Comparison of Methods for Estimating Unbound Intracellular-to-Medium Concentration Ratios in Rat and Human Hepatocytes Using Statins

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ABSTRACT

It is essential to estimate concentrations of unbound drugs inside the hepatocytes to predict hepatic clearance, efficacy, and toxicity of the drugs. The present study was undertaken to compare predictability of the unbound hepatocyte-to-medium concentration ratios (Kp,uu) by two methods based on the steady-state cell-to-medium total concentration ratios at 37°C and on ice (Kp,uu,V0) and based on their initial uptake rates (Kp,uu). Poorly metabolized statins were used as test drugs because of their concentrative uptake via organic anion-transporting polypeptides. Kp,uu,V0 values of these statins provided less interexperimental variation than the Kp,uu values, because only data at longer time are required for Kp,uu,V0. Kp,uu,V0 values for pitavastatin, rosuvastatin, and pravastatin were 1.2- to 5.1-fold Kp,uu in rat hepatocytes; Kp,uu,V0 values in human hepatocytes also tended to be larger than corresponding Kp,uu. To explain these discrepancies, theoretical values of Kp,uu,ss and Kp,uu,V0 were compared with true Kp,uu (Kp,uu,true), considering the inside-negative membrane potential and ionization of the drugs in hepatocytes and medium. Membrane potentials were approximately ~30 mV in human hepatocytes at 37°C and almost abolished on ice. Theoretical equations considering the membrane potentials indicate that Kp,uu,ss values for the statins are 0.85- to 1.2-fold Kp,uu,true, whereas Kp,uu,V0 values are 2.2- to 3.1-fold Kp,uu,true depending on the ratio of the passive permeability of the ionized to nonionized forms. In conclusion, Kp,uu,ss values of anions are similar to Kp,uu,true when the inside-negative membrane potential is considered. This suggests that Kp,uu,ss is preferable for estimating the concentration of unbound drugs inside the hepatocytes.

Introduction

According to the free drug hypothesis, only unbound drug is believed to interact with metabolic enzymes and pharmacological/toxicological target proteins. Knowing the intracellular unbound drug concentration is essential to estimate accurately the risk of drug–drug interactions involving drug-metabolizing enzymes and canalicular efflux transporters, and efficacy and toxicity of drugs when their targets are intracellular proteins. Extracellular unbound drug concentration is frequently assumed to be equivalent to the intracellular unbound concentration, especially when drugs are neutral or nonionized and freely permeable across the cytoplasmic membrane (Smith et al., 2010). However, this assumption cannot be applied to drugs whose tissue uptake is dominated by active transporters (Smith et al., 2005; Shugarts and Benet, 2009; Giacomini et al., 2010; Niemi et al., 2011; Shitara et al., 2013), in which their intracellular unbound concentration of drugs could be higher than the extracellular unbound concentration. For instance, unbound concentrations of pravastatin and rosuvastatin in the rat liver are 11- to 16-fold (Yamazaki et al., 1993; Nezasa et al., 2003) and 15-fold higher than unbound plasma concentrations, respectively, because of organic anion-transporting polypeptide (OATP)-mediated uptake (Nezasa et al., 2003).

Methods to estimate the unbound hepatocyte-to-medium concentration ratio (Kp,uu) are needed to predict the magnitude of drug–drug interactions involving drug-metabolizing enzymes, efflux transporters, and other intrahepatic target proteins in the liver. Brown et al. (2010) investigated the impact of transporters on the inhibition constant (K1) values of cytochrome P450 inhibitors by comparing their inhibitory effects using rat liver microsomes and freshly isolated rat hepatocytes. Using the hepatocytes, K1 values of clarithromycin and enoxacin with known hepatic transporter involvement were markedly smaller than those using the microsomes, which was consistent with their high cell-to-medium total concentration ratios (C/M ratios). The International Transporter Consortium published a review summarizing strategies to estimate intracellular drug concentrations (Chu et al., 2013). Among

Abbreviations: 1Ψ, membrane potential; AFE, average fold error; CLuptake, uptake clearance by hepatocytes; C/M ratio, cell-to-medium total concentration ratio; fC,cell,unbound fraction in liver homogenates; HBSS, Hank’s balanced salt solution; K1, inhibition constant; Kd, Michaelis-Menten constant; Kp,uu,ss, unbound hepatocyte-to-medium concentration ratio; Kp,uu,V0, unbound hepatocyte-to-medium concentration ratio based on initial uptake rate; Kp,uu,ss,ss, theoretically true unbound hepatocyte-to-medium concentration ratio; Kp,uu,V0, unbound hepatocyte-to-medium concentration ratio based on initial uptake rate; MRP, multidrug resistance-associated protein; OATP, organic anion-transporting polypeptide; PSdiff, influx intrinsic clearance by passive diffusion through sinusoidal membrane; TPP+, tetraphenylphosphonium; Vmax, maximum transport rate.
them, a strategy to estimate \( K_{p,uu,\text{true}} \) based on the initial uptake rate (\( K_{p,uu,\text{V0}} \)) calculated using active transport clearance (\( V_{\text{max}}/K_m \)), and passive diffusion clearance at various concentrations (Yabe et al., 2011) was introduced. In addition, we have proposed an alternative strategy to estimate \( K_{p,uu} \) under steady-state conditions (\( K_{p,uu,\text{ss}} \)), which can be calculated by dividing the C/M ratio at 37°C by that at a low temperature (on ice) or in the presence of ATP depletors, when active transport is stopped (Yamazaki et al., 1992; Shitara et al., 2013). Yamazaki et al. (1993) demonstrated that the uptake of pravastatin by rat hepatocytes was more greatly reduced at a low temperature than by ATP depletion.

In the present study, steady-state uptake of typical OATP substrates, pitavastatin, rosuvastatin, and pravastatin in rat and human hepatocytes was investigated at 37°C and on ice to evaluate their \( K_{p,uu,\text{ss}} \). In general, \( K_{p,uu} \) can be described by the C/M ratio of drugs in hepatocytes as follows:

\[
\frac{C}{M} \text{ ratio} = \frac{C_{\text{cell},\text{unbound}}}{M} = \frac{C_{\text{medium},\text{unbound}}}{M} = \frac{K_{p,uu,\text{true}}}{f_T} = \frac{f_T}{f_T^{(37)}}
\]

where \( f_T \) is the unbound fraction of the drugs in the blood (in vivo) or in the incubation medium (in vitro), and \( f_T^{(37)} \) is the unbound fraction of the drugs in the hepatocytes. The unbound fraction in hepatocytes at the steady state (\( f_T^{(37)} \)) obtained using our method should be validated because intracellular binding to cytosolic proteins/cellular organelles might be altered at low temperatures. Therefore, \( f_T^{(37)} \) and \( f_T \) were compared with \( K_{p,uu,\text{true}} \) in both rat and human hepatocytes. The difference between \( K_{p,uu,\text{true}} \) and \( K_{p,uu,\text{V0}} \) is discussed in the context of theoretical equations, considering the membrane potential (\( \Delta \psi \)) in hepatocytes and the fraction of ionized drugs at the designated pH. Finally, a method to predict \( K_{p,uu,\text{true}} \) from \( K_{p,uu,\text{ss}} \) and \( K_{p,uu,\text{V0}} \) obtained experimentally is proposed.

**Materials and Methods**

**Chemicals.** [H]Pitavastatin, [H]Rosuvastatin calcium, and [H]Pravastatin calcium were obtained from American Radiolabeled Chemicals (St. Louis, MO). [H]Diazepam was obtained from PerkinElmer Life Sciences (Boston, MA). Unlabeled diazepam, pitavastatin calcium, rosuvastatin calcium, and pravastatin sodium were obtained from Wako Pure Chemicals (Osaka, Japan). All other reagents and solvents were purchased from Invitrogen (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO), and Wako Pure Chemicals.

**Animals.** Male Sprague-Dawley rats were purchased from Charles River Japan (Shiga, Japan) and acclimatized for 7 days before the experiments. The rats were housed under conditions of controlled temperature and humidity with a 12-hour light/dark cycle with free access to standard laboratory rodent food (CE-2; CLEA Japan, Tokyo, Japan) and water. All animal experiments were approved by the Experimental Animal Care and Use Committee of the Mitsubishi Tanabe Pharma (Saitama, Japan) and conducted in accordance with the Declaration of Helsinki and the guidelines of the ethics committee.

**Isolation of Rat Hepatocytes.** Hepatocytes were isolated from male Sprague–Dawley rats (7–9 weeks old) using a procedure described previously (Baur et al., 1975). Isolated hepatocytes were suspended in albumin-free Krebs–Henseleit buffer with 12.5 mM HEPES (pH 7.4), and cell viabilities were determined using a trypan blue exclusion test. Hepatocytes obtained from three independent preparations with >80% viability were used for the uptake studies described below.

**Preparation of Human Hepatocytes.** Human biologic samples were obtained ethically, and their research use was in accordance with the terms of informed consent. Cryopreserved human hepatocytes from a donor (Lot Hu8075) were purchased from Life Technologies (Carlsbad, CA). Pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Lot TFF) were purchased from BioreclamationIVT (Baltimore, MD). Pooled cryopreserved human hepatocytes from 50 mixed-sex donors (Lot HUES05C) were purchased from Thermo Fisher Scientific (Waltham, MA). These hepatocytes were suspended in albumin-free Krebs–Henseleit buffer with 12.5 mM HEPES (pH 7.4), and viabilities were determined using a trypan blue exclusion test. Hepatocytes obtained from three independent preparations with >80% viability were used for the uptake studies described below.

**Determination of the Intracellular Volume of Hepatocytes.** The intracellular volume of rat hepatocytes (3.68 ± 1.37 μL/10⁶ cells) was estimated using published methods (Supplemental Table 1A) (Baur et al., 1975; Kletzien et al., 1975; Eaton and Klaasen, 1978; Kristensen and Folkle, 1984; Yamazaki et al., 1992; Miyazaki et al., 1993; Reinsos et al., 2001; Halifax and Houston, 2006). In brief, to determine the intracellular volume of human hepatocytes, cryopreserved human hepatocytes (Lot Hu8075) were suspended in Krebs–Henseleit buffer (pH 7.4) at 6.0 ± 10⁶ viable cells/mL and precultured at 37°C for 5 minutes. A reaction was initiated by adding an equal volume of buffer containing [H]water and [14C]Dextran at final concentrations of 2.5 μCi/mL and 0.5 μCi/mL, respectively. After incubation at 37°C for 10 minutes, during which the distribution of [H]water and [14C]Dextran reached a steady state, aliquots were removed and added to a narrow tube containing silicon–mineral oil (density: 1.015; Sigma-Aldrich) over aqueous 2 M sodium hydroxide, followed by centrifugation through the silicon–mineral oil layer to separate the cells from the medium. After the basic bottom layer was neutralized with 2 M hydrochloric acid, radioactivities in both cells and medium were determined using a Tri-Carb liquid scintillation counter (PerkinElmer, Shelton, CT). Thereby, the intracellular volume of human hepatocytes was estimated to be 2.28 ± 0.33 μL/10⁶ cells (Supplemental Table 1B).

**Determination of \( K_{p,uu,\text{true}} \) and \( f_T^{(37)} \).** The uptake studies described above. Based on Steady-State Uptake. To determine the incubation time for steady-state uptake into hepatocytes, transport studies were performed by using an oil–spin method (Iga et al., 1979) using suspended hepatocytes. Hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4) at 2.0 × 10⁶ viable cells/mL and precultured at 37°C for 5 minutes. A reaction was initiated by adding an equal volume of buffer containing each drug (pitavastatin, rosuvastatin, pravastatin, or diazepam previously used as a neutral drug with high membrane permeability for evaluating the uptake into isolated rat hepatocytes) (Ichikawa et al., 1992) at 1 μM. After incubation at 37°C for 0.5, 2, 5, 15, and 30 minutes (rat hepatocytes), 0.5, 5, 15, 30, and 60 minutes (human hepatocytes, Lot Hu8075), or 0.5, 1.5, 30, and 60 minutes (human hepatocytes, Lot TFF), aliquots were removed and added to a narrow tube containing silicon–mineral oil over aqueous 2 M sodium hydroxide and centrifuged through the silicon–mineral oil layer to separate the cells from the medium. To provide low temperature values, the uptake studies were performed on ice. After the basic bottom layer was neutralized with 2 M hydrochloric acid, radioactivities in both cells and medium was measured using the liquid scintillation counter.

The unbound hepatocyte-to-medium concentration ratio (\( K_{p,uu} \)) based on the steady-state uptake ratio at 37°C and on ice (\( K_{p,uu,\text{true}} \)) and the unbound fraction in hepatocytes based on the steady-state uptake (\( f_T^{(37)} \)) were defined as described in eqs. 2 and 3, respectively, based on the C/M ratio at 37°C and on ice. A part of the method to obtain \( f_T^{(37)} \) was reported previously (Yoshikado et al., 2016).

\[
K_{p,uu,\text{true}} = \frac{C_{\text{cell}}}{C_{\text{medium}}}.\text{Ratio}_{\text{on ice}} = \frac{C_{\text{cell},\text{on ice}}}{C_{\text{medium},\text{on ice}}} = \frac{C_{\text{cell},\text{unbound},\text{on ice}}}{C_{\text{medium},\text{unbound},\text{on ice}}} = \frac{f_T^{(37)} \cdot f_T^{(37)}}{f_T^{(37)}} = \frac{f_T^{(37)}}{f_T^{(37)}}
\]

\[
f_T^{(37)} = \frac{C_{\text{cell},\text{unbound},\text{on ice}}}{C_{\text{medium},\text{on ice}}} = \frac{1}{f_T^{(37)}}
\]

The following assumptions were made in calculating \( K_{p,uu,\text{true}} \) and \( f_T^{(37)} \): the active uptake in hepatocytes is abolished on ice (i.e., \( f_T^{(37)} \)) is equal to \( f_T^{(37)} \); and \( f_T^{(37)} \) is independent of temperature (i.e., \( f_T^{(37)} \)).
The uptake clearance by hepatocytes (CLuptake) was determined by the slope of the plot of C/M ratio versus time, and the initial uptake rate ($v$) was calculated by multiplying CLuptake with the initial substrate concentration. According to a method reported previously (Yabe et al., 2011), $v$ can be calculated using eq. 4:

$$v = \frac{V_{\text{max}} \cdot S}{K_m + S} + P_{\text{Sdiff}} \cdot S,$$

where $V_{\text{max}}$ is the maximum uptake rate, $K_m$ is the Michaelis constant, $P_{\text{Sdiff}}$ is the passive diffusion clearance, and $S$ is the substrate concentration in the medium. These kinetic parameters were optimized by fitting the equation to observed data using Phoenix WinNonlin version 6.3 (Pharsight Certara, St. Louis, MO). Because CLuptake consists of active uptake clearance (PS act) and passive diffusion clearance (PS dif), assuming that PSdif for the cellular uptake is equal to that for the efflux, $K_{p,uu}$ and $f_{T,cell}$ based on initial uptake rate ($K_{p,uu,V0}$, $f_{T,cell,V0}$) can be calculated using eqs. 5 and 6 (Yabe et al., 2011):

$$K_{p,uu} = \frac{P_{S act} + P_{S dif}}{P_{S dif}}$$

$$f_{T,cell,V0} = \frac{K_{p,uu,V0}}{C/M \text{ ratio}_{37^\circ C}}$$

where the C/M ratio$_{37^\circ C}$ in rat hepatocytes was obtained at 30 minutes, and that in human hepatocytes was obtained at 60 minutes.

**Determination of the Unbound Fraction in Human Liver Homogenates ($f_{T,\text{homogenate}}$) Using Equilibrium Dialysis.** Human liver samples were obtained from the Human and Animal Bridging Research Organization (Tokyo, Japan) with approval of the Ethics Committees of University of Tokyo and Human and Animal Bridging Research Organization. Three lots of liver samples were pooled and homogenized in 66.7 mM isotonic phosphate buffer at 1:3 (w/v) producing 25% homogenates. By diluting these homogenates, 12.5% and 6.25% homogenates were also prepared. Diazepam, pitavastatin, pravastatin, and rosuvastatin (final concentrations: 0.2 μM) were added to the compartment containing homogenates in a Rapid Equilibrium Dialysis plate (Thermo Fisher Scientific) and incubated for 12 hours at 37°C, or on ice.

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>$f_{i,ion}^{a}$</th>
<th>$f_{i,act}^{a}$</th>
<th>$f_{i,ion}^{a}$</th>
<th>$f_{i,act}^{a}$</th>
<th>$pK_a$</th>
<th>$PS_{\text{initiation,Caco-2}} \times 10^{-6}$</th>
<th>$\lambda^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitavastatin</td>
<td>0.857</td>
<td>0.143</td>
<td>0.905</td>
<td>0.095</td>
<td>4.46</td>
<td>3.387 ± 1.403</td>
<td>0.0282 ± 0.0204</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.986</td>
<td>0.014</td>
<td>0.991</td>
<td>0.009</td>
<td>4.6</td>
<td>7.14 ± 0.68</td>
<td>0.0112 ± 0.0035</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.986</td>
<td>0.014</td>
<td>0.991</td>
<td>0.009</td>
<td>4.6</td>
<td>1.11 ± 0.19</td>
<td>0.188 ± 0.038</td>
</tr>
</tbody>
</table>

*The $f_{i,ion}$ and $f_{i,act}$ were calculated based on Henderson–Hasselbalch equation assuming that intracellular pH and medium pH are 7.2 and 7.4, respectively. The $f_{i,ion}$ and $f_{i,act}$ were subtracted from $f_{i,ion}$ and $f_{i,act}$, respectively.

The $PS_{\text{initiation,Caco-2}}$ and $\lambda$ were determined by fitting eq. 9 to the pH-dependent permeation data of statins observed in Caco-2 cells (Supplemental Fig. 1).
The PS\textsubscript{dif,inf} is expressed as follows:

\[
PS\textsubscript{dif,inf} = f_{ion} \cdot PS\textsubscript{dif,ion} + f_{uion} \cdot PS\textsubscript{dif,uion}
\]

where the subscripts ion and uion represent the ionized and unionized (nonionized) form of a drug, respectively, and \( f_{ion} \) and \( f_{uion} \) are fractions of ionized and nonionized drug outside the cells, respectively. Subsequently, \( \lambda \) was defined as the ratio of passive diffusion influx clearance of the ionized drug to that of the nonionized drug, as follows:

\[
\lambda = \frac{PS\textsubscript{dif,ion}}{PS\textsubscript{dif,uion}}
\]

It is assumed that the \( \lambda \) value is not changed by temperature: the effect of low temperature on PS\textsubscript{dif,ion} is assumed to be the same as that on PS\textsubscript{dif,uion}, although these passive diffusion clearances should be affected by the change in membrane fluidity at low temperature (Kandusier et al., 2008). A part of the method to obtain \( \lambda \) was reported previously (Yoshikado et al., 2016). Using \( \lambda \), eq. 7 can be converted as follows:

\[
PS\textsubscript{dif,inf} = (1 - f_{uion}) \cdot PS\textsubscript{dif,inf} + f_{ion} \cdot PS\textsubscript{dif,inf} = (\lambda - (1 - f_{uion}) + f_{ion}) \cdot PS\textsubscript{dif,inf} = \lambda - (1 - \lambda) \cdot f_{ion} \cdot PS\textsubscript{dif,inf} = \lambda + (1 - \lambda) \cdot \frac{1}{C_{18}} \cdot PS\textsubscript{dif,inf}.
\]

The \( f_{ion} \) and \( f_{uion} \) for each statin were estimated using the pH of the medium (7.4) and pKa of the drug obtained from the manufacturer’s Information Forms, based on the Henderson–Hasselbalch equation (Table 1). Then, \( \lambda \) and PS\textsubscript{dif,ion} in Caco-2 cells were optimized by fitting eq. 9 to the pH-dependent membrane permeation of statins (Supplemental Fig. 1). Phoenix WinNonlin software version 6.3 (Pharsight Certara) was used to optimize the parameters. The obtained \( \lambda \) values for pitavastatin, rosuvastatin, and pravastatin are shown in Table 1.

### TABLE 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>( V_{\text{max,}^a} , \text{pmol/min/10}^6 \text{ Cells} )</th>
<th>( K_m^a , \mu M )</th>
<th>( V_{\text{max}}/K_m , \text{pmol/min/10}^6 \text{ Cells} )</th>
<th>( PS\textsubscript{u,}^a , \text{pmol/min/10}^6 \text{ Cells} )</th>
<th>( K_{\text{p,}u,\text{V0,}^a} )</th>
<th>( f_{\text{ion,}u,\text{V0,}^a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitavastatin</td>
<td>893 ± 124</td>
<td>5.43 ± 1.29</td>
<td>164 ± 1.45</td>
<td>3.00 ± 1.41</td>
<td>55.8 ± 29.9</td>
<td>0.172 ± 0.131</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>250 ± 80</td>
<td>2.86 ± 1.82</td>
<td>87.4 ± 2.64</td>
<td>1.16 ± 0.9</td>
<td>50.7 ± 48.0</td>
<td>0.649 ± 0.688</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>178 ± 11</td>
<td>20.3 ± 1.4</td>
<td>8.77 ± 0.8</td>
<td>8.56 ± 0.87</td>
<td>0.685 ± 0.427</td>
<td></td>
</tr>
</tbody>
</table>

The initial uptake rate in rat hepatocytes was calculated from the uptake of \[ ^{3} H \text{pitavastatin}, \] \[ ^{3} H \text{rosuvastatin}, \] and \[ ^{3} H \text{pravastatin} \] for 0.5–1.5 minutes. Kinetic parameters are obtained by fitting to the data at seven concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 \( \mu M \)) for all statins (Supplemental Fig. 2) using eq. 4.

The \( K_{\text{p,}u,\text{V0}} \) and \( f_{\text{ion,}u,\text{V0}} \) were calculated using eqs. 5 and 6, respectively. C/M ratios at 37°C (Table 2) were used for the calculation of the \( f_{\text{ion,}u,\text{V0}} \).
in rat hepatocytes was monitored. The uptake of diazepam in rat hepatocytes reached a steady state instantaneously because of its high permeability, whereas the uptake of pitavastatin, rosuvastatin, and pravastatin gradually increased over time and reached the steady state within 30 minutes (Fig. 1). On ice, C/M ratios of pitavastatin, rosuvastatin, and pravastatin were significantly smaller than the ratios at 37°C, whereas that for diazepam was not different from that at 37°C (Table 2). The $K_{P_{uu,ss}}$ for diazepam was 0.85, and the values for

\[
AFE = 10^{-\frac{\sum_{i=1}^{n} P_{cell,i}}{C_{12,12,12}}} \quad (11)
\]
Subsequently, based on a method reported previously (Yabe et al., 2011), the kinetic parameters (Vmax, Km, and PSdif) for pitavastatin, rosuvastatin, and pravastatin were determined by fitting eq. 4 to their initial uptake rate. The results are shown in Eadie–Hofstee plots (Supplemental Fig. 2). The obtained Kp,uu,V0 values for pitavastatin, rosuvastatin, and pravastatin were approximately 56, 51, and 8.6, respectively (Table 3). The calculated fT,cell,V0 for pitavastatin, rosuvastatin, and pravastatin were approximately 0.17, 0.65, and 0.69, respectively.

**Determination of Kp,uu,V0 and fT,cell,V0 in Rat Hepatocytes.**

Subsequently, based on a method reported previously (Yabe et al., 2011), the kinetic parameters (Vmax, Km, and PSdif) for pitavastatin, rosuvastatin, and pravastatin were determined by fitting eq. 4 to their initial uptake rate. The results are shown in Eadie–Hofstee plots (Supplemental Fig. 2). The obtained Kp,uu,V0 values for pitavastatin, rosuvastatin, and pravastatin were approximately 56, 51, and 8.6, respectively (Table 3). The calculated fT,cell,V0 for pitavastatin, rosuvastatin, and pravastatin were approximately 0.17, 0.65, and 0.69, respectively.

**Determination of Kp,uu,ss and fT,cell,ss in Human Hepatocytes.**

We investigated the time-dependent uptake of diazepam, pitavastatin, rosuvastatin, and pravastatin by human hepatocytes prepared from single donor (Lot Hu8075) (Fig. 2, A–D) and pooled human hepatocytes from 20 mixed-sex donors (Lot TFF) (Fig. 2, E–H). The uptake of all these statins increased over time and reached a steady state within 60 minutes, whereas that of diazepam reached a peak instantaneously. On ice, the C/M ratios for pitavastatin, rosuvastatin, and pravastatin were reduced, whereas that for diazepam was not dependent on temperature (Table 4). Although the Kp,uu,ss for diazepam was approximately 1 (1.2 and 0.41), the values were 13 and 6.9 for pitavastatin, 12 and 6.4 for rosuvastatin, and 2.0 and 1.3 for pravastatin in cells from Lot Hu8075 and Lot TFF, respectively (Table 4). The obtained fT,cell,ss values were approximately 0.028 and 0.046 for pitavastatin, 0.22 and 0.23 for rosuvastatin, and 0.55 and 0.48 for pravastatin in cells from Lot Hu8075 and Lot TFF, respectively.

**Determination of Kp,uu,V0 and fT,cell,V0 in Human Hepatocytes.**

Based on a method reported previously (Yabe et al., 2011), the kinetic parameters (Vmax, Km, and PSdif) for pitavastatin and rosuvastatin were determined by fitting eq. 4 to their initial uptake rate by cells from Lot Hu8075 (Supplemental Fig. 3, A and B) and Lot TFF (Supplemental Fig. 3, D and E); the kinetic parameters of pravastatin could be determined in cells from Lot Hu8075 (Supplemental Fig. 3C), but not from Lot TFF, because a saturation of the uptake of pravastatin was not observed clearly within the concentration range of 0.5–300 μM.

Calculated Kp,uu,V0 values were approximately 220 and 20 for pitavastatin; 200 and 3.5 for rosuvastatin in cells from Lot Hu8075 and Lot TFF, respectively; and 55 for pravastatin in cells from Lot Hu8075 (Table 5), which were higher in cells from Lot Hu8075 than from Lot TFF. Calculated fT,cell,V0 values were approximately 0.47 and 0.13 for pitavastatin; 3.8 and 0.13 for rosuvastatin in cells from Lot Hu8075 and Lot TFF, respectively; and 15 for pravastatin in cells from Lot Hu8075, although fT,cell should theoretically be less than 1. Thus, the

### Table 4

**Kp,uu,ss and fT,cell,ss in human hepatocytes**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lot</th>
<th>C/M Ratio</th>
<th>Kp,uu,ss</th>
<th>fT,cell,ss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>On ice</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>Hu8075</td>
<td>216 ± 20</td>
<td>181 ± 38</td>
<td>1.19 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>124 ± 53</td>
<td>303 ± 22</td>
<td>0.409 ± 0.247</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>Hu8075</td>
<td>471 ± 88</td>
<td>35.2 ± 26</td>
<td>13.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>150 ± 2.6</td>
<td>21.8 ± 3.2</td>
<td>6.92 ± 1.02</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Hu8075</td>
<td>52.2 ± 8.6</td>
<td>4.51 ± 0.41</td>
<td>11.6 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>27.2 ± 2.5</td>
<td>4.28 ± 0.53</td>
<td>6.36 ± 1.06</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>Hu8075</td>
<td>3.73 ± 0.73</td>
<td>1.84 ± 0.12</td>
<td>2.03 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>2.65 ± 1.29</td>
<td>2.07 ± 0.64</td>
<td>1.28 ± 0.89</td>
</tr>
</tbody>
</table>

### Table 5

**Kinetic parameters for the initial uptake of pitavastatin, rosuvastatin, and pravastatin in human hepatocytes**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lot</th>
<th>Vmax</th>
<th>Vmax/Km</th>
<th>PSdiff</th>
<th>Kp,uu,V0</th>
<th>fT,cell,V0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol/min/10^6 Cells</td>
<td>μM</td>
<td>μL/mm/min/10^6 Cells</td>
<td>μM</td>
<td>μL/mm/min/10^6 Cells</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>Hu8075</td>
<td>403 ± 84</td>
<td>4.77 ± 1.80</td>
<td>84.5 ± 36.4</td>
<td>0.388 ± 0.912</td>
<td>219 ± 521</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>148 ± 66.1</td>
<td>1.78 ± 1.04</td>
<td>83.1 ± 61.1</td>
<td>4.36 ± 0.70</td>
<td>20.1 ± 14.4</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Hu8075</td>
<td>198 ± 50</td>
<td>21.5 ± 7.8</td>
<td>9.21 ± 4.07</td>
<td>0.0462 ± 0.1547</td>
<td>200 ± 673</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>23.0 ± 8.6</td>
<td>4.49 ± 1.72</td>
<td>5.12 ± 2.75</td>
<td>2.07 ± 0.279</td>
<td>3.47 ± 1.41</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>Hu8075</td>
<td>93.8 ± 56.0</td>
<td>127 ± 81</td>
<td>0.739 ± 0.645</td>
<td>0.0137 ± 0.0922</td>
<td>54.8 ± 363.7</td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>Not determined</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*For the initial uptake rate in pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Lot TFF) was calculated from the uptake of [3H]pitavastatin, [3H]rosuvastatin, and [3H]pravastatin for 0.5–2.0 minutes. Kinetic parameters are obtained by fitting the data at several concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 μM for pitavastatin; 0.1, 0.3, 1, 3, 10, 30, and 300 μM for rosuvastatin; 1, 3, 100, and 300 μM for pravastatin; Supplemental Fig. 3, A–C) using eq. 4.*

*The initial uptake rate in pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Lot TFF) was calculated from the uptake of [3H]pitavastatin and [3H]rosuvastatin for 0.5–1.5 minutes. Kinetic parameters are obtained by fitting the data at several concentrations (0.1, 0.3, 1, 3, 10, 30, and 100 μM for pitavastatin; 0.1, 0.3, 1, 3, 10, 30, and 300 μM for rosuvastatin; Supplemental Fig. 3, D and E) using eq. 4.*

*The Kp,uu,ss and fT,cell,ss were calculated using eqs. 5 and 6, respectively. C/M ratios at 37°C (Table 4) were used for the calculation of the fT,cell,ss.*
values of $K_{p, uu,V0}$ and $f_{T, cell,V0}$ exhibited greater differences between lots.

Comparison of $K_{p, uu}$ and $f_{T, cell}$ Obtained by Different Methods.
In rat hepatocytes, $K_{p, uu,V0}$ values for pitavastatin, rosuvastatin, and pravastatin were, respectively, 5.1, 5.1, and 1.2 times those for $K_{p, uu,ss}$ (Fig. 3A). By contrast, in human hepatocytes (Lot Hu8075), $K_{p, uu,V0}$ values for pitavastatin, rosuvastatin, and pravastatin were, respectively, 16, 17, and 43 times those for $K_{p, uu,ss}$ (Fig. 3B). The differences between $K_{p, uu,V0}$ and $K_{p, uu,ss}$ in human hepatocytes from Lot TFF were smaller than those in Lot Hu8075: $K_{p, uu,V0}$ values for pitavastatin and rosuvastatin were, respectively, 2.9 and 0.55 times those for $K_{p, uu,ss}$ in cells from Lot TFF (Fig. 3B).

The $f_{T, cell,V0}$ values tended to be higher than $f_{T, cell,ss}$ in both rat (Fig. 3C) and human hepatocytes (Fig. 3D), except for rosuvastatin in human hepatocytes (Lot TFF). Both $K_{p, uu,V0}$ and $f_{T, cell,V0}$ Values thus exhibited quite larger interlot and interexperimental variabilities compared with $K_{p, uu,ss}$ and $f_{T, cell,ss}$ Values (comparison between Tables 2 and 3 for rats and Tables 4 and 5 for humans).

Comparison of $f_{T, cell}$ with the Measured Unbound Fraction in Human Liver Homogenates. For diazepam, pitavastatin, rosuvastatin, and pravastatin, the measured unbound fractions in human liver homogenates ($f_{T, homogenate}$) were measured using equilibrium dialysis (Table 6). The $f_{T, homogenate}$ values for these drugs obtained at 37°C ($f_{T, homogenate,37°C}$) were close to those obtained on ice ($f_{T, homogenate, on ice}$). Moreover, the $f_{T, homogenate, on ice}$ values for pitavastatin and rosuvastatin were comparable to those for $f_{T, cell,ss}$ (Table 4); the difference between $f_{T, homogenate, on ice}$ and $f_{T, cell,ss}$ for pravastatin was within threefold, whereas there was a larger discrepancy between $f_{T, homogenate, on ice}$ and $f_{T, cell,ss}$ for diazepam.

Measurement of the $\Delta \Psi$ Using TPP⁺. To evaluate whether $\Delta \Psi$ might affect the ratio of $PS_{dif,in}/PS_{dif,eff}$ and, accordingly, $K_{p, uu}$, the time-dependent uptake of TPP⁺ was examined in human hepatocytes under physiologic conditions and with amphotericin B, which is reported to abolish $\Delta \Psi$ specifically by 10-minute incubation with isolated rat hepatocytes (Saito et al., 1992). The C/M ratio of TPP⁺ gradually increased over time and reached the steady state between 30 and 60 minutes (Fig. 4). On ice, the C/M ratio of TPP⁺ was significantly smaller than that at 37°C. In addition, in the presence of amphotericin B, the C/M ratio of TPP⁺ was decreased significantly at 37°C compared with the condition without amphotericin B, whereas the C/M ratio on ice was not significantly changed with amphotericin B (Fig. 4). The C/M ratio (37°C) in the presence of amphotericin B at 60 minutes was lower than that at 30 minutes, suggesting some additional effects of amphotericin B (i.e., cytotoxicity) other than the loss of the $\Delta \Psi$. Therefore, based on the obtained data at 30 minutes and

<table>
<thead>
<tr>
<th>Drug</th>
<th>$f_{T, homogenate}$</th>
<th>$f_{T, homogenate,37°C}$</th>
<th>$f_{T, cell,ss}$ 4.0°C</th>
<th>$f_{T, cell,ss}$ 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>0.0259 ± 0.0018</td>
<td>0.0242 ± 0.0016</td>
<td>1.07 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.0301 ± 0.0017</td>
<td>0.0344 ± 0.0018</td>
<td>0.875 ± 0.067</td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.237 ± 0.037</td>
<td>0.206 ± 0.025</td>
<td>1.15 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Pravastatin</td>
<td>0.183 ± 0.022</td>
<td>0.167 ± 0.020</td>
<td>1.10 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>
eq. 10, ΔΨ values were calculated to be approximately −30 mV at 37°C and 5 mV on ice (Table 7).

Using Ψ = [exp(ξFΔΨ/R/T)] calculated from ΔΨ (Supplemental Equation 5) and physicochemical parameters calculated for statins (Table 1), theoretical values of Kp,uu,true, Kp,uu,V0, and Kp,uu,ss were calculated by Supplemental Equations 6, 8, and 11, respectively.

Furthermore, we aimed to understand better the quantitative relationship between Kp,uu,true and experimentally obtained Kp,uu,ss and Kp,uu,V0 (Table 8).

Discussion

We previously studied a method to estimate Kp,uu,true for anions in hepatocytes based on their steady-state C/M ratio at 37°C and the C/M ratio after suppressing active transport (Yamazaki et al., 1992; Shitara et al., 2013). Another method reported by Yabe et al. (2011) is to calculate AFs from uptake data at longer time (e.g., 0.5 or 1.5 h). Using Supplemental Equations 6, 8, and 11, respectively, with the use of intracellular pH parameters of statins (Table 1), theoretical values of Kp,uu,true, Kp,uu,V0, and Kp,uu,ss were calculated by eq. 7, as follows:

\[
R_{V0/true} = \frac{K_{p,uu,V0}}{K_{p,uu,ss}} = \frac{\Phi \cdot \lambda \cdot f_{i,ion} + f_{o,ion}}{f_{i,ion} + f_{o,ion}},
\]

and

\[
R_{in/tr} = \frac{K_{p,uu,ss}}{K_{p,uu,true}} = \frac{\Phi \cdot \lambda \cdot f_{i,ion} + f_{o,ion}}{f_{i,ion} + f_{o,ion}},
\]

where fi,ion and f o,ion are fractions of the ionized and nonionized forms of the drug in hepatocytes, respectively, with the use of intracellular pH (7.2) and pKa. R_V0/true was calculated to be 2.2–3.1 for statins, whereas R_in/tr was 0.85–1.2 (Table 8). Figure 5 also shows that simulated R_in/tr was closer to 1 than R_V0/true when λ values for pitavastatin, rosuvastatin, and pravastatin were approximately 0.028, 0.011, and 0.19, respectively (Table 1). Furthermore, experimentally obtained Kp,uu,V0 and Kp,uu,ss for statins were corrected using the calculated R_V0/true and R_in/tr values (Table 8) and eqs. 14 and 15:

\[
K_{p,uu,V0,corrected} = \frac{K_{p,uu,V0}}{R_{V0/true}}
\]

(14)

\[
K_{p,uu,ss,corrected} = \frac{K_{p,uu,ss}}{R_{in/tr}}
\]

(15)

Kp,uu,V0,corrected in rat hepatocytes approached Kp,uu,ss,corrected (Fig. 6A) compared with uncorrected Kp,uu,V0 versus Kp,uu,ss (Fig. 3A); the calculated AFs are 7.6 in Fig. 3A and 4.4 in Fig. 6A. Similarly, Kp,uu,V0,corrected in human hepatocytes approached corrected Kp,uu,ss,corrected (Fig. 3B and 6B); the calculated AFs are 132 in Fig. 3B and 41 in Fig. 6B. However, Kp,uu,V0,corrected in Lot Hu0705, a single donor lot selected because of its relatively high uptake ability, remained larger than Kp,uu,ss,corrected.

**TABLE 8**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Lot</th>
<th>Kp,uu,V0,a</th>
<th>Kp,uu,V0,b</th>
<th>Kp,uu,ss,a</th>
<th>Kp,uu,ss,b</th>
<th>R_V0/true</th>
<th>R_in/tr</th>
</tr>
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<tbody>
<tr>
<td>Pitavastatin</td>
<td>Hu8075</td>
<td>90.7</td>
<td>198</td>
<td>106</td>
<td>2.18</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>8.35</td>
<td>18.3</td>
<td>9.75</td>
<td>2.18</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>Hu8075</td>
<td>72.4</td>
<td>199</td>
<td>71.9</td>
<td>2.74</td>
<td>0.994</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>1.26</td>
<td>3.45</td>
<td>1.25</td>
<td>2.74</td>
<td>0.994</td>
<td></td>
</tr>
<tr>
<td>Pravastatin</td>
<td>Hu8075</td>
<td>17.9</td>
<td>54.5</td>
<td>15.2</td>
<td>3.05</td>
<td>0.850</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TFF</td>
<td>Not calculated</td>
<td></td>
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</tbody>
</table>

"Theoretical values of Kp,uu,V0,corrected and Kp,uu,ss,corrected were calculated by Supplemental Equations 6, 8, and 11, respectively, with the use of intracellular pH parameters of statins (Table 1), Vmax/Km (Table 5), and PS_diff,in,met in each lot of human hepatocytes determined by fi,ion, fo,ion, λ, and Ps,in (Table 5), accordingly to eq. 7, for the calculation.

R_V0/true = R_in/tr = Kp,uu,V0/Kp,uu,ss,corrected were calculated by eqs. 11 and 12.

"Not calculated for Lot TFF because kinetic parameters (Km, Vmax, and PS) of pravastatin were not determined (Table 5).

\[
K_{p,uu,V0,corrected} = \frac{PS_{act,inf} + PS_{diff,inf}}{PS_{act,eff} + PS_{diff,eff} + CL_{int,met} + CL_{ext,bile}}
\]

(16)

where PS_{act,inf}, PS_{act,eff}, CL_{int,met}, and CL_{ext,bile} represent the active influx clearance, the active efflux clearance, the intrinsic clearance for metabolism, and the intrinsic clearance for biliary excretion in an unchanged form, respectively. Based on eq. 16, a number of explanations can be considered for why Kp,uu,V0 Values for pitavastatin, rosuvastatin, and pravastatin tended to be larger than Kp,uu,ss in both rat and human hepatocytes (Fig. 3, A and B).
(i) As PS_{act,inf} is estimated by initial uptake rate for the calculation for K_{p,uu,V0}, it may include not only active transport, but also facilitated diffusion, which is also transporter mediated, but not by active (concentrated) transport. Thus, in this case, K_{p,uu,V0} can be larger than K_{p,uu,ss}. However, in the case of statins, hepatic OATPs are thought to be major uptake transporters, and the impact of facilitated diffusion on their overall uptake may be negligible.

(ii) The existence of non-negligible metabolism during the measurement of steady-state uptake (30–60 minutes) of a drug may lead to smaller K_{p,uu,ss} than K_{p,uu,V0} values, because metabolism might be negligible during short incubation times (~2 minutes) for the measurement of initial uptake rates. Considering that pitavastatin, rosuvastatin, and pravastatin are generally classified as statins metabolized poorly in humans (Shitara and Sugiyama, 2006), it is unlikely that metabolism accounts for the overestimation of K_{p,uu,V0}. However, a recent report suggested that pitavastatin undergoes lactonization by uridine 5'-diphospho-glucuronosyltransferases more extensively than other statins (Schirris et al., 2015), which may explain, at least in part, the overestimation of K_{p,uu,V0} compared with K_{p,uu,ss} (Fig. 3, A and B). In addition, pentenoic acid derivative was reported to be the major metabolite of rosuvastatin in rats (Nezasa et al., 2002; He et al., 2014). In our experiments using rat hepatocytes, the remaining amount of rosuvastatin after the 30-minute incubation was 87% of the initial amount, whereas little loss of rosuvastatin was observed after the 60-minute incubation in human hepatocytes. Therefore, the contribution of metabolism to the elimination of rosuvastatin in rats might be larger than that in humans, and the metabolism of rosuvastatin in rat hepatocytes might only slightly influence the estimation of its K_{p,uu,ss}.

(iii) PS_{act,eff} and CL_{int,bile} cannot be fully estimated by the short-term uptake of drugs. Thus, if a drug is a substrate of basolateral/apical efflux transporters, K_{p,uu,V0} may be overestimated. The expression of apical efflux transporters [P-glycoprotein, multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein] and basolateral efflux transporters (MRP3 and MRP4) was partly retained in cryopreserved human hepatocytes compared with fresh liver biopsies (Lundquist et al., 2014), which might contribute, at least in part, to the drug efflux from hepatocytes and lead to a discrepancy between K_{p,uu,V0} and K_{p,uu,ss}.

(iv) In most work, PS_{diff,inf} and PS_{diff,eff} are conventionally assumed to be equal for kinetic consideration of the cellular transport (Yabe et al., 2011), although PS_{diff,eff} might be larger than PS_{diff,inf} in the case of anions because of the inside-negative ΔΨ in normal cells. We calculated K_{p,uu,ss} by taking

Fig. 5. Theoretical simulation of K_{p,uu,V0}/K_{p,uu,true} (= R_{v,true}: blue) and K_{p,uu,V0}/K_{p,uu,ss} (= R_{v,ss}: red) using eqs. 12 and 13. (A) The solid line represents the simulation result for pitavastatin. (B) The broken line represents simulation results for rosuvastatin and pravastatin. Arrows represent the λ for statins estimated from our experiments with Caco-2 cells.

Fig. 6. Comparison of K_{p,uu,ss,corrected} and K_{p,uu,V0,corrected} corrected by eqs. 14 and 15 using R_{v,corrected} and R_{V,corrected} (Table 8). (A) Closed symbols: original K_{p,uu,V0} in rat hepatocytes were obtained from a previous report (Yabe et al., 2011). Open symbols: original K_{p,uu,V0} values were obtained in the present study. (B) Gray symbols: original K_{p,uu,V0} values were obtained in human hepatocytes from a single donor (Lot Hu8075). Open symbols: original K_{p,uu,V0} values were obtained in pooled human hepatocytes from 20 mixed-sex donors (Lot. TFF). 1, pitavastatin; 2, rosuvastatin; 3, pravastatin. Solid and dashed lines denote unity and threefold boundaries, respectively. The data are presented as mean ± S.D. for the x- and y-axis (n = 3).
asymmetric P_{DSf} into consideration with the assumption that ΔΨ is almost abolished on ice, as we confirmed experimentally (Table 8). Conversely, to calculate K_{P,uu,SS}, P_{DSf,eff} is set to be the same as P_{DSf,inf} as shown in Supplemental Equation 8. Therefore, theoretically, K_{P,uu,SS} should be larger than K_{P,uu,SL}, in the case of anions, which might mostly explain the observed discrepancy between K_{P,uu,SL} and K_{P,uu,SS}.

In this study, two assumptions were made. First, f_{T,cell,ss} is not affected by temperature; second, ΔΨ is abolished on ice. To investigate the first assumption, we sought to evaluate whether f_{T,cell,ss} was largely dependent on temperature, as mentioned in previous reports (Sugano et al., 1985; Fitz and Scharschmidt, 1987; Wondergem and Castillo, 1988; Yabe et al., 2011). Indeed, Yabe et al. (2011) estimated f_{T,cell,SS} for some drugs, including pravastatin, not by eq. 6, but by using in silico prediction with logD to obtain reasonable values for f_{T,cell,SS}.

The second assumption was investigated using TPP+ uptake studies with and without amphoterin B in human hepatocytes (Fig. 4), according to a method reported previously (Saito et al., 1992). Calculated ΔΨ at 37°C was similar to reported values (−35 to −39 mV) obtained in isolated rodent hepatocytes (Bradford et al., 1985; Edmonson et al., 1985; Fitz and Scharschmidt, 1987; Wondergem and Castillo, 1988; Weinman et al., 1989), and ΔΨ was almost abolished on ice under steady-state conditions at 30 minutes (Table 7). Collectively, our assumptions are valid for anions.

For the estimation of K_{P,uu,SL}, ATP depletors such as rotenone were also used to stop the active uptake (Yamazaki et al., 1992); however, it is difficult to optimize experimental conditions for the exposure to ATP depletors to abolish the active uptake without affecting cell viability, and the effect of ATP depletion is often required to be insufficient to maintain viability (Yamazaki et al., 1993). Thus, this approach may lead to underestimation of K_{P,uu,SL}. The use of transporter inhibitors is another strategy to stop the active transport. However, before this, we should understand the major transport mechanism of the drugs to be tested, and confirm that the contribution of other transporters to the overall active uptake is negligible. In the case of statins, because Na+-taurocholate cotransporting polypeptide is known to partly play a role in their hepatic uptake (Bi et al., 2013), the inhibition of Na+-taurocholate cotransporting polypeptide in addition to OATPs is required; otherwise, insufficient inhibition of the hepatic uptake causes an underestimation of K_{P,uu,SL}.

In conclusion, K_{P,uu} of statins were estimated in rat and human hepatocytes using two different methods based on steady-state uptake (K_{P,uu,SL}) and initial uptake rate (K_{P,uu,SS}). Considering the inside-negative ΔΨ and subsequent asymmetric diffusional clearance for influx and efflux, in theory, K_{P,uu,SL} should be similar to true K_{P,uu} (K_{P,uu,SS}), whereas K_{P,uu,SS} should be higher compared with K_{P,uu,SL}. This can explain, at least in part, the discrepancies observed between K_{P,uu,SL} and K_{P,uu,SS} for statins in the present study. Using the estimated K_{P,uu} Value and the ratio of diffusional uptake to active uptake determined in vitro, we can mathematically describe the relationship among permeation clearances across the sinusoidal membrane of hepatocytes, which can be applied for the construction of a physiologically-based pharmacokinetic model.

Although our current approach should be valid from the viewpoint of kinetic theory, we will need to practically validate our method for the in vitro–in vivo extrapolation of K_{P,uu}. One of the approaches is to compare the observed liver-to-blood total concentration ratio of positron emission tomography probes (e.g., [11C]dehydropravastatin) (Ijuin et al., 2012) in humans with that predicted from experimentally estimated hepatic K_{P,uu} and their unbound fractions in blood and hepatocytes. Another approach is to confirm whether the discrepancy of in vitro K_{P,uu} values with regard to the medium concentration of inhibitors obtained with hepatocytes and recombinant enzymes can be well explained by (in vitro K_{P,uu} when target proteins are located inside the hepatocytes (e.g., inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase by statins, inhibition of metabolic enzymes and efflux transporters by inhibitor drugs). Such validations should strengthen our theoretical considerations for in vitro K_{P,uu} estimation.

Authorship Contributions

Participated in research design: Yoshikado, Toshimoto, Nakada, Maeda, Sugiyama.

Conducted experiments: Yoshikado, Nakada, Ikejiri.

Performed data analysis: Yoshikado, Toshimoto, Nakada.

Wrote or contributed to the writing of the manuscript: Yoshikado, Toshimoto, Nakada, Kusuhara, Maeda, Sugiyama.

References


