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Prediction of the Overall Renal Tubular Secretion and Hepatic Clearance of Anionic Drugs and a Renal Drug-Drug Interaction Involving Organic Anion Transporter 3 in Humans by In Vitro Uptake Experiments[S]

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ABSTRACT:

The present study investigated prediction of the overall renal tubular secretion and hepatic clearances of anionic drugs based on in vitro transport studies. The saturable uptake of eight drugs, most of which were OAT3 substrates (rosuvastatin, pravastatin, pitavastatin, valsartan, olmesartan, trichlormethiazide, *p*-aminohippurate, and benzylpenicillin) by freshly prepared human kidney slices underestimated the overall intrinsic clearance of the tubular secretion; therefore, a scaling factor of 10 was required for in vitro-in vivo extrapolation. We examined the effect of gemfibrozil and its metabolites, gemfibrozil glucuronide and the carboxylic metabolite, gemfibrozil M3, on pravastatin uptake by human kidney slices. The inhibition study using human kidney slices suggests that OAT3 plays a predominant role in the renal uptake of pravastatin. Comparison of unbound concentrations and K_i values (1.5, 9.1, and 4.0 μM, for gemfibrozil, gemfibrozil glucuronide, and gemfibrozil M3, respectively) suggests that the mechanism of the interaction is due mainly to inhibition by gemfibrozil and gemfibrozil glucuronide. Furthermore, extrapolation of saturable uptake by cryopreserved human hepatocytes predicts clearance comparable with the observed hepatic clearance although fluvastatin and rosuvastatin required a scaling factor of 11 and 6.9, respectively. This study suggests that in vitro uptake assays using human kidney slices and hepatocytes provide a good prediction of the overall tubular secretion and hepatic clearances of anionic drugs and renal drug-drug interactions. It is also recommended that in vitro-in vivo extrapolation be performed in animals to obtain more reliable prediction.

Introduction

Prediction of the pharmacokinetic properties of drugs in humans in the preclinical stages of drug development is very important to avoid failure in the subsequent clinical stages because of poor pharmacokinetic properties. The liver and kidney are the major systemic clearance organs for drugs in the body. Drug-metabolizing enzymes and transporters play significant roles in the renal and hepatic elimination of drugs from the systemic circulation, and, therefore, these activities are a critical factor determining systemic drug exposure. It is well accepted that, because of large species differences in drug metabolism, the metabolic clearance determined in animal studies cannot always be directly extrapolated to humans. In vitro systems, such as liver microsomes and hepatocytes, have been developed to replace animal studies and provide reliable predictions of the hepatic metabolic clearance of drugs (Obach, 1999; Stringer et al., 2008; Chiba et al., 2009; Kilford et al., 2009).

Animal scale-up has been widely used to predict the renal clearance of drugs in humans (Adolph, 1949; Boxenbaum, 1982). Renal elimination occurs in the glomeruli and proximal tubules where filtration and secretion occur, respectively. Animal scale-up is undoubtedly useful for drugs that are eliminated in the urine by glomerular filtration because the glomerular filtration rate depends on the molecular size and conforms to allometric scaling across species. However, Mahmood (1998) reported outliers for this prediction. These may occur because of species difference in the tubular secretion of drugs mediated by renal transporters. We reported a species difference in the

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ABBREVIATIONS: OAT, organic anion transporter; DDI, drug-drug interaction; PAH, *p*-aminohippurate; LC, liquid chromatography; MS, mass spectrometry; IVIVE, in vitro-in vivo extrapolation.

transport activity of the basolateral renal organic anion transporter OAT3 (Tahara et al., 2005a) and in the contribution of organic anion and cation transporters to the renal uptake of H2 blockers (Tahara et al., 2005b). Therefore, an approach based on in vitro studies with a human-derived in vitro system will probably provide more reliable predictions of the renal clearance of drugs in humans.

Tubular secretion of organic anions involves uptake and subsequent efflux into the urine. In a recent study, we demonstrated that the renal uptake clearance of anionic drugs is close to the tubular secretion clearance in rats (Watanabe et al., 2009b). Hence, the uptake is the rate-determining process as observed in the hepatic elimination of statins (Watanabe et al., 2010a), indicating that in vitro-in vivo extrapolation of tissue uptake clearance could provide a reasonable estimate of renal clearance. Kidney slices have been used as an in vitro system to investigate the contribution of OAT1 and OAT3 to the net renal uptake of drugs in rats and humans (Hasegawa et al., 2003; Nozaki et al., 2007a). Although it was clear that the uptake clearance in rat kidney slices underestimates the in vivo renal clearance of drugs (Hasegawa et al., 2003), the introduction of a scaling factor improved the predictability (Watanabe et al., 2009b).

The present study evaluated the predictability of renal clearance of anionic drugs, statins, and sartans in freshly prepared human kidney slices as well as their hepatic clearance in cryopreserved hepatocytes. Human kidney slices have been used as an in vitro system to investigate the mechanisms of drug-drug interactions (DDIs) involving renal clearance (Nozaki et al., 2007b). The DDI between gemfibrozil and pravastatin, in which concomitant use of gemfibrozil reduced the renal clearance of pravastatin by approximately 40% (Kyrklund et al., 2003), is a unique transporter-based DDI; studies with human OAT-expressing systems suggest that the interaction involves inhibition of OAT3-mediated uptake of pravastatin not only by gemfibrozil but also by its metabolites (Nakagomi-Hagihara et al., 2007). In the present study, we investigated the mechanism of the DDI between pravastatin and gemfibrozil using human kidney slices.

Materials and Methods

Materials. [^{3}H]Pravastatin (44.5 Ci/mmol) and [^{3}H]olmesartan (79 Ci/mmol) and unlabeled pravastatin and olmesartan were kindly donated by Daiichi-Sankyo Co. (Tokyo, Japan). [^{3}H]Valsartan (81.0 Ci/mmol) and unlabeled valsartan were kindly donated by Novartis Pharma (Basel, Switzerland). [^{3}H]Pitavastatin (16 Ci/mmol) was kindly donated by Kowa Co. (Tokyo, Japan), and [^{3}H]rosuvastatin (79 Ci/mmol) was kindly donated by AstraZeneca (London, UK). Unlabeled pitavastatin was synthesized by Nissan Chemical Industries (Chiba, Japan). Unlabeled rosuvastatin and candesartan were purchased from Toronto Research Chemicals (North York, ON, Canada). [^{3}H]*p*-Aminohippurate (PAH) (4.1 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{14}C]Benzylpenicillin (59 mCi/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, England). Unlabeled PAH, gemfibrozil, and trichlormethiazide were purchased from Sigma-Aldrich (St. Louis, MO). Unlabeled benzylpenicillin was purchased from Wako Pure Chemicals (Osaka, Japan). A carboxylic metabolite of gemfibrozil, gemfibrozil M3 (purity 99.6%), was chemically synthesized by KNC Laboratories, Co. Ltd. (Kobe, Japan) (Shitara et al., 2004). Gemfibrozil 1-*O*-*β*-glucuronide was biosynthesized with dog liver microsomes by XenoTech, LLC (Lenexa, KS), as described previously (Hirouchi et al., 2009). All other chemicals were of analytical grade and commercially available.

Preparation of Human Kidney Slices and Uptake of Organic Anions by Human Kidney Slices. This study protocol was approved by the ethics review boards at 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. Intact renal cortical tissues were obtained from surgically nephrectomized patients with renal cell carcinoma at Tokyo Women's Medical University. Samples of human kidney from subjects were stored in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) on ice immediately after kidney removal. After 30-min transportation, kidney slices were prepared as described below.

Studies of uptake by human kidney slices were performed as described in previous reports (Watanabe et al., 2009b). Kidney slices (300 μm thick) from intact human cortical tissue were kept in ice-cold buffer before use. The buffer 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. One slice, weighing 2 to 10 mg, was incubated at 37°C on a 12-well plate with 1 ml of oxygenated buffer containing drugs in each well after a 5-min preincubation at 37°C. After incubation for designated periods, the uptake was terminated by transferring the slice to ice-cold drug-free buffer followed by washing twice with ice-cold buffer. The wet weight of the slice was measured before solubilization. Concentrations of the test drugs in the uptake studies were as follows: 0.1 and 100 μM for rosuvastatin, pitavastatin, and valsartan; 0.1 and 500 μM for pravastatin; 0.01 and 100 μM for olmesartan; 0.1 and 1000 μM for PAH; 1 and 1000 μM for benzylpenicillin; and 10 and 1000 μM for trichlormethiazide. The trace concentrations of all the drugs in the uptake assays were much lower than the K_m (IC_{50}) values for the basolateral uptake transporters, and the excess concentrations were high enough to saturate the transporter-mediated uptake process. K_m values of olmesartan, PAH, and benzylpenicillin for the uptake by human kidney slices are 0.12, 31.1 to 47.8, and 13.9 to 89.9 μM, respectively (Nozaki et al., 2007a; Yamada et al., 2007). K_m values of rosuvastatin, pravastatin, and pitavastatin for human OAT3 are 7.4, 27.7, and 3.3 μM, respectively (Fujino et al., 2005; Nakagomi-Hagihara et al., 2007; Windass et al., 2007). The IC_{50} value of valsartan for OAT3 is 0.2 μM, and IC_{50} values of trichlormethiazide for OAT1 and OAT3 are 19.2 and 71.2 μM, respectively (Hasannejad et al., 2004; Sato et al., 2008).

The concentrations of pravastatin, pitavastatin, rosuvastatin, valsartan, olmesartan, benzylpenicillin, and PAH were determined by measuring their radioactivity. The slice was dissolved in 1 ml of Soluene-350 (Perkin Elmer Life and Analytical Sciences). The radioactivity in the scintillation cocktail (Hionic-Fluor; Perkin Elmer Life and Analytical Sciences) was determined by liquid scintillation counting (liquid scintillation counter LS6000SE; Beckman Coulter, Fullerton, CA). The concentrations of candesartan and trichlormethiazide were determined by LC-MS, as described below. Phosphate-buffered saline (100 μl) was added to the slices, followed by sonication to break them down, and then these samples were used for the measurement of drug concentrations by LC-MS.

Uptake Study Using Human Cryopreserved Hepatocytes. Cryopreserved human hepatocytes were purchased from XenoTech, LLC, the Research Institute for Liver Disease (Shanghai, China), and In Vitro Technologies (Baltimore, MD). The uptake study was performed using a rapid separation method as described previously (Watanabe et al., 2010a). In brief, the uptake reaction was initiated by addition of an equal volume of buffer containing drugs (final concentration: 0.1 and 100 μM for rosuvastatin and olmesartan; 0.1 and 500 μM for valsartan; and 5 and 100 μM for candesartan) to the hepatocyte suspension after a 3-min preincubation at 37°C. After a designated time, the reaction was terminated by separating the cells from the medium using a centrifugal filtration technique. For this purpose, a 100-μl aliquot of incubation mixture was placed in a 0.4-ml centrifuge tube (Sarstedt, Numbrecht, Germany) containing 50 μl of 2 N sodium hydroxide for radiolabeled compounds or 100 μl of 5 M ammonium acetate for unlabeled compounds under a 100-μl layer of an oil mixture (density 1.05, mixture of silicone oil and mineral oil; Sigma-Aldrich). Samples were then centrifuged for 10 s in a Microfuge (Beckman Coulter). During this process, the hepatocytes pass through the oil layer into the aqueous solution (2 N sodium hydroxide or 5 M ammonium acetate). For unlabeled compounds, tubes were frozen in liquid nitrogen immediately after centrifugation and stored at −20°C until drug measurement.

The concentrations of rosuvastatin, valsartan, and olmesartan were determined by measuring their radioactivity. After overnight incubation at room temperature to dissolve the cells in alkali, the centrifuge tube was cut, and each compartment was transferred to a scintillation vial. The compartment containing dissolved cells was neutralized with 50 μl of 2 N hydrochloric acid and mixed with scintillation cocktail (Clearsol II; Nakalai Tesque, Kyoto, Japan), and the radioactivity was determined in a liquid scintillation counter. The concentration of candesartan was determined by LC-MS. An aliquot was taken from the upper portion of the medium and quenched in methanol, and the cells were taken from the centrifuge tube

and sonicated in a new tube containing methanol to disintegrate them. The samples were vortexed and centrifuged, and supernatant fractions from both the medium and cell portions were analyzed by LC-MS as described below.

LC-MS Analysis. Protein was precipitated with 3 volumes of methanol and removed by centrifugation at 15,000*g* at 4°C for 10 min. The supernatant fractions were subjected to LC-MS. The appropriate standard curves were prepared in the equivalent blank matrix and used for each analysis. The analysis of candesartan was performed with an LCMS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan), whereas the analysis of trichlormethiazide was performed with an Alliance HT 2795 separation module with an autosampler (Waters, Milford, MA) and a Micromass ZQ mass spectrometer (Waters). Chromatographic separation was performed on a CAPCELL PAK C18 MGII column (3 μm, 3.0 × 50 mm; Shiseido, Tokyo, Japan) in gradient mode. The column temperature and flow rate were 40°C and 0.4 ml/min, respectively. For the analysis of candesartan, 0.05% (v/v) formic acid and acetonitrile were used as the mobile phase. The acetonitrile concentration was 25% at 0 min, 50% at 3 min, and 25% from 3.01 to 5 min. Candesartan was detected at *m/z* 441 in electrospray positive ionization mode. 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 and 150°C, respectively. For the analysis of trichlormethiazide, 0.05% (v/v) formic acid and acetonitrile were used as the mobile phase. The acetonitrile concentration was 5% at 0 min, 90% at 4 min, and 5% from 4.01 to 7 min. Trichlormethiazide was detected at *m/z* 378 in electrospray negative ionization mode. The desolvation temperature, capillary voltage, and cone voltage were 350°C, 3200 V, and 20 V, respectively.

Determination of Kinetic Parameters. The in vitro intrinsic uptake clearance was calculated by dividing the initial uptake velocity by the drug concentration in the incubation buffer. The initial uptake velocity of the drugs was calculated as the slope of the uptake volume at 0.5 and 1 min or 0.5 and 2 min in hepatocytes and at 15 min in kidney slices. Because of the limitation in the supply of the human kidney, the uptake was determined only at one point (15 min). The linearity of the uptake of five drugs (rosuvastatin, pravastatin, pitavastatin, valsartan, and olmesartan) up to 15 min was examined using one batch of kidney slices for each drug. Intrinsic uptake clearances were scaled up to the in vivo value per body weight using the following physiological scaling factors, 1.2×10^8 cells/g liver, 24.1 g liver/kg b.wt., and 4.43 g kidney/kg b.wt. (Davies and Morris, 1993).

In vivo hepatic and renal secretion overall intrinsic clearances ($CL_{h,\,int,\,all}$ and $CL_{sec,\,int,\,all}$, respectively), which represent the elimination of drugs from circulating blood, were calculated from eqs. 1 to 4 using a dispersion model, because a drug showing blood flow-limited clearance was included:

$$CL_H(\text{or } CL_{sec}) = Q \cdot (1 - F) \tag{1}$$

$$F = \frac{4a}{(1+a)^2 \cdot \exp\{(a-1)/2/D_N\} - (1-a)^2 \cdot \exp\{-(a+1)/2/D_N\}} \tag{2}$$

$$a = (1 + 4R_N \cdot D_N)^{1/2} \tag{3}$$

$$R_N = f_B \cdot \frac{CL_{h,int,all}(\text{or } CL_{sec,int,all})}{Q} \tag{4}$$

where CL_H, CL_{sec}, Q, D_N, and f_B represent the hepatic clearance, renal secretion clearance, organ blood flow rate, dispersion number, and blood unbound fraction, respectively. The blood flow rate in the liver was set at 20.7 ml · min^{-1} · kg^{-1} b.wt. and in the kidney at 15.7 ml · min^{-1} · kg^{-1} b.wt. (Davies and Morris, 1993), and D_N was set at 0.17 (Roberts and Rowland, 1986; Iwatsubo et al., 1996). CL_{sec} was estimated by subtracting f_B × glomerular filtration rate (GFR) from the observed in vivo renal clearance with regard to the blood concentration, assuming that only unbound drug was cleared from plasma (CL_R) (eq. 5):

$$CL_{sec} = CL_R - f_B \times GFR \tag{5}$$

Inhibition constants (K_i) for gemfibrozil-related compounds were calculated from eq. 6, which is applicable to both competitive and noncompetitive inhibition, provided the substrate concentration is well below its K_m value (Ito et al., 1998):

$$\Delta CL_{uptake(+inhibitor)} = \frac{\Delta CL_{uptake(control)}}{1 + I/K_i} \tag{6}$$

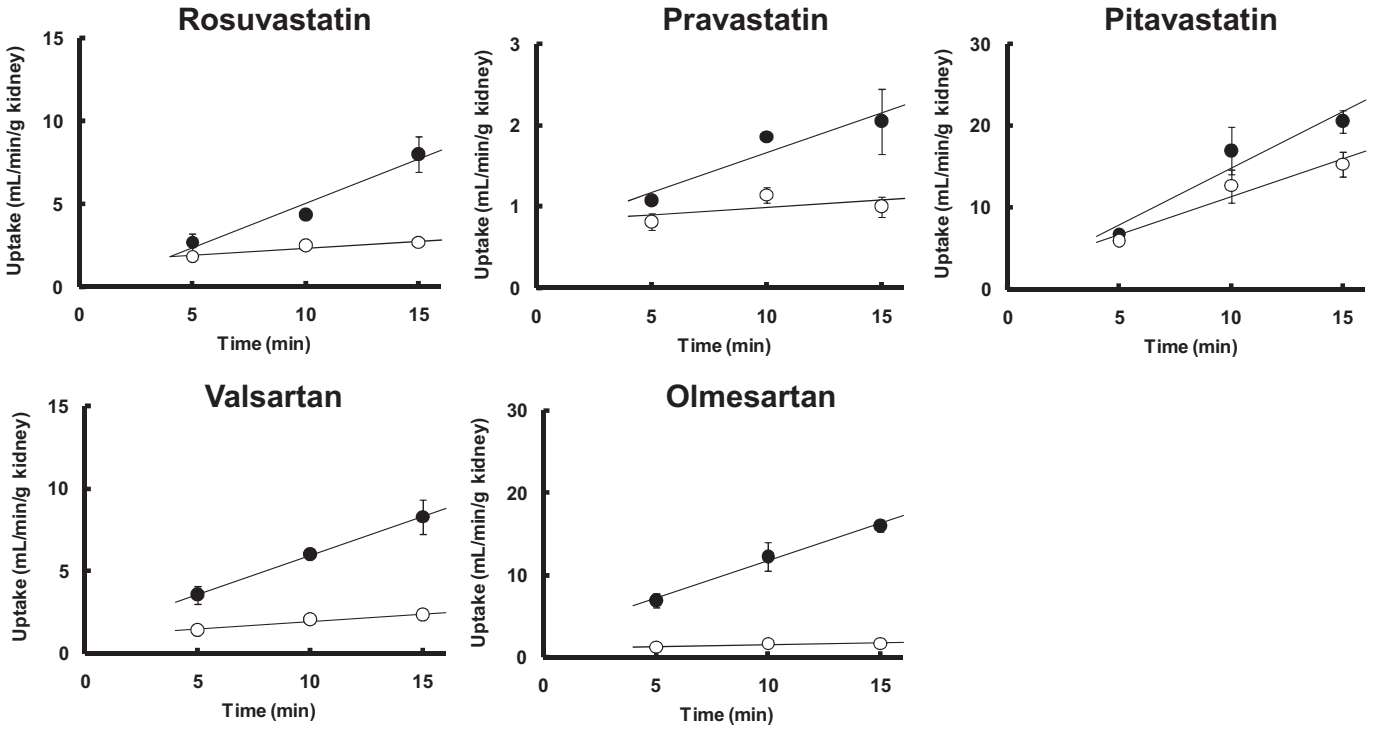

FIG. 1. Time profiles of the uptake of [^{3}H]rosuvastatin, [^{3}H]pravastatin, [^{3}H]pitavastatin, [^{3}H]valsartan, and [^{3}H]olmesartan by human kidney slices. The uptake of these drugs was determined at two concentrations at 37°C. Final concentrations of the drugs were as follows: 0.1 and 100 μM for rosuvastatin, pitavastatin, and valsartan; 0.1 and 500 μM for pravastatin; and 0.01 and 100 μM for olmesartan. Each point represents the mean ± S.E. (n = 3 slices from one kidney).

TABLE 1

Uptake clearance of drugs by human kidney slices

Uptake clearance of drugs by human kidney slices was determined at 37°C at two (trace and excess) concentrations. Final concentrations of the drugs were as follows: 0.1 and 100 μM for rosuvastatin, pitavastatin, and valsartan; 0.1 and 500 μM for pravastatin; 0.01 and 100 μM for olmesartan; 0.1 and 1000 μM for PAH; 1 and 1000 μM for benzylpenicillin; and 10 and 1000 μM for trichlormethiazide. The uptake clearance was calculated by dividing the uptake volume at 15 min by the drug concentration in the incubation buffer. Each value represents the mean ± S.E. (n = 3 slices). Benzylpenicillin was used as the standard compound throughout the study for normalization of the uptake of the test drugs by different batches of kidney slices.

	Batch No.	Uptake of Test Drug			Normalized Value[a]	$CL_{uptake, slice}$[b]
		Tracer (T)	Excess (E)	T − E		
		ml/g kidney/15 min				*ml · min^{-1} · kg^{-1}*
Rosuvastatin	1	8.02 ± 1.55	2.69 ± 0.06	5.33 ± 1.55	11.9	3.30
Pravastatin	2	2.86 ± 0.11	0.84 ± 0.05	2.02 ± 0.12	1.77 ± 0.13	
	3	2.62 ± 0.31	0.76 ± 0.04	1.86 ± 0.31	1.68 ± 0.38	0.447
	4	2.15 ± 0.10	0.71 ± 0.03	1.43 ± 0.10	1.71 ± 0.27	
	5	2.05 ± 0.40	1 ± 0.12	1.05 ± 0.42	1.29 ± 0.23	
Pitavastatin	6	20.6 ± 1.36	15.3 ± 1.52	5.27 ± 2.04	2.55	0.957
	8	15.3 ± 2.34	11.8 ± 1.04	3.50 ± 2.56	4.36 ± 0.24	
Valsartan	1	8.28 ± 1.06	2.34 ± 0.34	5.94 ± 1.11	13.3	2.38
	7	6.38 ± 1.88	3.34 ± 0.12	3.04 ± 1.89	3.88 ± 0.24	
Olmesartan	6	16.0 ± 0.72	1.67 ± 0.11	14.3 ± 0.73	6.93 ± 0.66	3.13
	7	13.9 ± 1.59	1.65 ± 0.04	12.2 ± 1.59	15.6 ± 0.76	
Trichlormethiazide	3	5.18 ± 0.79	2.12 ± 0.06	3.06 ± 0.79	2.77 ± 0.63	1.01
	4	6.72 ± 0.86	2.94 ± 0.33	3.78 ± 0.92	4.50 ± 0.71	
PAH	2	2.24 ± 0.31	0.55 ± 0.01	1.69 ± 0.31	1.48 ± 0.11	
	3	2.14 ± 0.22	0.60 ± 0.03	1.54 ± 0.22	1.40 ± 0.32	
	4	1.63 ± 0.17	0.63 ± 0.00	1.00 ± 0.17	1.19 ± 0.19	0.482
	5	2.98 ± 0.36	0.74 ± 0.32	2.24 ± 0.49	2.75 ± 0.49	
	6	4.98 ± 0.21	1.12 ± 0.06	3.86 ± 0.22	1.87 ± 0.18	
Fexofenadine[c]		2.60 ± 0.29	0.91 ± 0.21	1.68 ± 0.36	1.25 ± 0.19	0.347
Methotrexate[d]		1.26	0.51	0.75	0.61	0.168
Benzylpenicillin	1	1.56[e]	0.74[e]	0.82[e]		0.508
	2	2.70 ± 0.15	0.61 ± 0.02	2.09 ± 0.15		
	3	2.71 ± 0.46	0.69 ± 0.07	2.02 ± 0.46		
	4	2.21 ± 0.24	0.68 ± 0.00	1.54 ± 0.24	1.72[f] ± 0.09	
	5	2.00 ± 0.11	0.51 ± 0.24	1.49 ± 0.26		
	6	4.26 ± 0.27	0.46 ± 0.23	3.79 ± 0.36		
	7	2.17 ± 0.07	0.74 ± 0.02	1.43 ± 0.07		
	8	2.31[e]	0.84[e]	1.47[e]		

[a] Uptake of test drugs was corrected by the ratio of the saturable uptake of benzylpenicillin in the same batch of human kidney to the average value (1.83).
[b] Normalized uptake clearance were scaled up to the in vivo value per body weight using the following physiological scaling factor: 4.43 g kidney/kg b.wt. (Davies and Morris, 1993). Average values were used for the extrapolation for rosuvastatin, pravastatin, pitavastatin, valsartan, olmesartan, trichlormethiazide, and PAH.
[c] T − E of benzylpenicillin was 2.47 ml/g kidney/15 min (Matsushima et al., 2009).
[d] T − E of benzylpenicillin was 2.26 ml/g kidney/15 min (Nozaki et al., 2007b).
[e] Average value of two slices.
[f] Average value used for the normalization.

where ΔCL_{uptake} represents the CL_{uptake} for transporter-mediated uptake, which is the CL_{uptake} of radiolabeled pravastatin (0.1 μM) minus that measured in the presence of an excess of nonradiolabeled pravastatin (500 μM), $\Delta CL_{uptake\ (+inhibitor)}$ and $\Delta CL_{uptake\ (control)}$ are the ΔCL_{uptake} values estimated in the presence and absence of inhibitors, respectively, and I represents the inhibitor concentration. Data were fitted to eq. 6 by an iterative nonlinear least-squares method with use of the program MULTI (Yamaoka et al., 1981) to obtain the K_i value based on the nominal concentration, which was assumed to represent the free concentration. The input data were weighted as the reciprocal of the observed values and the algorithm for the fitting used the damping Gauss-Newton method.

The degree of inhibition of the renal tubular secretion clearance of pravastatin in humans was estimated by calculating the following R value, which represents the ratio of the renal tubular secretion clearance in the absence of inhibitor to that in its presence (eq. 7),

$$R = 1 + \frac{f_u \cdot C_{max}}{K_i} \quad (7)$$

where f_u represents the protein unbound fraction of the inhibitor in plasma and C_{max} represents the maximal plasma concentration of the inhibitor.

Results

Uptake Clearance of Anionic Drugs by Human Kidney Slices. Time profiles of the uptake of five drugs (rosuvastatin, pravastatin, pitavastatin, valsartan, and olmesartan) in human kidney slices are shown in Fig. 1. Uptake was linear for all the drugs up to 15 min at trace concentration. The uptake was saturated in the presence of an excess concentration of drugs, whereas the fraction of saturable com-

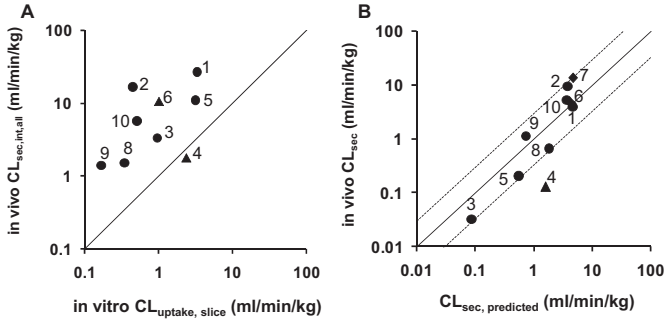

FIG. 2. Comparison between the uptake clearance by human kidney slices ($CL_{uptake, slice}$) and the observed renal secretion intrinsic clearance ($CL_{sec, int, all}$) (A) and between the predicted renal secretion clearance ($CL_{sec, predicted}$) and the observed renal secretion clearance (CL_{sec}) (B). $CL_{sec, int, all}$ was calculated on the basis of eqs. 1 to 4. $CL_{sec, predicted}$ was estimated from eqs. 1 to 4, assuming that $CL_{uptake, slice}$ was equal to the renal overall intrinsic clearance. Plots represent the following: 1, rosuvastatin; 2, pravastatin; 3, pitavastatin; 4, valsartan; 5, olmesartan; 6, trichlormethiazide; 7, PAH; 8, fexofenadine; 9, methotrexate; and 10, benzylpenicillin. The solid line and the dashed lines represent the line of unity and the lines of the 1:3 and 3:1 correlations, respectively. ● and ◆, drugs taken up into the kidney by mainly OAT3 and OAT1, respectively; ▲, drugs for which the dominant transporter is unclear.

TABLE 2

Kinetic parameters of drugs for hepatic and renal clearance

	$CL_{uptake,\ hep}$	$CL_{uptake,\ slice}$ [a]	f_u [b]	R_B [b]	f_B	$CL_{h,\ int,\ all}$ [b]	$CL_{sec,\ int,\ all}$ [b]	Scaling Factor [c]	CL_{sec} [b]
	$ml \cdot min^{-1} \cdot kg^{-1}$					$ml \cdot min^{-1} \cdot kg^{-1}$			$ml \cdot min^{-1} \cdot kg^{-1}$
Rosuvastatin	15.1	3.30	0.12	0.69	0.174	104	27.0	8.2	3.92
Pravastatin	13.8[d]	0.447	0.55	0.56	0.989	23.3	16.6	37	9.47
Pitavastatin	446[d]	0.957	0.0052	0.58	0.009	411	3.34	3.5	0.03
Valsartan	18.9	2.38	0.04	0.55	0.073	8.95	1.80	0.7	0.129
Olmesartan	12.1	3.13	0.01	0.55	0.018	18.6	11.1	3.5	0.204
Trichlormethiazide		1.01	0.29	0.55	0.527		10.7	11	4.54
PAH		0.482	0.83	0.70	1.19		N.A.	N.A.	13.6
Fexofenadine		0.347[e]	0.31	0.55	0.564		1.52	4.4	0.658
Methotrexate		0.168[f]	0.37	0.83	0.446		1.40	8.3	1.13
Benzylpenicillin		0.508	0.56	0.66	0.843		5.71	11	5.26
Candesartan	44.8		0.0027	0.55	0.005	67.2			
Atorvastatin	169[d]		0.0511	0.61	0.084	329			
Fluvastatin	351[d]		0.0037	0.52	0.007	3760			
Cerivastatin	746[g]		0.007	0.58	0.012	485			

$CL_{uptake,\ hep}$, uptake clearance by human hepatocytes; $CL_{uptake,\ slice}$, corrected saturable uptake clearance by human kidney slices; $CL_{h,\ int,\ all}$, observed hepatic overall intrinsic clearance; $CL_{sec,\ int,\ all}$, observed renal secretion overall intrinsic clearance; CL_{sec}, observed renal secretion clearance; N.A., not applicable.

[a] Saturable uptake clearance was corrected by uptake of benzylpenicillin. The details of this estimation are described in the text and Table 1.

[b] References cited are listed in Supplemental Table 1.

[c] Scaling factors were calculated by dividing $CL_{sec,\ int,\ all}$ by $CL_{uptake,\ slice}$.

[d] Data from Watanabe et al. (2010a).

[e] Data from Matsushima et al. (2009).

[f] Data from Nozaki et al. (2007b).

[g] Data from Shitara et al. (2003).

ponent of pitavastatin uptake at 100 μM was quite small. Table 1 shows the uptake clearance of trichlormethiazide, PAH, and benzylpenicillin as well as that of the five drugs in human kidney slices.

Comparison between In Vitro Uptake Clearance by Human Kidney Slices and In Vivo Renal Secretion Overall Intrinsic Clearance. Benzylpenicillin was used as the standard compound throughout the study for normalization of the uptake of the test drugs by different batches of kidney slices because OAT3 is predominantly involved in the renal uptake of benzylpenicillin (Nozaki et al., 2007a). The saturable uptake clearance of anionic drugs in human kidney slices was corrected by the ratio of the saturable uptake of benzylpenicillin in the same batch of human kidney to the average value to take into account the uptake by different batches of kidney slices (Table 1). This correction was based on our previous finding that the interbatch difference was very great; however, the uptake varied along with the mRNA expression of OAT3 (Nozaki et al., 2007a). The uptake of benzylpenicillin by the kidney slices ranged from 1.56 to 4.26 ml/g kidney/15 min in this study.

The corrected saturable uptake clearance was extrapolated to the in vivo value per body weight ($CL_{uptake,\ slice}$) using the physiological scaling factor, 4.43 g of kidney/kg b.wt. (Davies and Morris, 1993) (Table 1) and was compared with the in vivo renal secretion overall intrinsic clearance ($CL_{sec,\ int,\ all}$) (Fig. 2A). The uptake of methotrexate and fexofenadine by human kidney slices was taken from previous reports (Nozaki et al., 2007b; Matsushima et al., 2009). $CL_{uptake,\ slice}$ underestimated $CL_{sec,\ int,\ all}$, namely, $CL_{sec,\ int,\ all}$ was 10-fold greater than $CL_{uptake,\ slice}$ on average (Fig. 2A; Table 2). Because the renal clearance of PAH is close to the blood flow rate, no reliable intrinsic clearance can be estimated. Therefore, PAH was not included in Fig. 2A. The renal secretion clearance of the drugs was predicted from $CL_{uptake,\ slice}$ multiplied by the scaling factor of 10 using the dispersion model and was plotted against the observed renal secretion clearance (CL_{sec}) (Fig. 2B). The in vivo CL_{sec} of the test compounds including PAH, which has a blood flow-limited renal clearance, correlated with the uptake clearance by kidney slices. Because plasma protein binding of PAH is very low, $CL_{sec,\ predicted}$ of PAH was the highest of that of all the drugs even though it has moderate uptake clearance by kidney slices.

Effects of PAH, Benzylpenicillin, and Gemfibrozil and Its Metabolites on the Uptake of Pravastatin by Human Kidney Slices. The inhibitory effects of PAH and benzylpenicillin (selective inhibitors of human OAT1 and human OAT3, respectively) on the uptake of pravastatin by fresh human kidney slices were examined. PAH and benzylpenicillin inhibit both OAT1 and OAT3; however, for OAT1 substrates, PAH shows significant inhibition at lower concentrations than benzylpenicillin and vice versa for OAT3 substrates (Nozaki et al., 2007a). Both compounds inhibited the uptake of pravastatin by human kidney slices in a concentration-dependent manner and almost completely inhibited the saturable component of pravastatin uptake at 1 mM (Fig. 3). The IC_{50} value of benzylpenicillin was between 10 and 100 μM, which was lower than that of PAH (100 μM–1 mM).

The effect of gemfibrozil, gemfibrozil M3, and gemfibrozil glucuronide on the uptake of pravastatin (0.1 μM) by human kidney slices

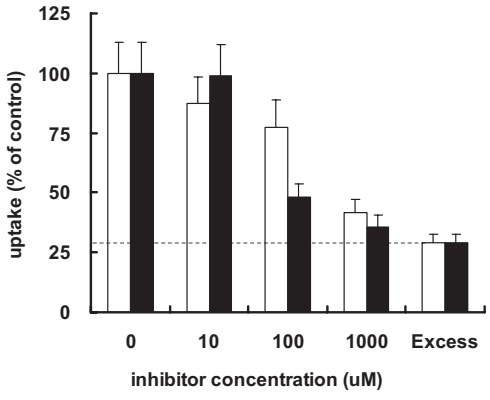

FIG. 3. Inhibitory effect of PAH and benzylpenicillin on the uptake of pravastatin by human kidney slices. The uptake of [^{3}H]pravastatin (0.1 μM) was determined in the presence and absence of unlabeled PAH (□) and benzylpenicillin (■) and in the presence of excess unlabeled pravastatin (500 μM) for 15 min at 37°C. The values are shown as a percentage of the uptake in the absence of inhibitors and an excess of pravastatin. Each value represents the mean ± S.E. (n = 6 slices). The absolute uptake clearance of pravastatin at 0.1 μM in the absence of inhibitors was 2.86 ± 0.11 ml/g kidney/15 min (mean ± S.E.).

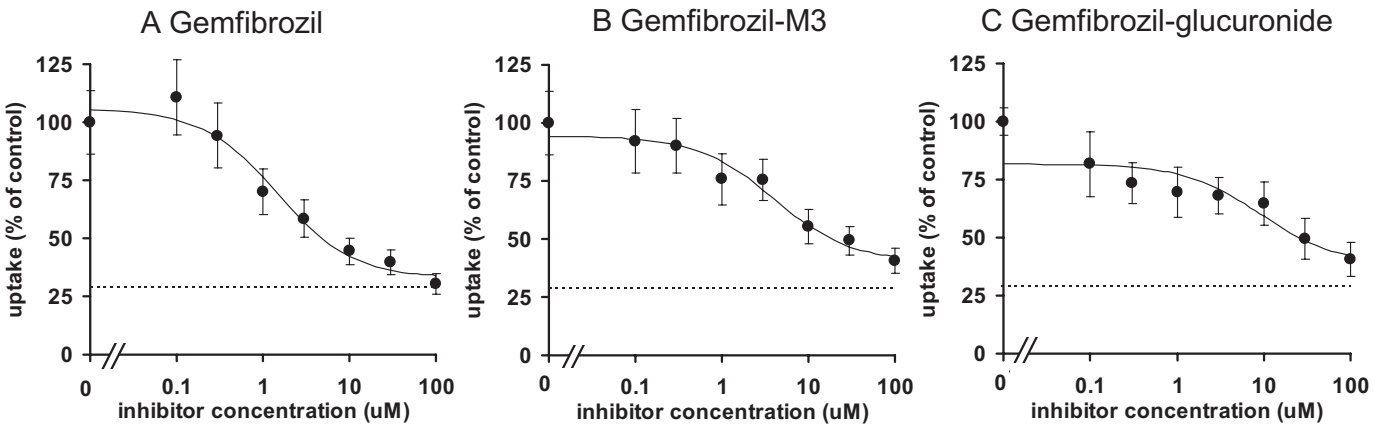

FIG. 4. Inhibitory effect of gemfibrozil and its metabolites, gemfibrozil M3 and gemfibrozil glucuronide, on the uptake of pravastatin by human kidney slices. The uptake of [^{3}H]pravastatin (0.1 μM) was determined in the presence and absence of unlabeled gemfibrozil (A), gemfibrozil M3 (B), and gemfibrozil glucuronide (C) for 15 min at 37°C. The values are shown as a percentage of the uptake in the absence of inhibitors. Each value represents the mean ± S.E. (n = 6 slices). The absolute uptake clearance of pravastatin at 0.1 μM in the absence of inhibitors was 2.86 ± 0.11 ml/g kidney/15 min (mean ± S.E.). Dashed lines represent the ratio of the uptake of [^{3}H]pravastatin determined in the presence of excess unlabeled pravastatin to that in the absence of excess pravastatin.

was examined (Fig. 4). Gemfibrozil and its two metabolites inhibited pravastatin uptake in a concentration-dependent manner. Because the concentration of pravastatin used in this inhibition study was 0.1 μM and was much lower than the K_m value for OAT3 (28 μM) (Nakagomi-Hagihara et al., 2007), the IC_{50} values of the inhibitors approximate their K_i values. The K_i values of gemfibrozil and its metabolites are summarized in Table 3. In consideration of the unbound concentration, gemfibrozil had the most potent effect on the renal uptake of pravastatin, whereas the metabolites produced a slight inhibition at the clinical dose of gemfibrozil (Table 3).

Comparison between In Vitro Uptake Clearance by Human Hepatocytes and In Vivo Hepatic Overall Intrinsic Clearance. The uptake clearance of rosuvastatin, valsartan, olmesartan, and candesartan by cryopreserved human hepatocytes was determined ($CL_{uptake, hep}$, microliters per minute per 10^6 cells) and found to be as follows: rosuvastatin, 5.21 ± 0.929 and 0.932 ± 1.32; valsartan, 6.53 ± 1.14 and 2.42 ± 0.642; olmesartan, 4.20 ± 0.120 and 2.07 ± 0.0333; and candesartan, 15.5 ± 0.743 and 1.98 ± 0.863 at trace and excess concentrations, respectively. The $CL_{uptake, hep}$ of each drug was considerably lower in the presence of an excess of the drug. $CL_{uptake, hep}$ at a trace concentration of drugs determined in this study as well as in previous studies (total of nine drugs) was scaled up to the in vivo value per body weight with the following physiological scaling factors: 1.2×10^8 cells/g liver and 24.1 g liver/kg b.wt. (Table 2). The $CL_{uptake, hep}$ values of the nine drugs were similar to or somewhat lower than in vivo hepatic overall intrinsic clearance ($CL_{h, int, all}$) (Fig. 5).

TABLE 3

Kinetic parameters of gemfibrozil-related compounds to predict the DDI between pravastatin and gemfibrozil

Inhibitory effect of gemfibrozil-related compounds on the uptake of pravastatin by human kidney slices was examined. K_i values were determined by nonlinear regression analysis and are represented as the mean ± S.D.

	K_i	C_{max}	f_u[a]	$C_{max, u}$	R
	μM	μM			
Gemfibrozil	1.47 ± 0.48	100–150[b,c]	0.00648	0.65–0.97	0.60–0.69
Gemfibrozil M3	3.97 ± 1.49	24[d]	0.0123	0.30	0.93
Gemfibrozil glucuronide	9.05 ± 8.43	20[c]	0.115	2.3	0.80

[a] Data from Shitara et al. (2004).
[b] Data from Backman et al. (2002).
[c] Data from Okerholm et al. (1976).
[d] Data from Nakagomi-Hagihara et al. (2007).

Discussion

We reported previously that hepatic uptake is the rate-determining process in the hepatic elimination of statins in rats and humans, and this probably holds true for the renal elimination of anionic drugs in rats although there are exceptions (Watanabe et al., 2009b, 2010a,b). These indicate the importance of measuring the uptake clearance for the prediction of hepatic and renal clearance based on in vitro experiments. The present study examined the predictability of renal and hepatic clearance using in vitro tools, such as freshly prepared human kidney slices and cryopreserved human hepatocytes.

The uptake of all drugs tested by kidney slices was saturable (Table 1). The clearance for the saturable uptake of the drugs by kidney slices ($CL_{uptake, slice}$) underestimated the in vivo intrinsic clearance for renal tubular secretion (Fig. 2A). Because the kidney slices consist of multilayered epithelial cells, limited diffusion into the slices from the incubation buffer may cause such an underestimation (Watanabe et al., 2009b). By introduction of the scaling factor of 10 for in vitro-in vivo extrapolation (IVIVE), the renal tubular secretion clearance of the test compounds including PAH correlated with the uptake clearance by kidney slices (Fig. 2B). As observed in rats (Watanabe et al., 2009b), these results suggest that the uptake is also rate-determining

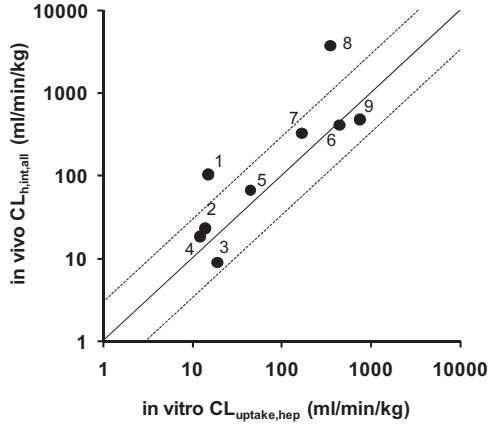

FIG. 5. Comparison between the uptake clearance by hepatocytes ($CL_{uptake, hep}$) and the observed hepatic overall intrinsic clearance ($CL_{h, int, all}$). Plots represent the following: 1, rosuvastatin; 2, pravastatin; 3, valsartan; 4, olmesartan; 5, candesartan; 6, pitavastatin; 7, atorvastatin; 8, fluvastatin; and 9, cerivastatin. The solid line and the dashed lines represent the line of unity and the lines of 1:3 and 3:1 correlations, respectively.

in the tubular secretion in the human kidney, and IVIVE will help to predict the renal clearance of anionic drugs. Among the drugs tested, valsartan was an outlier of the correlation for some unknown reason (Fig. 2), and it was also an outlier in rats (Watanabe et al., 2009b). We speculate that valsartan may undergo reabsorption from the urine to blood and/or significant basolateral efflux in the tubular secretion. It has been reported that the apical transporter, OAT4, interacts with valsartan; thus, OAT4 may mediate the reabsorption of valsartan in the kidney (Yamashita et al., 2006). Because of the large interbatch difference in the uptake of valsartan, further studies are necessary to elucidate the underlying mechanism. Most of the drugs used in the present study were reported to be substrates of human OAT3 but not of OAT1 (Fujino et al., 2005; Nakagomi-Hagihara et al., 2007; Windass et al., 2007; Yamada et al., 2007). PAH is the only OAT1-specific substrate in the present study, and, thus, further studies are necessary to elucidate the predictability of the tubular secretion of OAT1 substrates using kidney slices and to examine whether the scaling factor of 10 can be applied to OAT1 and OCT2 substrates.

Variations in the uptake activity in the major elimination organs greatly affect the systemic exposure (Kusuhara and Sugiyama, 2009). Inhibition of uptake has been reported as the underlying mechanism of DDIs in renal elimination (Tahara et al., 2006a,b; Nozaki et al., 2007b; Matsushima et al., 2009). In the present study, we investigated the mechanism of the DDI in the kidney between pravastatin and gemfibrozil using human kidney slices. The contribution of OAT-mediated uptake was examined using PAH and benzylpenicillin as inhibitors of OAT1 and OAT3. Benzylpenicillin was a more potent inhibitor of pravastatin uptake by human kidney slices than PAH (Fig. 3), suggesting that OAT3 is the main transporter (Nozaki et al., 2007a). This finding is consistent with a previous report showing that OAT3, but not OAT1, can accept pravastatin as a substrate (Nakagomi-Hagihara et al., 2007). Consistent with a previous report, not only gemfibrozil but also its metabolites, gemfibrozil M3 and gemfibrozil glucuronide, could inhibit the uptake of pravastatin by human kidney slices with K_i values similar to those for OAT3 (3.4, 2.7, and 9.9 μM) (Nakagomi-Hagihara et al., 2007) (Table 3). The unbound plasma maximum concentrations ($C_{max,u}$) of gemfibrozil and its glucuronide at clinical doses (600 mg twice a day) (Okerholm et al., 1976; Backman et al., 2002; Nakagomi-Hagihara et al., 2007) were enough to cause significant inhibition of pravastatin uptake (R values were 0.60–0.69 and 0.8, respectively), whereas that of gemfibrozil M3 was not high enough (R value was 0.93). Therefore, inhibition of OAT3 by gemfibrozil and gemfibrozil glucuronide could account for the DDI. It is worth mentioning that the inhibition by gemfibrozil glucuronide might appear to be biphasic (Fig. 4C), although such biphasic inhibition was not observed in OAT3-transfected cells (Nakagomi-Hagihara et al., 2007). Further studies are necessary to elucidate the mechanism underlying this discrepancy. Today, there is a growing interest in the safety of metabolites. The U.S. Food and Drug Administration has issued human metabolites in safety testing guidance to assure the safety of major drug metabolites. Caution must be paid to the incidence of DDIs caused by drug metabolites.

Taken together, our results show that freshly prepared kidney slices serve to predict renal clearance and DDIs quantitatively. Figure 5 shows the reliability of the prediction of hepatic clearance of anionic drugs using cryopreserved human hepatocytes in which the extrapolated hepatic uptake clearance of most anionic drugs was comparable with the overall hepatic intrinsic clearance. Rosuvastatin as well as fluvastatin was a outlier of the correlation (Fig. 5), and this result holds true in rats for an unknown reason (Watanabe et al., 2009b). As reported previously for fluvastatin (Watanabe et al., 2010), a rat scaling factor may be able to improve the prediction. However, because the hepatic elimination of rosuvastatin is blood flow-limited, the scaling factor could not be precisely obtained by rat studies. Taken together, in vitro uptake studies using human hepatocytes often lead to accurate prediction; however, it is recommended that an IVIVE in animal study be performed to obtain highly reliable prediction. In vitro transport experiments would be able to predict the elimination pathway of anionic drugs from the systemic circulation in the early stage of drug development.

In conclusion, the present study suggests that hepatic and renal clearances of anionic drugs are predictable on the basis of in vitro uptake studies using human cryopreserved hepatocytes and kidney slices. Moreover, kidney slices can be used for predicting clinically relevant DDIs caused by alterations in renal uptake activity. These in vitro experimental systems will be applicable to drug discovery and development studies for the prediction of organ clearances in the liver and kidney.

Authorship Contributions

Participated in research design: Ta. Watanabe, Kusuhara, To. Watanabe, Maeda, and Sugiyama.

Conducted experiments: Ta. Watanabe, To. Watanabe, Debori, Kondo, Nakayama, and Horita.

Contributed new reagents or analytic tools: Ogilvie, Parkinson, and Hu.

Performed data analysis: Ta. Watanabe, To. Watanabe, and Debori.

Wrote or contributed to the writing of the manuscript: Ta. Watanabe, Kusuhara, Ogilvie, Parkinson, and Sugiyama.

Other: Kusuhara and Sugiyama acquired funding for the research.

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