Integrated Approach of In Vivo and In Vitro Evaluation of the Involvement of Hepatic Uptake Organic Anion Transporters in the Drug Disposition in Rats Using Rifampicin as an Inhibitor

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ABSTRACT

Cumulative studies describe the importance of drug transporters as one of the key determinants of pharmacokinetics that necessitate investigation and assessment of the involvement of drug transporters in drug discovery and development. The present study investigated an integrated in vivo and in vitro approach to determine the involvement of organic anion transporting polypeptides (Oatps) in the disposition of drugs in rats using rifampicin as an inhibitor. When bromosulphophthalein (BSP) and HMG-CoA reductase inhibitors (statins), which were used as model substrates for Oatps, were administered intravenously (3 and 1 mg/kg, respectively) to rats pretreated with rifampicin orally (30 mg/kg), the total plasma clearance of BSP and statins was attenuated compared with that in control rats, suggesting the involvement of Oatps in the disposition of these drugs in vivo. On the other hand, the pharmacokinetics of midazolam, used as a model substrate of cytochrome P450 3a (Cyp3a), was unchanged between control rats and rifampicin-pretreated rats. The involvement of Oatps in the disposition of statins observed in vivo was further clarified by employing an in vitro hepatic uptake study and media-loss assay in the presence or absence of 100 μM rifampicin. Hepatic intrinsic clearance was reduced in the presence of rifampicin in both the media-loss assay and hepatocyte uptake study. The present study suggests in vivo investigations in rats using rifampicin together with in vitro investigations with a media-loss assay and/or uptake assay using rat hepatocytes can help determine whether a clinical drug-drug interaction study is necessary in drug development.

Introduction

Drug transporters as well as cytochrome P450 (Cyp) enzymes are one of the key determinants governing the pharmacokinetics of drugs. Because many drugs undergo metabolism and biliary excretion after sinusoidal uptake into the liver, hepatic uptake processes play an important role in the hepatic clearance of drugs. To date, a large number of studies have revealed the importance of organic anion transporting polypeptides (Oatps) in the disposition of many drugs such as HMG-CoA reductase inhibitors (statins) and angiotensin-converting enzyme inhibitors (Kallikoski and Niemi, 2009). Besides Cyp enzymes, drug-drug interactions (DDI) via transporters are a critical concern in drug development, as recently accelerated drug development.

The aforementioned publications recommend that new chemical entities (NCEs) be evaluated as potential substrates and inhibitors of transporters, regarding the NCE as the “perpetrator” or “victim” of a DDI. In cases in which the NCE is a victim of a DDI via an Oatp, in addition to in vitro studies using Oatp gene-expressing systems, the involvement of active hepatic uptake in the disposition of the NCE must be clarified to determine whether a clinical DDI study is necessary. However, what studies should be undertaken has not been concretely described to determine the involvement of active uptake, especially regarding in vivo contributions.

Besides in vitro studies, in vivo studies using preclinical species on the contribution of transporters to the disposition of NCEs are thought to be very helpful for making decisions on the necessity of clinical DDI studies, especially when discussions on the necessity of a DDI study take place before detailed human pharmacokinetics data are available. Our present study investigates an approach to easily determine the involvement of Oatps in vivo and in vitro in rats using inhibitors of Oatps.

ABBR EV I AT I ONS: ABT, 1-aminobenzotriazole; afe, average fold error; AUCB, area under the plasma concentration curve; BSP, bromosulphophthalein; CIinp, intrinsic clearance; CIhet,hepatic uptake, intrinsic clearance in hepatocyte uptake assay; CIhet,media-loss assay, intrinsic clearance in media-loss assay; CImet,metabolic stability assay, intrinsic clearance in metabolic stability assay; CIperf,vivo, hepatic intrinsic clearance; CIperf,renal plasma clearance; Cypt, cytochrome P450; DDI, drug-drug interactions; fo, unbound fraction in blood; HBSS, Hanks’ balanced salt solution; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRT, mean residence time; NCE, new chemical entity; Oatp, organic anion transporting polypeptide; Rp, blood-to-plasma concentration ratio; SD, Sprague-Dawley; statin, HMG-CoA reductase inhibitor; T1/2,e, terminal half-life; Vd,e, apparent volume of disposition at equilibrium; Xurine, amount of drug into urine.
Approaches using inhibitor(s) that can effectively inhibit transporters but not Cyp enzymes are preferable for measuring the in vivo involvement of transporters; well-known in vivo inhibitors of Oatps include cyclosporin A and rifampicin (Lau et al., 2006; Kalogutkar et al., 2007; Shiota et al., 2009). However, comprehensive studies determining the involvement of Oatps in vivo using certain inhibitors are still lacking. Rifampicin, used in our present study, is a well-known potent inhibitor of human and rat Oatps (Vavricka et al., 2002; Ye et al., 2008). Because rifampicin exhibits prolonged exposure around Cmax in mice once administered intravenously (Zaher et al., 2008), continuous exposure exceeding the inhibition constants (Ki) for transporters is expected to be easily achieved, which is high enough to inhibit Oatps. Although rifampicin potently inhibits Oatps, it also inhibits Cyp enzymes with a Ki value of 18.5 μM for CYP3A4 (Kajosaari et al., 2005). Thus, a detailed dosing regimen must be planned to use rifampicin as an inhibitor for Oatps in vivo without affecting Cyp3a.

In vitro approaches using hepatocytes—namely, the media-loss and hepatocyte uptake assays—provide information about the involvement of transporters. Soars et al. (2007) developed the media-loss assay, which provides information about the involvement of transporters in combination with metabolism by examining loss from the media in a time-dependent manner. In contrast, the hepatocyte uptake assay can directly determine the uptake clearance of test substrates via passive diffusion and/or transporters. In principle, both methods provide information about the involvement of transporters by using transporter inhibitors.

In our present study, we constructed an integrated in vivo and in vitro approach to clarify the involvement of Oatps in the disposition of NCE in rats, using rifampicin as an inhibitor. In vivo, bromosulphophthalein (BSP) and midazolam were used as model substrates to optimize rifampicin dosing to inhibit Oatps but not Cyp3a. Furthermore, to validate the methodology of rifampicin as an inhibitor to determine the involvement of Oatps in vivo, pharmacokinetics studies of statins were conducted. In parallel, an in vitro approach using media-loss and hepatocyte uptake assays of statins was performed to confirm in vivo observations regarding the involvement of Oatps in drug disposition.

Materials and Methods

Materials. Rifampicin, BSP, and pravastatin were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam was purchased from Wako Pure Chemicals (Osaka, Japan). We purchased 1-aminobenzotriazole (ABT) from Tokyo Chemical Industry (Tokyo, Japan), and atorvastatin, fluvoxamine, and rosuvastatin from Toronto Research Chemicals (North York, ON, Canada). Pitavastatin was purchased from AK Scientific (Mountain View, CA). Cervinatidin was purchased from Sequoia Research Products (Oxford, UK). We obtained [3H]atorvastatin (10 Ci/mmol), [3H]pitavastatin (10 Ci/mmol), [3H]pravastatin (15 Ci/mmol), and [3H]rosuvastatin (10 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals and reagents were of analytic grade and are available from commercial sources.

Pharmacokinetics Studies in Rats. Animal care and in vivo procedures were conducted in accordance with the National Institutes of Health Guidelines for Laboratory Animal Welfare (Institute of Laboratory Animal Resources, NRC, 1996). The institutional animal care and use committee approved the protocols. Female Sprague-Dawley (SD) rats weighing 150–200 g obtained from Japan SLC (Shizuoka, Japan) were used for the experiments. All animals were fasted overnight before dosing, and water was provided ad libitum. In the experiments investigating the effects of rifampicin and ABT on the pharmacokinetics of BSP and midazolam, BSP or midazolam was administered to control, rifampicin-treated, and ABT-treated rats. Rifampicin was administered orally at 3, 30, or 100 mg/kg 1 hour before BSP or midazolam administration. ABT was administered intraperitoneally at 50 mg/kg 2 hours before midazolam administration. BSP was administered intravenously at 3 mg/kg via the jugular vein. Midazolam was administered orally at 10 mg/kg.

For the pharmacokinetics studies of statins, statins were administered intravenously at 1 mg/kg via the jugular vein to the control and rifampicin-pretreated rats. Rifampicin was given orally at 30 mg/kg 1 hour before statin administration.

To calculate renal plasma clearance, rats were housed individually in metabolic cages, and 24-hour urine samples were collected. Blood was drawn from the jugular vein in which test compounds were not administered, and plasma was generated by centrifugation at 10,000 g for 5 minutes. Plasma and urine samples were stored at −20°C until analysis.

Rat Hepatocyte Preparation. Rat hepatocytes were isolated according to the two-step in situ collagenase perfusion method. The hepatic portal vein of female SD rats was cannulated, and liver perfusion medium (Intravention, Burlington, ON, Canada) was perfused via the hepatic portal vein until the liver became clear for about 10 minutes. Liver digestion medium (Intravention) containing 200 mg/l of collagenase (Roche, Mannheim, Germany) was then perfused for another 5 minutes. The liver was subsequently dissected, filtered, and placed into hepatocyte wash buffer (Intravention). The cell suspension was centrifuged at 50g for 2 minutes at 4°C, the supematant was discarded, and the cells were then placed into the hepatocyte wash buffer. After repeating the centrifugation procedure, the resultant pellets were suspended in Hanks’ balanced salt solution (HBSS) containing 10 mM HEPES adjusted to pH 7.4.

Metabolic Stability Assay Using Rat Hepatocytes. A vial containing rat hepatocytes (8 × 10^6 cells/ml) in HBSS buffer containing HEPES (10 mM; pH 7.4) was preincubated for 5 minutes at 37°C along with a vial containing 2 μM test substrates and either dimethylsulfoxide (DMSO) or rifampicin (200 μM) in the HBSS buffer. The reaction was initiated by adding an equal volume of buffer-containing drugs to the hepatocyte suspension. Aliquots of 40 μl were removed at 0, 10, 20, 30, and 60 minutes. The reactions were terminated by adding the aliquots into the ice-cold acetonitrile containing 100 nM warfarin as an internal standard for subsequent liquid chromatography–tandem mass spectrometry (LC-MS/MS) measurements.

After centrifugation at 10,000g for 5 minutes, the supernatants were removed and analyzed by LC-MS/MS. Intrinsic clearance in the metabolic stability assay (CLint,metabolic stability assay) was determined as V × k, where V is the incubation volume and k is the elimination rate constant from the suspension of hepatocytes and medium. The drug disappearance rates in log concentration–time plots starting from 0–30 minutes for atorvastatin, pravastatin, rosuvastatin, and cerivastatin; 0–10 minutes for fluvoxamine; and 0–20 minutes for pitavastatin were used to estimate the elimination rate in the metabolic stability assay. Physiologic scaling factors of 1.2 × 10^6 cells/g liver and 40 g liver/kg were used to scale up to in vivo clearance values per unit body weight (Davies and Morris, 1993; Iwatsubo et al., 1997).

Media-Loss Assay Using Rat Hepatocytes. The media-loss assay was performed as described previously elsewhere with some modifications (Soars et al., 2007). After incubating the hepatocyte suspension with buffer-containing drugs as described previously, 80-μl aliquots were removed at 0.25, 1, 2, 5, 10, 20, 30, and 60 minutes and placed into centrifuge tubes. Aliquots were centrifuged at 2000g for 15 seconds, and 40 μl supernatant was used to scale up to in vivo clearance values per unit body weight (Davies and Morris, 1993; Iwatsubo et al., 1997).

Evaluation of the Involvement of Hepatic Uptake Transporters
designated time, the reaction was terminated by separating the cells from the substrate solution.

To examine the uptake of isotope-labeled compounds (atorvastatin, pitavastatin, pravastatin, and rosuvastatin), a 100-μl aliquot of reaction mixture was placed in a 0.4-ml centrifuge tube containing 50 μl 3 N KOH under a layer of 100 μl oil (density 1.015, a mixture of silicone oil and mineral oil; Sigma-Aldrich) and was centrifuged at 10,000g for 10 seconds using a tabletop centrifuge (Beckman Microfuge E; Beckman Coulter). During this process, hepatocytes passed through the oil layer into the alkaline solution. After incubation in alkaline solution to dissolve the hepatocytes, the centrifuge tube was cut, and each compartment was transferred to a scintillation vial. The compartment containing the dissolved cells was mixed with a scintillation cocktail, and the radioactivity was measured in a liquid scintillation counter.

To examine the uptake of non-isotope-labeled compounds, a 100-μl aliquot of incubation mixture was placed in a 0.4-ml centrifuge tube containing 50 μl 5 M ammonium acetate under a 100-μl layer of oil mixture, and the sample tubes were then centrifuged. The tubes were frozen in liquid nitrogen immediately after centrifugation and stored at −20°C until quantification. An aliquot was taken from the upper media portion and quenched in methanol, and immediately after centrifugation and stored at −20°C until quantification. An aliquot was taken from the upper media portion 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 supernatants from both the media and cell portions were analyzed by LC-MS/MS.

Intrinsic clearance in the hepatocyte uptake assay (CL_{int/hepatocyte uptake}) (μl per 10^6 cells per minute) was determined by calculating the slope of the uptake volume (V_u) (μl per 10^6 cells) between 0.5 and 1 minute using the following equation.

\[
CL_{int/hepatocyte \ uptake} = \frac{V_{u,1min} - V_{u,0.5min}}{1 - 0.5}
\]

Physiologic scaling factors of 1.2 × 10^8 cells/g liver and 40 g liver/kg were used to scale up to in vivo clearance values per unit body weight.

**Pharmacokinetics Analysis.** The plasma concentration–time data were analyzed by noncompartmental analysis using WinNonlin software (version 5.2.1; Pharsight Corporation, Mountain View, CA). V_{ss}, T_{1/2}, and T_{max} were taken directly from the concentration–time profile. The terminal half-life (T_{1/2}) was calculated as ln2/C_{0}. The area under the plasma concentration curve (AUC_{p}) was calculated using the trapezoidal rule from time 0–24 hours. Total plasma clearance (CL_{tot,p}) was estimated as dose/AUC_{p}, and renal plasma clearance (CL_{renal,p}) was calculated by urine0–24/AUC_{p}, where X_{urine,0–24} represents the cumulative amount of drugs excreted into urine from time 0–24 hours. The apparent volume of disposition at equilibrium (V_{d,app}) was calculated as V_{d,app} = CL × MRT, where MRT is the mean residence time after the intravenous bolus dose. Nonrenal clearance was calculated as CL_{tot,p} − CL_{renal,p} and was regarded as the hepatic clearance (CL_{hep}) used for the following estimation of hepatic intrinsic clearance (CL_{int,vivo}). Hepatic clearance based on the blood concentration (CL_{B}) was estimated as CL_{B,RB}, CL_{int,vivo} was calculated from CL_{B,hep} using a parallel tube model as follows:

\[
CL_{int,vivo} = \frac{Q_0}{V_0} \times \frac{\ln(Q_{B} - CL_{B,hep})}{Q_0}
\]

where Q_0 is the fraction unbound in blood and Q_{B} is the blood flow in rats (60 ml/min/kg).

**LC-MS/MS Analysis.** Mass spectrometry was performed with the AP4000 mass spectrometer system (AB Sciex, Foster City, CA) interfaced with Shimadzu Ultra Fast Liquid Chromatography (UFLC) system (Kyoto, Japan). The analytical column was an Atlantis T3 column (30 × 2.1 mm, 3 μm) (Waters, Milford, MA). The flow rate and column temperature were set at 0.6 ml/min and 40°C, respectively. The mobile phase consisted of 10 mM ammonium formate/0.1% formic acid and acetonitrile.

For atorvastatin, pitavastatin, rosuvastatin, and midazolam, separation was performed using a linear gradient of the organic phase as follows: initial concentration of the organic phase (5%) for 0.5 minutes, followed by a linear gradient of the organic phase up to 95% for 0.2 minutes, and maintenance of 95% of the organic phase for 1.4 minutes. Separation for cerivastatin, fluvastatin, and pravastatin was performed as follows: the initial concentration of the organic phase (5%) for 0.7 minutes, followed by a linear gradient of the organic phase up to 95% for 0.1 minutes, and maintenance of 95% of the organic phase for 1.4 minutes.

Data were acquired in the positive (for atorvastatin, pitavastatin, rosuvastatin, midazolam, and warfarin) or negative (for cerivastatin, fluvastatin,
pravastatin, and warfarin) ion mode with electrospray ionization voltages of 5.5 and −4.5 kV, respectively. Selected reaction monitoring was used to simultaneously monitor the analyte and internal standard. The following selected reaction monitoring transitions were used for the detection: 559.2→440.1 m/z for atorvastatin, 482.2→258.1 m/z for rosuvastatin, 326.0→291.0 m/z for midazolam, 458.3→396.0 m/z for cerivastatin, 410.2→348.2 m/z for fluvastatin, 423.2→320.1 m/z for pravastatin, 309.2→163.1 m/z for warfarin (internal standard; positive ion mode), and 307.0→161.0 m/z for warfarin (internal standard; negative ion mode). The collision energies for atorvastatin, pitavastatin, rosuvastatin, midazolam, cerivastatin, fluvastatin, and pravastatin were set to 31, 39, 45, 39, −24, −22, and −17 eV, respectively. For warfarin as an internal standard, the collision energies were set to 21 and −26 eV for the positive and negative ion modes, respectively.

Statistical Analysis. Student’s t test was used to analyze differences between groups. P < 0.05 was considered statistically significant.

Accuracy of Predictions. The accuracy of predictions was assessed using the average fold error (aFE) with the geometric mean of prediction error, providing an equal value for both underprediction and overprediction estimated as follows:

\[ aFE = 10^{-\frac{1}{N} \sum \log \left( \frac{\text{Predicted}}{\text{Observed}} \right)} \]

For regression analyses, the regression equation and correlation coefficient (r²) were derived, and P < 0.05 was considered statistically significant.

Results

Effect of Rifampicin on Statin Pharmacokinetics of BSP and Midazolam in Rats. In vivo pharmacokinetics was investigated using BSP and midazolam as model substrates for Oatps and Cyp3a, respectively, with the aim of optimizing the dosing of rifampicin to exclusively inhibit Oatps. The time profiles of the plasma concentration of BSP after intravenous administration at 3 mg/kg in control, rifampicin-pretreated, and ABT-pretreated SD rats are shown in Fig. 1A. Pretreatment with rifampicin reduced the CLtot,p of BSP in a dose-dependent manner by 80%–98%. The Vdss of BSP tended to be attenuated by rifampicin pretreatment (Table 1).

To examine the inhibitory effect of rifampicin on Cyp3a, pharmacokinetics studies were performed using midazolam as a probe substrate of Cyp3a in control, rifampicin-pretreated, and ABT-pretreated SD rats (Fig. 1B). When midazolam was administered orally, the AUCp increased markedly by 54-fold with ABT pretreatment (50 mg/kg), whereas rifampicin (30 or 100 mg/kg) exhibited a minimal effect on the pharmacokinetics of midazolam (Table 2).

The time profiles of the plasma concentration of rifampicin are shown in Fig. 1C. After an oral dosing exceeding 30 mg/kg, rifampicin exhibited prolonged exposure around Cmax for up to 7 hours after administration.

Effect of Rifampicin on Statin Pharmacokinetics in Rats. To validate the methodology of using rifampicin as an inhibitor to determine the involvement of Oatps in rats, five different studies of statins were conducted using several statins; the involvement of Oatps is well documented in the drug disposition of statins (Kalliokoski and Niemi, 2009). Because 30 mg/kg of rifampicin resulted in a 31-fold increase in exposure to BSP without affecting the pharmacokinetics of midazolam, the dosage of rifampicin was set to 30 mg/kg and used for further pharmacokinetics studies of statins. The effect of rifampicin 30 mg/kg pretreatment on the pharmacokinetics of statins is shown in Fig. 2. As depicted in Table 3, intravenous administration of statins to rifampicin-pretreated SD rats significantly reduced the CLtot,p of all statins tested (62%–84%) in comparison with control SD rats. This implies that rifampicin treatment (30 mg/kg) can successfully evaluate the involvement of Oatps in the disposition of statins in vivo.

Reduction of Vdss was observed by rifampicin pretreatment as for pitavastatin, cerivastatin, and fluvastatin (54%–79%). To exclude the possibility that the decrease in the CLtot,p of statins caused by rifampicin treatment was due to the inhibition of renal uptake transporters, the CLrenal,p was determined. CLrenal,p was negligible for all statins except pravastatin.

Effect of Rifampicin on the Hepatic Uptake of Statins in the Media-Loss and Metabolic Stability Assays Using Freshly Isolated Rat Hepatocytes. In an attempt to confirm that the effect of rifampicin on the pharmacokinetics of statins is due to the inhibition of Oatps rather than Cyp enzymes, in vitro studies were conducted using media-loss and metabolic stability assays with freshly isolated SD rat hepatocytes (Fig. 3; Table 4). All statins tested exhibited significant disappearance from the media (i.e., in the media-loss assay) compared with that from the suspensions (i.e., in the metabolic stability assay) (Fig. 3). These results suggest the involvement of Oatps in their hepatic clearance.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Single-dose intravenous pharmacokinetic parameters of BSP in SD rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Data are presented as the mean ± S.E. of three female rats.</td>
</tr>
<tr>
<td></td>
<td>BSP</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td></td>
</tr>
<tr>
<td>+Rifampicin, 3 mg/kg</td>
<td>3</td>
</tr>
<tr>
<td>+Rifampicin, 30 mg/kg</td>
<td>3</td>
</tr>
<tr>
<td>+Rifampicin, 100 mg/kg</td>
<td>2</td>
</tr>
</tbody>
</table>

* P < 0.01 versus values for the same dosing in the absence of rifampicin.
* P < 0.05 versus values for the same dosing in the absence of rifampicin.

For regression analyses, the regression equation and correlation coefficient (r²) were derived, and P < 0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Single-dose oral pharmacokinetic parameters of midazolam in SD rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Data are presented as the mean ± S.E. of three female rats.</td>
</tr>
<tr>
<td></td>
<td>Midazolam</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Alone</td>
<td></td>
</tr>
<tr>
<td>+Rifampicin, 30 mg/kg</td>
<td>2.72 ± 0.50</td>
</tr>
<tr>
<td>+Rifampicin, 100 mg/kg</td>
<td>3.53 ± 1.85</td>
</tr>
<tr>
<td>+ABT, 50 mg/kg</td>
<td>15.43 ± 2.53a</td>
</tr>
</tbody>
</table>

* P < 0.05 versus values for the same dosing in the absence of ABT.
* P < 0.01 versus values for the same dosing in the absence of ABT.
The CL_{int,hepatocyte uptake} values obtained from the hepatocyte uptake assay and metabolic stability assay (CL_{int,metabolic stability}) are shown in Table 4. The parameters of CL_{int,media-loss assay} and CL_{int,metabolic stability} are expressed as values per kg body weight using the following physiologic scaling factors for comparison with CL_{int,vivo} values: 1.2 \times 10^{6} cells/g liver and 40 g liver/kg. The CL_{int,media-loss assay} values among the statins tested ranged from 34.4 ml/min/kg for pravastatin to 533 ml/min/kg for rosuvastatin; these values are concordant with or slightly lower than CL_{int,vivo} values. In contrast, the CL_{int,metabolic stability} values were much lower than the CL_{int,vivo} values.

To further support the involvement of Oatps in vitro, the effect of rifampicin on the disappearance of statins from the media was investigated. The CL_{int,media-loss assay} values of statins obtained by the initial disappearance rates in the media-loss assay were attenuated in the presence of 100 \mu M rifampicin. On the other hand, rifampicin treatment only slightly altered the initial disappearance rate of midazolam (unpublished data).

### Effect of Rifampicin on the Uptake of Statins into Freshly Isolated Rat Hepatocytes

The involvement of Oatps in the hepatic uptake of statins was further clarified by hepatocyte uptake assay using freshly isolated female SD rat hepatocytes (Fig. 4; Table 4). The hepatocyte uptake assay directly measures the cellular uptake of investigational drugs, thereby directly determining hepatic active uptake clearance. The cellular uptake of the statins tested increased in a time-dependent manner, which was remarkably attenuated in the presence of cyclosporin A (20 \mu M) or rifampicin (100 \mu M), both potent inhibitors of Oatps (Fig. 4).

The CL_{int,hepatocyte uptake} values obtained from the hepatocyte uptake assay are shown in Table 4. CL_{int,hepatocyte uptake} values are expressed as value per kg body weight using the following physiologic scaling factors for comparison with CL_{int,vivo} values: 1.2 \times 10^{6} cells/g liver and 40 g liver/kg. The CL_{int,hepatocyte uptake} values for all statins tested were attenuated in the presence of rifampicin, suggesting the involvement of active uptake into the liver.

### In Vivo–In Vitro Correlation of Hepatic Intrinsic Clearance

To clarify the correlation of CL_{int} values in vivo and in vitro, the relationships between CL_{int,media-loss assay}, CL_{int,hepatocyte uptake}, CL_{int,metabolic stability}, and observed CL_{int,vivo} were investigated (Fig. 5). It is reported that the media-loss assay can better predict clearance in rats than the metabolic stability assay (Gardiner and Paine, 2011). Indeed, in our study, the CL_{int,media-loss assay} values were much more correlated with observed CL_{int,vivo} values ($r^2 = 0.85, P = 0.009$) than CL_{int,metabolic stability} ($r^2 = 0.007, P = 0.872$). Although statistically not significant, the CL_{int,hepatocyte uptake} values tended to be correlated with observed CL_{int,vivo} values to some extent ($r^2 = 0.44, P = 0.1497$). The media-loss assay provided the most accurate prediction of CL_{int, viva} (afe = 1.87), followed by the hepatocyte uptake assay (afe = 3.17) and metabolic stability assay (afe = 127).

### Summary of the Pharmacokinetics Parameters of Statins in Rats after a Single Intravenous Administration (1 mg/kg) with and without Oral Rifampicin (30 mg/kg)

Data are presented as the mean ± S.E. of three female rats.

<table>
<thead>
<tr>
<th>Statin</th>
<th>$T_{1/2}$ h</th>
<th>AUC$_{0\infty}$</th>
<th>V$_{ss}$ ml/kg</th>
<th>CL$_{int}$ ml/min/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>0.324 ± 0.013</td>
<td>0.838 ± 0.111</td>
<td>0.591 ± 0.056</td>
<td>34.7 ± 4.1</td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>0.642 ± 0.038a</td>
<td>2.68 ± 0.40b</td>
<td>0.493 ± 0.051</td>
<td>11.0 ± 1.7a</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.817 ± 0.024</td>
<td>4.99 ± 0.33</td>
<td>0.547 ± 0.034</td>
<td>7.14 ± 0.45</td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>1.29 ± 0.027a</td>
<td>28.3 ± 3.0a</td>
<td>0.250 ± 0.020a</td>
<td>3.14 ± 0.14a</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.134 ± 0.022</td>
<td>1.77 ± 0.30</td>
<td>0.139 ± 0.017</td>
<td>22.4 ± 4.4</td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>0.501 ± 0.125b</td>
<td>4.50 ± 0.45a</td>
<td>0.142 ± 0.010</td>
<td>8.44 ± 0.79b</td>
</tr>
<tr>
<td>Cervastatin</td>
<td>0.565 ± 0.199</td>
<td>1.17 ± 0.19</td>
<td>0.392 ± 0.123</td>
<td>28.7 ± 4.6</td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>2.93 ± 0.97</td>
<td>3.69 ± 0.24a</td>
<td>0.279 ± 0.045</td>
<td>8.61 ± 0.64b</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>3.57 ± 0.19</td>
<td>4.12 ± 0.57</td>
<td>1.25 ± 0.16</td>
<td>8.70 ± 1.21</td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>2.49 ± 0.03a</td>
<td>25.4 ± 2.3a</td>
<td>0.296 ± 0.015a</td>
<td>1.38 ± 0.12a</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>1.99 ± 0.18</td>
<td>8.86 ± 0.69</td>
<td>1.20 ± 0.16</td>
<td>4.30 ± 0.31</td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>2.82 ± 0.21b</td>
<td>39.4 ± 4.0a</td>
<td>0.250 ± 0.020a</td>
<td>0.987 ± 0.096a</td>
</tr>
</tbody>
</table>

*a P < 0.01 versus values for the same dosing in the absence of rifampicin.

*b P < 0.05 versus values for the same dosing in the absence of rifampicin.
Optimal dosing regimen was determined using BSP as a model Oatp substrate. To validate the methodology of using rifampicin as an inhibitor and confirm the involvement of Oatps in vivo, pharmacokinetics studies of statins, in which the involvement of uptake transporters in drug disposition is well documented, were conducted. In parallel, the inhibitory effect of rifampicin on the hepatic uptake transporters was investigated by hepatic uptake study and media-loss assay in vitro. Furthermore, the in vivo-in vitro correlation was examined.

Oral rifampicin pretreatment in rats reduced the CL\textsubscript{m,v} of BSP in a dose-dependent manner (Table 1). Considering BSP is mainly cleared by hepatic transporters with minimal contribution from Cyp enzyme (Fujiyama et al., 2007), this observation can be accounted for by the inhibition of hepatic uptake transporters, presumably Oatps. This is supported by the observation that the average plasma concentration of rifampicin (from 1–7 hours) achieved after an oral dose of 30 mg/kg was 31.4 μM, which is high enough to inhibit Oatps considering the IC\textsubscript{50} value of rifampicin for rat Oatps (3.2 μM) (Zaher et al., 2008) and the unbound fraction in plasma (0.25) (unpublished data). Although rifampicin is well known as a potent inhibitor of P-glycoprotein (Fardel et al., 1995), the involvement of intestinal P-glycoprotein inhibition by rifampicin was negligible in our study because model substrates (i.e., BSP and statins) were administered intravenously; thus, the effect of rifampicin was due to the inhibition of Oatps and/or Cyp enzymes.

However, it is still possible that rifampicin treatment affects Cyp enzymes in addition to inhibiting Oatps. Thus, the effect of rifampicin on the pharmacokinetics of midazolam (a probe substrate of Cyp3a) was investigated to clarify whether rifampicin inhibits Cyp3a via oral administration (30 and 100 mg/kg) (Fig. 1B; Table 2). When administered orally, the AUC\textsubscript{p} of midazolam increased markedly with the multispecific Cyp inhibitor ABT (50 mg/kg); meanwhile, rifampicin (30 mg/kg) pretreatment minimally affected the pharmacokinetics of midazolam. Although the values were not statistically significant, rifampicin pretreatment (100 mg/kg) produced higher exposure to midazolam than that in control rats. This observation suggests rifampicin does not inhibit Cyp3a at a dose of 30 mg/kg but can partially inhibit Cyp3a at high dosages as much as 100 mg/kg.

Considering these in vivo findings of BSP and midazolam together, the results show that when rifampicin was administered orally at

### TABLE 4

<table>
<thead>
<tr>
<th>Statin</th>
<th>R\textsubscript{0}</th>
<th>f\textsubscript{0}</th>
<th>CL\textsubscript{m,v}</th>
<th>CL\textsubscript{m,k}</th>
<th>CL\textsubscript{m,v}</th>
<th>CL\textsubscript{m,k}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>1.2a 0.047a</td>
<td>851 ± 137</td>
<td>2480 ± 238</td>
<td>403</td>
<td>7.39</td>
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<tr>
<td>+Rifampicin</td>
<td>211 ± 35b</td>
<td>663 ± 225c</td>
<td>38.1</td>
<td>3.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.65a 0.021a</td>
<td>571 ± 40</td>
<td>2940 ± 327</td>
<td>347</td>
<td>1.23</td>
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</tr>
<tr>
<td>+Rifampicin</td>
<td>91.6 ± 10.3c</td>
<td>1040 ± 101c</td>
<td>105</td>
<td>1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.59a 1.2a</td>
<td>41.8 ± 11.7</td>
<td>74.3 ± 17.7</td>
<td>34.4</td>
<td>9.29</td>
<td></td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>9.95 ± 2.04</td>
<td>0.147 ± 11.21b</td>
<td>7.16</td>
<td>2.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>0.65d 0.065d</td>
<td>1480 ± 562</td>
<td>1040 ± 90</td>
<td>533</td>
<td>6.65</td>
<td></td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>219 ± 20</td>
<td>276 ± 12</td>
<td>196</td>
<td>6.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>0.7e 0.083e</td>
<td>169 ± 26</td>
<td>2930 ± 934</td>
<td>262</td>
<td>0.336</td>
<td></td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>24.2 ± 2.0c</td>
<td>1310 ± 745</td>
<td>174</td>
<td>0.310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.53a 0.019a</td>
<td>458 ± 35</td>
<td>705 ± 1070</td>
<td>194</td>
<td>2.90</td>
<td></td>
</tr>
<tr>
<td>+Rifampicin</td>
<td>98.9 ± 9.9c</td>
<td>613 ± 572</td>
<td>136</td>
<td>&lt;0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Watanabe et al., 2010.
* P < 0.05 versus values in the absence of rifampicin.
* P < 0.01 versus values in the absence of rifampicin.
* Calculated from the unbound fraction in the plasma (f\textsubscript{0}) and the blood-to-plasma concentration ratio (R\textsubscript{0}) data (Paine et al., 2008).
a dose of 30 mg/kg, plasma exposure of rifampicin was high enough to inhibit Oatps; moreover, rifampicin pretreatment (30 mg/kg) did not affect Cyp3a activity. Thus, rifampicin 30 mg/kg is considered optimal for exclusively inhibiting Oatps without affecting Cyp3a enzymes.

The involvement of Oatps in vivo could be successfully evaluated using an in vivo approach with or without rifampicin treatment (30 mg/kg); this would enable the evaluation of compounds with no metabolism or those undergoing Cyp3a metabolism. However, the possibility that rifampicin has an inhibitory effect on metabolic enzymes other than Cyp3a cannot be excluded. In the case of compounds undergoing extensive metabolism, the involvement of Oatps in the pharmacokinetics should be judged carefully, not only from in vivo studies using rifampicin as an inhibitor but together with in vitro media-loss assays and uptake assays.

We investigated the inhibitory effects of rifampicin 30 mg/kg on the pharmacokinetics of statins in an attempt to validate the methodology of using rifampicin as an inhibitor to confirm the involvement of Oatps in rats in vivo (Fig. 2; Table 3). Intravenous statin administration into rifampicin-pretreated rats led to the significant attenuation of CLtot,p in all statins tested in comparison with control rats. Because the possibility that rifampicin inhibits organic anion transporters (i.e., Oats) in the kidneys cannot be excluded, nonrenal clearance, which was regarded as hepatic clearance, was calculated by subtracting the plasma renal clearance from the plasma total clearance, and the effect of rifampicin was examined. In addition, the nonrenal clearance of statins decreased markedly in the presence of rifampicin. This suggests that the uptake transporter of organic anions in the liver, presumably Oatps, is involved in the disposition of statins. Regarding renal excretion, renal clearance (CLrenal,p) was negligible for all statins tested except pravastatin. The CLrenal,p of pravastatin was observed with 14% of total plasma clearance. This is concordant with the report indicating that in rats pravastatin is eliminated from the kidneys as well as the liver (Takada et al., 2004) while other statins are predominantly eliminated from the liver (Boberg et al., 1998; Nezasa et al., 2002; Watanabe et al., 2010). Taken together, these findings indicate that the involvement of Oatps in vivo can be assessed by comparing the nonrenal clearance of test compounds or for compounds with minimal renal excretion by comparing the total clearance between the presence and absence of rifampicin.

To confirm that the effect of rifampicin on the pharmacokinetics of statins observed in rats in vivo was due to the inhibition of Oatps rather
Evaluation of the Involvement of Hepatic Uptake Transporters

than Cyp enzymes, we conducted in vitro studies using media-loss and hepatic uptake assays in rat hepatocytes (Figs. 3 and 4). In the media-loss assay, the involvement of transporters is proved by the following observations: 1) the initial drug disappearance rate from the media obtained from the media-loss assay is larger than that obtained from the conventional metabolic stability assay; 2) the initial drug disappearance rate is attenuated in the presence of inhibitors of uptake transporters, such as rifampicin. The intrinsic clearance rates of statins calculated from the initial disappearance rate in the media-loss assay were much greater than those obtained from the metabolic stability assay. In addition, the initial disappearance rates of statins in the media-loss assay were markedly attenuated in the presence of 100 μM rifampicin, suggesting that Oatps are involved in the disappearance of these statins. Rifampicin treatment minimally altered the initial disappearance rate of midazolam (unpublished data).

The involvement of Oatps in the pharmacokinetics of these statins was also investigated by the hepatic uptake assay, which directly measures the uptake of test drugs in hepatocytes and provides information about hepatic uptake clearance (Fig. 4). The cellular uptake of statins exhibited a time-dependent manner that was attenuated in the presence of cyclosporin A (20 μM) or rifampicin (100 μM). These findings support the in vivo and in vitro observations of the media-loss assay.

To clarify the correlation between CLint in vivo and in vitro, we investigated the relationships between CLint,media-loss assay, CLint,hepatocyte uptake, and observed CLint,vivo (Fig. 5). We found that CLint,media-loss assay was more closely correlated with observed CLint,vivo (r² = 0.85, P = 0.009) than CLint,hepatocyte uptake. In addition, the accuracy of prediction of CLint,vivo was better in the media-loss assay (afe = 1.87) than the hepatocyte uptake assay (afe = 3.17). The lower accuracy of prediction of CLint,vivo by the hepatocyte uptake assay may be due to the time-dependent disappearance of the drug from the media during the assay, which was not taken into consideration when calculating CLint,hepatocyte uptake by the hepatocyte uptake assay. The accuracy of predictions was worst with the use of the metabolic stability assay (afe = 127), presumably because the active uptake process determines the clearance of these statins. Indeed, uptake is reported to be the rate-determining process for pravastatin, pitavastatin, atorvastatin, and fluvastatin (Watanabe et al., 2010). Although not included in the present study, the metabolic stability assay as well as the media-loss assay would be expected to provide accurate predictions of the clearance of compounds whose clearance is governed by metabolism. Taken together, these findings indicate the media-loss assay is the preferable method for evaluating the hepatocellular clearance of an NCE.

In summary, our study provides the in vitro and in vivo methodology to determine the involvement of Oatps in the disposition of anionic drugs in rats using rifampicin as an inhibitor in the preclinical stage. In vivo studies using rats revealed rifampicin pretreatment (30 mg/kg) serves to evaluate the involvement of Oatps in drug disposition without the involvement of transporters for ES in rats with hepatic first-pass by oral administration. In vitro studies using rats revealed rifampicin pretreatment (30 mg/kg) serves to evaluate the involvement of Oatps in drug disposition without the involvement of transporters for ES in rats with hepatic first-pass by oral administration.

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Authorship Contributions

Participated in research design: Imaoka, Mikkaichi, Abe, Hirouchi. Conducted experiments: Imaoka, Mikkaichi, Abe, Hirouchi. Performed data analysis: Imaoka, Mikkaichi. Wrote or contributed to the writing of the manuscript: Imaoka, Mikkaichi, Abe, Okudaira, Izumi.

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Disclosure: The authors declare no competing interests.