

6 β -hydroxycortisol is an endogenous probe for evaluation of drug–drug interactions involving a multispecific renal organic anion transporter, OAT3/SLC22A8, in healthy subjects

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Abbreviations:

6 β -OHF, 6 β -hydroxycortisol; AUC, area under the concentration-time curve; CL_{renal, 6 β -OHF}, renal clearance of 6 β -OHF; CL_{6 β -OHF}, apparent cortisol 6 β -hydroxylation clearance; CYP, cytochrome P450; 11 β -HSD, 11 β -hydroxysteroid dehydrogenase; TEA, tetraethylammonium; I_{u, max}, maximum unbound plasma concentration; HEK, human embryonic kidney; MRM, multiple reaction monitoring

Abstract

6 β -hydroxycortisol (6 β -OHF) is a substrate of the organic anion transporter 3 (OAT3) and the multidrug and toxin extrusion proteins, MATE1 and MATE-2K, in the corresponding cDNA-transfected cells. This study aimed to examine the contribution of OAT3 and MATEs to the urinary excretion of 6 β -OHF in humans using the appropriate *in vivo* inhibitors, probenecid and pyrimethamine, for OAT3 and MATEs, respectively. Oat3(-/-) mice showed significantly reduced renal clearance of 6 β -OHF ($CL_{\text{renal, 6}\beta\text{-OHF}}$) compared with wild-type mice (18.1 ± 1.5 versus 7.60 ± 1.8 ml/min/kg). 6 β -OHF uptake by human kidney slices was inhibited significantly by probenecid to 20–45% of the control values and partly by 1-methyl-4-phenylpyridinium. 6 β -OHF plasma concentration and the urinary excretion of 6 β -OHF ($X_{6\beta\text{-OHF}}$) were measured in healthy subjects enrolled in drug–drug interaction studies of benzylpenicillin alone or with probenecid (Study 1), adefovir alone or with probenecid (Study 2), and metformin alone or with pyrimethamine (Study 3). Probenecid treatment caused a 57% and 76% increase in the area under the plasma concentration–time curve for 6 β -OHF ($AUC_{6\beta\text{-OHF}}$) in Study 1 and 2, respectively, but did not affect $X_{6\beta\text{-OHF}}$. Consequently, $CL_{\text{renal, 6}\beta\text{-OHF}}$ (ml/min) decreased significantly from 231 ± 11 to 135 ± 9 and from 225 ± 26 to 141 ± 12 after probenecid administration in Study 1 and 2, respectively. By contrast, neither $AUC_{6\beta\text{-OHF}}$ nor $CL_{\text{renal, 6}\beta\text{-OHF}}$ was significantly altered by pyrimethamine administration. Taken together, these data suggest that OAT3 plays a significant role in the urinary excretion of 6 β -OHF, and that 6 β -OHF can be used to investigate the perpetrators of the pharmacokinetic drug interactions involving OAT3 in humans.

Introduction

Pharmacokinetic drug–drug interactions (DDIs) involving drug transporters that cause accumulation of victim drugs in the body can potentiate the pharmacological effect or cause adverse reactions (Giacomini et al., 2010; DeGorter et al., 2012; König et al., 2013; Yoshida et al., 2013). Typically, appropriate probe drugs are used when quantitatively evaluating the magnitude of inhibition by test drugs of drug transporters. Apart from drugs, endogenous substances and food-derived compounds have been identified as substrates of drug transporters. These compounds include creatinine, thiamine, and *N*-methylnicotinamide for the renal organic cation transporters, OCT2 and MATEs (Gorboulev et al., 1997; Urakami et al., 2004; Masuda et al., 2006; Tanihara et al., 2007), and various endogenous anionic metabolites for OATs (Deguchi et al., 2004; Wikoff et al., 2011). Plasma or urinary levels of such substances enable the monitoring of drug transporter activities without the need to administer substrate drugs (Fromm, 2012; Kusuha et al., 2013); for example, when using 6 β -hydroxycortisol (6 β -OHF) and 4 β -cholesterol to evaluate hepatic CYP3A4 activity (Galteau and Shamsa, 2003; Diczfalusy et al., 2011). We have reported previously that the renal clearance of *N*-methylnicotinamide and thiamine decreased markedly in humans who received an oral dose of pyrimethamine, a potent inhibitor of MATE1/*SLC47A1* and MATE2-K/*SLC47A2* (Imamura et al., 2011; Ito et al., 2012; Kato et al., 2013).

6 β -OHF is produced from cortisol by hepatic CYP3A4 (Abel et al., 1992; Peng et al., 2011), one of the major drug-metabolizing enzymes in the liver and small intestine. Because 6 β -OHF is excreted into the urine, the apparent cortisol 6 β -hydroxylation clearance ($CL_{6\beta\text{-OHF}}$) is used as a quantitative index for hepatic CYP3A4 activity in clinical DDI studies (Furuta et al., 2003; Peng et al., 2011). $CL_{6\beta\text{-OHF}}$ is defined as the ratio of the amount of urinary excretion of 6 β -OHF to the area under the plasma concentration time curve of cortisol (AUC_{cortisol}). Cortisol is one endogenous substrate of OAT3 (Asif et al., 2005). During the pharmacokinetic analysis of 6 β -OHF in the quantitative assessment of drug interactions involving CYP3A4 using a fluoroquinolone DX-619, we found that the urinary excretion mechanisms of 6 β -OHF involved active tubular secretion in humans and that

OAT3/*SLC22A8* and MATEs efficiently transported 6 β -OHF as a substrate into the corresponding cDNA-transfected cells (Imamura et al., 2013). OAT3 is a multispecific organic anion transporter that plays a major role in the renal uptake of a variety of anionic drugs, zwitterions, and some cationic drugs as well as endogenous metabolites including uremic toxins in the kidney (Nigam et al., 2007; Burckhardt, 2012; Kusuhara et al., 2013; Wang and Sweet, 2013). It is possible that 6 β -OHF is an endogenous probe that may be useful for evaluating the activity of OAT3 and/or MATEs in the kidney.

The primary purpose of this study was to examine the effects of OAT3 and MATE inhibitors on renal clearance of 6 β -OHF ($CL_{\text{renal, 6}\beta\text{-OHF}}$) in healthy subjects to demonstrate directly the sensitivity of 6 β -OHF plasma and urine concentrations to the activity of these transporters. We measured 6 β -OHF plasma and urinary concentrations in biological specimens from healthy subjects enrolled in a pharmacokinetic interaction study involving probenecid or pyrimethamine. In Study 1, subjects were given benzylpenicillin alone (400,000 U, p.o.) or concomitantly with probenecid (750 mg, p.o.); in Study 2, they were given adefovir alone (10 mg, p.o.) or concomitantly with probenecid (750 mg, p.o.); and in Study 3, they were given metformin alone (100 μ g, p.o.) or concomitantly with pyrimethamine (50 mg, p.o.). Probenecid is a well-known, potent inhibitor of OAT1 and OAT3 (Cihlar and Ho, 2000; Tahara et al., 2005a; Nozaki et al., 2007b; Takahara et al., 2013) and significantly reduces the renal clearance of various drugs that undergo tubular secretion by OATs (Masereeuw and Russel, 2010; Kusuhara et al., 2013). Pyrimethamine is a potent MATE inhibitor (Ito et al., 2010), and at its therapeutic dose, it significantly reduces the renal clearance of metformin (Kusuhara et al., 2011).

Materials and Methods

Chemicals and reagents

Cortisol, 6 β -OHF, probenecid, pyrimethamine and carbenoxolone were purchased from Sigma-Aldrich (St. Louis, MO). d4-Cortisol and d4-6 β -OHF were purchased from Toronto Research Chemicals (ON, Canada). Pooled human liver microsomes were purchased from BD Gentest (Woburn, MA). All other chemicals and reagents were of analytical grade and are commercially available.

Animals

Oat3(-/-) mice were obtained from Deltagen, Inc. (San Carlos, CA). Wild-type and Oat3(-/-) mice (female, 23 to 25 weeks old, and 21 to 27 grams) were used in this study. All animals were maintained under standard conditions with a reverse dark-light cycle. Food and water were available ad libitum. Oat3(-/-) mice does not show any abnormality except lack of the ability of uptake from extracellular space and secretion of anionic compounds into the urine (Sweet et al., 2002; Vanwert et al., 2007) and 10 to 15% lower blood pressure than wild-type mice (Vallon et al., 2008). The studies were conducted in accordance with the guidelines provided by the Institutional Animal Care Committee at the Graduate School of Pharmaceutical Sciences, the University of Tokyo, Tokyo, Japan.

Determination of renal clearance of 6 β -hydroxycortisol in wild-type and Oat3(-/-) mice

After anesthesia with isoflurane, the urinary bladder was catheterized for urine collection. 6 β -OHF (5.8 or 7 nmol/min/kg) was infused via the jugular vein. Blood samples were collected via the jugular vein at 30, 60, and 90 min after administration and centrifuged to obtain plasma. Urine specimens were collected at 30 to 60 and 60 to 90 min. At the end of the experiment, the kidneys and liver were removed. Drug concentration was determined by LC-MS/MS analysis as described below.

The fractional urinary excretion ratio (F_{urine}), the total body clearance ($CL_{\text{tot}, 6\beta\text{-OHF}}$), the renal clearance ($CL_{\text{renal}, 6\beta\text{-OHF}}$), and kidney-to-plasma concentration ratio ($K_{\text{p,kidney}}$) of $6\beta\text{-OH}$ were calculated using the following equations:

$$F_{\text{urine}} = V_{\text{urine}} / I \times 100 \quad \text{Eq. 1}$$

$$CL_{\text{tot}, 6\beta\text{-OHF}} = I / C_{\text{p},60\text{-}90\text{min}} \quad \text{Eq. 2}$$

$$CL_{\text{renal}, 6\beta\text{-OHF}} = V_{\text{urine}} / C_{\text{p},60\text{-}90\text{min}} \quad \text{Eq. 3}$$

$$K_{\text{p,kidney}} = C_{\text{kidney}} / C_{\text{p},90\text{min}} \quad \text{Eq. 4}$$

where V and I represent urinary excretion and infusion rates, respectively, and C represents the concentration in the kidney and plasma (C_{kidney} and C_{p} , respectively).

Preparation of human kidney slices and uptake of $6\beta\text{-OHF}$ by human kidney slices

Experimental procedures were performed according to the guidelines of the charitable, state-controlled foundation, Human Tissue and Cell Research (HTCR, Regensburg, Germany), with informed patient's consent approved by the local ethics committee of the University of Regensburg, Germany. Human renal samples were obtained from 3 patients (1 female and 2 male patients) who underwent surgical nephrectomy for cancer and the intact renal cortical segments were obtained from the specimens.

The human renal sections were provided by Hepacult GmbH (Regensburg, Germany) after being commissioned by HTCR and Daiichi Sankyo Europe GmbH (Munich, Germany). Uptake studies with human kidney slices (200 μm thick) were carried out following previous reports (Nozaki et al., 2007a; Matsushima et al., 2009). 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 $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.5). One slice per tube, weighing 7 to 23 mg, was selected and incubated at 37°C on Metalblock-Thermostat (Gebr. Liebisch GmbH & Co. KG, Bielefeld, Germany) with oxygenated buffer containing $6\beta\text{-OHF}$ with or without inhibitors. After 20 min incubation, slices were washed three times in ice-cold HBSS buffer, blotted on filter paper, weighed, and homogenated with 0.5 ml of HBSS.

For monitoring OAT1, OAT3 and OCT2 activity, uptake of the corresponding reference compounds, ^3H -adefovir (0.2 $\mu\text{Ci/ml}$), ^{14}C -benzylpenicillin (0.1 $\mu\text{Ci/ml}$), or ^{14}C -TEA (0.1 $\mu\text{Ci/ml}$),

was determined. Washed slices were solubilized with Solvable™ (Packard BioScience BV, Groningen, Nederland) at 37 °C overnight and subsequently analyzed in a liquid scintillation counter (Tri-Carb, PerkinElmer, Germany). 6β-OHF in the slices was extracted by acetonitrile with internal standard (d4-6β-OHF), and the specimens were subjected to LC-MS/MS analysis as described below.

Effect of probenecid and pyrimethamine on CYP3A4 and 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) activity

CYP3A4-mediated Midazolam 1'-hydroxylation was determined in human liver microsomes in the absence and presence of probenecid or pyrimethamine (100 μM), and with or without preincubation for 5, 10, 20 and 30 min, as reported previously, (Imamura et al., 2013). Activity of 11β-HSD2 was determined using microsomes prepared from Sf9 cells expressing 11β-HSD2, as described earlier (Imamura et al., 2013). Shortly, 11β-HSD2-mediated production of cortisone was measured after 20 min incubation in the presence and absence of probenecid or pyrimethamine (300 μM). Quantification was done by LC-MS/MS as described below.

Cell culture and in vitro transport and inhibition studies

Construction of HEK293 cells stably expressing hOAT3 and hMATE1 and *in vitro* transport studies were performed as described previously (Enomoto et al., 2003; Ohta et al., 2009). The data obtained from the inhibition study (Figure 2A) can be fitted to the following equation to calculate the half maximal inhibitory concentration (IC₅₀):

$$CL_{\text{uptake}(+\text{pyrimethamine})} = CL_{\text{uptake}(\text{control})} / (1 + I / IC_{50}) \quad \text{Eq. 5}$$

where $CL_{\text{uptake}(+\text{pyrimethamine})}$ and $CL_{\text{uptake}(\text{control})}$ are the uptake clearance determined in the presence or absence of pyrimethamine, 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.

Clinical samples for probenecid/ benzylpenicillin and probenecid /adefovir co-administration in healthy subjects

The following randomly allocated clinical study had been designed to evaluate the effect of probenecid on the urinary excretion of benzylpenicillin and adefovir in Japanese healthy male subjects. The clinical study protocol was approved by the Ethics Review Boards of both the Graduate School of Pharmaceutical Sciences of the University of Tokyo (Tokyo, Japan) and the Clinical Trial Center, Kitasato University East Hospital (Kanagawa, Japan). All participants provided their written informed consent. Determination of cortisol and 6β -OHF in the biological samples was approved by the Ethics Review Boards of both the Graduate School of Pharmaceutical Sciences of the University of Tokyo and the Clinical Trial Center, Kitasato University East Hospital.

Subjects were between 20 and 35 years old and had a body mass index between 18.5 and 28.5 kg/m². Twelve healthy subjects were randomly divided into two groups and six subjects in each group were treated with one of four combinations of substrates (benzylpenicillin or adefovir) and probenecid. The clinical drug interaction study was performed four times in the same subjects (phase 1 (probe drug alone), -2 (probe + 500 mg of probenecid), -3 (probe + 750 mg of probenecid) and -4 (probe + 1500 mg of probenecid) (Supplemental Table 1). Plasma and urine specimens analyzed in this study was from phase 1 (probe drug alone) and phase 3 (probe drug with 750 mg of probenecid). The medical products used in this study were Bicillin® G granules 400,000 units (400,000 U benzylpenicillin benzathine hydrate; Banyu Pharmaceutical Co., Ltd, Tokyo, Japan), Hepsara® 10 mg tablets (10 mg of adefovir dipivoxil; GlaxoSmithKline plc., Middlesex, UK) and Benecid® 250 mg tablets (250 mg probenecid; Kaken Pharmaceutical Co., Ltd, Tokyo, Japan). The doses of the inhibitors, benzylpenicillin adefovir dipivoxil, and probenecid were 400,000 U (p.o.), 10 mg (p.o.) and 750 mg (p.o.), respectively.

After an overnight fast, each subject received an oral dose of 400,000 U of benzylpenicillin or 10 mg of adefovir with 150 ml of water. Probenecid was administered orally 2 h before the administration of benzylpenicillin or adefovir. Venous blood samples were collected into heparin-pretreated tubes before and at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h after the administration of the

probe drugs and centrifuged. Urine samples were collected in the predose period and periods 0–4 h, 4–8 h, and 8–24 h after treatment with the probe drugs. All of the plasma and urine samples were stored at –80 °C until analysis.

Clinical samples for metformin/pyrimethamine co-administration in healthy subjects

Cortisol and 6 β -OHF were quantified in the plasma and urine specimens from subjects who had been enrolled in the aforementioned DDI study with pyrimethamine and metformin (Supplemental Table 1) (Kusuhara et al., 2011). The study protocol was approved by the ethics review boards of both the Graduate School of Pharmaceutical Sciences of the University of Tokyo and the Clinical Investigation Center, Kitasato University East Hospital. All participants provided their written informed consent.

Quantification of cortisol and 6 β -OHF in the biological samples by LC-MS/MS

Centrifuged plasma or urine samples were mixed with internal standard mixture (d4-cortisol and d4-6 β -OHF) and applied to the Oasis HLB μ Elution SPE plates (Waters Corporation, Milford, MA). Cortisol and 6 β -OHF concentration were measured using a UFLC system (Shimadzu, Japan) coupled with 5500 QTRAP mass spectrometer (AB-SCIEX, Framingham, MA) using an Accucore RP-MS 2.6 μ m 50 \times 2.1-mm column (Shiseido Co., Ltd., Tokyo, Japan). The mobile phase comprised solvent A (12 mM ammonium acetate dissolved in 10% methanol) and solvent B (12 mM ammonium acetate dissolved in 90% methanol); the A/B gradient was 80:20 (0–3 min) followed by 20:80 (7.5 min) at a flow rate of 0.3 ml/min. The analysis was performed in MRM mode with monitoring of m/z 361.2 \rightarrow 331.2 for cortisol, m/z 377.2 \rightarrow 347.0 for 6 β -OHF, m/z 365.2 \rightarrow 335.2 for d4-cortisol and m/z 381.2 \rightarrow 351.0 for d4-6 β -OHF. The calibration curve for 6 β -OHF was constructed over the concentration range of 0.15-500 for plasma and 0.1-500 ng/ml for urine. The calibration curve for cortisol was constructed over the concentration range of 0.15-500 ng/ml for plasma. The inter-day variability for the measurement of these compounds was within 15%.

Solubilized kidney slice samples were mixed with internal standard (d4-6 β -OHF). 6 β -OHF concentration were measured using a Waters Acquity I-Class UPLC system coupled with Waters

Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation) using a BEH C18 1.7 μm , 100 mm \times 2.1 mm column (Waters Corporation), and the mobile phase comprised solvent A (10 mM ammonium acetate and 0.1% formic acid dissolved in 10% acetonitrile) and solvent B (10 mM ammonium acetate and 0.1% formic acid dissolved in 90% acetonitrile); the A/B gradient was 100:0 (0–0.5 min) at a flow rate of 0.4 ml/min followed by 40:60 (0.5–3 min) at 0.35 ml/min. The analysis was performed in MRM mode with monitoring of m/z 379.4 \rightarrow 325.2 for 6 β -OHF and m/z 383.4 \rightarrow 329.2 for d4-6 β -OHF. The calibration curve for 6 β -OHF was constructed over the concentration range of 0.15–320 pmol/ml within a 15% coefficient of variation and accuracy margin.

Calculation of $CL_{\text{renal, 6}\beta\text{-OHF}}$ and $CL_{6\beta\text{-OHF}}$

$CL_{\text{renal, 6}\beta\text{-OHF}}$ and $CL_{6\beta\text{-OHF}}$ were calculated as follows:

$$CL_{\text{renal, 6}\beta\text{-OHF}} = X_{6\beta\text{-OHF}} / AUC_{6\beta\text{-OHF}} \quad \text{Eq. 6}$$

$$CL_{6\beta\text{-OHF}} = X_{6\beta\text{-OHF}} / AUC_{\text{cortisol}} \quad \text{Eq. 7}$$

where $X_{6\beta\text{-OHF}}$ is the amount of 6 β -OHF excreted into the urine and $AUC_{6\beta\text{-OHF}}$ and AUC_{cortisol} are the area under the time-concentration profiles (AUC) of plasma 6 β -OHF and cortisol during the designated time intervals, respectively.

Statistical analysis

Statistically significant differences in this study were determined using two-tailed unpaired t -tests. Differences were considered to be significant at $p < 0.05$ and < 0.01 .

Results

Effect of impaired Oat3 on the urinary excretion rate of 6 β -OHF in mice

Endogenous level of 6 β -OHF was below the lower limit of quantification both in the plasma and urine specimens. Plasma concentrations and urinary excretions of 6 β -OHF were determined in mice given 6 β -OHF by constant intravenous infusion. Pharmacokinetic parameters were summarized in Table 1. The urinary excretion rate of 6 β -OHF accounts for 33 \pm 2% of the infusion rate in wild-type mice (Table 1). The urinary excretion rate was significantly lower in Oat3(-/-) than in wild-type mice although there was no difference in the plasma concentrations (Figure 1). Consequently, the CL_{renal,6 β -OHF} was significantly lower in Oat3(-/-) mice, whereas its non-renal clearance was unchanged between wild-type and Oat3(-/-) mice (34.8 \pm 3.1 and 38.1 \pm 3.1 ml/min/kg, respectively) (Table 1). The kidney-to-plasma concentration ratio of 6 β -OHF was decreased by 23% in Oat3(-/-) mice ($p < 0.05$).

Effects of probenecid and MPP⁺ on the uptake of 6 β -OHF by human kidney slices

Human kidney slices used in the transport studies were obtained from three subjects. The activities of OAT1, OAT3 and OCT2 in the human kidney slices were monitored routinely using their typical substrates, ³H-adefovir, ¹⁴C-benzylpenicillin and ¹⁴C-TEA, respectively. The uptake of ³H-adefovir was reduced to 7.2 – 13% of the control value by 1 mM of probenecid, the uptake of ¹⁴C-benzylpenicillin was reduced to 29 – 36% by 1 mM of probenecid, and the uptake of ¹⁴C-TEA was reduced to 4.6 – 7.9% by 1 mM of MPP⁺ (Supplemental Table 2).

The concentration of endogenous 6 β -OHF in the blank kidney slices was less than 1% of the amount 6 β -OHF that accumulated in the kidney slices after incubating the kidney slices in the presence of 20 μ M of exogenous 6 β -OHF. 6 β -OHF uptake was inhibited to 41% ($p < 0.01$), 25% ($p < 0.01$), and 20% ($p < 0.01$) of the corresponding control values in the presence of probenecid, and to 68% ($p < 0.01$), 58%, and 58% of the corresponding control values in the presence of MPP⁺ (Figure 2).

Effects of probenecid and pyrimethamine on CYP3A4 and 11 β -hydroxysteroid dehydrogenase 2 (11 β -HSD2)activities

The inhibition potency of probenecid and pyrimethamine for CYP3A4 and 11 β -HSD2, which are involved in the production of 6 β -OHF from cortisol and conversion of 6 β -OHF to 6 β -hydroxycortisone, respectively, was examined *in vitro*. Midazolam 1'-hydroxylation in human liver microsomes, which is catalyzed predominantly by CYP3A4, was decreased to 29.6 \pm 1.1% of the control value by ketoconazole (0.1 μ M, direct inhibitor). Probenecid and pyrimethamine (100 μ M) had no effect on midazolam 1'-hydroxylation in human liver microsomes.

To examine whether probenecid and pyrimethamine are irreversible inhibitors of CYP3A4, human liver microsomes were preincubated in the presence of inhibitors for 30 min prior to the addition of midazolam. Midazolam 1'-hydroxylation in human liver microsomes was decreased to 52.7 \pm 1.2% after preincubation in the presence of troleandomycin (30 μ M), which is the irreversible type inhibitor of CYP3A4 (Chan et al., 1998), whereas neither probenecid nor pyrimethamine affected the activity (data not shown).

The activity of 11 β -HSD2 (14.8 \pm 0.3 pmol/h/mg protein) decreased to 0.426 \pm 0.021 pmol/h/mg protein by carbenoxolone (1 μ M), which is an inhibitor of 11 β -HSD2 (Brown et al., 1996), whereas neither probenecid nor pyrimethamine affected 11 β -HSD2 activity by 300 μ M (data not shown).

Effect of pyrimethamine and probenecid on OAT3- and MATE1-mediated transport of 6 β -OHF in their cDNA transfected cells

OAT3-mediated 6 β -OHF uptake in OAT3-HEK293 cells (17.1 \pm 1.6 μ l/min/mg protein) was reduced to 7.8 \pm 2.0% of control (1.3 \pm 0.3 μ l/min/mg protein) by probenecid (1 mM) (Figure 3A). Pyrimethamine inhibited OAT3-mediated 6 β -OHF uptake with IC₅₀ of 32.0 \pm 6.2 μ M (Figure 3A). Since the concentration of 6 β -OHF in the uptake buffer (5 μ M) was low enough compared with its K_m value for OAT3 (183 μ M, Imamura et al., 2013), IC₅₀ of 6 β -OHF for OAT3 is theoretically close to the corresponding Ki value.

MATE1-mediated 6 β -OHF uptake of 16.2 \pm 0.3 μ l/min/mg protein in MATE1-HEK293 cells was reduced to 1.1 \pm 0.1 μ l/min/mg protein (6.5 \pm 0.9% of control) by pyrimethamine (100 μ M) (Figure 3B). Only a weak inhibition of MATE1 was detected in the presence of probenecid (300 μ M) (Figure 3B).

Effects of pyrimethamine and probenecid on the plasma and urinary excretion of 6 β -OHF in healthy subjects

The maximum unbound plasma concentration of probenecid ($I_{u, \max \text{ probenecid}}$) was calculated as the product of the unbound fraction of probenecid in the plasma, which ranges from 0.05 to 0.17 (Selen et al., 1982), and the maximum plasma concentration observed in the study. They ranged from 12.2 \pm 0.9 to 41.6 \pm 3.0 μ M in the probenecid-benzylpenicillin treated groups (Study1), and 11.6 \pm 0.9 – 39.4 \pm 3.0 μ M in the probenecid-adeфовir treated groups (Study 2) (Supplemental Table 1). The plasma concentrations of 6 β -OHF were significantly increased when co-administered with probenecid (Figures 4A and 5A). $AUC_{6\beta\text{-OHF}}$ from 0 to 24 hours were increased 1.7- and 1.8-fold higher in the probenecid treated groups (Study No1 and 2, respectively) (Figure 4B, 5B and Table 2), whereas the amount of 6 β -OHF excreted into the urine was unchanged (Figures 4C and 5C). Consequently, $CL_{\text{renal}, 6\beta\text{-OHF}}$ was decreased significantly in the probenecid treated groups (Figure 4D and 5D). Compared with the corresponding values in the corresponding control groups, $CL_{\text{renal}, 6\beta\text{-OHF } 0\text{-}24\text{h}}$ was decreased by 42 and 37%, and the renal secretion clearance of 6 β -OHF ($CL_{\text{renal sec}, 6\beta\text{-OHF}}$) was decreased by 46 and 41% by probenecid (Study No1 and 2, respectively, Table 2). The absolute value of $CL_{\text{renal}, 6\beta\text{-OHF}}$ was lower in the interval from 8 to 24 h compared with other intervals, and the effect of probenecid was moderate or weak in this interval (Figure 4D and 5D).

The maximum unbound plasma concentrations of pyrimethamine ($I_{u, \max \text{ probenecid}}$) were determined in the previous study which were ~300 nM (Kusuhara et al., 2011). Plasma 6 β -OHF was unaffected by pyrimethamine (Figure 6A), and AUC of 6 β -OHF from 0 to 12 hours in control and pyrimethamine treated groups were unchanged (Figure 6B, Table 2). $X_{e, 6\beta\text{-OHF } 0\text{-}12\text{h}}$ was decreased by 19% in the pyrimethamine-treated group than that of the control group (Figure 6C, Table 2) although the difference did not reach statistical significance. $CL_{\text{renal}, 6\beta\text{-OHF}}$ and $CL_{\text{renal sec}, 6\beta\text{-OHF}}$ were slightly

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decreased by 23 and 25%, respectively by pyrimethamine without statistically significance (Table 2).

Discussion

6 β -OHF was found to be a substrate of OAT3 and MATEs in the corresponding cDNA-transfected cells (Imamura et al., 2013). We speculate that the tubular secretion of 6 β -OHF in the kidney involves these transporters and that 6 β -OHF may be an endogenous *in vivo* probe that can be used to monitor the activities of these transporters. The present study examined the effect of *in vivo* inhibitors of these transporters on the plasma concentrations and urinary excretion of 6 β -OHF to demonstrate its usefulness as an *in vivo* probe for OAT3 and MATEs in humans.

To examine the contribution of Oat3 to the urinary excretion of 6 β -OHF, an *in vivo* study using Oat3(-/-) mice was conducted. CL_{renal, 6 β -OHF} was significantly lower in Oat3(-/-) mice compared with wild-type mice (Table 1). Because CL_{renal, 6 β -OHF} accounted for at most 34% of the systemic elimination in wild-type mice (Table 1), it is reasonable that the reduction in the urinary excretion did not cause significant accumulation of 6 β -OHF in the plasma in Oat3(-/-) mice.

To evaluate the contribution of drug transporters to the renal uptake process, an inhibition study was conducted using human kidney slices, which are used with appropriate inhibitors to investigate the contribution of uptake transporters in the kidney (Nozaki et al., 2007b; Matsushima et al., 2009). The uptake of 6 β -OHF was inhibited significantly by probenecid and partly by MPP⁺ (Figure 2). The marked inhibition of the uptake by probenecid in kidney slices is consistent with our previous prediction that OAT3 makes a greater contribution to the renal uptake of 6 β -OHF than does OCT2 (Imamura et al., 2013). Inhibition of the uptake by MPP⁺ also suggests that OCT2 contributes to the uptake. Whether 6 β -OHF is an OCT2 substrate remains controversial. hOCT2-HEK cells showed significantly greater uptake of 6 β -OHF than did mock-vector transfected cells; however, this uptake was not inhibited by the representative inhibitor (Imamura et al., 2013). We also found that MPP⁺ inhibited OAT3-mediated uptake of estrone-3-sulfate and 6 β -OHF by 58% and 42%, respectively, in hOAT3-HEK cells at 1 mM (data not shown). The effect of MPP⁺ in kidney slices partly involved inhibition of OAT3, and the actual contribution of OCT2 was smaller than that for the inhibition by MPP⁺.

To confirm the clinical data, the specificity of probenecid and pyrimethamine was measured *in vitro*. These drugs had no effect on CYP3A4 and 11 β -HSD2 activity, suggesting that these inhibitors affect neither 6 β -OHF production from cortisol nor 6 β -OHF metabolism to 6 β -hydroxycortisone. Pyrimethamine inhibited OAT3 *in vitro* (Figure 3), but the inhibition of OAT3 by pyrimethamine was negligible *in vivo* because its K_i value (32 μ M) was greater than the clinical unbound concentration (\sim 300 nM, Kusuhara et al., 2011). Probenecid had no effect on MATE1. Therefore, among the known enzymes and transporters that might be involved in the disposition of 6 β -OHF, probenecid and pyrimethamine are potential inhibitors of OAT3 and MATEs, respectively (Table 3). The plasma 6 β -OHF concentration was significantly higher in the probenecid-treated group, whereas the amount excreted into the urine did not differ between groups (Figure 4). This was also observed in another group (Figure 5). $CL_{\text{renal, 6}\beta\text{-OHF}}$ decreased significantly in these two independent studies (Table 2). Taken together, the *in vivo* data obtained from Oat3(-/-) mice (Figure 1) and *in vitro* data from human kidney slices (Figure 2) suggest that the reduction in the $CL_{\text{renal, 6}\beta\text{-OHF}}$ was probably attributable to inhibition of OAT3-mediated uptake of 6 β -OHF by probenecid.

Because the control subjects received a therapeutic dose of benzylpenicillin or adefovir, the possibility that benzylpenicillin or adefovir modifies the effect of probenecid needs further discussion. The K_m value of benzylpenicillin for OAT3 was reported as 52 μ M in cDNA-transfected cells (Tahara et al., 2005b) and as 14–90 μ M in human kidney slices (Nozaki et al., 2007a). We confirmed that adefovir at a concentration of 300 nM had no inhibitory effect on OAT3-mediated 6 β -OHF uptake (data not shown). Considering the total plasma concentrations of benzylpenicillin and adefovir in the clinical studies (at most 1 μ M and 100 nM, respectively), their effect on OAT3 (*i.e.*, saturation) can be excluded. To provide concrete evidence, a clinical study in which subjects receive only probenecid should be conducted. We note that the tubular secretion of 6 β -OHF was still greater than its glomerular filtration even in the probenecid-treated groups. The K_i value of probenecid was greater for the OAT3-mediated 6 β -OHF transport than for that determined using other substrates, although it remained below the unbound plasma concentration of probenecid during the experiments (Table 3).

Higher doses of probenecid may be needed to achieve complete inhibition of the tubular secretion of 6 β -OHF.

The present study also showed that the magnitude of inhibition of $CL_{\text{renal, 6}\beta\text{-OHF}}$ by probenecid depends on the time after probenecid treatment. $CL_{\text{renal sec, 6}\beta\text{-OHF}}$ was decreased by probenecid to 37 \pm 5% (0–4 h, $p < 0.01$), 42 \pm 6% (4–8 h, $p < 0.01$), and 72 \pm 8% (8–24 h, $p < 0.05$) of the corresponding control values in Study 1 and to 45 \pm 2% (0–4 h, $p < 0.01$), 47 \pm 3% (4–8 h, $p < 0.01$), and 78 \pm 11% (8–24 h, $p = 0.2128$) of the corresponding control values in Study 2 (Figures 4C and 5C). This may be explained partly by a reduction in probenecid concentration with time. In addition, the absolute value of $CL_{\text{renal, 6}\beta\text{-OHF}}$ in the control group was lower during the 8–24 h interval compared with the other intervals, suggesting that OAT3 is under regulation by circadian rhythm, which would lower its contribution to the net urinary excretion.

Unlike probenecid, the effect of pyrimethamine on $CL_{\text{renal, 6}\beta\text{-OHF}}$ was not changed although 6 β -OHF is a MATE1 substrate *in vitro* (Imamura et al., 2013). The significant inhibition of MATEs by pyrimethamine (>70%) is supported by the significant reduction in the renal clearance of *N*-methylnicotinamide and thiamine (Kusuhara et al., 2011; Ito et al., 2012; Kato et al., 2013). In addition to the lower than expected contribution to the efflux of MATEs, there is another possible reason for this discrepancy. When the luminal efflux is far greater than the basolateral efflux, the influx clearance on the basolateral membrane approximates the overall tubular secretion clearance (Kusuhara and Sugiyama, 2009). Under such conditions, as far as the assumption is satisfied, the inhibition of luminal efflux hardly affects the overall tubular secretion clearance. In fact, the magnitude of inhibition of the tubular secretion clearance of metformin by pyrimethamine was at most 20–30% (Kusuhara et al., 2011). Therefore, the importance of MATE1 in the luminal efflux of 6 β -OHF cannot be excluded based only on these results; however, it is evident that 6 β -OHF is inferior to the MATE substrates mentioned above in terms of their sensitivity to MATE activities.

The present study highlights the importance of OAT3 in the urinary excretion of 6 β -OHF. Both the plasma concentration and renal clearance of 6 β -OHF are highly sensitive to OAT3 activity. Caution is needed when the plasma concentrations alone are used to evaluate OAT3 activity because

the plasma concentration and amount of 6 β -OHF excreted into the urine are also influenced by CYP3A4 activity. DX-619 decreased CL_{renal, 6 β -OHF} in healthy subjects, however the plasma concentration of 6 β -OHF was unchanged because of the reduced production rate by mechanism-based inhibition of CYP3A4 (Imamura et al., 2013). It is recommended that CL_{renal, 6 β -OHF} should be determined when evaluating OAT3 activity with drugs that potentially inhibit or induce CYP3A4 in the liver.

The apparent cortisol 6 β -hydroxylation clearance (CL_{6 β -OHF}) is used as a quantitative index of hepatic CYP3A4 activity. Theoretically, CL_{6 β -OHF} involves the total body and renal clearance of 6 β -OHF (CL_{tot, 6 β -OHF} and CL_{renal, 6 β -OHF}, respectively) (Imamura et al., 2013), as shown in equation 8:

$$CL_{6\beta\text{-OHF}} = X_{6\beta\text{-OHF}} / AUC_{\text{cortisol}} = CL_{\text{CYP3A4}} \cdot CL_{\text{renal, } 6\beta\text{-OHF}} / CL_{\text{tot, } 6\beta\text{-OHF}} \quad \text{Eq.8}$$

where CL_{CYP3A4} is the metabolic clearance of 6 β -OHF by CYP3A4. Therefore, unless urinary excretion is the major elimination pathway, pharmacokinetic interaction could influence CL_{6 β -OHF} and thereby affect the estimation of the magnitude of DDI involving hepatic CYP3A4. To address this issue, we calculated CL_{6 β -OHF} in the control, and probenecid or pyrimethamine-treated groups. Probenecid and pyrimethamine had no effect on the plasma cortisol concentrations (Supplemental Figure 1), and CL_{6 β -OHF} (Supplemental Table 3), indicating that CL_{renal, 6 β -OHF}/CL_{tot, 6 β -OHF} is near unity, supporting the idea that CL_{6 β -OHF} can be used to investigate hepatic CYP3A4 irrespective of the interaction with OAT3. Species differences in the effect of OAT3 inhibition or impairment on the plasma concentrations of 6 β -OHF might be explained by the contribution of renal clearance to total body clearance. In our previous report, we could not conclude whether DX-619, a CYP3A4 mechanism-based inhibitor, significantly inhibited hepatic CYP3A4 based on CL_{6 β -OHF} because CL_{renal, 6 β -OHF} was also significantly decreased during DX-619 administration (Imamura et al., 2013). The present study supports the idea that DX-619 administration caused significantly inhibition of CYP3A4 (~72% inhibition) at that dose.

These data suggest that OAT3 plays a significant role in the urinary excretion of 6 β -OHF, and that 6 β -OHF can be used to investigate the perpetrators of the pharmacokinetic drug interactions involving OAT3, but not MATEs, in humans.

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Declaration of Conflicting Interests

Yuichiro Imamura, Nobuyuki Murayama, Noriko Okudaira, Atsushi Kurihara and Takashi Izumi are employees of Daiichi Sankyo Co., Ltd. Katja Damme and Dominik Heer are employees of Daiichi Sankyo Europe GmbH. Other authors declare no conflict of interest.

Footnote

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Figure Legends

Figure 1 Plasma concentration and urinary excretion rates of 6 β -OHF in wild-type and Oat3(-/-) mice.

Wild-type and Oat3(-/-) mice were given 6 β -OHF intravenously (7 nmol/min/kg). Plasma concentrations were determined at designated time, and urinary excretion rates were determined during the interval from 30 to 60, and 60 to 90 min. Each point and bar represents the mean and S.E. (n=6-7) ** $p < 0.01$

Figure 2 Effects of probenecid and MPP⁺ on the uptake of 6 β -OHF by human kidney slices.

The uptake of 6 β -OHF (20 μ M) by human kidney slices from three different volunteers (Panel A; male 70 years, Panel B; female 51 years and Panel C male 75 years) for 20 min was determined at 37°C. Three slices were used in each batch of human kidney. Each point and bar represent the mean \pm SE (n=3). * $p < 0.05$, ** $p < 0.01$.

Figure 3 Effects of probenecid and pyrimethamine on OAT3- and MATE1-mediated 6 β -OHF uptake

(A) The uptake of 6 β -OHF (5 μ M) by hOAT3 for 5 min was determined in the presence of probenecid (1mM) and pyrimethamine at designated concentrations at 37°C. The solid line represents the fitted line obtained by nonlinear regression analysis. (B) The uptake of 6 β -OHF (10 μ M) by hMATE1 for 1 min was determined in the presence of pyrimethamine (100 μ M) and probenecid at designated concentrations at 37°C. Each value represents the mean \pm SE (n=3). ** $p < 0.01$

Figure 4 Effect of probenecid on the plasma concentration and urinary excretion of 6 β -OHF and on plasma concentration of cortisol in healthy subjects.

(A) Plasma concentration–time profile, (B) AUC and (C) the amount excreted into the urine of 6 β -OHF ($X_{6\beta\text{-OHF}}$) were determined in healthy subjects who received benzylpenicillin alone (control) or concomitantly with probenecid (probenecid-treated group). Open and closed symbols in (A)-(C)

represent the control and probenecid-treated group, respectively. (D) Renal clearance of 6 β -OHF ($CL_{\text{renal, 6}\beta\text{-OHF}}$) was calculated in the designated time intervals in control and probenecid-treated group (PRB) as described in Materials and Methods. Each point and bar represent the mean \pm SE (n = 6). * p < 0.05, ** p < 0.01.

Figure 5 Effect of probenecid on the plasma concentration and urinary excretion of 6 β -OHF and on plasma concentration of cortisol in healthy subjects.

(A) Plasma concentration–time profile, (B) AUC and (C) $X_{6\beta\text{-OHF}}$ were determined in healthy subjects who received benzylpenicillin with or without pretreatment of probenecid. Open and closed symbols in (A)-(C) represent the control and probenecid-treated group, respectively. (D) Renal clearance of 6 β -OHF ($CL_{\text{renal, 6}\beta\text{-OHF}}$) was calculated in the designated time intervals in control and probenecid-treated group (PRB) as described in Materials and Methods. Each point and bar represent the mean \pm SE (n = 6). * p < 0.05, ** p < 0.01.

Figure 6 Effect of pyrimethamine on the plasma concentration and urinary excretion of 6 β -OHF and on plasma concentration of cortisol in healthy subjects.

(A) Plasma concentration–time profile, (B) AUC and (C) $X_{6\beta\text{-OHF}}$ were determined in healthy subjects who received metformin with or without pyrimethamine treatment. Open and closed symbols in (A)-(C) represent the control and probenecid-treated group, respectively. (D) Renal clearance of 6 β -OHF ($CL_{\text{renal, 6}\beta\text{-OHF}}$) was calculated in the designated time intervals in control and probenecid-treated group (PYR) as described in Materials and Methods. Each point and vertical bar represent the mean \pm SE (n = 8).

Table 1 Pharmacokinetic parameters of 6 β -OHF in wild-type and Oat3(-/-) micePharmacokinetic parameters of 6 β -OHF were determined as described in Materials and Methods. Data were taken from Figure 1.* $p < 0.05$, ** $p < 0.01$

	C_p 60-90min ¹⁾ (nmol/L)	$V_{urine,30-60}$ (nmol/min/kg)	F_{urine} (%)	$CL_{tot, 6\beta-OHF}$ (mL/min/kg)	$CL_{renal,6\beta-OHF}$ (mL/min/kg)	$C_{p,90min}$ (nmol/L)	C_{kidney} (nmol/L)	$K_{p,kidney}$
Wild-type	122±8	2.18±0.17	33.5±1.7	52.9±4.0	18.1±1.5	131±17	217±35	1.70±0.18
Oat3(-/-)	152±13	1.07±0.23**	18.1±3.7**	45.8±3.73	7.60±1.8**	161±19	210±27	1.30±0.09*

¹⁾ Mean value of the plasma concentrations at 60 and 90 min

Table 2 Summary of kinetic indices of 6 β -OHF and cortisol after co-administration of benzylpenicillin/probenecid, adefovir/probenecid or metformin/pyrimethamine in healthy subjects

Study	Groups	AUC _{6β-OHF} ¹⁾	X _{6β-OHF} ¹⁾	CL _{renal, 6β-OHF}	CL _{renal sec, 6β-OHF} ²⁾	f _{sec} ³⁾
		(ng • h/ml)	(μ g)	(ml/min)	(ml/min)	(%)
1 [¶]	control	72.0 \pm 7.6	995 \pm 108	231 \pm 11	211 \pm 11	91 \pm 1
	+probenecid (750 mg)	113 \pm 6**	917 \pm 91	135 \pm 9**	114 \pm 9**	50 \pm 5**
2 [‡]	control	74.0 \pm 6.9	990 \pm 130	225 \pm 26	205 \pm 26	90 \pm 1
	+probenecid (750 mg)	130 \pm 9**	1072 \pm 53	141 \pm 12*	121 \pm 12*	55 \pm 5**
3 [‡]	control	36.2 \pm 1.7	480 \pm 30	226 \pm 19	206 \pm 19	90 \pm 1
	+pyrimethamine (50 mg)	41.1 \pm 4.2	387 \pm 42	174 \pm 32	154 \pm 32	67 \pm 10*

Healthy subjects received oral dose of [†]benzylpenicillin (400,000 U) in Study 1, [†]adefovir (10 mg) in Study 2, or [‡]metformin (100 μ g) in Study 3.

*; $p < 0.05$; **; $p < 0.01$ (vs. without inhibitor)

Each parameter represents mean \pm S.E.

AUC: area under the plasma concentration-time curve from 0 to 24 hours

$X_{6\beta\text{-OHF}}$: cumulative amount of 6 β -OHF in the urine up to 24 hours after administration of probe drug

¹⁾ calculated time interval was from 0 to 12 hours. ²⁾ secretion clearance of 6 β -OHF in the kidney with regard to the plasma concentration using $f_p \cdot \text{GFR}$ of 6 β -OHF (20.4 ml/min (Imamura et al., 2011)), ³⁾ fraction of 6 β -OHF secreted with regard to $\text{CL}_{\text{renal}, 6\beta\text{-OHF}}$

Table 3 Summary of unbound plasma concentration of probenecid and pyrimethamine and their inhibitory potential for 6 β -OHF-related transporters and enzymes.

Transporter/enzyme	Probenecid (I_u 12.2 \pm 0.9 – 41.6 \pm 3.0 μ M)			Pyrimethamine (I_u ~300 nM)		
	Substrate	K_i (μ M)	Reference	Substrate	K_i or IC_{50} (μ M)	Reference
OAT3	6 β -OHF	12.1 \pm 3.8	(Imamura et al., 2013)	6 β -OHF	32.0 \pm 6.2 ¹⁾	(this study)
	Estrone-3-sulfate	4.9 \pm 1.4	(Tahara et al., 2006)			
	Fexofenadine	1.3 \pm 0.3	(Tahara et al., 2006)			
OCT2	Cimetidine	> 1,000	(Tahara et al., 2005)	TEA	10 \pm 1	(Ito et al., 2010)
	Famotidine	>1,000	(Tahara et al., 2005)			
MATE1	6 β -OHF	> 300	(this study)	6 β -OHF	0.281 \pm 0.033	(Imamura et al., 2013)
				TEA	0.077 \pm 0.013	(Ito et al., 2010)
MATE2-K		Not tested		TEA	0.046 \pm 0.006	(Ito et al., 2010)
CYP3A4	Midazolam	> 100	(this study)	Midazolam	> 100 ¹⁾	(this study)
11 β -HSD2	Cortisol	> 300	(this study)	Cortisol	> 300 ¹⁾	(this study)

¹⁾ representing IC_{50} value

Figure 1

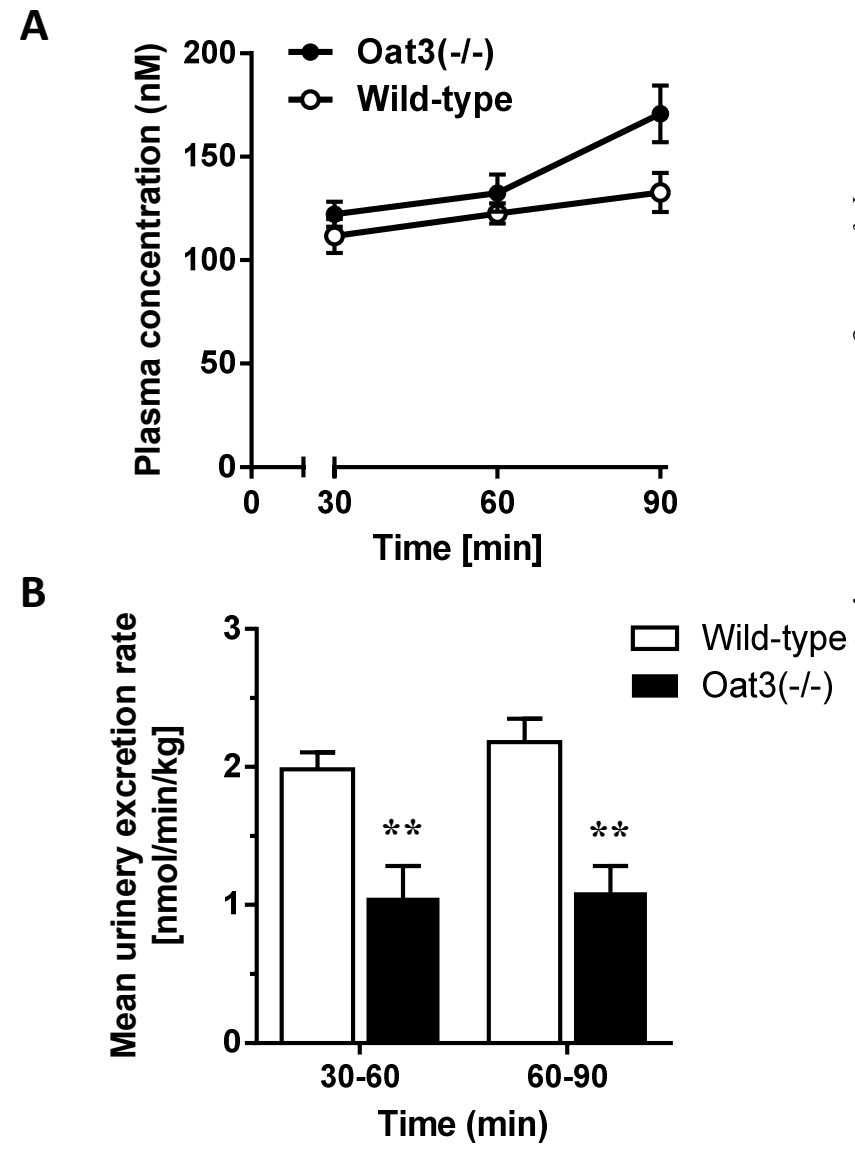


Figure 2

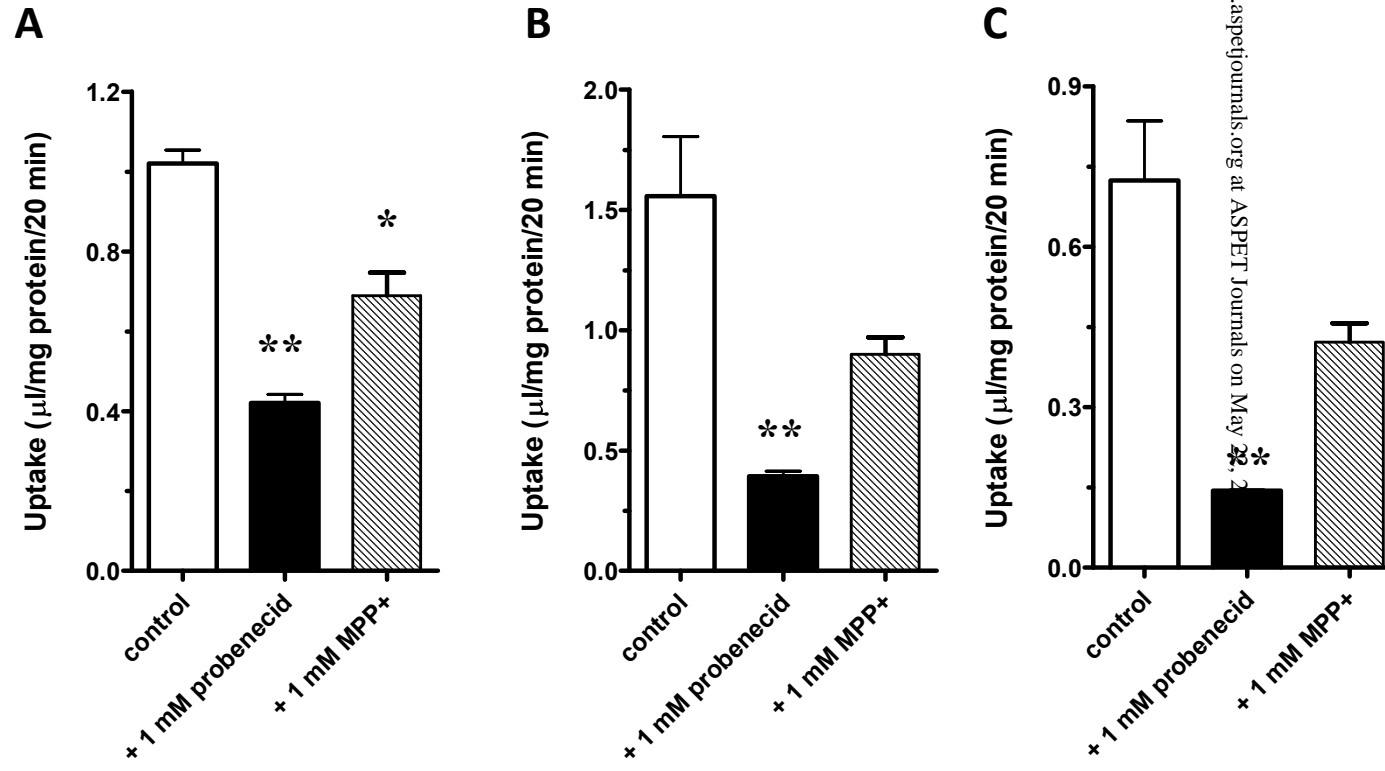
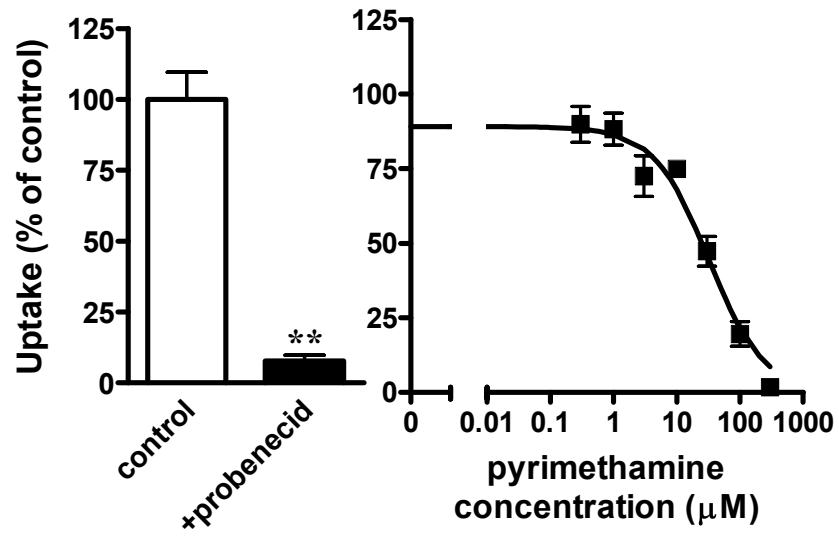


Figure 3

A



B

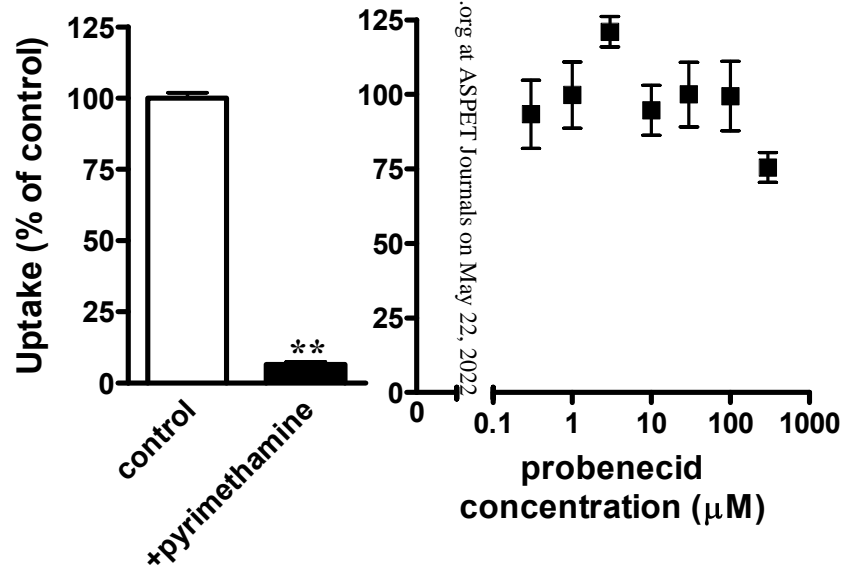


Figure 4

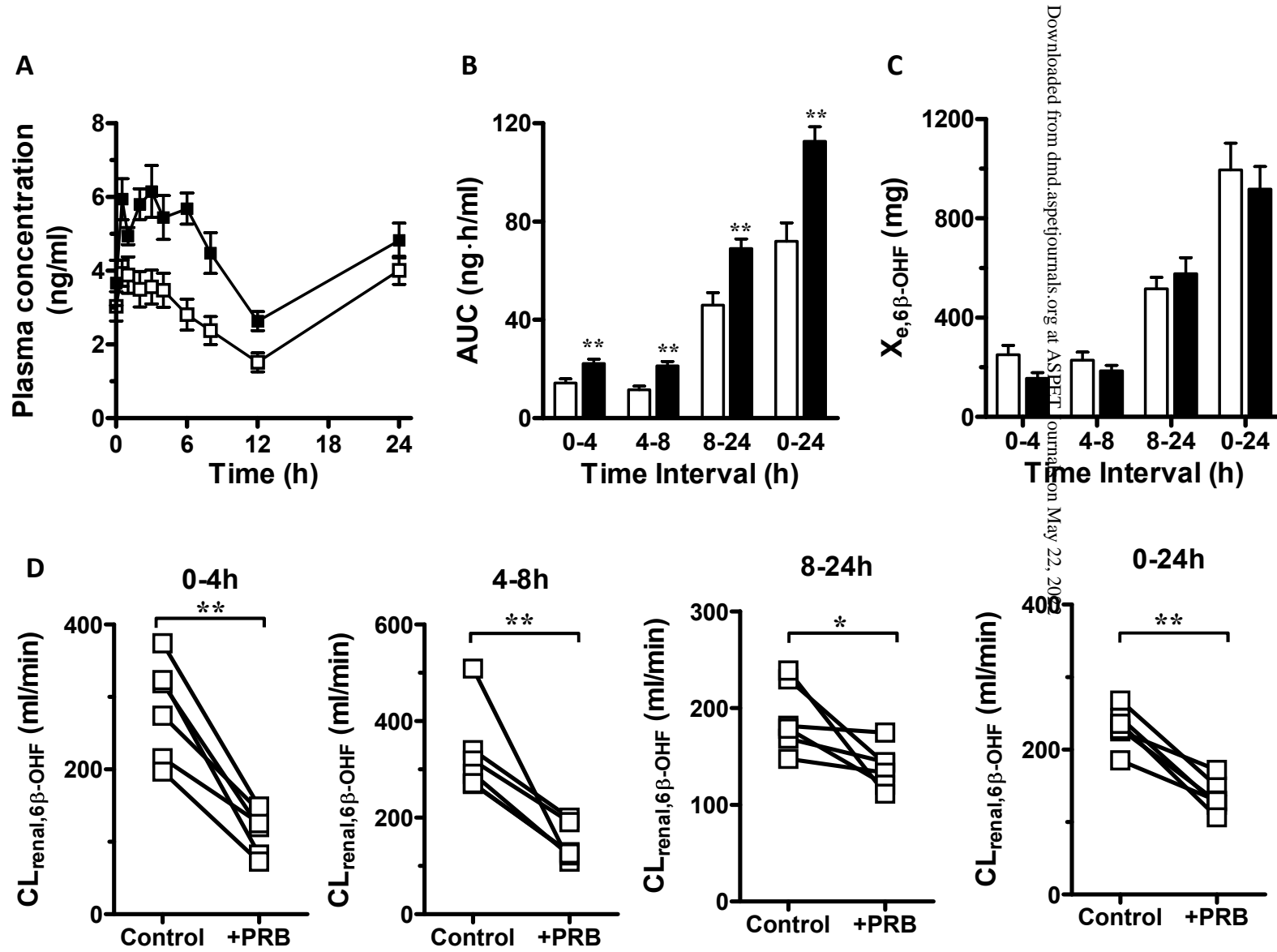


Figure 5

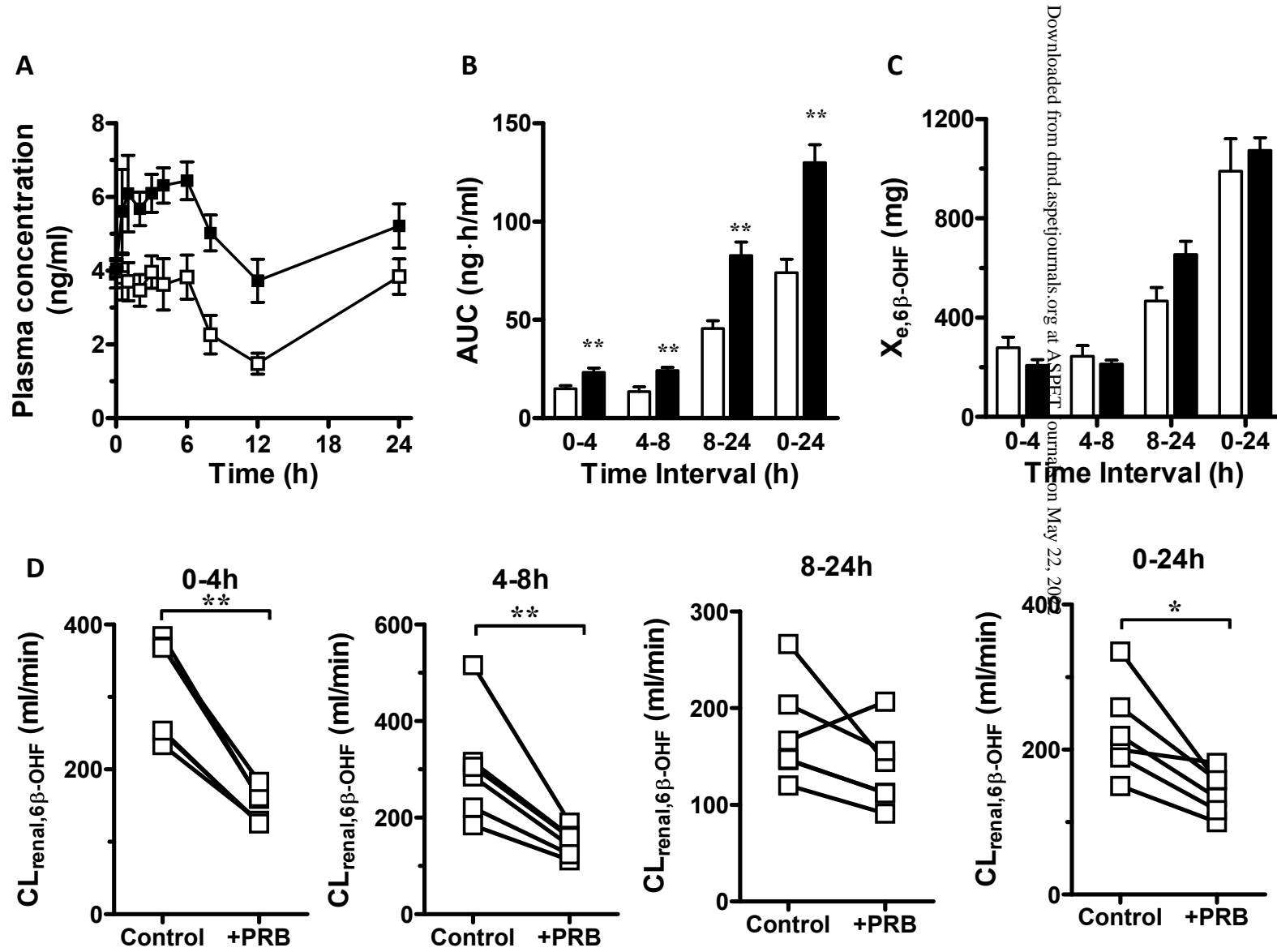
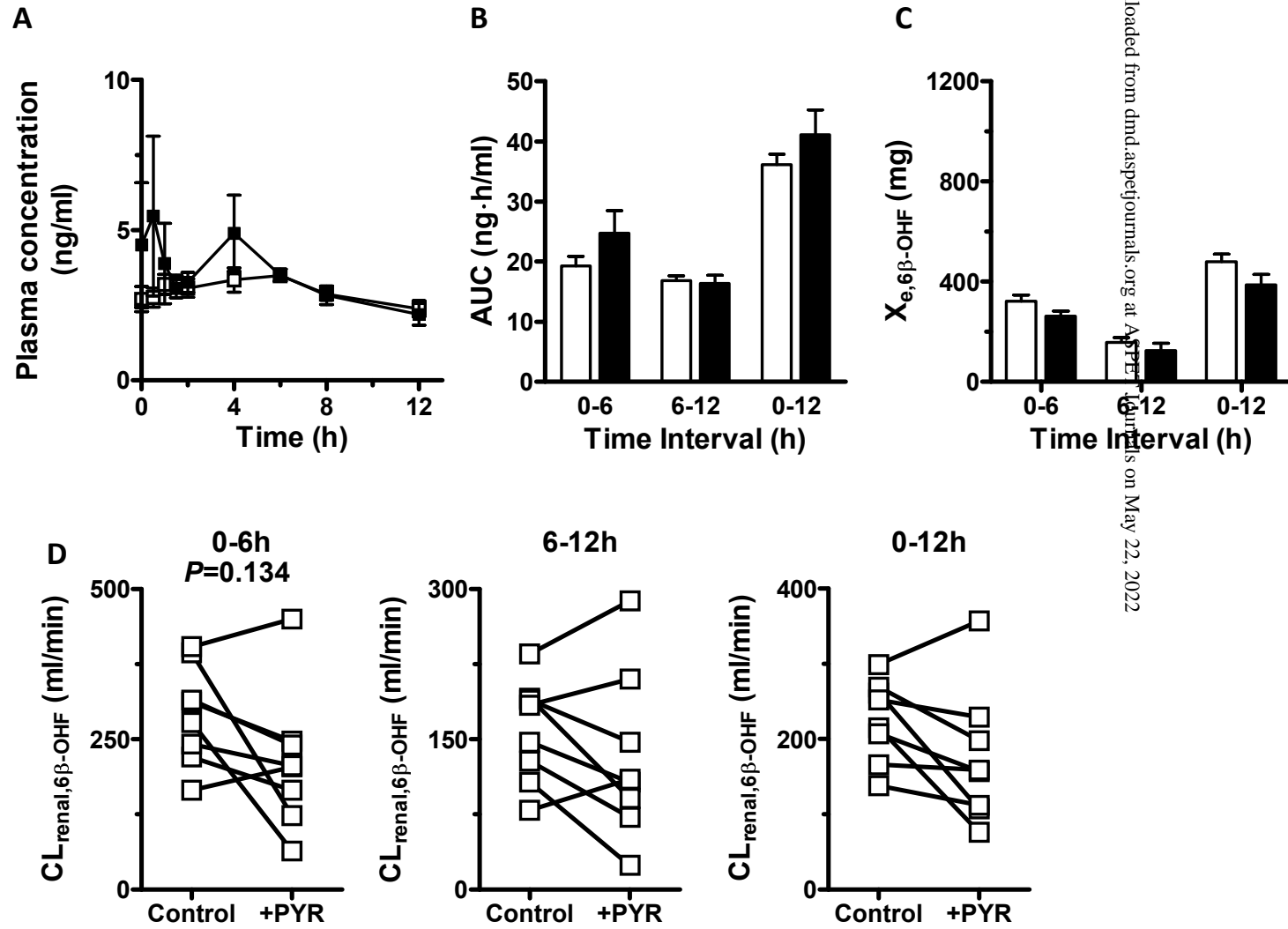


Figure 6



6 β -hydroxycortisol is an endogenous probe for evaluation of drug–drug interactions involving a multispecific renal organic anion transporter, OAT3/*SLC22A8*, in healthy subjects

Yuichiro Imamura, Yuri Tsuruya, Katja Damme, Dominik Heer, Yuji Kumagai, Kazuya Maeda, Nobuyuki Murayama, Noriko Okudaira, Atsushi Kurihara, Takashi Izumi, Yuichi Sugiyama and Hiroyuki Kusuhara

Drug Metabolism and Disposition

Supplemental Table 1 Outlines of clinical studies the plasma and urine samples from which were used in this study

The specimens from the underlined study were used for the analysis in this study. Probenecid was given 2 h before the administration of the probe drugs (adefovir or benzylpenicillin), and pyrimethamine was given 1 h before the administration of metformin. Plasma and urine specimens used in this study were from underlined drug treatments.

Study	Number of subjects	Phase number	Drug administered	Plasma specimens	Urine specimens	Reference
1	6	Phase 1 Phase 2	<u>Benzylpenicillin alone (400,000 U, po)</u> :control group in this study	Plasma samples were collected at	Urine samples were collected in	Maeda K et al., submitted

		<p><u>Phase 3</u></p> <p>Phase 4</p>	<p>Benzylpenicillin+probenecid (500 mg, po)</p> <p><u>Benzylpenicillin+probenecid (750 mg, po)</u></p> <p>Benzylpenicillin+probenecid (1,500 mg, po)</p>	<p>0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h after the administration of the probe drugs</p>	<p>the predose period and during the periods 0–4 h, 4–8 h, and 8–24 h after treatment with the probe drugs.</p>	
2	6	<p>Phase 1</p> <p>Phase 2</p> <p><u>Phase 3</u></p> <p>Phase 4</p>	<p><u>Adefovir alone (10 mg, po) :control group in this study</u></p> <p>Adefovir + probenecid (500 mg, po)</p> <p><u>Adefovir + probenecid (750 mg, po)</u></p> <p>Adefovir + probenecid (1,500 mg, po)</p>			
3	8	<p>Phase 1</p> <p>Phase 2</p>	<p><u>metformin alone (100 µg, po) :control group in this study</u></p> <p><u>metformin + pyrimethamine (50 mg,</u></p>	<p>Plasma samples were collected at 0.5, 1, 1.5, 2, 4, 6, 8,</p>	<p>Urine samples were collected in the predose</p>	<p>Kusuhara et al., 2011</p>

			<u>po)</u>	and 12h after the administration of the probe drugs	period and during the periods 0–6 h, 6–12 h, and 12–24 h after treatment with the probe drugs.	
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Drug Metabolism and Disposition

Supplemental Table 2 The uptake of reference compounds in human kidney slices, and the effect of typical inhibitors

The uptake of 6 β -OHF (20 μ M) by human kidney slices from three different volunteers (Panel A; male 70 years, Panel B; female 51 years and Panel C male 75 years) for 20 min was determined at 37°C. Three slices were used in each batch of human kidney. Uptake represents the mean \pm SE (n=3). * $P < 0.05$, ** $P < 0.01$.

Donor information	reference compounds	Uptake (ml/20 min/g kidney)	
		Control	+inhibitor ^a
male 70 years (panel A)	³ H-adefovair	5.47 \pm 0.57	0.68 \pm 0.01**
	¹⁴ C-benzylpenicillin	2.09 \pm 0.36	0.75 \pm 0.01*
	¹⁴ C-TEA	12.9 \pm 2.2	0.60 \pm 0.03**
female 51 years (panel B)	³ H-adefovair	3.97 \pm 0.26	0.46 \pm 0.06**
	¹⁴ C-benzylpenicillin	1.50 \pm 0.11	0.55 \pm 0.07**
	¹⁴ C-TEA	8.10 \pm 0.05	0.37 \pm 0.00**
male 70 years (panel C)	³ H-adefovair	8.33 \pm 0.09	0.60 \pm 0.05**
	¹⁴ C-benzylpenicillin	1.44 \pm 0.17	0.41 \pm 0.05**
	¹⁴ C-TEA	3.81 \pm 1.16	0.30 \pm 0.03*

^a Probenecid (1mM) was used for the uptake of ³H-adefovir and ¹⁴C-benzylpenicillin, and MPP⁺ (1mM) was used for the uptake of ¹⁴C-TEA.

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Drug Metabolism and Disposition

Supplemental Table 3 Comparison of apparent formation clearance of 6 β -OHF (CL_{6 β -OHF})

CL_{6 β -OHF} was calculated using X_{6 β -OHF} and AUC of cortisol (AUC_F) following equation (7) in Materials and Methods.

Study	Groups	AUC _F ¹⁾	X _{6β-OHF} ¹⁾	CL _{6β-OHF}
		($\mu\text{g} \cdot \text{h/ml}$)	(μg)	(ml/min)
1 [¶]	control	8.66 \pm 0.60	995 \pm 108	1.90 \pm 0.12
	+probenecid (750 mg)	8.75 \pm 0.41	917 \pm 91	1.76 \pm 0.18
2 [†]	control	7.94 \pm 0.73	990 \pm 130	2.10 \pm 0.27
	+probenecid (750 mg)	8.86 \pm 0.45	1072 \pm 53	2.04 \pm 0.13
3 [‡]	control	4.45 \pm 0.34	480 \pm 30	1.90 \pm 0.24
	+pyrimethamine (50 mg)	4.59 \pm 0.37	387 \pm 42	1.44 \pm 0.15

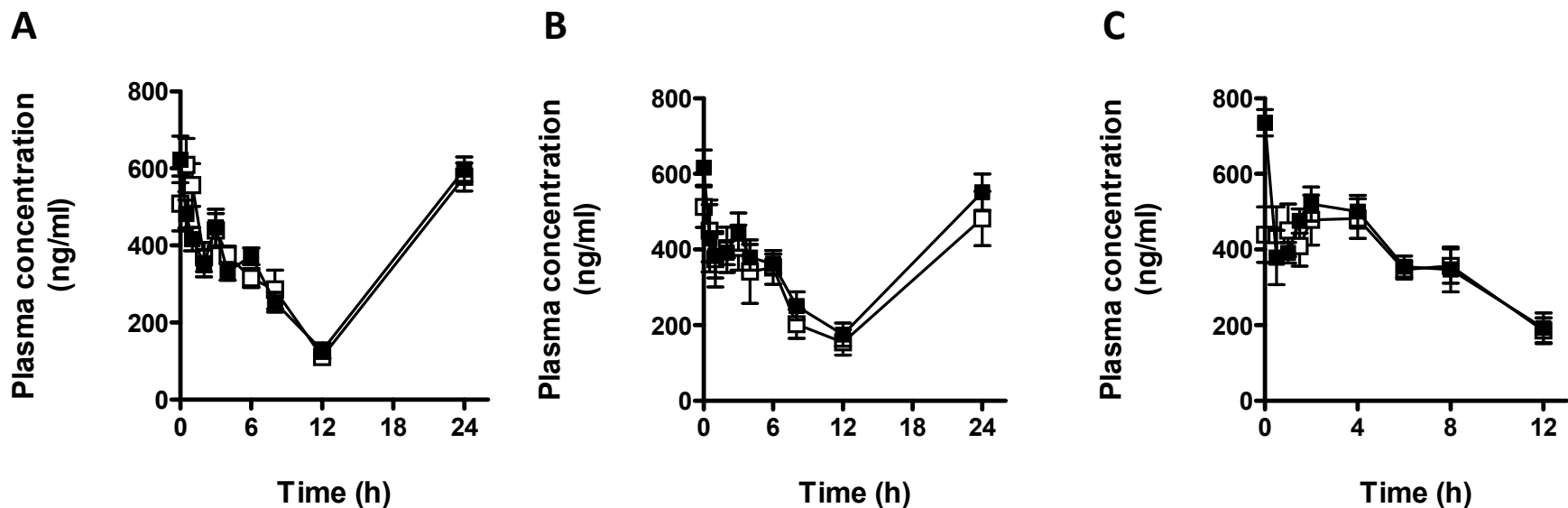
Healthy subjected received oral dose of [¶]benzylpenicillin (400,000 U), [†]adefovir (10 mg), and [‡]metformin (100 μg). Each parameter represents mean \pm S.E (n=6 or 8).

AUC: area under the plasma concentration-time curve from 0 to 24 (Study 1 and 2) or to 12 hours (Study No 3). X_{6 β -OHF}: cumulative amount of 6 β -OHF in the urine up to 24 (Study 1 and 2) or 12 hours (Study No 3) after administration of probe drug

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Drug Metabolism and Disposition



Supplemental Figure 1 Effect of probenecid and pyrimethamine on the plasma concentrations of cortisol in healthy subjects.

Plasma concentrations of cortisol were determined in healthy subjects at designated time points. Open and closed symbols represent control or inhibitor treated groups (panel A; Study 1, panel B Study 2 and panel C Study 3). Each point and bar represent the mean \pm SE (panel A and B n = 6 and panel C n = 8).