

Investigation of endogenous compounds applicable to drug–drug interaction studies involving the renal organic anion transporters, OAT1 and OAT3, in humans

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Abbreviations:

AUC_p, area under the plasma concentration–time curve; CL_R, renal clearance with regard to the plasma concentration; DDI, drug–drug interaction; EMA, European Medicines Agency; FDA, US Food and Drug Administration; GCDCA-S, glycochenodeoxycholate sulfate; HEK, human embryonic kidney; HPLC, high pressure liquid chromatography; LC-MS/MS, liquid chromatography–tandem mass spectrometry; OAT, organic anion transporter; PAH, *p*-aminohippurate; PMDA, Pharmaceuticals and Medical Devices Agency of Japan

Abstract

The present study was a comprehensive analysis of metabolites in plasma and urine specimens from subjects who received probenecid, a potent inhibitor of renal organic anion transporters (OATs). Taurine and glycochenodeoxycholate sulfate (GCDCA-S) could be identified using authentic standards. Probenecid had no effect on the area under the plasma–concentration time curves (AUC_p) of taurine and GCDCA-S, whereas it significantly inhibited their urinary excretion in a dose-dependent manner. The renal clearance (CL_R) values of taurine and GCDCA-S were decreased by 45% and 60%, 59% and 79%, and 70% and 88% by probenecid at 500, 750, and 1,500 mg, p.o., respectively. The CL_R values correlated strongly ($r > 0.96$) between the test compounds (benzylpenicillin, 6β -hydroxycortisol, taurine, and GCDCA-S). Taurine and GCDCA-S were substrates of OAT1 and OAT3, with K_m values of 379 ± 58 and 64.3 ± 3.9 μ M, respectively. The K_i values of probenecid for the OAT1- and OAT3-mediated uptake of taurine and GCDCA-S (9.49 ± 1.27 and 7.40 ± 0.70 μ M, respectively) were similar to those of their typical substrate drugs. The magnitude of the reduction in the CL_R of taurine and GCDCA-S by probenecid could be explained reasonably using the geometric mean of unbound probenecid concentration and K_i values. These results suggest that taurine and GCDCA-S can be used as probes for evaluating

pharmacokinetic drug–drug interactions involving OAT1 and OAT3, respectively, in humans.

Introduction

Pharmacokinetic drug–drug interaction (DDI) is one factor that increases the risk of adverse reactions through altered systemic exposure of victim drugs. The magnitude of inhibition of drug-metabolizing enzymes and transporters by drugs at their clinically relevant concentration should be considered to avoid combinations of drugs that can cause severe events in the clinical setting. The US Food and Drug Administration (FDA), European Medicines Agency (EMA), and Pharmaceuticals and Medical Devices Agency of Japan (PMDA) have issued their draft guidance/guidelines for pharmacokinetic DDIs (FDA, 2012; EMA, 2012; PMDA, 2012). These guidelines state that, when an index for the pharmacokinetic DDI risk calculated using the *in vitro* inhibition constant (K_i) for drug enzymes and transporters and clinically relevant unbound concentration is over the threshold, a clinical interaction study should be conducted using probe drugs for the enzyme and transporter of interest for confirmation.

We advocate the application of endogenous or food-derived compounds to pharmacokinetic DDI studies involving drug transporters because such compounds allow the evaluation of drug transporter activities without the need to administer the probe drugs to the subjects (Imamura et al., 2011; Ito et al., 2012; Imamura et al., 2013; Imamura et al., 2014; Kato et al., 2014). This advantage allows for the evaluation of

the pharmacokinetic DDI risk in healthy subjects in phase I studies (Imamura et al., 2011). Early evaluation of the DDI risk in humans saves costs and time when conducting clinical DDI studies with investigational new drugs that show weak or negligible DDI impact, and the information gathered may help in the design of phase II studies by setting the exclusion criteria for coadministered drugs whose pharmacokinetics might be affected by the investigational new drug. We have demonstrated that *N*-methylnicotinamide and thiamine were superior to the prototypical substrate drug metformin in terms of the sensitivity to the activities of organic cation transporters (MATE1 and MATE2-K) in healthy subjects (Ito et al., 2012; Kato et al., 2014).

This approach needs endogenous compounds for other drug transporters, at least those suggested by the International Transporter Consortium (Giacomini et al., 2010; Tweedie et al., 2013). In this study, we focused on organic anion transporter 1 (OAT1)/*SLC22A6* and OAT3/*SLC22A8*, which are multispecific OATs with slightly overlapping substrate specificities. These transporters mediate the basolateral uptake of various anionic drugs, a weak cationic compound famotidine, and zwitterions such as fexofenadine in the overall tubular secretion in the kidney (Nigam et al., 2007; Emami Riedmaier et al., 2012; Konig et al., 2013; Kusuhara et al., 2013; Pelis and Wright,

2014). Because of their broad substrate specificity, and importance in the kidney, these two transporters deserve evaluation of their risk of pharmacokinetic DDIs as perpetrators and the relevance of the pharmacokinetics of investigational drugs to safety information.

Kidney failure causes accumulation of so-called nephrotoxic compounds, such as hippurate, indole acetate, and indoxyl sulfate, which are OAT1 and OAT3 substrates (Deguchi et al., 2004; Miyamoto et al., 2011). However, whether such compounds can be used as pharmacokinetic DDI probes under normal conditions remains unknown. Previously, we demonstrated that 6 β -hydroxycortisol, a cortisol metabolite produced by CYP3A4 (Ged et al., 1989), is an endogenous substrate of OAT3 and that its renal clearance was decreased significantly by probenecid administration in healthy subjects (Imamura et al., 2014). To expand understanding of the endogenous OAT1 and OAT3 substrates, we conducted a comprehensive analysis of those compounds in the plasma and urine specimens that are significantly altered by probenecid administration in healthy subjects. Probenecid is a potent inhibitor of OAT1 and OAT3, and, at therapeutic doses, can inhibit both OAT1 and OAT3, and cause DDIs. Administration of probenecid decrease the renal clearance of various drugs, such as benzylpenicillin, diuretics (furosemide and bumetanide), and antiviral drugs (adefovir, cidofovir and

Ro64-0802) (Emami Riedmaier et al., 2012; Konig et al., 2013; Kusuhara et al., 2013).

Probenecid inhibits the basolateral uptake of the OAT1/OAT3 substrate, Tc-99m-MAG-3 in healthy subjects, consistent with an interaction that involves inhibition of basolateral uptake from the blood circulation (Takahara et al., 2013).

Materials and Methods

Materials and cell lines

Glycochenodeoxycholate-3-sulfate (GCDCA-S) was synthesized from glycochenodeoxycholate (Sigma-Aldrich, St. Louis, MO) as described previously (Parmentier and Eyssen, 1977). The purity was confirmed as >97% by reverse-phase high pressure liquid chromatography (HPLC). [³H]*p*-Aminohippurate (PAH) (4.56 Ci/mmol), [³H]estrone sulfate (45.0 Ci/mmol), and [³H]taurine (22.7 Ci/mmol) were purchased from Perkin Elmer Life Science (Boston, MA). All other chemicals and reagents were of analytical grade and are commercially available.

Human embryonic kidney 293 (HEK293) cells stably expressing human OAT1 (hOAT1) and hOAT3 were established previously (Deguchi et al., 2004). For transient expression, hOAT1-pcDNA3.1(+) vector, hOAT3-pIRES2-enhanced green fluorescent protein vector, and empty vector that had been constructed previously (Deguchi et al.,

2004) were used. The vector (0.5 µg/well) was transfected using polyethylenimine “Max” (Mw 40,000) and Opti-Mem® (Polysciences, Inc., Warrington, PA) according to the manufacturer’s protocol. Twenty-four hours after the transfection, 5 mM butyrate was added to the culture medium. The cells were used for the uptake study 48 h after the transfection.

Clinical samples

This study 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.

The plasma and urine specimens were those from our previously conducted clinical DDI study (Maeda et al., 2014). In brief, the study was a single-arm, four-phase study where eight healthy male subjects (ages, 20~35 years old; body mass index, 18.5~28.5 kg/m²) were enrolled. Within one month before starting the clinical study, a medical history was obtained from each candidate subject, who then underwent a physical examination, electrocardiography, routine blood testing, and urinalysis.

Benzylpenicillin (400,000 U p.o.) was administered to the subjects as the OAT3 probe in all phases. After the washout period, benzylpenicillin and probenecid were

administered simultaneously with probenecid dose escalation at 500, 750, and 1,500 mg, p.o.

Selection of compounds present in the plasma and urine specimens for further analysis

The plasma and urine specimens from subjects who received benzylpenicillin with or without probenecid administration (750 mg, p.o.) were subjected to metabolome analysis. The plasma and urine specimens were diluted 5- and 100-fold in Milli-Q water followed by addition of a 4-times volume of acetonitrile/methanol (9:1, v/v) for deproteinization. The supernatant was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis.

The LC system was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA) equipped with an XBridge HILIC column (3.5 μ m, 50 \times 4.6 mm; Waters, Milford, MA). The mobile phase comprised 5 mM ammonium acetate as solvent A and acetonitrile as solvent B, and was delivered at 1.0 mL/min using 95% solvent B between 0 and 2 min followed by a linear gradient to 70% B at 12 min and 50% B at 13 min, and then isocratic to 14.5 min with 50% B. The MS data were acquired using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The samples were analyzed using negative ion-heated electrospray ionization. The MS

conditions were set as follows: spray voltage, 2,500 V; scanning range, mass-to-charge ratio (m/z), 100–800; resolving power, 30,000; and normalized collision energy, 35%. The freely available MZmine software (Pluskal et al., 2010) was used to align the data in the time domain and to integrate the peak area automatically. The HMDB (Wishart et al., 2009) and KEGG (Kanehisa and Goto, 2000) databases were used for structural searching based on accurate masses.

The criteria for candidate peak selection were as follows: 1) the product of the peak area and the urine volume divided by the plasma concentrations was decreased by 50% of the corresponding control values; 2) the decrease was observed in all volunteers; and 3) the peak intensity was strong enough to obtain the product ion spectrum.

Quantification of the compounds in the plasma and urine specimens

The plasma and urine concentrations of compounds were measured by LC–MS/MS in the subjects who receive an oral dose of penicillin with or without probenecid (500, 750, and 1,500 mg, p.o.). The plasma and urinary excretion rates of 6 β -hydroxycortisol were also determined in the subjects treated with an oral dose of probenecid (500 and 1,500 mg) as described previously (Imamura et al., 2014). An AB SCIEX QTRAP 5500 mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Prominence LC system (Shimadzu, Kyoto, Japan), operated in the

electron spray ionization mode, was used for the analysis. The conditions are summarized in Supplemental Table 1. Urine specimens were diluted 10-fold with Milli-Q water. A 2-times volume of acetonitrile was added to the plasma and diluted urine specimens for deproteinization. After centrifugation at 20,000g for 10 min, the supernatant was diluted with a 4-times volume of Milli Q water and then subjected to LC-MS/MS analysis.

Measurement of the unbound fraction of GCDCA-S and probenecid in human plasma

Blank control plasma purchased from Cosmo Bio Co. (Tokyo, Japan) was spiked with [³H]taurine or GCDCA-S, and the sample was incubated for 1 h at 37°C. The bound and unbound forms of [³H]taurine and GCDCA-S and probenecid were separated using a Centrifree Ultrafiltration Device (Merck Millipore, Darmstadt, Germany). The concentrations of test compounds in the plasma specimens and eluents were determined using a scintillation counter or LC-MS/MS. To examine the effect of probenecid on the unbound fraction of GCDCA-S in human plasma (f_p), probenecid was added to the plasma specimens at 70, 200, and 600 μ M.

In vitro uptake study using stable or transient transfectants

An in vitro uptake study was conducted as reported previously (Deguchi et al.,

2004). Briefly, after a 15-min preincubation with test compound-free Krebs–Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO₃, 4.8 mM KCl, 1.0 mM KH₂PO₄, 1.2 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose, and 1.5 mM CaCl₂, pH 7.4), uptake assays were initiated by addition of transport buffer containing the test compounds. The uptake was terminated at the designated time by adding ice-cold Krebs–Henseleit buffer, followed by three washes with ice-cold buffer. To determine the uptake of radiolabeled compounds, the cells were solubilized with 0.2 N NaOH overnight. Following addition of 0.4 N HCl to neutralize the pH, aliquots of the lysates were transferred to scintillation vials containing scintillation cocktail (Clear-soll, Nacalai Tesque, Kyoto, Japan), and the radioactivity was measured in a liquid scintillation counter (LS6000SE, Beckman Instruments, Inc., Fullerton, CA). To determine the uptake of nonradiolabeled compounds, the cells were recovered in Milli-Q water using a scraper and disrupted using a Bioruptor (UCD-250HSA, Cosmo Bio Co., Ltd.). A 2-times volume of acetonitrile was added for deproteinization, and the specimens were centrifuged for 10 min at 20,000g. The supernatant was diluted with a 4-times volume of Milli-Q water and subjected to LC-MS/MS analysis. The remaining cell lysates were used to determine the protein concentration using the Lowry method with bovine serum albumin as the standard.

Kinetic analysis of in vitro data

The transporter-mediated uptake was obtained by subtracting the uptake in the empty vector-transfected cells from the uptake in the transporter-expressing cells. The kinetic parameters were determined using the iterative nonlinear least-squares method assuming the Michaelis–Menten equation (Eq. 1):

$$v = \frac{V_{\max} \cdot S}{K_m + S} \quad \text{Eq. 1}$$

where v , K_m , S , and V_{\max} represent the uptake velocity, Michaelis constant, substrate concentration, and maximum transport velocity, respectively. The inhibition constants of probenecid for OAT1 and OAT3 were calculated as shown in Eq. 2:

$$CL_{\text{uptake (+Inhibitor)}} = \frac{CL_{\text{uptake (control)}}}{1+I/K_i} \quad \text{Eq. 2}$$

where $CL_{\text{uptake (+Inhibitor)}}$ and $CL_{\text{uptake (control)}}$ represent the uptake in the presence and absence of probenecid, respectively, and I and K_i represent the probenecid concentration and inhibition constant, respectively. The fitting using Eq. 1 and 2 was conducted by iterative non-linear least square method using the MULTI program (Yamaoka et al., 1981) and the damped Gauss–Newton algorithm. The substrate concentrations were designed to be below the K_m value in the inhibition experiments so that the IC_{50} would approximate K_i .

Calculation of the renal clearance of the test compounds in humans

The renal clearance with regard to the plasma concentration (CL_R) was calculated as follows:

$$CL_R = X_{\text{urine}}/AUC_p \quad \text{Eq. 3}$$

where X_{urine} represents the amount of the compound excreted into the urine, and AUC_p represents the area under the plasma–concentration time curve. The AUC was calculated using the trapezoidal rule from 0.5 to 8 h after the oral administration of benzylpenicillin.

Statistical analysis

The data are presented as the mean \pm standard error of the mean (SEM). One-way analysis of variance with Tukey’s test or Dunnett’s multiple-comparison test was used to identify significant differences between control and probenecid groups. One-way analysis of variance with Tukey’s test was used to examine significant differences between the groups in the in vitro uptake study. p-values <0.05 were considered significant.

Results

Investigation of candidate compounds in plasma and urine specimens

In the plasma and urine specimens, 1,087 and 782 peaks could be detected, respectively; 230 of the peaks, m/z values, and retention times were identical between plasma and urine specimens. Nine compounds satisfied the criteria (Table 1). Database searching based on the accurate m/z values suggested 5 compounds as candidates. Because the retention times of peaks numbers 6 and 8 were identical, peak no. 6 peak could have been a fragment ion of peak no. 8. Using the authentic standards, compounds were identified as taurine (no. 1), GCDCA-S (no. 4), and inosine (no. 5). The pharmacological target of probenecid as an antiuricosuric drug is URAT1, which mediates the reabsorption of uric acid (Enomoto et al., 2002). In this study, the enhancement effect on the urinary excretion of uric acid was observed in subjects who received probenecid treatment (data not shown).

Quantification of taurine, GCDCA-S, and inosine in plasma and urine specimens

Taurine, GCDCA-S, and inosine concentrations in the plasma and urine specimens from control and the probenecid groups (500, 750, and 1,500 mg p.o.) were quantified using LC-MS/MS. The plasma concentrations of taurine and GCDCA-S were not significantly changed by probenecid at any dose (Figure 1). The amount of taurine and

GCDCA-S excreted into the urine was decreased in a dose-dependent manner during the intervals from 0 to 4 and from 4 to 8 h after administration. The reduction in X_{urine} of taurine was significant at 750 mg from 0 to 4 h, and at 500 and 1500 mg from 4 to 8 h (Figure 1A). The X_{urine} of taurine recovered from 8 to 24 h, but was not significant (Figure 1A). The reduction in X_{urine} of GCDCA-S was significant at 750 mg from 0 to 4 h, and at all the doses tested from 4 to 8 h (Figure 1B). The X_{urine} of GCDCA-S recovered from 8 to 24 h was only significantly reduced at 1500 mg (Figure 1B). The amounts of taurine and GCDCA-S excreted into the urine from time zero to 8 h were significantly lower than their corresponding control values (Table 2). The CL_R values for both taurine and GCDCA-S were decreased in a probenecid dose-dependent manner (Figure 1). On the other hand, the plasma concentrations and urinary excretion rates of inosine were unchanged (data not shown).

The plasma concentration and urinary excretion rate of 6β -hydroxycortisol in the subjects treated with an oral dose of probenecid (500 and 1,500 mg) were also determined. The plasma concentration was significantly higher in the probenecid-treated group, but the urinary excretion rate was unchanged (Supplemental Figure 1). The control values and their corresponding values in the subjects treated with an oral dose of probenecid (750 mg) were reported previously (Imamura et al.,

2013).

The correlations between the renal clearances of the probe administered (benzylpenicillin), taurine, 6 β -hydroxycortisol, and GCDCA-S are shown in Figure 2. The correlation coefficients were high: $r > 0.97$.

Unbound fractions of [³H]taurine and GCDCA-S in human plasma

The f_p values for [³H]taurine and GCDCA-S were determined in duplicate and were 1.01 (0.992 and 1.02) and 0.0190 (0.0191 and 0.0189), respectively. The f_p values for probenecid were determined to be 0.0694 (0.0703 and 0.0685), 0.0710 (0.0713 and 0.0708), and 0.0909 (0.0949 and 0.0869) at 70, 200, and 600 μ M, respectively. Probenecid did not affect the f_p of GCDCA-S at the concentrations examined; the values were 0.0177 (0.0182 and 0.0172), 0.0159 (0.0177 and 0.0141), and 0.0201 (0.0210 and 0.0192) at 70, 200, and 600 μ M, respectively. The values in parentheses represent the individual value.

Uptake of [³H]taurine and GCDCA-S by hOAT1- and hOAT3-transfected cells

Transient expression of hOAT1 induced the uptake of [³H]taurine by HEK293 cells which was significantly inhibited by probenecid, whereas transfection of mock-vector or vector containing hOAT3 cDNA was not (Figure 3A). The uptake of GCDCA-S was significantly higher in HEK293 cells stably expressing hOAT3 than in

mock vector-transfected cells. Addition of 100 μM probenecid diminished the uptake to the levels found in mock-vector transfected cells (Figure 3A). Whereas, the uptake of GCDCA-S in HEK293 cells stably expressing hOAT1 was identical to that in mock vector-transfected cells (Figure 3A). Transient expression of hOAT1 did not affect the uptake of GCDCA-S in HEK293 cells (data not shown).

Based on the time profiles of the uptake of [^3H]taurine and GCDCA-S (Figure 3B), the uptake of taurine by hOAT1 and that of GCDCA-S by hOAT3 for 2 min was determined at various substrate concentrations. The concentration dependence was shown in Eadie-Hofstee plot (Figure 3C). K_m and V_{max} values of [^3H]taurine for hOAT1 were $379 \pm 58 \mu\text{M}$ and $2320 \pm 28 \text{ pmol/mg protein/min}$, respectively, and those of GCDCA-S for hOAT3 were $64.3 \pm 3.9 \mu\text{M}$ and $545 \pm 23 \text{ pmol/mg protein/min}$, respectively (Figure 3C).

The K_i of probenecid (μM) was determined as 9.49 ± 1.27 for OAT1-mediated taurine uptake. The K_i values for the uptake of PAH and furosemide were determined as 11.8 ± 1.6 and 11.4 ± 1.7 , respectively (Figure 4). The K_i values (μM) of probenecid were determined as 7.40 ± 0.70 for OAT3-mediated GCDCA-S uptake and as 7.25 ± 0.81 , 7.25 ± 0.87 , 7.28 ± 0.53 , and 5.41 ± 0.82 for the uptake of benzylpenicillin, rosuvastatin, pravastatin, and furosemide, respectively (Figure 4).

Comparison of the observed and predicted magnitude of interaction with renal clearance

$CL_{R,0-8h}$ values were calculated using $CL_{R,0-8h}$ under control conditions ($CL_{R,0-8h,control}$), the K_i values (this study), and the geometric mean values of the unbound probenecid plasma concentrations from time zero to 8 h were as reported previously (Maeda et al., 2014) ($CL_{R,0-8h}(I) = CL_{R,0-8h,control} / [1 + I_u / K_i]$). The calculated $CL_{R,0-8h}(I)$ of taurine slightly underestimated the observed CL_R , but the values were within the $CL_{R,0-8h}(I)$ value calculated using a 2-fold greater K_i value (Figure 5). The calculated $CL_{R,0-8h}(I)$ of GCDCA-S was comparable with the observed values (Figure 5).

Discussion

The purpose of this study was to identify the compounds endogenously present in the plasma and urine that could be applied to DDI studies involving the renal OATs, OAT1 and OAT3, as probes in healthy subjects.

The metabolomic analysis of the plasma and urine specimens using LC-MS suggested that three compounds (taurine, GCDCA-S, and inosine) are potential OAT probes in humans (Table 1), although the result for inosine could not be reproduced in the subsequent quantification analysis for unknown reasons. Taurine is an amino acid

derived from food or produced from cysteine by cysteine dioxygenase and cysteine sulfinate decarboxylase in the body. Among the amino acids, its urinary excretion rate is the highest (Held et al., 2011). GCDCA-S is a major sulfate conjugate of bile acids in both plasma and urine, and accounts for 28% of urinary bile acid sulfate conjugates, and 24% of plasma bile acid sulfate conjugates (Bathena et al., 2013). Other major urinary bile acid sulfate conjugates are glycodeoxycholate sulfate (35%) and glyoursodeoxycholate sulfate (13%), which were not selected in the LC-MS analysis. Because glycodeoxycholate is an isomer of GCDCA, it is possible that glycodeoxycholate sulfate was detected as GCDCA-S in the analysis. An *m/z* ratio identical to that of indole acetate, a uremic toxin that is an OAT1 substrate, was also detected in LC-MS analysis. In one urine specimen from the probenecid-treated group, the peak intensity was fairly low, and the mean value for the magnitude of reduction in the urine-to-plasma concentration ratio of indole acetate by probenecid was at most 20% (data not shown). Therefore, in the subsequent analysis, we focused on taurine and GCDCA-S.

Unlike 6 β -hydroxycortisol (Imamura et al., 2014) (Supplemental Figure 1), probenecid had no effect on the mean AUC_p values of taurine and GCDCA-S (Figure 1). Whereas, it significantly decreased the amount of taurine and GCDCA-S excreted into

the urine in a dose-dependent manner and, consequently, the CL_R values of both taurine and GCDCA-S were decreased with an increase in probenecid dose. The CL_R values of 6 β -hydroxycortisol, taurine and GCDCA-S correlated strongly each other, and also they also showed a good correlation with the CL_R of benzylpenicillin, a reference OAT3 substrate (Figure 2). Thus, the CL_R values of the endogenous compounds measured in this study provide a quantitative index for the DDI with probenecid.

Unexpectedly, taurine and GCDCA-S happened to be OAT1 and OAT3 substrates, respectively. The K_m values for taurine and GCDCA-S were greater than their unbound plasma concentrations, suggesting that OAT1- and OAT3-mediated transport occurs under linear conditions. Assuming the competitive inhibition, for which the K_m value can be theoretically regarded as the K_i value, inhibition of OAT1- and OAT3-mediated uptake of drugs by these compounds is unlikely to occur under physiological conditions. We note that there was a discrepancy in the specific uptake of taurine by OAT1 between the stable and transient expression systems (Figure 3 and Supplemental Figure 2). Part of the mechanism may be the higher transport activity of OAT1 in the transient expression system and the low uptake activity of taurine in HEK293 cells used as the host to study the transient expression of OAT1.

K_i values sometimes show substrate dependence. Substrate-dependent K_i values

have been reported for OCT2 (Hacker et al., 2015), MATE1 (Martinez-Guerrero and Wright, 2013; Lechner et al., 2016), and OATP1B1 (Noe et al., 2007; Izumi et al., 2015). This motivated us to compare the K_i values of probenecid for taurine and GCDCA-S uptake with those for other OAT1 and OAT3 substrates, respectively. As far as the test substrates are concerned, the inhibition constants of probenecid for drugs were identical to those determined using endogenous substrates; this finding indicates the usefulness of taurine and GCDCA-S in these studies (Figure 4). Finally, we examined whether these in vitro K_i values could predict the magnitude of DDI for the CL_R of taurine and GCDCA-S using the geometric mean value of unbound probenecid concentrations. The calculated CL_R were in a good agreement with the observed values (Figure 5). The somewhat lower predictability for the CL_R of taurine compared with that of GCDCA-S is attributable to the greater contribution of glomerular filtration. Using a glomerular filtration rate (GFR) of 2.1 mL/min/kg (Maeda et al., 2014), the CL_R of taurine was 16% of its average GFR (range 11%–25%), whereas the GFR of GCDCA-S was 120% (range 42%–234%). However, assuming that 1500 mg probenecid completely inhibited the tubular secretion of taurine and GCDCA-S, the fraction of taurine and GCDCA-S reabsorbed from urine was calculated to be 98% and 87%, respectively, by comparing the control CL_R value with the product of f_p and GFR.

Using these values, the tubular secretion clearance was calculated to be 2.4- and 7.8-fold greater than the product of f_p and GFR.

Both taurine and GCDCA-S undergo extensive reabsorption from the urine. Based on the results of Taut/Slc6a6-knockout mice (Huang et al., 2006), we consider that Taut is the primary candidate responsible for taurine reabsorption in the human kidney. The transporters responsible for GCDCA-S reabsorption remain unknown. Because GCDCA-S turned out to be an OAT4 substrate with a lower transport activity than its typical substrate, estrone sulfate (Supplemental Figure 3), OAT4 is one candidate. These transporters provide other sites of drug interactions that could influence their CL_R .

The magnitude of the reduction in the CL_R of taurine by probenecid (3.5-fold) is greater than that of adefovir (2-fold) (Maeda et al., 2014). The CL_R values of GCDCA-S and 6 β -hydroxycortisol by probenecid (4.5- and 9.0-fold, respectively) are similar to or greater than that of benzylpenicillin (4.4-fold) (Maeda et al., 2014). The sensitivity of the CL_R of taurine, GCDCA-S, and 6 β -hydroxycortisol to drug transporter inhibition is not inferior to that of the probe drugs. Taken together, our results suggest that taurine can be applied in the evaluation of OAT1-mediated DDIs and that GCDCA-S and 6 β -hydroxycortisol can be applied to the evaluation of OAT3-mediated

DDIs. It is noteworthy that the plasma concentration of 6 β -hydroxycortisol also provides a quantitative index of OAT3 activity (Supplemental Figure 1).

Previously, the CL_R of [³H]chenodeoxycholate-3-sulfate following an intravenous dose in cholestatic patients was significantly decreased by an oral dose of probenecid (500 mg, p.o.) (Corbett et al., 1981). Kidney slices from Oat3(-/-) mice exhibit a lower [³H]taurocholate uptake in vitro than those from wild-type mice (Sweet et al., 2002). Nonsulfated bile acids are detected in both human urine and serum (Bathena et al., 2013). The urine-to-plasma concentration ratios of glycine- and taurine-conjugates were relatively low compared with those of sulfated bile acids, whereas among nonconjugated bile acids, 12-Oxo-CDCA and muricholate showed similar or higher ratio. It is possible that we can expand the use of endogenous OAT3 probes to other bile acids once the analytical method is established.

The K_i of probenecid for MRP2 is 45 μ M (Horikawa et al., 2002), for OATP1B1 is 76 μ M (Hirano et al., 2006), and for OATP1B3 is 130 μ M (Matsushima et al., 2008). Considering the geometric means of unbound probenecid (Figure 5), it is likely that probenecid inhibits MRP2, OATP1B1 and OATP1B3. Since MRP2 is expressed in the brush border membranes of the proximal tubules (Schaub et al., 1999), and mediates sulfated bile acid transport (Akita et al., 2001), inhibition of the urinary excretion of

GCDCA-S may involve inhibition of MRP2. According to a recently published paper, the signals tentatively annotated as GCDCA-S were 2-fold higher in the subjects treated with cyclosporine A (Yee et al., in press). Considering the limited impact on AUC_p (Table 2), the inhibition of OATP1B1 and OATP1B3 by probenecid is unlikely to be remarkable.

We have reported on endogenous substrates that can be applied in the study of pharmacokinetic DDIs involving renal drug transporters such as OCT2, MATE1/MAT2-K, OAT1, and OAT3. Comparison of the CL_R values of these compounds before and after administration of the drugs of interest or in the clinical settings provides a means to assess the activities of multiple drug transporters in the kidney in the same subjects without administration of probe substrates. For hepatic drug transporters, the plasma concentrations of DHEAS, bilirubin and its glucuronide, and some bile acids were suggested as endogenous probes for OATPs, whose plasma concentration was significantly increased in monkeys treated with rifampicin, an OATP inhibitor (Chu et al., 2015; Watanabe et al., 2015). Furthermore, recent studies demonstrated an increase in the systemic exposure of endogenous OATP substrates by administration of rifampicin or cyclosporin A (Lai Y et al., *in press*; Yee et al., *in press*).

Thiamine was found to be a substrate of OCT1, whose plasma concentration was

significantly increased in Oct1/2(-/-) mice (Chen et al., 2014; Kato et al., 2015). These findings need to be confirmed in humans. Accumulation of further clinical examples that show good correlations between the magnitude of changes in the pharmacokinetics of endogenous and exogenous substrates may motivate regulatory authorities to introduce this new decision rule into the decision tree between in vitro and clinical studies using probe drugs into the DDI guidance/guidelines.

In conclusion, the present study demonstrated that taurine and GCDCA-S are substrates of OAT1 and OAT3, respectively, and that they can be applied as probes in clinical DDI studies involving OAT1 and OAT3.

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Author Contributions

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Contributed new reagents or analytic tools: none

Performed data analysis: Tsuruya, Kato, Kusuhara

Wrote or contributed to the writing of the manuscript: Tsuruya, Sugiyama, Kusuhara

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Footnotes

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Legends of Figures

Figure 1 Effect of probenecid on the plasma concentrations, urinary excretion, and renal clearance of taurine (A) and GCDCA-S (B) in healthy subjects.

Following oral administration of probenecid (500, 750 or 1,500 mg, p.o.), benzylpenicillin (40,000 units) was administered to six subjects orally. Blood samples were taken from the subjects just before administration of the drugs, and 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, and 24.0 h after benzylpenicillin administration. Urine specimens were collected at the designated times. The renal clearance of taurine and GCDCA-S was calculated as described in the *Materials and Methods* section. Each symbol and bar represent the mean value and SEM, respectively (n=6). *p<0.05, **p<0.01 versus control values

Figure 2 Correlations between the renal clearance of taurine, GCDCA-S, 6 β -hydroxycortisol, and benzylpenicillin.

The CL_R of benzylpenicillin was taken from Maeda et al. (2014), and the CL_R of 6 β -hydroxycortisol was taken from Imamura et al. (2014) and was determined in this study. Each symbol represents the mean and SEM (n=6). The rigid and dotted lines represent the fitted line and 95% confidence interval of the fitted line, respectively.

Figure 3 Uptake of taurine and GCDCA-S in HEK293 cells expressing hOAT1 and hOAT3.

A Uptake of [³H]taurine (5 nM) for 5 min was determined in HEK293 cells transiently expressing hOAT1 or hOAT3 in the presence (closed bar) and absence (open bar) of 100 μM probenecid. Uptake of 1 μM GCDCA-S for 2 min was determined in HEK293 cells stably expressing hOAT1 or hOAT3 in the presence and absence of probenecid (100 μM). Each symbol and bar represents the mean and SEM, respectively (n=3).

*p<0.05, **p<0.01

B Uptake of [³H]taurine (5 nM) and GCDCA-S (1 μM) was determined at the designated times in HEK293 cells transiently expressing hOAT1, in empty vector-transfected cells, in HEK293 cells stably expressing hOAT3, and in the corresponding empty vector-transfected cells.

C Uptake of [³H]taurine and GCDCA-S by hOAT1 and hOAT3 for 2 min was determined at various substrate concentrations from 1 to 3,000 μM of unlabeled taurine, and from 1 to 1,000 μM of GCDCA-S. The concentration dependence on OAT1- and OAT3-mediated taurine and GCDCA-S uptake is shown in the Eadie-Hofstee plot.

Figure 4 Effects of probenecid on OAT1- and OAT3-mediated uptake of various test substrates.

A Uptake of test compounds for 2 min in HEK293 cells transiently expressing hOAT1, and empty vector-transfected cells was determined in the absence and presence of probenecid at the designated concentrations. The substrate concentrations were 5 nM for [³H]taurine, 20 nM for [³H]PAH, and 0.25 μM for furosemide. The specific uptake of substrates by hOAT1 was calculated by subtracting the uptake by mock vector-transfected cells from the uptake by hOAT1-expressing HEK293T cells. The solid line represents the fitted line obtained by nonlinear regression analysis as described in the *Materials and Methods* section. Each point represents the mean value, and error bars represent the SEM (n=3).

B Uptake of test compounds for 2 min in HEK293 cells stably expressing hOAT3 and in empty vector-transfected cells was determined in the absence and presence of probenecid at the designated concentrations. The substrate concentrations were 1 μM for GCDCA-S, benzylpenicillin, rosuvastatin, and pravastatin, and 0.25 μM for furosemide. The specific uptake of substrates by hOAT3 was calculated by subtracting the uptake by mock vector-transfected cells from the uptake by hOAT3-expressing HEK293T cells. The solid line represents the fitted line obtained by nonlinear

regression analysis as described in the *Materials and Methods* section. Each point represents the mean value, and error bars represent the SEM (n=3).

Figure 5 Comparison of the observed and calculated CL_R values of taurine and GCDCA-S.

The CL_R of taurine and GCDCA-S values were calculated using control values, K_i for OAT1 or OAT3, and the geometric mean of the unbound probenecid concentrations from time zero to 8 h. Three K_i values were used for calculation: in vitro K_i , $2 \times K_i$, and $0.5 \times K_i$.

Table 1 List of endogenous compounds of interest in the metabolomics analysis performed in healthy subjects treated with or without probenecid

Peak number	Identity	Retention time (min)	Protonated molecule (<i>m/z</i>)	Fold change (probenecid treated/control)		
				Plasma	Urine	Urine/plasma
1	Taurine	7.32	124.0073	0.9±0.3	0.2±0.1	0.3±0.1
2	U305 ^a	7.33	206.0100	1.0±0.1	0.3±0.2	0.2±0.2
3	U118 ^a	7.29	297.9724	0.9±0.3	0.2±0.2	0.2±0.2
4	GCDCA-S	4.28	528.2634	1.9±1.8	0.2±0.3	0.2±0.4
5	Inosine ^{a,b}	5.03	267.0731	5.6±2.0	0.8±0.5	0.13±0.08
6	U172 ^a	7.28	126.0031	1.0±0.3	0.13±0.06	0.13±0.08
7	U67 ^a	7.30	271.0036	1.0±0.3	0.12±0.07	0.12±0.06
8	U42 ^a	7.30	249.0216	1.0±0.4	0.06±0.04	0.06±0.04
9	U682 ^a	7.28	286.9776	0.9±0.3	0.04±0.05	0.04±0.05

^a structurally unidentified

^b suggested compound

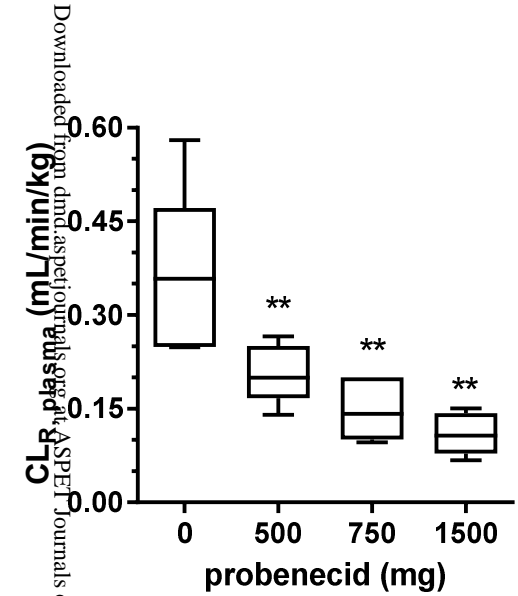
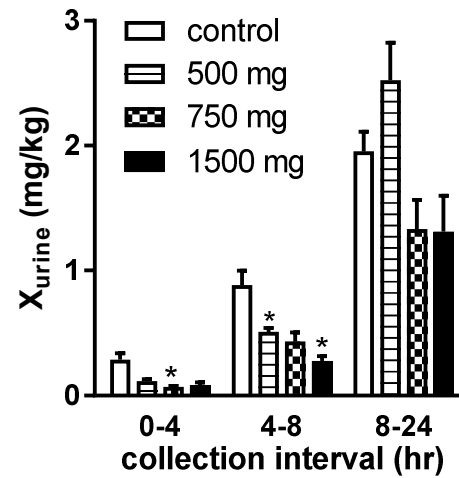
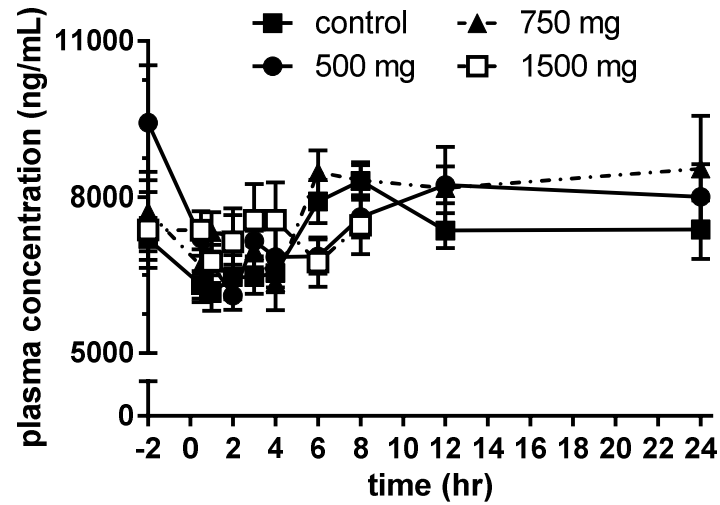
Table 2 Pharmacokinetic parameters of taurine and GCDCA-S in healthy subjects

Compound	Groups	AUC _p ($\mu\text{g/mL}\times\text{h}$)	X _{urine} (mg/kg)	CL _R (mL/min/kg)
Taurine	Control	53.1 \pm 2.6	1.17 \pm 0.15	0.372 \pm 0.054
	+500 mg probenecid	51.7 \pm 2.3	0.625 \pm 0.042**	0.204 \pm 0.019**
	+750 mg probenecid	52.2 \pm 2.6	0.502 \pm 0.076**	0.147 \pm 0.019**
	+1,500 mg probenecid	53.9 \pm 3.8	0.358 \pm 0.059**	0.109 \pm 0.014**
GCDCA-S	Control	1.79 \pm 0.29	4.80 \pm 1.06	0.0517 \pm 0.0140
	+500 mg probenecid	1.89 \pm 0.39	2.15 \pm 0.49*	0.0197 \pm 0.0029*
	+750 mg probenecid	1.79 \pm 0.17	1.17 \pm 0.20**	0.0110 \pm 0.0014**
	+1,500 mg probenecid	2.45 \pm 0.21	0.877 \pm 0.146**	0.00591 \pm 0.00067* *

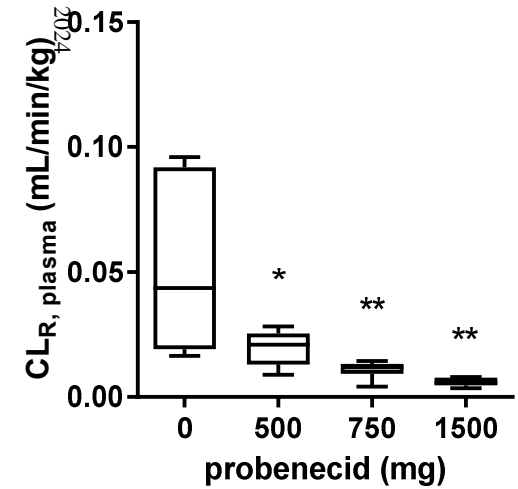
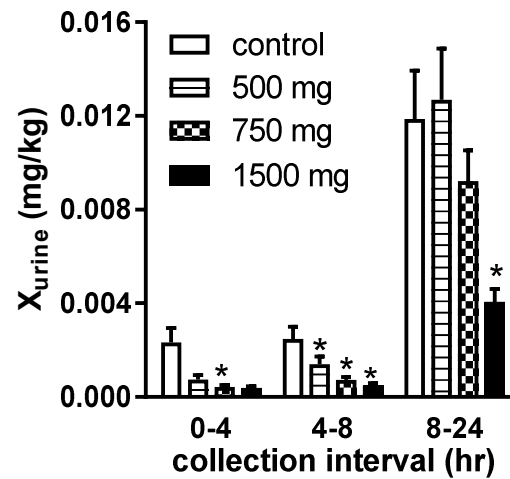
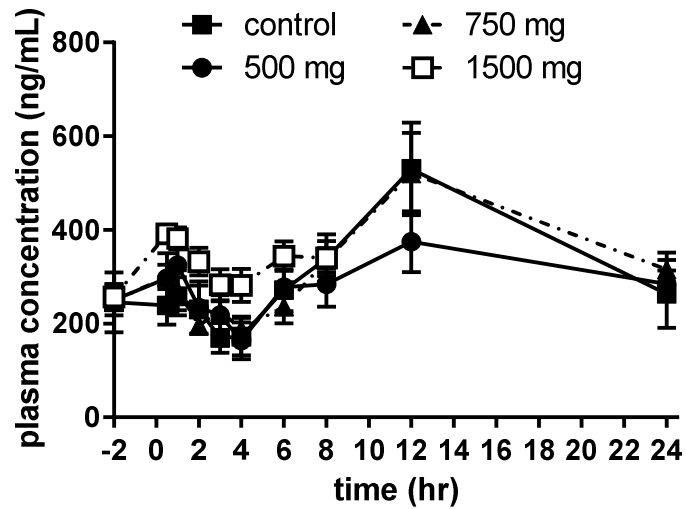
AUC_p of plasma concentration from time zero to 8 h was calculated using the trapezoidal rule. X_{urine} represents the urinary excretion from time zero to 8 h.

Figure 1

A taurine



B GCDCA-S



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Figure 2

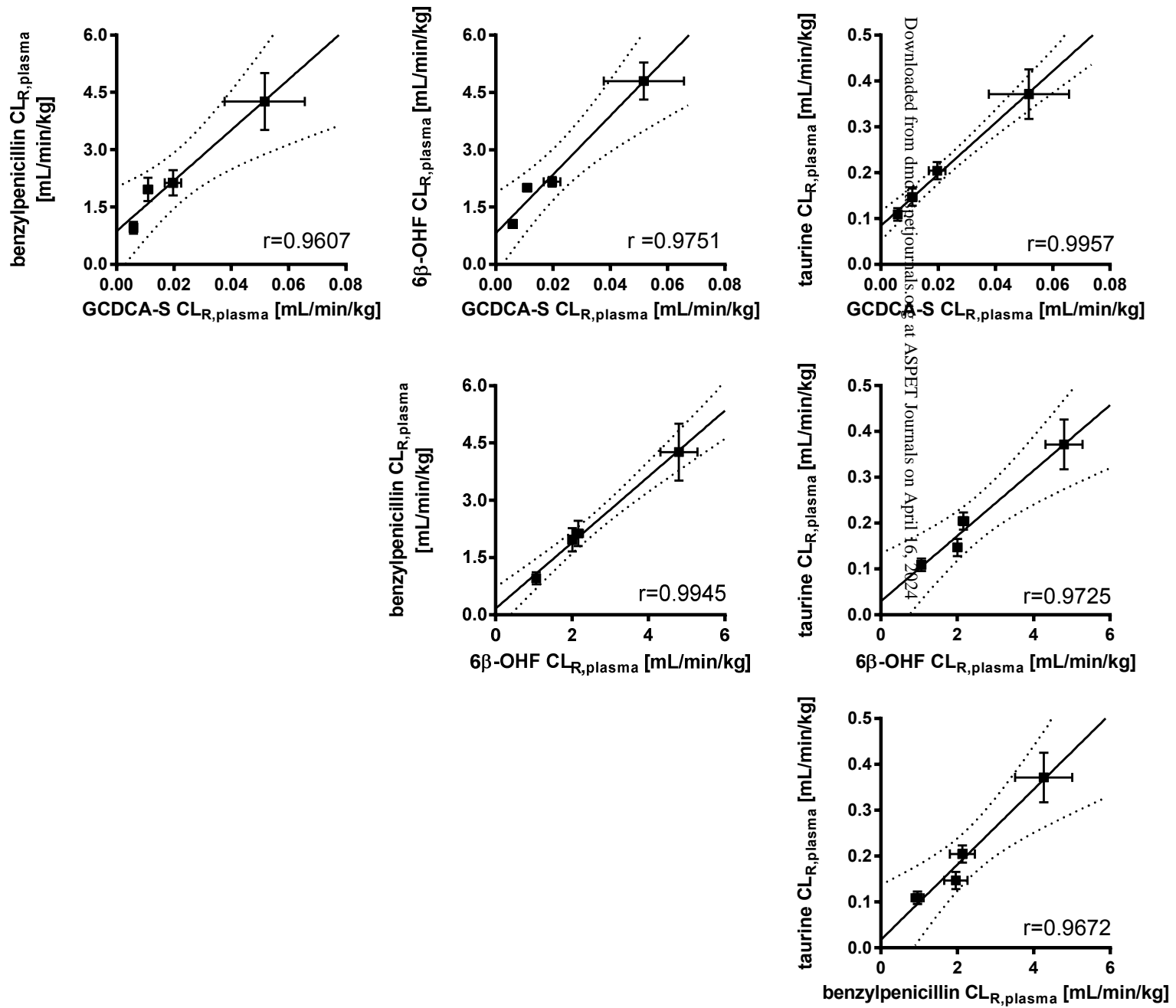


Figure 3

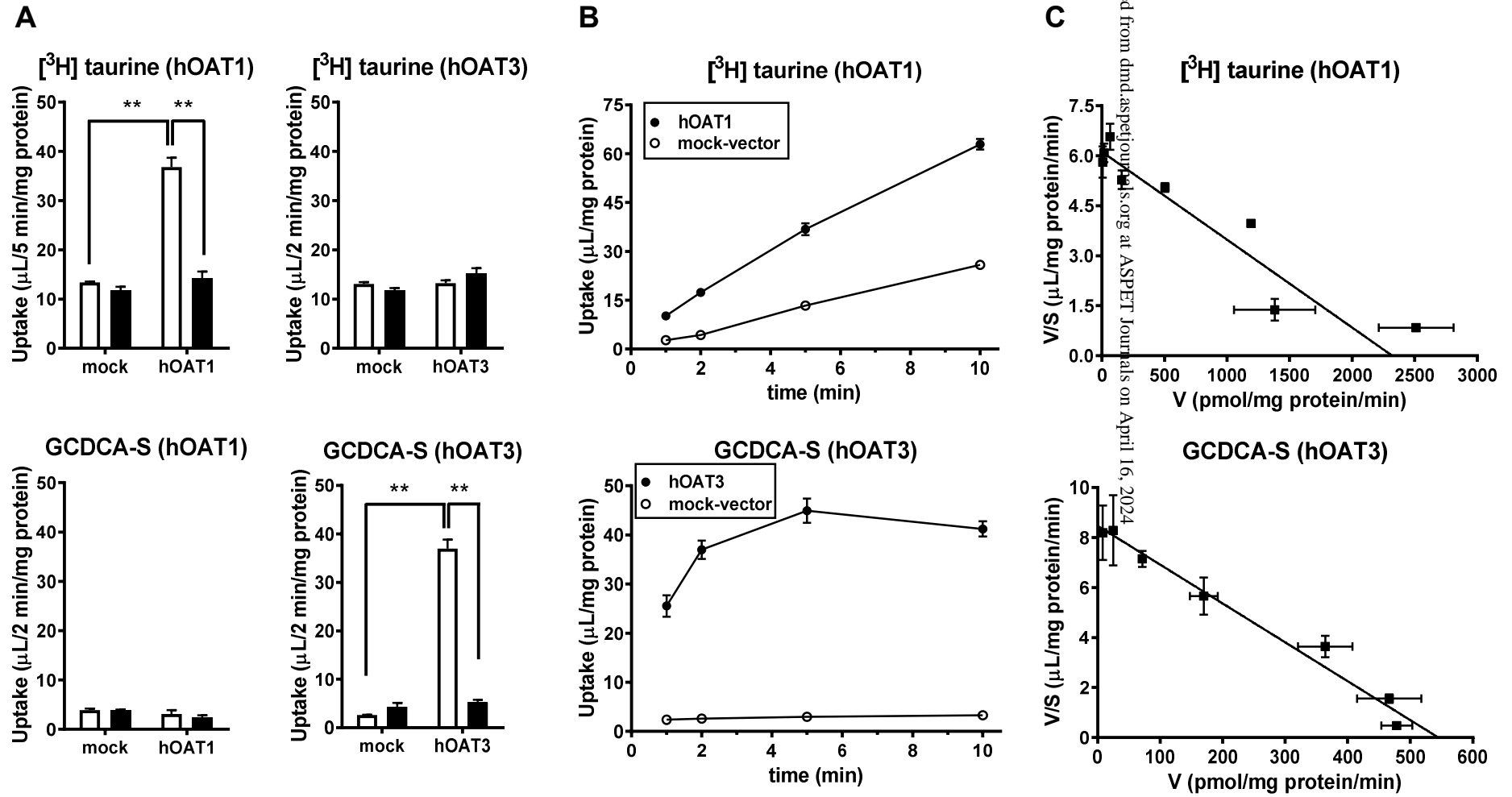


Figure 4

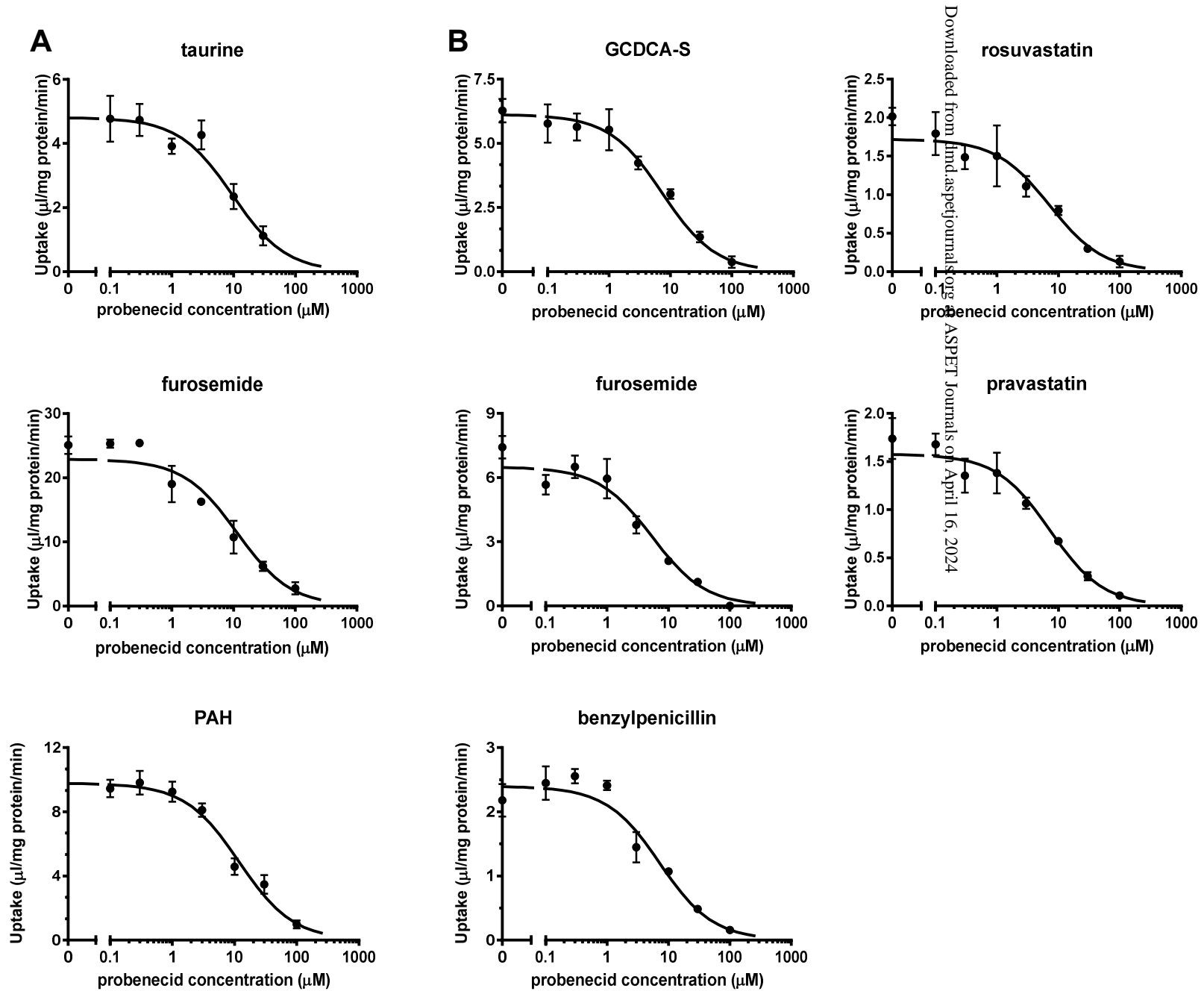
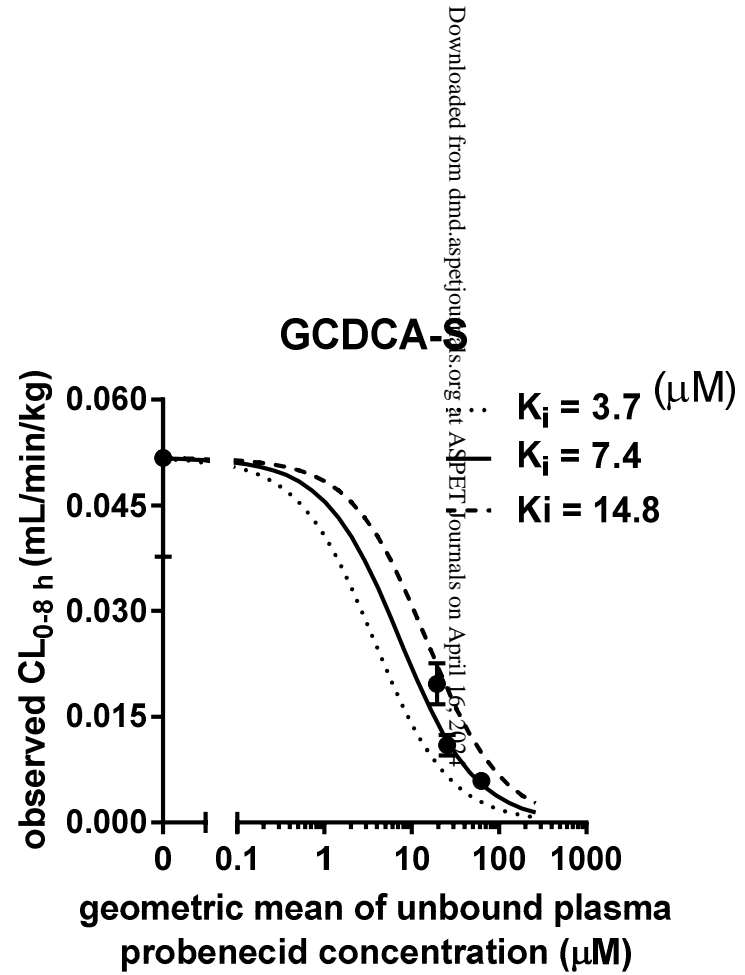
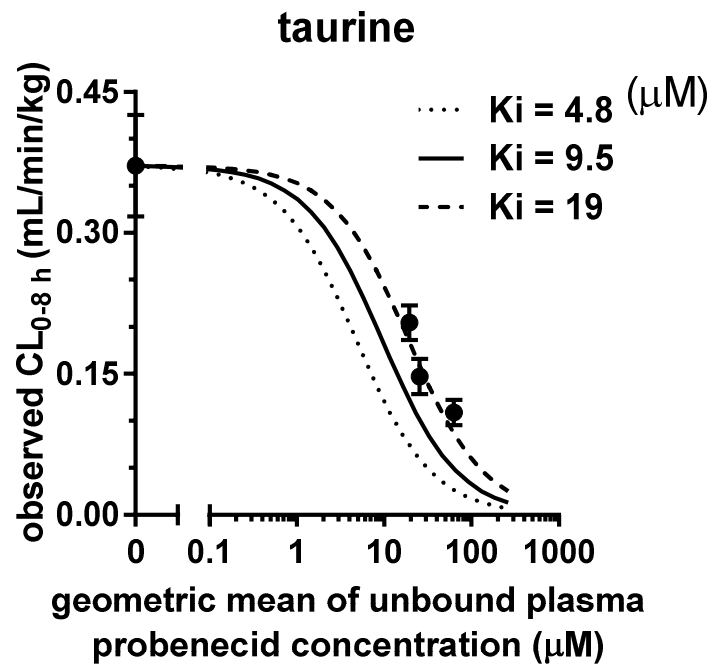


Figure 5



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