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Potent and specific inhibition of mMate1-mediated efflux of type I organic cations in the liver and kidney by pyrimethamine

Sumito Ito, Hiroyuki Kusuhara, Yushun Kuroiwa, Chunyong Wu, Yoshinori Moriyama, Katsuhisa Inoue, Tsunenori Kondo, Hiroaki Yuasa, Hideki Nakayama, Shigeru Horita, Yuichi Sugiyama

Laboratory of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan (S.I., H.K., Y.K., C.W., Y.S.)

Department of Membrane Biochemistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 1-1, Tsushima-Naka 1-chome, Okayama 700-8530, Japan (Y.M.)

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan (K.I., H.Y.)

Department of Urology, Kidney Center, Tokyo Women's Medical University, 8-1 Kawadacho, Shinjuku-ku, Tokyo 162-8666, Japan (T.K., H.N., S.H.)

Running title: PYR is a potent inhibitor of MATE proteins

Corresponding author:

Yuichi Sugiyama, Ph.D.

Address: Laboratory of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Phone : +81-3-5841-4770

Fax : +81-3-5841-4766

E-mail : sugiyama@mol.f.u-tokyo.ac.jp

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Abbreviations:

AMPK, AMP-activated protein kinase; BBMV, brush-border membrane vesicle; CMV, canalicular membrane vesicles; LC–MS, liquid chromatography combined with mass spectrometry; MATE, multidrug and toxin extrusion; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; TEA, tetraethylammonium

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Abstract

This report describes a potent and selective inhibitor of multidrug and toxin extrusion (MATE) protein, pyrimethamine (PYR) and examined its effect on the urinary and biliary excretion of typical Mate1 substrates in mice. *In vitro* inhibition studies demonstrated that PYR is a potent inhibitor of mMate1 (K_i 145 nM) among renal organic cation transporters, mOctn1 and mOctn2 (K_i >30 μM), mOct1 (K_i 3.6 μM) and mOct2 (K_i 6.0 μM). PYR inhibited the uptake of metformin by kidney brush-border membrane vesicles (BBMVs) (K_i 41 nM) and canalicular membrane vesicles in the presence of outward gradient of H^+. PYR treatment significantly increased the kidney-to-plasma ratio of tetraethylammonium, and both the liver-, and kidney-to-plasma ratios of metformin in mice, while it did not affect their plasma concentrations and urinary excretion rates. Furthermore, the plasma lactate concentration, a biomarker for inhibition of gluconeogenesis by metformin, was significantly higher in the PYR-treated group than in the control group. These results not only suggest the importance of mMate1 in the efflux of organic cations into the urine and bile in mice, but also the importance of canalicular efflux mediated by MATE proteins for the therapeutic efficacy of metformin. PYR is a potent inhibitor of hMATE1 and hMATE2-K (K_i 77 and 46 nM, respectively), and H^+ and organic cation exchanger in human kidney BBMVs (K_i 31 nM) in the presence of outward gradient of H^+. Taken together, PYR can be used as a potent probe inhibitor of human MATE transporters.

Introduction

The pharmacokinetics of drugs, which involves drug absorption, distribution, metabolism, and excretion are indispensable for the understanding of pharmacological action and adverse reactions of drugs. Drug transporters mediate the tissue distribution of drugs and facilitate their elimination from the systemic circulation, and thereby play a significant role in the drug response (Giacomini and Sugiyama, 2005). The role of the organic cation transporter (OCT/*SLC22*) family has already been accumulated for the tissue uptake from the systemic circulation in the liver and kidney for type I organic cations, such as tetraethylammonium (TEA) and metformin (Wang et al., 2002; Wright, 2005; Koepsell et al., 2007). Cumulative studies have demonstrated the indispensable role of organic cation transporter 1 (Oct1/*SLC22A1*) and Oct2/*SLC22A2* in the basolateral uptake of hydrophilic organic cations in rodent kidney (Jonker et al., 2003), and hOCT2 in human kidney (Motohashi et al., 2002). OCT1 is the predominant isoform in the liver, and its importance in the pharmacological action and the adverse reactions of metformin have been reported in animal and clinical studies (Wang et al., 2003; Shu et al., 2007; Shu et al., 2008).

Unlike the basolateral side which is mediated by facilitated diffusion, *in vitro* transport studies using brush border membrane vesicles (BBMVs) from the kidney suggest involvement of active transport (Inui et al., 1985). Since the outward gradient of H^+ stimulates the uptake of typical organic cations like TEA by BBMV, an organic cation/H^+ exchanger has been considered as the efflux

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transporter. Organic cation/carnitine transporter 1 (OCTN1/*SLC22A4*) and 2 (OCTN2/*SLC22A5*) were reported to mediate an exchange of H^+ and TEA (Tamai et al., 1997; Wu et al., 1998). Animal and clinical studies suggest that these transporters mediate luminal efflux of drugs in the kidney. In jvs mice, which exhibit systemic carnitine deficiency caused by a defect of Octn2, show a significant reduction in the renal clearance of TEA and cephaloridine accompanied by a significant increase in their kidney accumulation (Ohashi et al., 2001; Kano et al., 2009). In addition, a recent clinical study reported that healthy subjects homozygous for the L503F variant of OCTN1, which is associated with decreased OCTN1 function, exhibited smaller tubular secretion of gabapentin in the kidney compared with those homozygous for the reference allele (Urban et al., 2008). However, their importance in the luminal efflux of type I organic cations remains unclear.

On the other hand, there is a growing interest in multidrug and toxin extrusion (MATE) proteins as alternative candidate transporter for the efflux of type I organic cations in the kidney. MATEs were isolated from the kidney of various species (Otsuka et al., 2005; Masuda et al., 2006). MATE1 is expressed both in the liver and the kidney, while another isoform, MATE2/MATE2-K, is specific to the kidney (Moriyama et al., 2008; Terada and Inui, 2008). Both MATE1 and MATE2/MATE2-K exhibit polyspecific substrate specificities for a variety of organic cations, including TEA and metformin, with an extensive overlap (Tanihara et al., 2007). Since MATE1 and MATE2/MATE2-K are expressed in the apical membrane of the epithelial cells, they have been

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considered as further candidate proteins accounting for the luminal efflux of cationic drugs with an exchange of H^+ in the kidney (Moriyama et al., 2008; Terada and Inui, 2008). A single-nucleotide polymorphism (SNP) in the intron (rs2289669) of hMATE1 has been found to be associated with an enhanced pharmacological effect of metformin (HbA_{1c} reduction) in diabetic patients (Becker et al., 2009), but it does not cause a variation of the systemic exposure of metformin(Tzvetkov et al., 2009). Recently, *Mate1*$^{-/-}$ have been generated and *in vivo* studies using *Mate1*$^{-/-}$ mice showed a significant reduction in the urinary excretion of metformin compared with wild-type mice (Tsuda et al., 2009a).

The present report describes a potent and selective inhibitor of MATE transporters, pyrimethamine (PYR), and examines its effect on the urinary and biliary excretion of typical MATE1 substrates TEA and metformin, as well as the pharmacological response of metformin in mice. In mice, only one isoform, MATE1, corresponds to MATE1 and MATE2-K in humans (Hiasa et al., 2006), while another isoform identified in rodents, Class III MATE protein, was found to be specifically expressed in the testis (Hiasa et al., 2007). The plasma concentrations and urinary and biliary excretion rates were determined under steady-state conditions. Kidney and liver concentrations were also determined in order to compare the intrinsic efflux activities across the BBM and canalicular membrane in mice, where MATE1 is expressed, with and without PYR. Furthermore, the effect of PYR on the plasma lactate concentration was examined to elucidate the importance of MATE1 in the pharmacological action of metformin.

Methods

Materials

[^{14}C]TEA (3.2 mCi/mmol) was purchased from PerkinElmer Life Science (Boston, MA), [^{14}C]metformin (26 mCi/mmol) was purchased from Moravec Biochemicals (Brea, CA), and [^{3}H]inulin (102 mCi/gm) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled TEA, metformin, and LabAssay ALP were purchased from Wako Pure Chemicals (Osaka, Japan). An L-Lactate Assay Kit was purchased from Biomedical Research Service Center (Buffalo, NY). All other chemicals used were commercially available and of analytical grade.

Animals

Male ddY mice were purchased from Japan SLC (Shizuoka, Japan). All animals were maintained under standard conditions with a reverse dark–light cycle and were kept for at least 7 days before pharmacological experiments. Food and water were available ad libitum. The mice used in the present study were from 8 to 9 weeks old. The studies were carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

Construction of stable transfectants of mMate1, mOct1 and mOct2 in HEK293 cells and cell cultures

m*Mate1* tagged with *myc* cDNA (NM_026183) was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). cDNA of m*Oct1* and m*Oct2* open reading frame were ligated into pENTR/D-TOPO

vector (Invitrogen), and then recombined into pEF-DEST51 vector (Invitrogen) by LR recombination reaction of a Gateway system following the manufacturer's protocol. The plasmids were transfected into human embryonic kidney 293 (HEK293) cells by lipofection with FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The transfectants were selected by culturing in the presence of G418 sulfate (800 μg/ml) (Gibco BRL, Gaithersburg, MD). Transporter-expressing or vector-transfected HEK293 cells were grown in low glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic–antimycotic (Gibco BRL) and incubated at 37 °C with 5% CO_2 and 95% humidity.

In vitro transport study using cDNA transfectants

hMATE1-HEK293, hMATE2-K-HEK293, hOCT1-HEK293, hOCT2-HEK293, mOctn1-HEK293, and mOctn2-HEK293 were already constructed previously (Busch et al., 1998; Tamai et al., 2000; Muller et al., 2005; Matsushima et al., 2009). 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 1.5×10^5 cells per well. For the transport study, 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 transporters. The transport study was carried out as described previously (Hirano et al., 2004). Uptake was initiated by the addition of radiolabeled substrates, after the cells had been washed twice and preincubated with

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Krebs–Henseleit buffer at 37 °C for 15 min. The Krebs–Henseleit buffer consisted of 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$ and was adjusted to pH 7.4. 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 aliquots was measured by liquid scintillation counting. For the examination of the inhibitor effect on the uptake by PYR, PYR was added to the incubation buffer. The protein concentration was determined using the Lowry method with bovine serum albumin as the protein standard as described previously (Lowry et al., 1951).

Transport study using kidney slices

Uptake studies were carried out as described in a previous report (Hasegawa et al., 2002). Slices (300 μm in thickness) of whole kidneys from male ddY mice were kept in ice-cold oxygenated buffer before use. The buffer for the present study consisted of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4 adjusted to pH 7.5. Two slices were selected and then incubated at 37 °C on a 12-well plate with 1ml oxygenated buffer containing 3.8 μM [^{14}C]metformin in each well after preincubation of slices for 5 min at 37 °C. After incubation for 15 min, slices were rapidly removed from the incubation buffer, washed twice with ice-cold buffer, blotted on filter paper, weighed, and dissolved in 1 ml Soluene-350 (Packard Instruments, Downers

Grove, IL) at 55 °C for 12 h. The radioactivity in the specimens was determined in a scintillation counter after adding scintillation cocktail (Hionic Fluor, Packard Instruments). In every experiment, the uptake activity of Oct1 and Oct2 in the mouse kidney slices was checked with TEA as the positive control.

Transport study using kidney BBMVs

The BBMVs were isolated from the kidney of human and male ddY mice by the Ca^{2+} precipitation method as described previously (Shah et al., 1979) with several modifications. Human kidney samples were obtained from surgically nephrectomized patients with renal cell carcinoma at Tokyo Women's Medical University. The protocol was approved by the Ethics Review Boards of both the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan) and Tokyo Women's Medical University (Tokyo, Japan). All participants provided written informed consent. Samples were stored in Dulbecco's modified Eagle's medium and kept on ice, immediately after kidney excision.

The kidney samples were homogenized in a 30-fold (w/v) volume of solution containing 30 mM mannitol, 10 mM $CaCl_2$, buffered with 10 mM Tris-HEPES, pH 7.4, and after standing for 15 min, the homogenate was centrifuged at 3,000 rpm (TS-7 rotor, TOMY) for 15 min. Then, the supernatant was centrifuged at 40,000 rpm (50.2 Ti rotor, Beckman) for 60 min. The vesicle pellet was resuspended in an experimental buffer to give a final protein concentration of 7 mg/ml using a

25-gauge needle. The experimental buffer consisted of 100 mM mannitol, 100 mM potassium chloride, and 10 mM MES (pH 6.0) or HEPES (pH 8.0), and the pH was adjusted with potassium hydroxide. The enrichment was determined by alkaline phosphatase activity using the LabAssay ALP, which was 6- to 8-fold higher compared with the homogenate. All steps were performed either on ice or at 4 °C.

The uptake of [^{14}C]metformin by BBMVs was measured using a rapid filtration technique. The uptake was initiated by the addition of 80 μl of a transport buffer containing 50 μM [^{14}C]metformin (final concentration 40 μM) to 20 μl of membrane suspension at 20 °C. After 30 seconds, the incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold buffer. The mixture was then poured immediately onto Millipore filters (HA, 0.45 μm), and the filters were washed twice with 5 ml of ice-cold buffer. In separate experiments, the nonspecific association of the ligand with filters was estimated by the absence of membrane vesicles. This value was subtracted from the uptake data to evaluate the specific uptake of the ligand. The radioactivity trapped in membrane vesicles was determined by liquid scintillation counting.

Transport study using bile canalicular membrane vesicles (CMV)

The CMVs were isolated from the liver of male ddY mice as described previously (Niinuma et al., 1999; Hayashi and Sugiyama, 2007). The vesicle pellet was resuspended in an experimental buffer using a 25-gauge needle. The experimental buffer consisted of 100 mM mannitol, 100 mM

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potassium chloride, and 10 mM MES (pH 6.0) or HEPES (pH 8.0), and the pH was adjusted with potassium hydroxide. The uptake of [^{14}C]metformin (100 μM) for 2 min by CMVs was measured at 37 °C using a rapid filtration technique as described above.

Steady-state infusion study of TEA and metformin in mice treated with and without PYR

After anesthesia with intraperitoneal sodium pentobarbital (51.8 mg/kg, for TEA) or isoflurane (for metformin), the urinary bladder or bile duct were catheterized. In the PYR-treated group, a bolus dose of PYR (0.2, 0.5, or 2 μmol/kg in 0.9% NaCl and 10% ethanol) was administered via the jugular vein 30 min prior to the start of infusion. [^{14}C]TEA (128 nmol/min/kg) and [^{3}H]inulin (49 pmol/min/kg) or metformin (200 nmol/min/kg) were then also infused via the jugular vein. Blood samples were collected via the jugular vein at 30, 50, 70, and 90 min (for [^{14}C]TEA) or 60, 80, 100, and 120 min (for metformin) after administration and centrifuged to obtain plasma. Urine specimens were collected at 30 to 50, 50 to 70, and 70 to 90 min (for [^{14}C]TEA), and urine or bile specimens were collected at 60 to 80, 80 to 100, and 100 to 120 min (for metformin). At the end of the experiment, the kidneys and liver were removed. To determine the glomerular filtration rate (GFR), [^{3}H]inulin (for TEA) or creatinine (for metformin) renal clearance was measured. Drug concentration was determined by liquid scintillation counting (for TEA and inulin) and LC–MS analysis (for creatinine and metformin).

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Quantification of metformin by LC–MS in biological samples

The kidney and liver were homogenized in a 4-fold volume of PBS. The urine specimens were diluted with a 100-fold volume of water. All specimens (10 μl) were mixed with 10 μl of methanol and 80 μl of acetonitrile. Mixed solutions were centrifuged at 20,000 g for 10 min. The supernatants were mixed with a 4-fold volume of water, centrifuged at 20,000 g for 10 min and were subjected to LC–MS analysis. In the case of creatinine, the plasma specimens (10 μl) were mixed with 20 μl of acetonitrile and centrifuged at 20,000 g for 10 min. The supernatants (10 μl) were evaporated, and the pellets were reconstituted with 80 μl of water. The reconstituted samples were then centrifuged at 20,000 g for 10 min to remove particles, and an aliquot of the supernatants was used for LC–MS analysis. The urine specimens were diluted with a 2000-fold volume of water and centrifuged twice at 20,000 g for 10 min. The supernatants were then used for LC–MS analysis.

An LCMS2010EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. The interface voltage was –3.5 kV, and the nebulizer gas (N_2) flow was 1.5 l/min. The heat block and curved desolvation line temperatures were 200 °C and 150 °C, respectively. Detailed LC conditions and mass-to-charge ratios are shown in Supplemental Table 1.

Kinetic analyses

The data obtained from the inhibition study can be fitted to the following equation to calculate the inhibition constant (K_i):

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$$CL_{uptake(+Inhibitor)} = \frac{CL_{uptake(control)}}{1+I/K_i} + P_{dif} \quad \text{(eq. 1)}$$

where $CL_{uptake\ (+Inhibitor)}$ and $CL_{uptake\ (control)}$ are the uptake clearance determined in the presence or absence of the inhibitor, P_{dif} is the nonsaturable uptake clearance, and I is the concentration of the inhibitor. Fitting was performed by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981), as well as the Damping Gauss–Newton Method algorithm for fitting.

Pharmacokinetic analysis

The fractional urinary excretion ratio (F_{urine}), the fractional biliary excretion ratio (F_{bile}), the total body clearance ($CL_{tot,\ plasma}$), the renal clearance with respect to the plasma concentration ($CL_{renal,\ plasma}$), the secretion clearance with respect to the concentration in the kidney ($CL_{renal,\ kidney}$), the biliary clearance with respect to the plasma concentration ($CL_{bile,\ plasma}$), and the biliary clearance with respect to the concentration in the liver ($CL_{bile,\ liver}$) were calculated using the following equations:

$$F_{urine} = (V_{urine}/I) \times 100 \quad \text{(eq. 2)}$$

$$F_{bile} = (V_{bile}/I) \times 100 \quad \text{(eq. 3)}$$

$$CL_{tot,\ plasma} = Dose/AUC_p \quad \text{(eq. 4)}$$

$$CL_{renal,\ plasma} = X_{urine}/AUC_p \quad \text{(eq. 5)}$$

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$$CL_{renal,\ kidney} = (CL_{renal,\ plasma} - f_p \times GFR)/K_{p,\ kidney} \quad \text{(eq. 6)}$$

$$CL_{bile,\ plasma} = X_{bile}/AUC_p \quad \text{(eq. 7)}$$

$$CL_{bile,\ liver} = CL_{bile,\ plasma}/K_{p,\ liver} \quad \text{(eq. 8)}$$

where V and I represent urinary or biliary excretion rate and infusion rate. Dose and X represent the amount of ligand administered in 1 h and excreted into urine or bile from 30 to 90 min (for TEA) or 60 to 120 min (for metformin), respectively. AUC_p represents the area under the time–plasma concentration curve for ligands from 30 to 90 min (for TEA) or 60 to 120 min (for metformin). The apparent tissue-to-plasma concentration ratio ($K_{p,\ tissue}$) was calculated using the following equation:

$$K_{p,\ tissue} = C_{tissue}/C_p \quad \text{(eq. 9)}$$

where C_{tissue} and C_p represent tissue and plasma concentrations of ligands at 90 or 120 min after administration for TEA or metformin, respectively. Glomerular filtration rate (GFR) was determined by [^{3}H]inulin (for TEA) or creatinine (for metformin) renal clearance.

Plasma lactate levels following metformin treatment in mice

Because isoflurane causes an increase in the plasma lactate level in mice, pentobarbital was used for anesthesia. Mice were anesthetized with intraperitoneal sodium pentobarbital (51.8 mg/kg). PYR (2 μmol/kg, i.v.) was given to mice 30 min before starting the intravenous infusion of metformin at the

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rate of 15 μmol/min/kg for 4 h through the jugular vein. Blood samples were collected in the same way at 0 (just prior to administration), 150, 180, 210, and 240 min. The blood was centrifuged and the plasma samples were used for lactate determination with an L-Lactate Assay Kit according to the manufacturer's protocol. After sampling at 240 min, the mice were sacrificed, and the liver was removed immediately. Plasma and liver concentrations of metformin were determined as described above.

Statistical analysis

Data are presented as the mean ± standard error (SE). Student's two-tailed unpaired *t* test and a one-way ANOVA followed by Dunnett's post hoc test were used to identify significant differences between groups where appropriate.

Results

Inhibition potency and selectivity of PYR for the mouse renal organic cation transporters

mMate1-HEK constructed in this study exhibited similar transport characteristics (Supplemental Figure 1) to results reported previously (Hiasa et al., 2006). Intracellular accumulation of TEA, metformin, cimetidine, creatinine, carnitine, and acyclovir were significantly greater in mMate1-HEK compared with mock cells (Supplemental Figure 2). The effect of PYR on mMate1, mOct1 and mOct2, mOctn1 and mOctn2 was investigated for the uptake of their model substrate (TEA). TEA uptake by mMate1, mOct1, and mOct2 was strongly inhibited by PYR, while it had little or negligible effect on TEA transport by mOctn1 and mOctn2 (Figure 1). The K_i values are summarized in Table 1. The results clearly show that PYR is a 25- and 42-times more potent inhibitor for mMate1 than for mOct1 and mOct2, respectively.

Inhibitory effect of PYR on metformin uptake by mouse kidney slices

The inhibitory effect of PYR on metformin uptake by mouse kidney slices is shown in Figure 2A. Saturable uptake of metformin was clearly observed in mouse kidney slices. PYR significantly inhibited the uptake of metformin at 10 and 100 μM.

Inhibitory effect of PYR on metformin uptake by renal BBMVs and CMVs

The effect of PYR on metformin uptake in the presence of a H^+ gradient by renal BBMVs from mouse kidney was examined. Nonspecific binding to filters, which accounted for 12 % of the total

uptake, was identical in the presence and absence of PYR. The uptake of metformin determined at pH 8.0 was markedly reduced in the presence of excess metformin (16 mM), showing saturation, and inhibited by PYR (8 μM) (Figure 2B). PYR significantly inhibited the uptake of metformin by BBMVs in a concentration-dependent manner with a K_i value of 41 ± 6 nM (Figure 2C).

Similarly, the uptake of metformin by CMVs determined at pH 8.0 was markedly reduced in the presence of excess metformin (10mM), showing saturation, and inhibited by PYR (10 μM) (Figure 3). Nonspecific binding to filters, which accounted for 19% of the total uptake, was identical in the presence and absence of PYR.

Effect of PYR on the urinary and biliary excretion of TEA and metformin in mice

According to our preliminary study, PYR shows a long half-life in the systemic circulation (> 4 h), and therefore, PYR was given to mice by a bolus injection 30 min before starting the infusion of substrate drugs, resulting in a near-constant plasma concentration during the experiments. The plasma, kidney, and liver concentrations of PYR (μM) were 0.230 ± 0.037, 4.99 ± 0.49 and 2.79 ± 0.37, respectively, at a dose of 2 μmol/kg. Considering that unbound fractions of PYR in the plasma, kidney, and liver were 8.1%, 2.6%, and 1.1%, respectively, the unbound concentrations of PYR in the plasma, kidney, and liver are estimated at about 10–30 nM, 50–150 nM, and 20–50 nM.

TEA was administered to mice by constant intravenous infusion, and the concentrations of TEA in the plasma and kidney, as well as the urinary excretion rate, were measured under steady-state

conditions with and without pretreatment by PYR. PYR treatment caused a significant increase in the kidney-to-plasma ratio ($K_{p, kidney}$), even though both plasma concentrations and urinary excretion rates of TEA were similar (Figure 4). Kinetic parameters of TEA in control and PYR-treated mice are summarized in Table 2. The secretion clearance with regard to the kidney concentration was significantly decreased by PYR in a dose-dependent manner from 0.5 to 2 μmol/kg (Table 2).

Metformin was administered to mice with and without PYR pretreatment (2 μmol/kg) through constant intravenous infusion. The effect of PYR on the urinary and biliary excretion of metformin was examined in separate experiments. PYR significantly increased the kidney concentration of metformin (3.7-fold), whereas it did not affect the plasma concentrations and urinary excretion rates of metformin (Figure 5). The secretion clearance of metformin with respect to the kidney concentration was significantly decreased by PYR (Table 3). Metformin was found to undergo biliary excretion, the rate of which was 621-fold smaller than its urinary excretion rate (Table 3). PYR also significantly increased the liver concentration of metformin (2.6-fold) (Figure 5). Because the biliary excretion rate of metformin was unchanged, the biliary clearance with regard to the liver concentration of metformin was significantly decreased in mice treated with PYR (Table 3). In mice whose bile duct was cannulated, the total body clearance was 60% of that in mice whose bladder was cannulated by unknown reason. The operation of bile duct ligation may affect the renal blood flow rate since the renal clearance of metfomin is blood flow limited.

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Effect of PYR on plasma lactate concentration in mice treated with metformin

The time profiles of the plasma lactate concentrations on constant infusion of a dose of 15 μmol /min/kg of metformin are shown in Figure 6A, and the simultaneously determined plasma concentrations of metformin are shown in Figure 6B. There was a marked difference in the response to metformin between the metformin-administered group and the combined metformin- and PYR-administered group after 240 min of intravenous infusion of metformin (Figure 6A), although the plasma concentrations of metformin were comparable in both sample sets (Figure 6B). At 240 min, the plasma lactate concentration was 1.8-fold greater in mice receiving both metformin and PYR than that in mice receiving only metformin (54.8 ± 4.4 vs. 31.2 ± 2.6 mg/dl). After the mice were sacrificed, the concentration of metformin in the liver showed a significant increase in PYR-treated mice (1.05 ± 0.14 vs. 2.98 ± 0.30 μmol/g liver, $p < 0.01$).

Inhibition potency and selectivity of PYR for human renal organic cation transporters, and metformin uptake by BBMVs from human kidney samples

Significant uptake of TEA, metformin, cimetidine, carnitine, and acyclovir were observed both in hMATE1 and hMATE2-K expressed cell lines. Creatinine was revealed to be specific substrate of hMATE1, but not of hMATE2-K (Supplemental Figure 2). The effect of PYR on hMATE1, hMATE2-K, hOCT1, and hOCT2 was investigated for the uptake of their model substrate (TEA). PYR inhibited the uptake of TEA by hMATE1, hMATE2-K, hOCT1 and hOCT2 in a

concentration-dependent manner (Figure 7). The K_i values are summarized in Table 1. PYR is not an isoform-selective inhibitor of hMATE1 and hMATE2-K. PYR is a more potent inhibitor for hMATE1 and hMATE2-K than for basolateral organic cation transporters, hOCT1 and hOCT2.

The effect of PYR on the metformin uptake by BBMVs from human kidney was examined. Nonspecific binding to filters, which accounted for 12 % of the total uptake, was identical in the presence and absence of PYR. The uptake of metformin by BBMVs was determined in the presence of a H^+ gradient and it was shown that the uptake determined at pH 8.0 was markedly reduced in the presence of excess metformin (24 mM) (Figure 8A). PYR significantly inhibited the uptake of metformin by BBMVs in a concentration-dependent manner, with a K_i value of 31 ± 4 nM in humans (Figure 8B).

Discussion

We examined the role of MATE1 in the disposition of typical type I organic cations, TEA and metformin, and in the pharmacological action of metformin using a potent MATE1 inhibitor, PYR. MATE transporters are organic cation transporters driven by an exchange of H^+, and have been a candidate transporter mediating the efflux of type I organic cations in the liver and kidney. Inhibition of MATE proteins could prolong the systemic and tissue exposure of its substrate drugs, such as metformin, enhancing the pharmacological action and the adverse reaction.

First, we examined the selectivity and inhibition potency of PYR in cDNA transfectants, mouse kidney slices and BBMVs. Among the organic cation transporters, PYR can specifically block mMate1 (Figure 1). Even though PYR inhibited both mOct1 and mOct2, the potency was quite low compared with mMate1 (Table 1). In other words, PYR can specifically inhibit the luminal efflux mediated by mMate1 without inhibiting the uptake process mediated by mOct1 and mOct2. In fact, PYR could not inhibit the uptake of metformin by kidney slices at the concentration (1 μM) adequately greater than the K_i value for mMate1 (Figure 2A). The uptake of metformin by BBMVs determined in the presence of a H^+ gradient was completely inhibited by PYR (Figure 2B) with a K_i value similar to that observed in mMate1-expressed HEK293 cells (Figure 2C). Therefore, mMate1 accounts for the major part of the H^+-coupled organic cation transport in the kidney. Furthermore, CMVs shows saturable uptake of metformin in the presence of outward gradient of H^+, which was

inhibited by PYR. This is in a good agreement with the membrane localization of mMate1 in the liver (Hiasa et al., 2006).

Second, the effect of PYR was examined *in vivo* in mice. PYR could significantly increase the kidney-to-plasma ratio of TEA and metformin, which is ascribed to an inhibition of the efflux into the urine across the BBM because the secretion clearance with respect to the kidney concentrations was significantly reduced in PYR-treated mice (Tables 2 and 3). Absence of an effect of PYR on the renal clearance with respect to the plasma concentrations in spite of significant inhibition of the luminal efflux seems to contradict the fact that both TEA and metformin are exclusively eliminated into the urine by tubular secretion as well as by glomerular filtration (Tables 2 and 3). The following points can explain this discrepancy: 1) the renal elimination of TEA and metformin is blood-flow limited, and 2) the fraction inhibited by PYR is not large enough to disturb the directional transport across the proximal tubules involving the transporters both in the uptake and in the subsequent efflux processes. In fact, the renal clearance of TEA and metformin was close to the previously reported renal blood flow rate (65 ml/min/kg, (Davies and Morris, 1993)). The unbound concentration of PYR in the kidney was 150 nM at the highest dose examined, which was similar to the K_i value of PYR for mMate1, and thus, PYR could reduce mMate1-mediated efflux to 40% of the control.

Third, PYR highlighted the significance of mMate1 in the liver (Figure 5F). Metformin was found to be eliminated into the bile, although the biliary excretion made a negligible contribution to

the systemic elimination (Table 3). PYR significantly inhibited the efflux of metformin across the bile canalicular membrane, and thereby increased the liver-to-plasma concentration ratio (Table 3). In fact, Na^+/H^+ exchanger 3 protein is present on the canalicular membrane of hepatocytes and generates a H^+ gradient (Mennone et al., 2001). Considering that the pharmacological target of metformin for inhibition of gluconeogenesis is located inside the hepatocytes, it is reasonable to announce that PYR could enhance the pharmacological action of metformin (Figure 6). Considering that a marked reduction in the hepatic concentration of metformin caused by the deletion of the Oct1 gene resulted in a reduced effect of metformin on AMP-activated protein kinase (AMPK) phosphorylation (Shaw et al., 2005), PYR will increase AMPK phosphorylation following metformin treatment. Recently, it was reported that a SNP in the intron (rs2289669) of hMATE1 is associated with enhanced pharmacological effects of metformin (HbA_{1c} reduction) in diabetic patients (Becker et al., 2009). Since hMATE1 is also expressed in the canalicular membrane of human hepatocytes (Otsuka et al., 2005), it is most probable that MATE1 can be a determining factor in the pharmacological action of metformin through the regulation of liver concentrations in human. In addition, MATE1 can potentially be the site of a drug–drug interaction between metformin and concomitant drugs. For instance, cimetidine has been reported to inhibit the renal elimination, and thereby enhance the pharmacological action of metformin in healthy volunteers (Somogyi et al., 1987). Considering the fact that cimetidine is a clinically relevant inhibitor of MATE1 (Matsushima

et al., 2009; Tsuda et al., 2009b), it is possible that the interaction involves the inhibition of the canalicular efflux as well as inhibition of renal elimination. *In vitro* inhibition studies using cDNA transfectant of hMATE1 and concomitant drugs will contribute to avoid severe drug-drug interaction by comparing the inhibition constant with the unbound concentration of the concomitant drugs at clinical dose. It should be noted that, unlike in mice, the human kidney expresses two MATE transporters, MATE1 and MATE2-K. Considering that MATE1 SNP was not associated with the variation of systemic exposure of metformin,(Tzvetkov et al., 2009) presumably because of compensation of MATE2-K, specific inhibition of MATE1 in the kidney by concomitant drugs will not affect the renal elimination of metformin.

Finally, PYR was found to be a significantly potent inhibitor of MATE1 and MATE2-K (Table 1). The uptake of metformin by BBMVs in the presence of a H^+ gradient was completely inhibited by PYR with a K_i value similar to those for MATE1 and MATE2-K (Figure 8). Thus, the metformin transport driven by an exchange of H^+ is likely accounted for by MATE1 or MATE2-K. PYR is used clinically as an antimalarial and antitoxoplasmic drug (Daraprim, fansidar (fansidar is the sulfadoxine–pyrimethamine combined drug)). The unbound plasma concentration of PYR at the clinical dosage is 200 nM on average, adequately greater than the K_i values for hMATE1 and hMATE2-K, but lower than its K_i values for hOCT1 and hOCT2 (Table 1). Therefore, the clinical dose of PYR would be sufficient to inhibit MATE1 and MATE2-K in humans, without affecting the

uptake process. To our knowledge, there is no report on any drug–drug interaction involving PYR except creatinine. PYR decreases the renal clearance of endogenous creatinine without affecting the renal clearance of inulin, a true GFR marker (Opravil et al., 1993). Creatinine is known to undergo tubular secretion in the kidney that involves hOCT2 as well as hMATE1 and hMATE2-K (Urakami et al., 2004; Tanihara et al., 2007). Reduction of renal clearance of creatinine may be ascribed to an inhibition of MATE1 or MATE2-K in the kidney by PYR.

In summary, MATE proteins play a significant role in the urinary and biliary excretion of metformin. MATE1 is particularly important for the pharmacological action of metformin through the regulation of liver concentration. PYR is a potent and selective inhibitor of mMate1, MATE1 and MATE2-K, and is likely a potent probe inhibitor to investigate the significance of MATE1 and MATE2-K in the disposition of type I organic cations in humans.

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Footnotes

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Legends for Figures

Figure 1 Inhibitory potency of PYR on the uptake of [^{14}C]TEA by mouse renal organic cation transporters

Effect of PYR on the uptake of [^{14}C]TEA by HEK293 cells stably expressing mMate1 (A), mOct1 (B), mOct2 (C), mOctn1 (D), and mOctn2 (E) were examined. HEK293 cells were incubated with [^{14}C]TEA (30 μM) for 5 min at 37 °C in the presence of PYR at the designated concentrations. The specific uptake of [^{14}C]TEA was calculated by subtracting the uptake by mock cells from the uptake by cDNA transfectants. Each point represents the mean value and SE (n = 3).

Figure 2 Effect of PYR on metformin uptake by kidney slices and mouse BBMVs

(A) Effect of PYR on the uptake of [^{14}C]metformin (0.1 μCi/ml, 3.8 μM) by kidney slices was examined. Freshly prepared slices from mouse kidney were incubated with [^{14}C]metformin (0.1 μCi/ml) for 15 min at 37 °C in the absence and presence of excess amount of metformin (30 mM) or PYR at the designated concentrations. Each bar represents the mean value and SE (n = 3).

(B, C) Membrane vesicles (20 μl) were suspended in MES buffer (pH_{in} 6.0). The uptake study was initiated by an addition of 80 μl of HEPES (pH_{out} 8.0, open bar) or MES (pH_{out} 6.0, closed bar) buffer containing [^{14}C]metformin (1.3 μCi/ml, 50 μM). After 30 s incubation at 20 °C, the uptake was terminated by rapid filtration technique. The uptake of [^{14}C]metformin was determined in the presence of an excess amount of unlabelled metformin (16 mM) or PYR (8 μM) (B). Concentration dependence

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of the effect of PYR was examined for the uptake of metformin by BBMVs (C). The specific uptake was calculated by subtracting the uptake in pH_{out} = 6.0 from that in pH_{out} = 8.0 in panel B. Each point represents the mean value and SE (*n* = 4).

Figure 3 Effect of PYR on metformin uptake by mouse CMVs in the presence of a H^+ gradient

Membrane vesicles (20 μl) were suspended in MES buffer (pH_{in} 6.0). The uptake study was initiated by an addition of 80 μl of HEPES (pH_{out} 8.0, open bar) or MES (pH_{out} 6.0, closed bar) buffer containing [^{14}C]metformin (3.3 μCi/ml, 125 μM). After 2 min incubation at 37 °C, the uptake was terminated by rapid filtration technique. The uptake of [^{14}C]metformin was determined in the presence of an excess amount of unlabelled metformin (10 mM) or PYR (10 μM). Each point represents the mean value and SE (*n* = 3).

Figure 4 Effect of PYR on the plasma and kidney concentrations, and urinary excretion rate of [^{14}C]TEA in mice

Plasma concentrations (A) and urinary excretion rates (B) of [^{14}C]TEA were determined in control (○) and PYR-treated mice (□, 0.2 μmol/kg; ●, 0.5 μmol/kg; ■, 2 μmol/kg). PYR was given to mice by a bolus injection 30 min before starting the intravenous infusion of [^{14}C]TEA (128 nmol/min/kg). Blood specimens were collected at designated times, and urine specimens were collected at 20 min intervals from 30 to 90 min. At the end of the experiment, kidneys were removed, and drug concentrations in

the plasma, urine, and kidney were determined by liquid scintillation counting. Each point represents the mean value and SE (n = 4–5).

Figure 5 Effect of PYR on the plasma, kidney, and liver concentrations, and urinary and biliary excretion rates of metformin in mice

(A–C) Plasma concentrations (A), urinary excretion rates (B), and kidney concentrations (C) of metformin were determined in control (○) and PYR-administered mice (■) whose bladders were cannulated for the collection of urine. PYR (2 μmol/kg) was given to mice by a bolus injection 30 min before starting intravenous infusion of metformin (200 nmol/min/kg). Blood specimens were collected at designated times, and urine specimens were collected at 20 min intervals from 60 to 120 min. At the end of the experiment, kidneys were excised. (D–F) Plasma concentrations (D), biliary excretion rates (E), and liver concentrations (F) of metformin were determined in control (○) and PYR-administered mice (■) whose bile ducts were cannulated for the collection of bile. PYR (2 μmol/kg) was given to mice as a bolus injection 30 min before starting intravenous infusion of metformin (200 nmol/min/kg). Blood specimens were collected via the jugular vein at designated times, and bile specimens were collected at 20 min intervals from 60 to 120 min. At the end of the experiment, the liver was excised. Drug concentrations in the plasma, urine, bile, kidney, and liver were determined by LC–MS. Each point represents the mean value and SE (n = 3–4).

Figure 6 Effect of PYR on the plasma lactate concentration in mice treated with

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metformin

PYR (2 μmol/kg) was given to mice by bolus injection 30 min before starting intravenous infusion of metformin (15 μmol/min/kg). Plasma concentrations of lactate (A) and metformin (B) were determined in control mice (○), mice treated with PYR alone (●), mice treated with metformin alone (□), and mice treated with both metformin and PYR (■). Blood was collected via the jugular vein at designated times. At the end of the experiment, the liver was removed, and metformin concentrations in the plasma and the liver were determined by LC–MS. Plasma concentration of lactate was determined by using a Lactate Assay Kit. Statistical analysis was performed using a one-way ANOVA followed by Dunnett's test. Significant difference at $p < 0.05$: *****, saline-treated versus metformin-treated mice; $p < 0.01$: ##, PYR-treated versus PYR and metformin-treated mice; and ††, metformin-treated versus PYR and metformin-treated mice, respectively. Each point represents the mean value and SE ($n = 4$).

Figure 7 Inhibitory potency of PYR on the uptake of [^{14}C]TEA by human renal organic cation transporters

Effect of PYR on the uptake of [^{14}C]TEA by HEK293 cells stably expressing hMATE1 (A), hMATE2-K (B), hOCT1 (C), and hOCT2 (D) was examined. HEK293 cells were incubated with [^{14}C]TEA (30 μM) for 5 min at 37 °C in the absence and presence of PYR at the designated concentrations. The specific uptake of [^{14}C]TEA was calculated by subtracting the uptake by mock

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cells from the uptake by cDNA transfectants. Each point represents the mean value and SE (n = 3).

Figure 8 Effect of PYR on metformin uptake by human BBMVs in the presence of a H^+ gradient

Membrane vesicles (20 μl) were suspended in MES buffer (pH_{in} 6.0). The uptake study was initiated by an addition of 80 μl of HEPES (pH_{out} 8.0, open bar) or MES (pH_{out} 6.0, closed bar) buffer containing [^{14}C]metformin (1.3 μCi/ml, 50 μM). After 30 s incubation at 20 °C, the uptake was terminated by rapid filtration technique. The uptake of [^{14}C]metformin was determined in the presence of an excess amount of unlabelled metformin (24 mM) or PYR (2.4 μM) (A). Concentration dependence of the effect of PYR was examined for the uptake of metformin by BBMVs (B). The specific uptake was calculated by subtracting the uptake in pH_{out} = 6.0 from that in pH_{out} = 8.0 in panel B. Each point represents the mean value and SE (n = 4).

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Table 1 Inhibition constant of PYR for the transport of [^{14}C]TEA by HEK293 cells expressing organic cation transporters

Data shown in Figures 1 and 7 were used to determine these parameters calculated using nonlinear regression analysis as described in the Materials and Methods section. Each parameter represents the mean value ± SE.

Transporter	Mouse	Human
MATE1	145 ± 36 nM	77 ± 13 nM
MATE2-K	-	46 ± 6 nM
OCTN1	> 10 μM	N.D.
OCTN2	> 30 μM	N.D.
OCT1	3.6 ± 0.5 μM	3.8 ± 0.3 μM
OCT2	6.0 ± 1.5 μM	10 ± 1 μM

N.D. not determined

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Table 2 Pharmacokinetic parameters of TEA in control and PYR-treated mice

Each value was determined from the data shown in Figure 4 and the equations described in the Materials and Methods section. Each value represents the mean ± SE (n = 4–5) *p < 0.05, **p < 0.01

PYR dose (μmol/kg)	0	0.2	0.5	2
$C_{p,ss}$ (μM)	1.73 ± 0.07	1.72 ± 0.09	1.92 ± 0.09	1.64 ± 0.12
C_{kidney} (nmol/g kidney)	10.6 ± 0.8	15.4 ± 2.5	20.8 ± 1.7*	27.8 ± 3.5**
C_{liver} (nmol/g liver)	20.9 ± 2.1	25.3 ± 2.3	35.1 ± 4.5*	22.1 ± 2.8
V_{urine} (nmol/min/kg)	109 ± 7	96 ± 5	89 ± 6	106 ± 11
F_{urine} (%)	85 ± 3	75 ± 5	69 ± 4	83 ± 2
$CL_{tot,plasma}$ (mL/min/kg)	76 ± 7	76 ± 7	68 ± 5	79 ± 5
$CL_{renal,\ plasma}$ (mL/min/kg)	65 ± 8	57 ± 7	47 ± 6	66 ± 5
$CL_{renal,\ kidney}$ (mL/min/kg)	7.8 ± 1.1	5.1 ± 0.9	3.1 ± 0.3**	3.0 ± 0.4**
GFR (mL/min/kg)	17 ± 1	14 ± 2	14 ± 1	17 ± 1
$K_{p,\ kidney}$ (mL/g kidney)	6.2 ± 0.3	9.0 ± 1.3	11 ± 1*	17 ± 1**
$K_{p,\ liver}$ (mL/g liver)	12 ± 1	15 ± 1	18 ± 1	14 ± 2

Abbreviations: $C_{p,\ ss}$, steady-state concentration; C_{kidney}, drug concentration in the kidney at 90 min; C_{liver}, drug concentration in the liver at 90 min; V_{urine}, urinary excretion rate; F_{urine}, fractional urinary excretion ratio; CL_{tot}, total body clearance; $CL_{renal,\ plasma}$, renal clearance with respect to the concentration in circulating plasma; $CL_{renal,\ kidney}$, renal clearance with respect to the kidney concentration; GFR, glomerular filtration rate; $K_{p,\ kidney}$, kidney-to-plasma concentration ratio; $K_{p,\ liver}$, liver-to-plasma concentration ratio

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Table 3 Pharmacokinetic parameters of metformin in control and PYR-treated mice

Each value was determined from the data shown in Figure 5 and the equations described in the Materials and Methods. Each value represents the mean ± SE (n = 3–4) *p < 0.05, **p < 0.01

	urinary excretion		biliary excretion	
PYR dose (μmol/kg)	0	2	0	2
$C_{p,ss}$ (μM)	3.47 ± 0.15	3.65 ± 0.17	6.18 ± 0.58	6.64 ± 1.06
C_{kidney} (nmol/g kidney)	15.1 ± 1.3	55.8 ± 8.5*	41.1 ± 3.5	89.6 ± 12.4**
C_{liver} (nmol/g liver)	5.33 ± 0.81	21.5 ± 2.4**	12.4 ± 1.2	32.5 ± 6.0*
V_{urine} (nmol/min/kg)	190 ± 5	203 ± 7	N.D.	N.D.
F_{urine} (%)	95 ± 3	101 ± 4	N.D.	N.D.
V_{bile} (nmol/min/kg)	N.D.	N.D.	0.306 ± 0.057	0.336 ± 0.048
F_{bile} (%)	N.D.	N.D.	0.15 ± 0.03	0.17 ± 0.02
$CL_{tot,plasma}$ (mL/min/kg)	57 ± 2	56 ± 3	33 ± 3	34 ± 8
$CL_{renal,\ plasma}$ (mL/min/kg)	55 ± 1	55 ± 1	N.D.	N.D.
$CL_{renal,\ kidney}$ (mL/min/kg)	9.6 ± 1.1	3.4 ± 0.7**	N.D.	N.D.
$CL_{bile,\ plasma}$ (mL/min/kg)	N.D.	N.D.	0.050 ± 0.010	0.051 ± 0.003
$CL_{bile,\ liver}$ (mL/min/kg)	N.D.	N.D.	0.026 ± 0.003	0.012 ± 0.002**
GFR (mL/min/kg)	15 ± 2	12 ± 1	N.D.	N.D.
$K_{p,\ kidney}$ (mL/g kidney)	4.3 ± 0.5	15 ± 2**	6.3 ± 0.1	13 ± 3
$K_{p,\ liver}$ (mL/g liver)	1.5 ± 0.3	5.7 ± 0.5**	1.9 ± 0.2	4.5 ± 0.5**

Abbreviations: C_{kidney}, drug concentration in the kidney at 120 min; C_{liver}, drug concentration in the liver at 120 min; V_{bile}, biliary excretion rate; F_{bile}, fractional biliary excretion ratio; $CL_{bile,\ plasma}$, hepatic clearance with respect to the concentration in circulating plasma; $CL_{bile,\ liver}$, hepatic clearance with respect to the liver concentration; ND, not determined

Figure 1

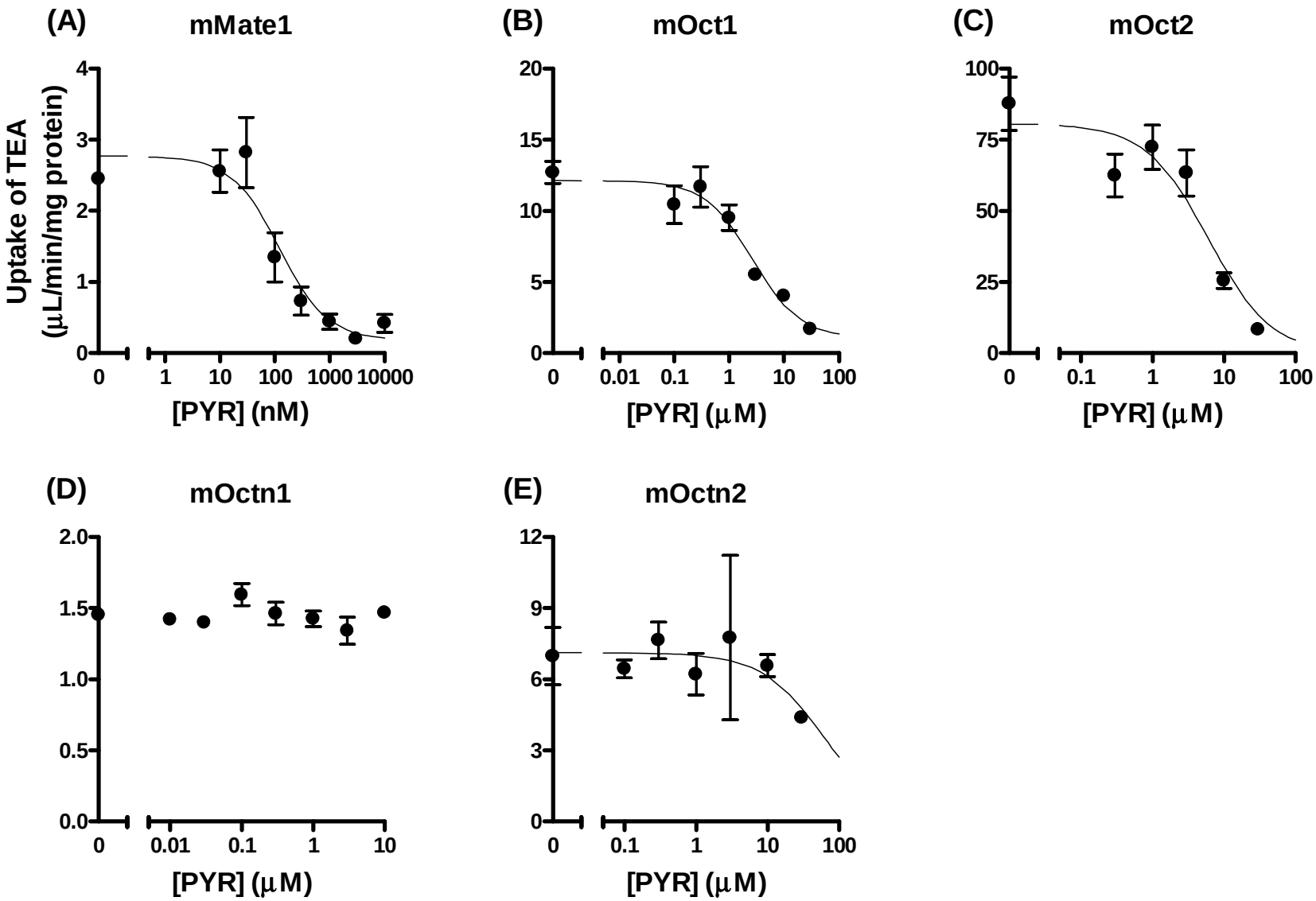

Figure 2

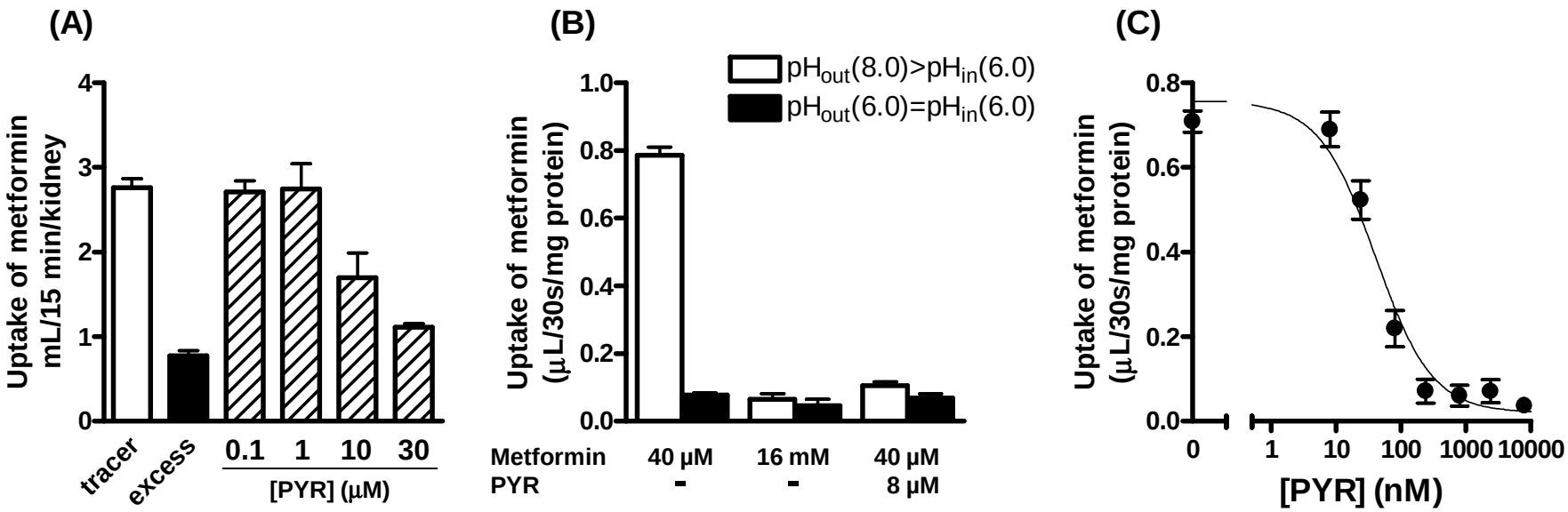

Figure 3

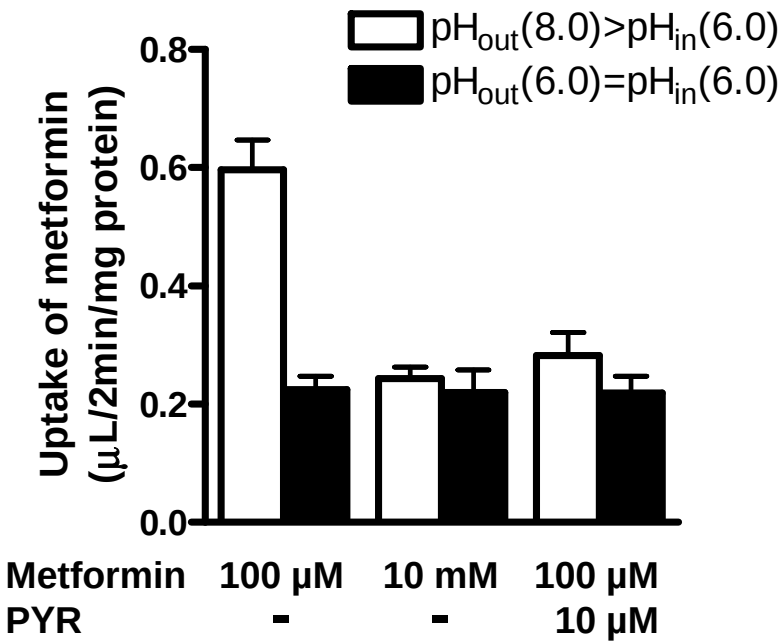

Figure 4

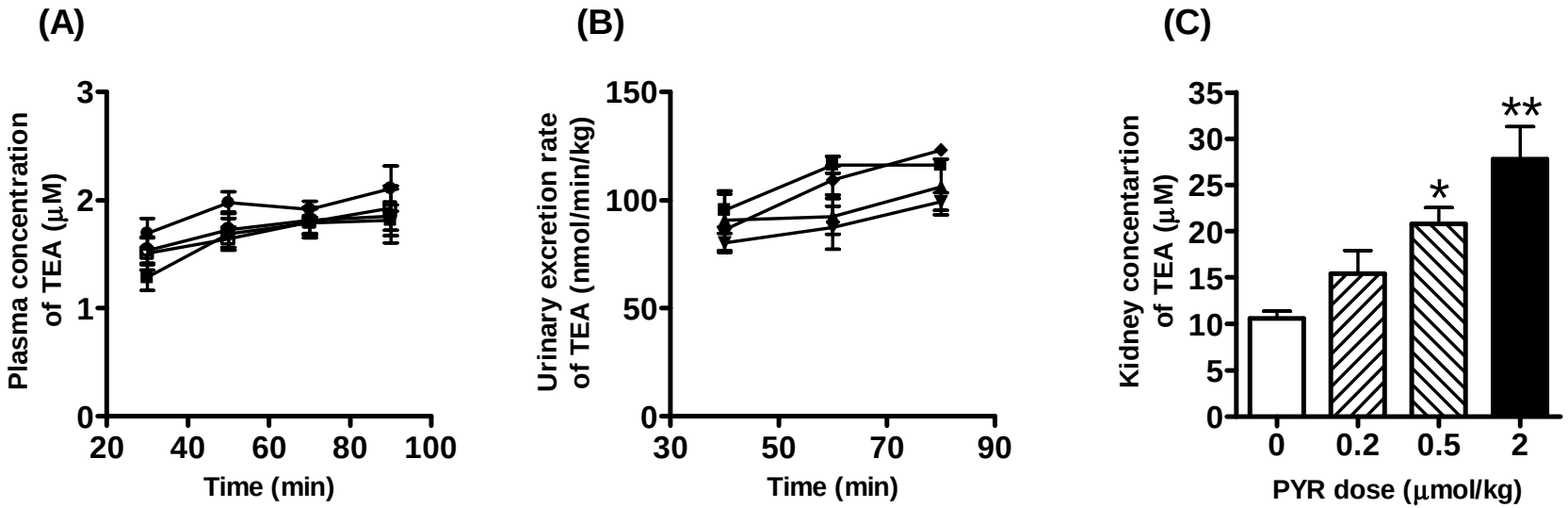

Figure 5

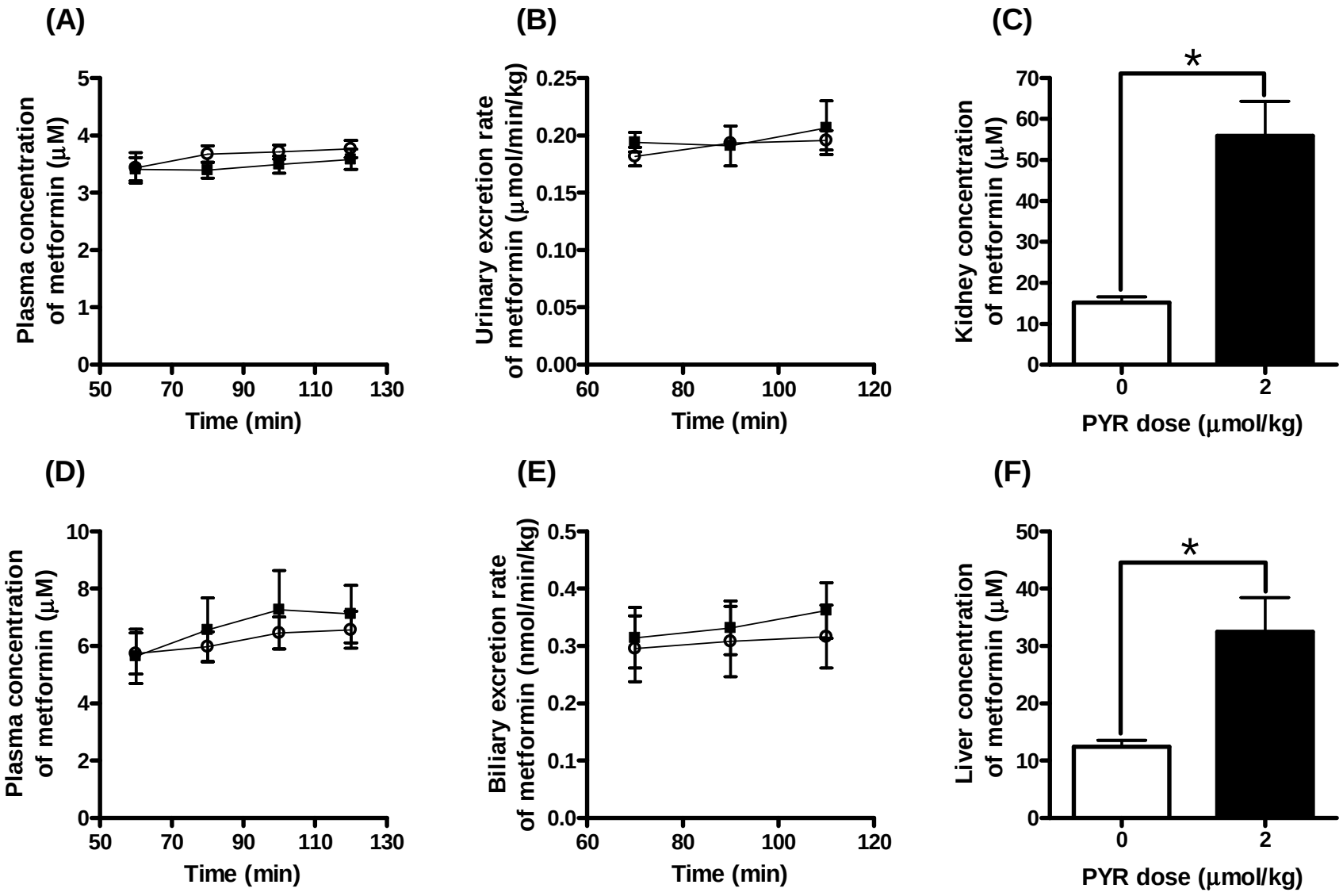

Figure 6

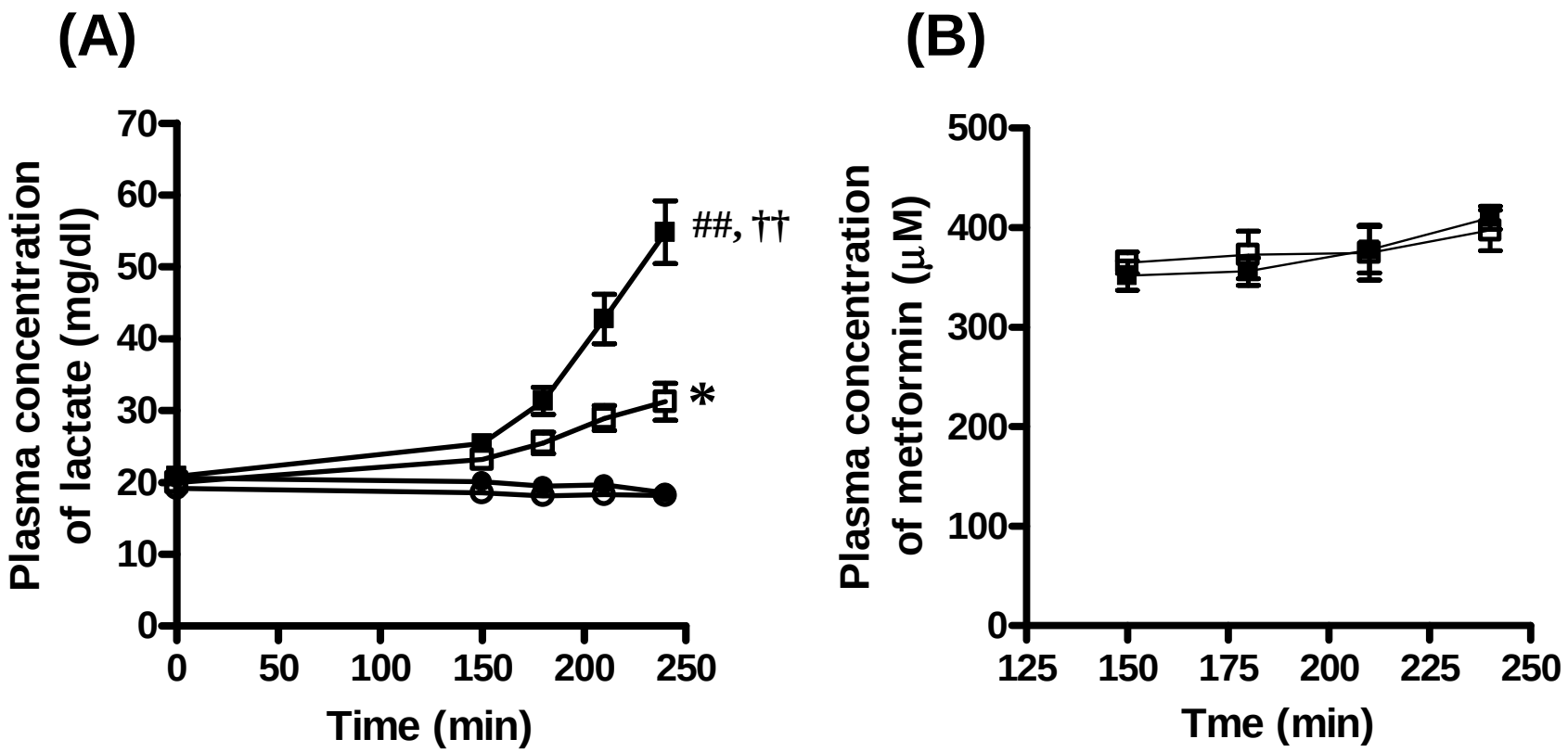

Figure 7

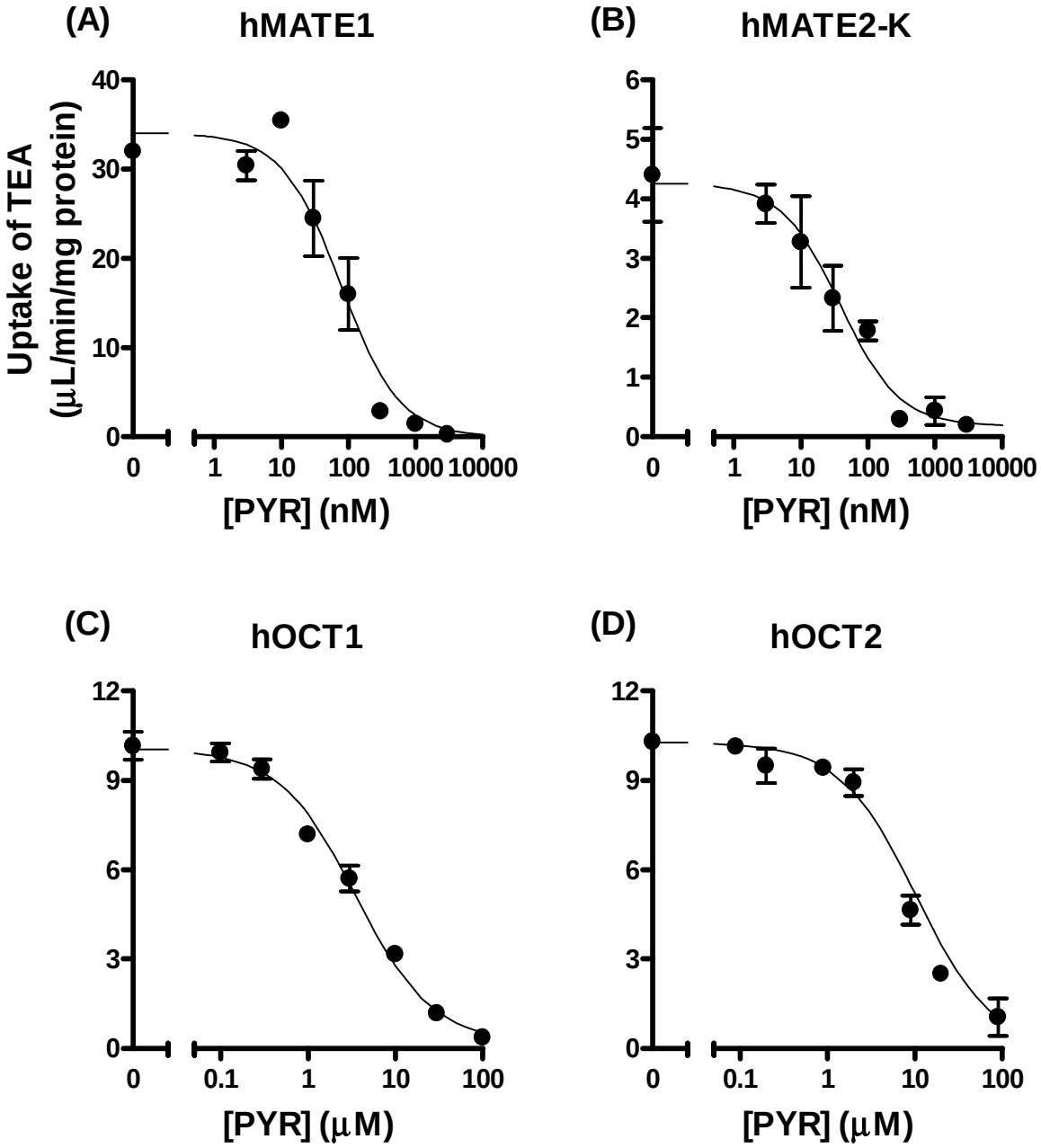

Figure 8