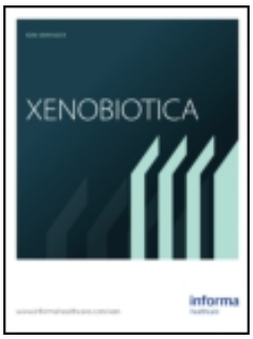

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RESEARCH ARTICLE

Use of uptake intrinsic clearance from attached rat hepatocytes to predict hepatic clearance for poorly permeable compounds

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Abstract

1. We previously reported that the accuracy of clearance (CL) prediction could be differentiated by permeability. CL was drastically under-predicted by *in vitro* metabolic intrinsic clearance (CL_{int}) for compounds with low permeability ($<5 \times 10^{-6}$ cm/s).
2. We determined apparent uptake CL_{int} by measuring initial disappearance from medium using attached rat hepatocytes and metabolic CL_{int} by measuring parent depletion in suspended rat hepatocytes (cells and medium).
3. Uptake and metabolic CL_{int} were comparable for highly permeable metabolic marker compounds. In contrast, uptake CL_{int} was 3- to 40-fold higher than metabolic CL_{int} for rosuvastatin, bosentan, and 15 proprietary compounds, which had low permeability, suggesting that uptake could be a rate-determining step in hepatic elimination for these poorly permeable compounds.
4. The prediction of hepatic CL was improved significantly when using uptake CL_{int} for the compounds with low permeability. The average fold error was 2.2 and 6, as opposed to >11 and >47 by metabolic CL_{int}, with and without applying a scaling factor of 4, respectively.
5. Uptake CL_{int} from attached hepatocytes can be used as an alternative approach to predict hepatic clearance and to understand the significance of hepatic uptake in elimination in an early drug discovery setting.

Keywords: Permeability, clearance prediction, OATP, scaling factor

Introduction

Successful prediction of *in vivo* clearance (CL) from *in vitro* metabolic intrinsic clearance (CL_{int}) has been reported for some compounds (Obach 1999; Ito & Houston 2004; Riley et al. 2005; Brown et al. 2007). However, it remains a challenge to accurately predict *in vivo* CL from *in vitro* data during drug discovery and development. Previously, we examined the relationship between passive permeability, efflux status, and predictability of rat CL from CL_{int} for a set of 41 proprietary compounds. We found that the accuracy of CL prediction from metabolic CL_{int} using rat liver microsomes or fresh rat hepatocytes varied drastically among the compounds tested, depending on their permeability and efflux status (Huang et al. 2010). For compounds that displayed high passive permeability and were not good substrates for efflux transporters, metabolic CL_{int} provided reasonably good prediction of *in vivo* CL. In contrast, CL was significantly under-predicted for transporter substrates, in particular, those with apparent permeability $<5 \times 10^{-6}$ cm/s. None of the compounds with low permeability was predicted within 3-fold error; the average fold error (AFE) was 54.4 and 29.2 for rat liver microsomes and fresh hepatocytes, respectively.

Several uptake transporters including organic anion transporting polypeptides (OATP), sodium taurocholate co-transport polypeptides, and organic anion and cation transporters are expressed in the basolateral membranes of hepatocytes (Chandra & Brouwer 2004). The impact of hepatic uptake transporters on drug disposition has

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been demonstrated using Oatp1a/1b knockout mice (van de Steeg et al. 2010). The absence of OATPs led to a significant increase in plasma concentration and drastic decrease in liver exposure of methotrexate, which exhibited poor passive permeability ($<1 \times 10^{-6}$ cm/s) (Xia et al. 2007). Liver to plasma concentration ratio was 43.1 and 0.33 in the wild-type and knockout mice, respectively, at 15 min following an intravenous bolus administration of methotrexate (van de Steeg et al. 2010). These data indicate that hepatic uptake could be a rate-determining step in hepatic elimination for compounds with poor permeability.

In order to provide better prediction of *in vivo* hepatic CL from *in vitro* data, the process of hepatic uptake must be taken into account for *in vitro* CL_{int} determination for compounds with poor permeability. Traditionally, hepatic uptake was determined by the oil centrifugation method (Petzinger & Fuckel 1992). Hepatocyte suspension is loaded over a silicone oil layer, which is overlaid on another layer of sodium hydroxide, and rapidly centrifuged. The layer of sodium hydroxide associated with cells is then collected and counted for radioactivity. A few years ago, Soars et al. (2007) published the "media loss" method by measuring disappearance of parent from the incubation medium after rapid centrifugation. Although the method does not require radiolabeled compounds, it still presents technical challenges to have reasonable throughput due to the centrifugation step. Furthermore, it is difficult to have accurate sampling time due to multiple transferring steps of aliquot and the centrifugation.

In this study, we determined apparent uptake CL_{int} by measuring initial depletion of parent from medium using attached rat hepatocytes. The step of separating hepatocytes from medium by rapid centrifugation was eliminated. Our aims were to (1) explore this method for assessing hepatic uptake CL_{int} using attached rat hepatocytes in an early drug discovery setting, (2) understand the significance of uptake in hepatic elimination for compounds with low permeability, and (3) compare clearance prediction between uptake CL_{int} from attached hepatocytes and metabolic CL_{int} from suspended hepatocytes for compounds with low permeability.

Materials and methods

Plasma from male Sprague-Dawley rats was purchased from Bioreclamation, Inc. (Westbury, NY). Type IV collagenase was purchased from Sigma-Aldrich (St Louis, MO). Phenol red-free Dubelco's Modified Eagle's Medium (DMEM), DMEM-GlutaMax, L-glutamine, 0.05% and 0.25% trypsin–1 mM ethylenediaminetetraacetic acid (EDTA), Hank's balanced salt solution (HBSS), and heat-inactivated fetal bovine serum (FBS) were purchased from Invitrogen Corp (Carlsbad, CA). Noncollagen-coated 24-well Transwells (0.4-μm pore size, 0.7 cm^2 surface area) were purchased from Millipore Corp (Billerica, MA). BioCoat collagen I six-well plates were obtained from BD bioscience (Bedford, MA). Bosentan was purchased from Bosche Scientific LLC (New Brunswick, NJ). ^{3}H-estrone sulfate and ^{3}H-taurocholate were purchased from PerkinElmer (Waltham, MA). Proprietary compounds were synthesized by the Department of Medicinal Chemistry at Amgen (Cambridge, MA). All other chemicals were purchased from Sigma-Aldrich. The Madin-Darby canine kidney cells were obtained from American-Type Culture Collection (ATCC, Manassas, VA).

Animals

All animal procedures were conducted under protocols approved by the Amgen Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 300–325 g were obtained from Charles River Laboratories (Wilmington, MA). For pharmacokinetic studies, rats were purchased from the vendor with catheters implanted in the femoral artery and vein. The rats were housed in a temperature- and humidity-controlled environment subject to a 12:12-hr light:dark cycle and had access to water and a standard laboratory rodent diet *ad libitum*. Animals were allowed to acclimate for 1 week prior to use.

Isolation of rat hepatocytes

Rat hepatocytes were isolated using the two-step collagenase method described by Seglen (1976), with some modifications. Briefly, the rat was euthanized via CO_2 asphyxiation and the liver was exposed, and the vena cava was cannulated caudal to the hepatic vein using PE-60 tubing with the portal vein bisected within 3 min. The liver was perfused with calcium-free-HBSS supplemented with HEPES, sodium bicarbonate, 1 mM EDTA, and 1% BSA until it was cleared of blood. The liver was perfused further with HBSS containing 0.05% type IV collagenase and 3 mM calcium chloride until extensive dissociation was evident. The liver was agitated gently to release cells from the capsule into ice-cold phenol-free-DMEM supplemented with 10% FBS and 2 mM glutamine. The cells were then filtered through sterile gauze and centrifuged at 50 *g* for 5 min at 4°C. The re-suspended cells were mixed with equal volume of Percoll in phosphate-buffered saline and centrifuged at 200 *g* for 10 min. The pellet was washed a final time and re-suspended in DMEM supplemented with 2 mM glutamine. The viability was determined using the trypan blue exclusion method, and only cell preparations with viability of greater than 85% were used.

Determination of apparent uptake and metabolic CL_{int} in rat hepatocytes

Uptake CL_{int} was determined in duplicate as the initial disappearance of parent from the incubation medium of attached rat hepatocytes. Briefly, freshly isolated rat hepatocytes were plated to collagen-coated six-well plates at 1 million cells/well in DMEM supplemented with 5% FBS.

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Plates were incubated in a 5% CO_2 humidified incubator at 37°C for ~2 h. Approximately, 90% of hepatocytes were attached when examined under microscope. DMEM (1 mL) containing test compounds was added to each well after the cells were washed three times with 2 mL of prewarmed DMEM to remove dead or unattached hepatocytes. At 0, 1, 3, 5, 10, and 15 min, aliquots of 20-μL medium were taken to a 96-well plate containing 100 μL of solvent (66% acetonitrile, 0.1% formic acid, and 33% water). The 0-min samples were usually taken 10–20 s after the compounds were added to each well. Apparent uptake CL_{int} was calculated from 0 to 5 min unless the loss of parent was <10% over 5 min; in this case, CL_{int} was determined from 0 to 15 min. For the inhibition studies, attached rat hepatocytes were pre-incubated with DMEM containing 0.1% dimethyl sulfoxide (control) or inhibitors for 15 min.

Metabolic CL_{int} was estimated in duplicate by measuring parent depletion in the incubations of suspended hepatocytes as described previously (Huang et al. 2010). Briefly, freshly isolated hepatocytes in suspension were dispensed into 24-well tissue culture plates at 0.5 million viable cells/well (0.8 mL/well) and were pre-incubated in a 5% CO_2 humidified incubator at 37°C for 15–30 min. Metabolism was initiated by addition of 200 μL of DMEM containing test compound (37°C, final incubation volume of 1 mL). The plates were incubated without shaking under 5% CO_2 at 37°C. At designated time points, the plates were shaken gently and aliquots (50 μL) were transferred to a 96-well plate, which contained 200 μL of acetonitrile with 0.1% formic acid per well. The 0-min samples were usually taken 1 min after the compounds were added to each well.

The samples were analyzed on the liquid chromatography tandem mass spectrometry method (LC-MS/MS) described below. For each compound, peak areas at each time point were converted to the natural log relative to the 0-min samples (Obach 1999). The resulting slope of these values relative to time (k) was converted to *in vitro* $t_{1/2}$, where $t_{1/2} = -0.693/k$. CL_{int} was calculated using the following relationship: $CL_{int} = (0.693/t_{1/2}) \times (1/M)$, where M is the concentration of hepatocytes in the incubation.

For the scaling of rat CL_{int}, values of 40 g liver/kg body weight (Davies & Morris 1993) and 120×10^6 hepatocytes/g liver (Naritomi et al. 2003) were assumed.

Determination of fraction unbound in rat plasma, hepatocyte suspension, and blood-to-plasma concentration ratio

Fraction unbound in rat plasma (fu_p) or in the incubations of suspended hepatocytes (fu_{inc}) was determined in duplicate by equilibrium dialysis, using the Rapid Equilibrium Dialysis device (Thermo Scientific, Rockford, IL). For plasma protein binding, plasma was spiked with test compounds at a final concentration of 5 μM. For hepatocyte binding experiments, the dead hepatocyte suspensions (0.5 million cells/mL) that underwent a freeze-thaw cycle were spiked with test compounds to achieve a final concentration of 1 μM. Plasma or hepatocyte suspension dialyzed against 1.7 volumes of plasma water (ultrafiltrate) or DMEM (hepatocyte) for 5 hr at 37°C. Preliminary experiments with selected compounds indicated that a 5-hr incubation time was sufficient to achieve equilibrium. For plasma protein binding, aliquots of the dialysate were transferred into an equal volume of blank plasma and extracted with five volumes of acetonitrile. Aliquots of the original spiked plasma were mixed with an equal volume of plasma and two volumes of plasma water (ultrafiltrate), and extracted with 10 volumes of acetonitrile. For hepatocyte binding, aliquots of the dialysate were transferred into an equal volume of blank hepatocyte suspension and extracted with two volumes of acetonitrile. Samples of the dialyzed hepatocyte suspensions were mixed with an equal volume of DMEM and extracted with two volumes of acetonitrile. All samples from the binding studies were centrifuged at 3500 *g* for 15 min and analyzed by LC-MS/MS as described below.

Blood-to-plasma concentration ratio (R_b) was determined in triplicate using the method described by Yu et al. (2005). Heparinized fresh rat whole blood was spiked with test compounds at a final concentration of 1 μM. The blood samples were processed for plasma after 1-hr incubation at 37°C. Reference plasma was also spiked with test compound to a final concentration of 1 μM. Samples were extracted with six volumes of acetonitrile containing an internal standard, centrifuged, and analyzed on the LC-MS/MS system described below. The blood-to-plasma concentration ratio was calculated by dividing the peak area ratio of the analyte over the internal standard observed in the reference plasma (representing nominal blood concentration) by the peak area ratio of the analyte over the internal standard observed in the treated plasma (representing plasma concentration).

Determination of passive permeability in MDCK cells

Passive permeability was determined in duplicate as the apparent permeability (Papp) in the apical to basolateral direction in MDCK cells as described previously (Huang et al. 2010). Briefly, compounds were tested at 5 μM in the presence of the known P-glycoprotein inhibitor GF120918 (2 μM), to eliminate the contribution of endogenous P-glycoprotein. Transport studies were conducted at 37°C in a humidified incubator with shaking (70 rpm) for 120 min. Samples were analyzed by LC-MS/MS as described below.

The apparent permeability values (Papp) were calculated according to the following equation (Huang et al. 2010).

$$\text{Papp} = (1/A * C_0) \times (dQ/dt)$$

Where A is the membrane surface area, C_0 is the donor drug concentration at t = 0, and dQ/dt is the amount of drug transported within a given time period.

Intravenous pharmacokinetic studies in male rats

Rats were administered a single bolus dose (the dose listed in Table 4) of test compounds (formulated in dimethyl sulfoxide) by intravenous injection into the femoral vein catheter ($n=2$ for compounds 3, 4, 6, 7, 8, and 9; $n=3$ for rosuvastatin, bosentan, compounds 1, 2, 5, 10, 11, 12, 13, 14 and 15; and $n=5$ for propranolol). The catheter was flushed with 50% propylene glycol in water or saline to ensure the full dose was given. Blood samples were collected from the femoral artery catheter at 0.033 (2 min, rosuvastatin, bosentan, compounds 1, 3, and 4), 0.05 (3 min, compounds 5, 6, 7, 8, 9, and 15), 0.083 (5 min, propranolol and compound 2), 0.117 (7 min, rosuvastatin, bosentan, compounds 1, 3, and 4), 0.13 (7.8 min, compounds 10, 11, 12, 13, and14), 0.25, 0.5 1, 2, 4, 6, 8, and 24-hr postdose. Plasma was separated from blood cells by centrifugation and stored at –80°C. For rosuvastatin, plasma samples were mixed 1:1 with 0.1 M (pH 4.0) sodium acetate buffer and stored at –80°C (Schneck et al. 2004). Plasma samples were extracted by the addition of acetonitrile containing 0.1% formic acid and an internal standard. Plasma samples were analyzed using the LC-MS/MS system described below.

Raw data were collected using PE/Sciex software Analyst 1.4. The area under the concentration versus time curve from time zero to infinity (AUC_{0-inf}) was calculated in Small Molecules Discovery Assay (SMDA) Watson® (version 7.0.01, InnaPhase Corp., Philadelphia, PA) using the linear/log trapezoidal method. CL was calculated in SMDA Watson according to the equation:

$$CL = Dose/AUC_{0-inf}$$

Prediction of hepatic clearance

Predicted CL_h was calculated according to the well-stirred model (Ito & Houston 2004) as follows:

$$CL_h = \frac{Q_h \times uCL_{int} \times fu_p / R_b}{Q_h + uCL_{int} \times fu_p / R_b}$$

$$CL_h = \frac{Q_h \times mCL_{int} \times fu_p / (funic \times R_b)}{Q_h + mCL_{int} \times fu_p / (funic \times R_b)}$$

where CL_h is hepatic blood clearance, uCL_{int} is the scaled uptake CL_{int} from attached hepatocytes, fu_p is the fraction of drug unbound in plasma, R_b is blood to plasma concentration ratio, mCL_{int} is the scaled metabolic CL_{int} from suspended hepatocytes, fu_{inc} is the fraction of unbound drug in the incubations of suspended hepatocytes, and Q_h is the hepatic blood flow in rats. Q_h ranging from 55.2 and 80 mL/min/kg has been reported (Davies & Morris 1993; Houston 1994) and was set to be 70 mL/min/kg (4.2 L/hr/kg) here (Soars et al. 2007). The overall accuracy of clearance prediction was determined by AFE as follows (Obach 1999):

$$AFE = 10^{\frac{\sum \log (predicted/actual)}{N}}$$

LC-MS/MS analysis

The LC-MS/MS system consisted of 2 LC-10AD HPLC pumps and a DGU-14A degasser (Shimadzu, Columbia, MD), a CTC PAL autoinjector (Leap Technologies, Carrboro, NC), and either an API3000 or API4000 mass spectrometer (Applied Biosystems, Foster City, CA), according to the requirements of the compound. For each analyte, the mass spectrometer electronics were tuned to the most intense mass transition.

Samples from *in vitro* experiments were injected onto a Sprite Armor C18 analytical column (20 × 2.1 mm, 10-μm pore size; Analytical Sales and Products, Pompton Plains, NJ) with a 0.5-μm polyetherketone guard filter. Analytes were separated using a gradient solvent system consisting of two components, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The percentage of solvent B was increased in a linear fashion from 2% to 95% over 2 min; flow rate was 0.4 mL/min.

Samples from *in vivo* studies were injected onto a Waters YMC ODS A column (20 × 2.0 mm, 5-μm pore size; Waters Corporation, Milford, MA) and separated using the mobile phase gradient described above.

Calculated physicochemical properties

Physicochemical descriptors of the 15 proprietary compounds were calculated using commercial software packages: Daylight (Daylight Chemical Information Systems, Aliso Viejo, CA) for polar surface area (PSA) and cLogD calculation, and Pipeline Pilot (Accelrys Software Inc, San Diego, CA) for calculating molecular weight and the number of hydrogen bond donors and acceptors.

Results

Compound depletion in attached and suspended rat hepatocytes

In the incubations of suspended hepatocytes, rosuvastatin and bosentan (0.1 μM) were stable. In contrast, rosuvastatin and bosentan were rapidly disappeared from the medium in the incubations of attached hepatocytes; approximately, 50%–60% of the compounds were depleted after 5 min. The loss of rosuvastatin was approximately linear for up to 5 min, whereas bosentan disappearance was linear up to 15 min. While verapamil (1 μM) was rapidly depleted, tolbutamide (1 μM) was stable in both attached and suspended incubations (Figure 1) .

Metabolic and uptake CL_{int} were comparable for the seven commonly used probe substrates of metabolism including verapamil, phenacetin, dextromethorphan, diclofenac, propranolol, tolbutamide, and 7-OH coumarin (Table 1). These metabolic marker compounds displayed high permeability (Table 1). Metabolic CL_{int} was corrected for hepatocyte binding, although their

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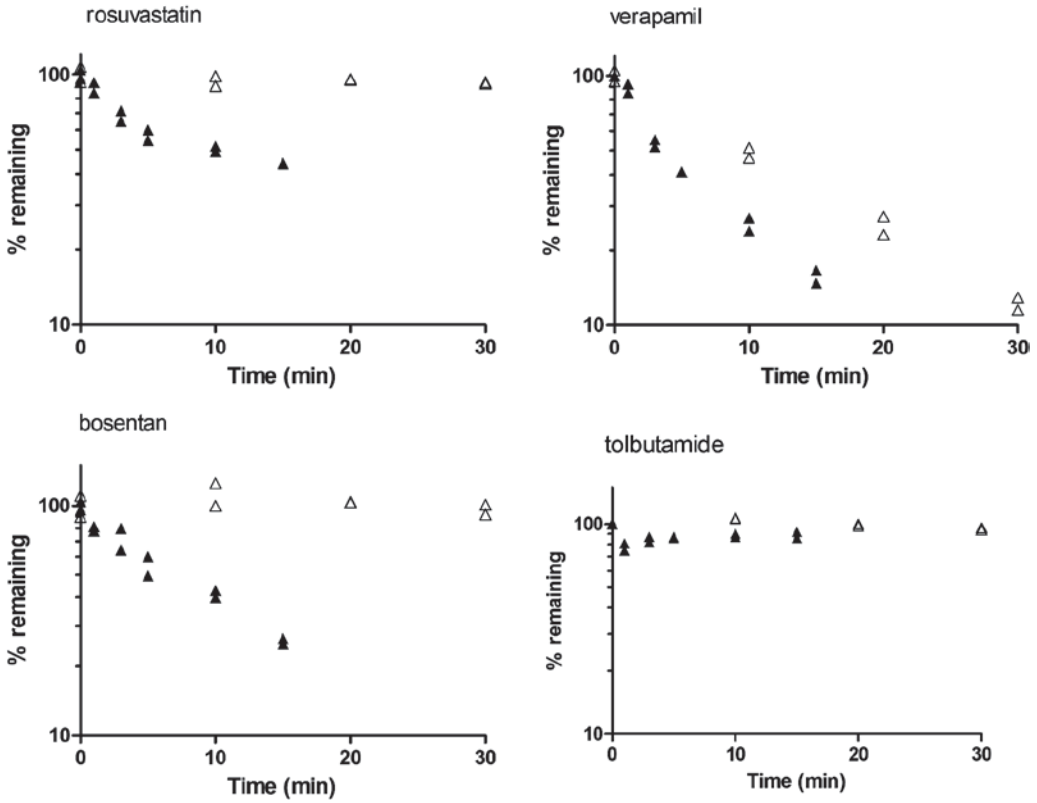

Figure 1. Compound depletion in the incubations of rat hepatocytes. Open triangles, suspended hepatocytes; solid triangles, attached hepatocytes.

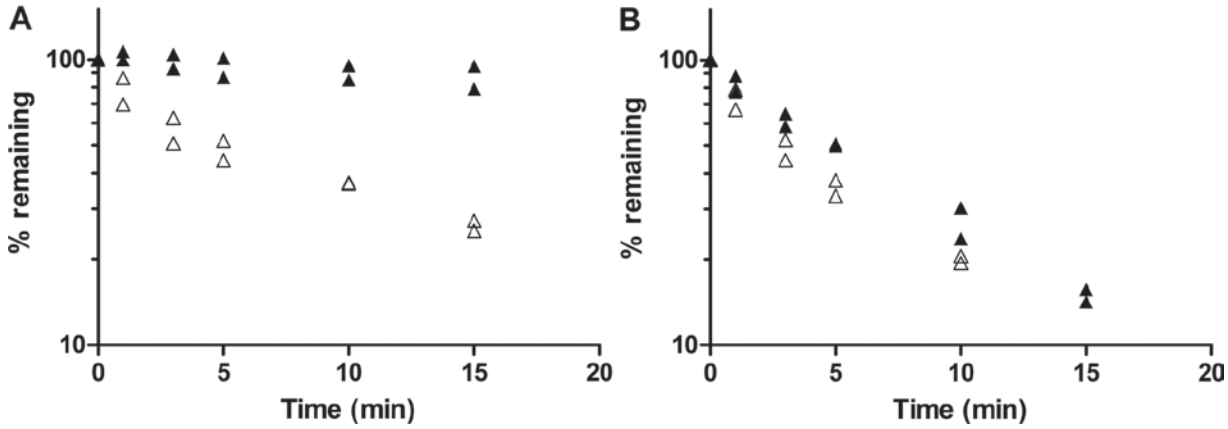

Figure 2. Effect of cyclosporine on the depletion of rosuvastatin (A) and verapamil (B) in the incubations of attached hepatocytes. Open triangles, control; solid triangles, 10 μM cyclosporine.

binding to hepatocytes was minimal (fu_{inc} ranged from 0.8 to 1).

Apparent uptake CL_{int} from attached rat hepatocytes ranged from 74 to 197 μL/min/million cells for bosentan, rosuvastatin, estrone-3-sulfate, and taurocholate, marker compounds of hepatic uptake transporters (Ho et al. 2006; Treiber et al. 2007; Kitamura et al. 2008) (Table 1). Uptake CL_{int} was 6- to 15-fold higher than unbound metabolic CL_{int} for rosuvastatin and bosentan (Table 1).

Effect of quinidine, rifampicin, and cyclosporine on rosuvastatin, bosentan, and verapamil uptake in attached hepatocytes

The ability of quinidine (100 μM), rifampicin (50 μM), and cyclosporine (10 μM) to inhibit uptake in rat hepatocytes was examined. Figure 2 showed rosuvastatin and verapamil depletion over time in the presence and absence of cyclosporine from a representative experiment. Rosuvastatin and bosentan uptake from 0 to 15 min was inhibited 70%–90% by cyclosporine and quinidine. While rifampicin

Table 1. Comparison between uptake and metabolic CL_{int} for marker compounds.

Compounds	CL_{int} (μL/min/million cells)			Papp (10^{-6} cm/s)
	Conc (μM)	Unbound metabolic	Uptake	
^{3}H-estrone sulfate	0.2	ND	197	ND
^{3}H-taurocholate	0.2	ND	188	ND
Bosentan	0.1	<6.3	97 ± 22[a]	3.6[c]
Bosentan	1	9	76	
Rosuvastatin	0.1	14	135 ± 13[a]	1.1
Rosuvastatin	1	19	112	
Dextromethorphan	1	202	217	36
Diclofenac	1	86	100	50[c]
Phenacetin	1	27	31	46
Propranolol	1	308	220	28
Tolbutamide	1	<5	<7.8	33[c]
Verapamil	1	151 ± 49[b]	190 ± 72[a]	24[c]
7-OH coumarin	1	218 ± 51[b]	176 ± 32[a]	46

ND, not determined, Papp, apparent permeability.
Values are the average of duplicate from a single experiment.
Metabolic and uptake CL_{int} were measured at the same day using the same batch of cells.
[a] Mean ± standard deviation from 6 to 11 experiments.
[b] Mean ± standard deviation from 3 to 4 experiments.
[c] Data taken from Huang et al. 2010.

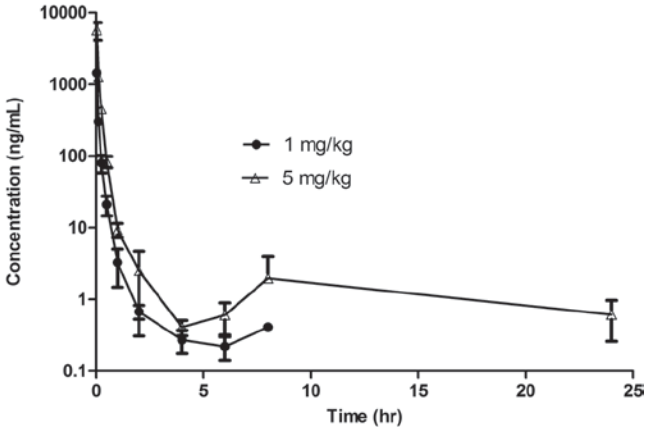

Figure 3. Plasma concentration–time profiles of rosuvastatin following a single intravenous bolus administration. Values are mean ± standard deviation, $n = 3$.

had a profound effect on bosentan uptake (~77% inhibition), its effect on rosuvastatin uptake was moderate (43% inhibition). The depletion of verapamil, a probe substrate of metabolism, was moderately affected by quinidine, but not by cyclosporine and rifampicin (Table 2).

Physical chemical properties, fu_p, R_b, fu_{inc} metabolic CL_{int}, and uptake CL_{int} of the 15 proprietary compounds

Most of the proprietary compounds tested were acids or acidic molecules with high molecular weight, cLogD ≥ 1, large PSA, 1–3 hydrogen bond donors, and 5–9 hydrogen bond acceptors (Table 3). In general, these compounds did not partition into rat blood cells extensively and were highly bound in rat plasma with fu_p ranging from 0.001 to 0.159. In the incubations of suspended hepatocytes, metabolic CL_{int} was low for the majority of the compound set and fu_{inc} ranged from 0.1 to 0.87. These compounds

Table 2. Effect of quinidine (100 μM), rifampicin (50 μM), and cyclosporine (10 μM) on rosuvastatin, bosentan, and verapamil depletion in attached rat hepatocytes.

Compounds	Inhibition (%)		
	Quinidine	Rifampicin	Cyclosporine
Bosentan	84 ± 13*	77 ± 16*	90 ± 8.8*
Rosuvastatin	74 ± 14*	43 ± 11*	82 ± 11*
Verapamil	42 ± 4.0*	2.2 ± 2.1	5.3 ± 3.5

Values are mean ± standard deviation, $n = 4$–6.
For the control, average loss from 0 to 15 min was 67%, 65%, and 91% for bosentan (0.2 μM), rosuvastatin (0.2 μM), and verapamil (1 μM), respectively.
* $p < 0.01$, unpaired t-test, two tailed.

were rapidly removed from the medium in attached hepatocytes. Uptake CL_{int} ranged from 84 μL/min/million cells for compound 14 to 210 μL/min/million cells for compound 3 (Table 4).

In vivo pharmacokinetics

In vivo pharmacokinetic studies were conducted for propranolol, rosuvastatin, bosentan, and the 15 proprietary compounds following a single intravenous bolus administration. Plasma CL was converted to blood CL using blood-to-plasma concentration ratios obtained *in vitro*. In general, these compounds showed high CL. Blood CL exceeded hepatic blood flow for 11 out of 18 compounds including propranolol and rosuvastatin (Table 4).

A number of compounds including rosuvastatin, compounds 1, 5, 9, 10, and 14 exhibited bi-exponential decline. Plasma concentrations of rosuvastatin declined rapidly initially followed by a slower decrease in drug concentration in the second phase (Figure 3). At the dose of 1 mg/kg, rosuvastatin concentration fell below 1 ng/mL, which was close to detection limit, after 1 hr. Rosuvastatin was further administered at 5 mg/kg in

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Table 3. Physical chemical properties of the proprietary compounds examined.

Compounds	Molecular weight	Number of acid	Number of base	cLogD (pH = 7.4)	PSA	Number of hydrogen bond acceptors	Number of hydrogen bond donors
1	504	0	1	NC	126	9	2
2	434	0	0	2.2	123	8	2
3	459	0	0	0.2	153	9	3
4	508	1	1	1.3	117	7	2
5	532	1	0	2.2	107	7	1
6	470	1	0	2.9	110	6	2
7	480	1	0	1.8	153	8	2
8	519	1	0	2.8	95	5	1
9	505	1	0	2.4	106	5	2
10	583	2	0	2.1	122	6	3
11	516	1	0	2.6	109	6	2
12	465	1	0	1.6	99	5	2
13	563	2	0	2.4	122	6	3
14	580	2	0	1.0	144	8	3
15	581	2	0	1.2	131	7	3

PSA, polar surface area.

Table 4. Summary of *in vivo* CL and *in vitro* data for the compounds examined.

	Rat *in vivo* CL			CL_{int} in hepatocytes (μL/min/million cells)[b]						Predicted hepatic CL (L/hr/kg)			
										Without a scaling factor		With a scaling factor of 4	
Compounds	Dose (mg/kg)	Blood CL (L/hr/kg)	Hepatic blood CL[a] (L/hr/kg)	Metabolic	Uptake	R_b	fu_p	fu_{inc}	Permeability (10^{-6} cm/s)	Metabolic	Uptake	Metabolic	Uptake
Propranolol	1.0	5.62	4.20	305	166	1.2[c]	0.093	0.88	28	2.72	1.97	3.70	3.27
Rosuvastatin	1.0	8.10	4.20	11	135	0.7[c]	0.078	0.81	1.1	0.39	2.13	1.23	3.38
Bosentan	1.0	0.88	0.88	<5	97	1.04	0.01	0.79	3.6[d]	<0.02	0.25	<0.07	0.86
1	2.0	4.88	4.20	<5	91	0.82	0.11	0.85	<1	<0.22	1.91	<0.75	3.23
2	0.25	4.74	4.20	45	129	0.69	0.025	0.73	3.1	0.56	1.02	1.60	2.36
3	0.5	1.35	1.35	24	210	0.80	0.009	0.60	1.8	0.13	0.59	0.46	1.65
4	0.5	11.67	4.20	<5	157	0.70	0.159	0.74	4.8	<0.4	2.98	<1.24	3.81
5	0.5	5.78	4.20	<5	176	0.59	0.022	0.72	3.2	<0.07	1.30	<0.28	2.70
6	0.5	1.66	1.66	<5	202	0.50	0.003	0.36	<1	<0.02	0.32	<0.09	1.05
7	0.5	0.46	0.46	<5	148	0.62	0.003	0.55	<1.5	<0.01	0.20	<0.05	0.69
8	0.5	5.94	4.20	<5	143	0.69	0.002	0.26	<1.7	<0.02	0.12	<0.06	0.43
9	0.5	6.42	4.20	<5	178	0.81	0.001	0.23	<2.7	<0.01	0.06	<0.03	0.24
10	2.0	0.66	0.66	<5	130	0.59	0.003	0.80	<1.4	<0.01	0.18	<0.04	0.64
11	5.0	4.81	4.20	<5	143	0.57	0.01	0.10	<2.2	0.24	0.62	0.24	1.71
12	5.0	4.64	4.20	12	165	0.58	0.004	0.32	<1.5	0.07	0.30	0.28	1.00
13	0.5	0.48	0.48	8.2	102	0.56	0.001	0.72	3	0.01	0.05	0.02	0.20
14	5.0	7.40	4.20	<5	84	0.57	0.013	0.87	<1	<0.04	0.49	<0.15	1.45
15	1.0	3.82	3.82	13	109	0.57	0.002	0.74	<1	0.02	0.11	0.07	0.40

[a] Hepatic CL = blood CL for compounds with CL < 4.2 L/hr/kg; hepatic CL = 4.2 L/hr/kg for those with blood CL ≥ 4.2 L/hr/kg.
[b] Obtained at 0.1 or 0.2 μM except propranolol (1 μM).
[c] Data taken from Berry et al. 2010.
[d] Data taken from Huang et al. 2010.

order to better characterize its terminal phase. Systemic exposure of rosuvastatin increased in proportion to the dose from 1 to 5 mg/kg, and CL exceeded hepatic blood flow for both dose groups.

Prediction of rat hepatic CL

To examine the accuracy of prediction, blood CL was considered to be hepatic CL for those compounds with CL less than hepatic blood flow, assuming no extrahepatic elimination. For compounds with blood CL exceeding hepatic blood flow, hepatic CL was set to be 4.2 L/hr/kg, the rat hepatic blood flow.

Predicted CL was calculated without and with a scaling factor of 4 for both uptake and metabolic CL_{int}. Hepatic CL of propranolol was well predicted using either metabolic or uptake CL_{int} from attached hepatocytes, regardless of the scaling factor.

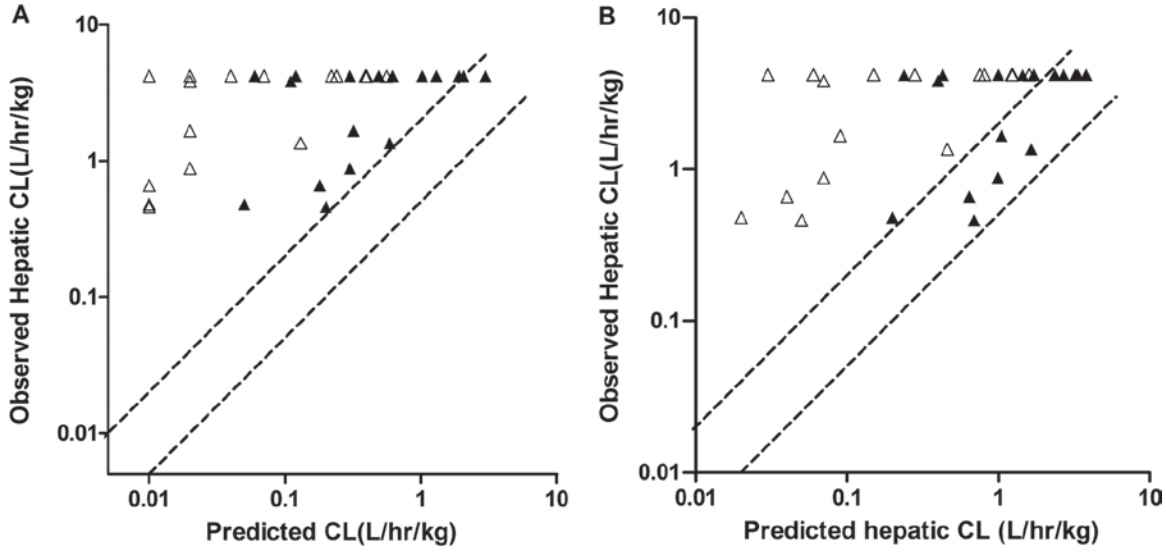

Figure 4. Observed versus predicted rat hepatic CL for rosuvastatin, bosentan, and the 15 proprietary compounds without (A) and with (B) a scaling factor of 4. Open triangles, predicted from metabolic CL_{int}; solid triangles, predicted from uptake CL_{int}. Dashed lines represent 2-fold boundaries.

For bosentan, rosuvastatin, and the 15 proprietary compounds that had low permeability, CL was drastically under-predicted by metabolic CL_{int} (Table 4, Figure 4). None of the compounds were predicted within 2-fold error even with the scaling factor of 4. The AFE was >47 and >13 without and with the scaling factor, respectively. Uptake CL_{int} obtained from attached hepatocytes significantly improved CL prediction; the AFE was 2.2 and 6 with and without applying the scaling factor. With the scaling factor, CL was predicted within 2-fold error for 59% of the compounds (10 out of 17) using uptake CL_{int}. However, compounds 8, 9, 11, 12, 13, 14, and 15, which were extremely highly bound in rat plasma ($fu_p \leq 0.01$ except compound 14, which had fu_p of 0.013), were still under-predicted by more than 2-fold with the scaling factor (Table 4, Figure 4).

Discussion

We routinely use *in vitro* metabolic CL_{int} for compound progression in drug discovery. However, metabolic CL_{int} often under-predicts *in vivo* CL, especially for compounds with permeability $<5 \times 10^{-6}$ cm/s (our internal cutoff for defining low permeability) (Huang et al. 2010). In order to understand the significance of hepatic uptake in the elimination of poorly permeable compounds, we determined apparent uptake CL_{int} by measuring initial disappearance of parent from medium using attached rat hepatocytes. This approach is consistent with the *in vivo* pharmacokinetic evaluation, in which systemic circulation is sampled. Our method eliminated the step of centrifugation, which made it easier to have accurate sampling time points and provided higher throughput compared with the traditional uptake assay using the oil centrifugation method (Petzinger & Fuckel 1992) and the medium loss method reported by Soars et al. (2007). The incubation medium of attached hepatocytes was presumably protein free since cells were washed thoroughly with protein-free medium and protein-free incubation medium was used. Compared with the traditional suspension method for CL_{int} determination, our method eliminated the need to measure fu_{inc}.

Apparent uptake CL_{int} reflects the processes of passive diffusion, transporter-mediated uptake, and metabolism. Highly permeable compounds are presumably to cross cellular membranes efficiently without the assistance of transporters. Since rapid equilibrium between incubation medium and intracellular compartments can be reached for compounds with high permeability, their apparent uptake CL_{int} would be mainly determined by metabolic clearance. As expected, apparent uptake CL_{int} into attached hepatocytes and metabolic CL_{int} in suspended hepatocytes were similar for the metabolic marker compounds examined, which displayed high passive permeability. Our data suggest that activities of metabolic enzymes were well maintained in attached hepatocytes after 2 to 3 hr culturing relative to fresh hepatocytes in suspension. Griffin and Houston (2005) reported much lower CL_{int} in attached hepatocytes than in suspended hepatocytes for several metabolic marker compounds including propranolol and dextromethorphan. The difference between their findings and ours may be attributed to the differences in experimental conditions such as incubation medium. Griffin and Houston used Williams' E medium containing 0.03% of BSA for suspended hepatocytes and a medium containing 5% fetal calf serum for attached hepatocytes. Protein binding could be a contributing factor to the apparent difference between attached and suspended hepatocytes and the authors did not correct CL_{int} for binding. In contrast, we used the same incubation medium (DMEM, without any protein) for both suspended and attached hepatocytes.

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Apparent uptake CL_{int} would be determined mainly by the process of active uptake for poorly permeable compounds such as rosuvastatin and bosentan. The mean uptake CL_{int} for rosuvastatin was 135 μL/min/million cells (corresponding to 16.2 mL/min/g liver using a scaling factor of 120 million cells/g liver), which was similar to the value previously reported by Nezasa et al. (2003) (17.1 mL/min/g liver, obtained by the traditional oil centrifugation method). Our values for rosuvastatin and bosentan were slightly higher than or comparable to those obtained by measuring appearance of parent into attached rat hepatocytes (Ménochet et al. 2011; 84.6 ± 9.2 μL/min/million cells for rosuvastatin and 77.8 ± 15 μL/min/million cells for bosentan), but different from the values reported by Yabe et al. (2011) using the oil centrifugation method (418 ± 319 μL/min/million cells for rosuvastatin and 18.7 ± 12.6 μL/min/million cells for bosentan).

Uptake of rosuvastatin and bosentan, known OATP substrates (Ho et al. 2006; Treiber et al. 2007; Kitamura et al. 2008), was reduced by OATP inhibitors cyclosporine, rifampicin, and quinidine (Cvetkovic et al. 1999; Hirano et al. 2006), although the extent of inhibition varied. Rifampicin only inhibited rosuvastatin uptake by ~40%, whereas bosentan uptake was reduced by 77% at the nominal concentration of 50 μM. The moderate inhibitory effect of rifampicin on rosuvastatin uptake could be: (1) transporters other than OATPs are involved in hepatic uptake of rosuvastatin; rosuvastatin has been identified as a substrate not only for OATPs, but organic anion transporter and sodium taurocholate co-transport polypeptides as well (Ho et al. 2006; Windass et al. 2007) and (2) rifampicin is a relatively specific OATP inhibitor. Co-administration of rifampicin increases plasma concentration and decreases liver concentration of methotrexate in wild-type mice, but it does not affect plasma and liver level of methotrexate in Oatp1a/1b knockout mice (van de Steeg et al. 2010). Verapamil showed high permeability and can rapidly get into hepatocytes by passive diffusion. Uptake CL_{int} of verapamil was not affected by cyclosporine and rifampicin, but by quinidine, which inhibits CYP2D6 and CYP 1A (Sai et al. 2000).

Proprietary compounds were selected from four different projects mainly based on their permeability ($<5 \times 10^{-6}$ cm/s) and structure diversity. Although the selected compounds were structurally different and hydrophobic (cLogD ≥ 1 except compound 1), they possess some physical chemical properties similar to typical OATP substrates such as statins. Most of them are acids or acidic molecules, have high PSA, and contain a relatively large number of hydrogen bond acceptors/donors. In general, the proprietary compounds were metabolically stable (metabolic CL_{int} < 5 μL/min/million cells), which was consistent with the trend described by Wu and Benet (2005) that poorly permeable compounds tend to be mainly excreted into bile and/or urine as parent. In contrast, these compounds were rapidly removed from the medium in the incubations of attached rat hepatocytes. The profound differences between uptake and metabolic CL_{int} suggest that these proprietary compounds are likely taken into rat hepatocytes by transporters, and hepatic uptake could be a rate-determining step in their elimination. Several of these proprietary compounds were further evaluated for OATP substrate status. Their uptake CL_{int} in attached rat hepatocytes was inhibited by OATP inhibitors and their uptake into the cells transfected with human *OATP1B1* gene was significantly higher than that into the control cells (data not shown).

In vivo CL exceeded hepatic blood flow for many of the compounds examined including propranolol and rosuvastatin. Pulmonary first-pass elimination could be a contributor to the high CL observed for propranolol (Iwamoto et al. 1987). However, the reasons for the CL exceeding hepatic blood flow are not well understood for the rest. The compound set should be stable in rat plasma since good recovery was observed in the experiments of fu_p determination, in which compounds were incubated with plasma for 5 h (data not shown). Tang et al. (2008) have suggested that sampling scheme can have a profound effect on the calculation of CL. The addition of a 2-min time point was reported to reduce CL by more than 2-fold. Therefore, a 2- or 3-min time point was added as the first sampling point for many of the compound set to minimize the potential under-estimation of systemic exposure. Initially, we thought that the inability to characterize terminal phase might contribute to the high CL obtained at low doses. Rosuvastatin CL still exceeded hepatic blood flow at 5 mg/kg, although its terminal phase was better characterized at the dose. CL of higher than hepatic blood flow (127 mL/min/mg, female Sprague-Dawley rats, 0.5 mg/kg) has also been reported for rosuvastatin (Watanabe et al. 2009a). Consistent with what has been reported (Nezasa et al. 2002), renal excretion was minimal (<1% of the dose) for rosuvastatin in our study (data not shown). Urinary excretion accounted for 14% of the dose for compound 1, but it was minimal for compounds 5 and 15; biliary excretion as parent was a major elimination pathway for compounds 1, 5, and 15 (data not shown). Further investigation is required to understand the reasons for the CL of greater than hepatic blood flow for the compounds examined.

Uptake CL_{int} obtained from hepatocytes has been used to predict hepatic CL (Soars et al. 2007; Watanabe et al. 2009a; Watanabe et al. 2010). Watanabe et al. (2009a) compared *in vitro* uptake CL_{int} from rat hepatocytes with *in vivo* uptake CL_{int} obtained from integration plot analyses for 12 compounds. Good correlation was observed for the seven compounds with low to moderate hepatic extraction ratio, although pravastatin, pitavastatin, rosuvastatin, temocaprilate, and benzyl penicillin, which had hepatic extraction of greater than 0.7, were excluded. The authors believed that they could not obtain accurate *in vivo* uptake CL_{int} for the compounds with such high hepatic extraction ratio. Watanabe et al. (2009b) and Poirier et al. (2009a, 2009b) incorporated *in vitro* uptake data obtained from hepatocytes into physiologically based pharmacokinetic modeling for pravastatin, valsartan, napsagatran, and

fexofenadine. A factor of 3.7–10 was applied to scale *in vitro* uptake CL_{int}, suggesting that a scaling factor may be required for *in vitro–in vivo* extrapolation of uptake CL_{int}. The need of a scaling factor for extrapolating uptake CL_{int} to *in vivo* could be due to: (1) the difference in the orientation of efflux transporters between *in vivo* and *in vitro*. *In vivo* efflux transporters are predominantly expressed on the canalicular membranes of hepatocytes; compounds can be continuously pumped into bile by efflux transporters after they are taken into hepatocytes. On the contrary, *in vitro* hepatocytes are not polarized and efflux transporters on the cell membrane may transport drugs back to the medium; (2) activities of uptake transporters *in vitro* may be decreased relative to *in vivo* due to the isolation and handling of hepatocytes.

For compounds with low permeability, uptake could be a rate-determining step in hepatic elimination. Similar to what has been reported (Soars et al. 2007; Watanabe et al. 2010), uptake CL_{int} provided better prediction than metabolic CL_{int} for rosuvastatin, bosentan, and the proprietary compounds. However, CL was generally under-predicted by uptake CL_{int} without a scaling factor (Figure 4A). Therefore, a scaling factor of 4, an approximated value of the scaling factor used for pravastatin (Watanabe et al. 2009b), was applied. With the scaling factor, CL were well predicted (<2-fold error) for 59% of the compounds using uptake CL_{int} (Figure 4B), although seven compounds were still under-predicted by more than 2-fold error. These seven compounds were extremely highly bound in rat plasma, which may complicate the ability to accurately determine their unbound fraction. The inaccuracy in fu_p measurement likely contributes to the under-prediction of these highly bound compounds. Another potential reason could be due to the limitation of using uptake CL_{int} for clearance prediction, which does not take the process of biliary efflux into account.

Conclusion

In summary, uptake CL_{int} from attached rat hepatocytes significantly improved CL prediction for the compounds with low permeability. Our method can be used as an alternative approach to predict hepatic clearance and to understand the significance of hepatic uptake in elimination in an early drug discovery setting.

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Declaration of interest

The authors report no declarations of interest.

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