Simultaneous Assessment of Uptake and Metabolism in Rat Hepatocytes: A Comprehensive Mechanistic Model

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ABSTRACT

Kinetic parameters describing hepatic uptake in hepatocytes are frequently estimated without appropriate incorporation of bidirectional passive diffusion, intracellular binding, and metabolism. A mechanistic two-compartment model was developed to describe all of the processes occurring during the in vitro uptake experiments performed in freshly isolated rat hepatocytes plated for 2 h. Uptake of rosuvastatin, pravastatin, pitavastatin, valsartan, bosentan, telmisartan, and repaglinide was investigated over a 0.1 to 300 μM concentration range at 37°C for 2 or 45–90 min; nonspecific binding was taken into account. All concentration-time points were analyzed simultaneously by using a mechanistic two-compartment model describing uptake kinetics [unbound affinity constant (Km,u), maximum uptake rate (Vmax,u), unbound active uptake clearance (CLactive,u)], passive diffusion [unbound passive diffusion clearance (Pdiff,u)], and intracellular binding [intracellular unbound fraction (fu_cell)]. When required (telmisartan and repaglinide), the model was extended to account for the metabolism [unbound metabolic clearance (CLmet,u)]. The CLactive,u ranged 8-fold, reflecting a 11-fold range in uptake Km,u with telmisartan and valsartan showing the highest affinity for uptake transporters (Km,u < 10 μM). Both Pdiff,u and fu_cell span over two orders of magnitude and reflected the lipophilicity of the drugs in the dataset. An extended incubation time allowed steady state to be reached between media and intracellular compartment concentrations and reduced the error in certain parameter estimates observed with shorter incubation times. Active transport accounted for >70% of total uptake for all drugs investigated and was 4- and 112-fold greater than CLmet,u for telmisartan and repaglinide, respectively. Modeling of uptake kinetics in conjunction with metabolism improved the precision of the uptake parameter estimates for repaglinide and telmisartan. Recommendations are made for uptake experimental design and modeling strategies.

Introduction

For many years, research has focused on reducing the metabolic liability of new chemical entities (NCEs). Consequently, a shift in physicochemical properties has occurred, and more NCEs show a reduced ability to cross membranes readily and rely on active transport for their disposition (Wu and Benet, 2005; Funk, 2008). Organic anion-transporting proteins (OATPs) are expressed on the sinusoidal membrane of hepatocytes and have been identified as important contributors to the disposition of statins, angiotensin II receptor inhibitors, and antidiabetics (Hagenbuch and Gui, 2008; International Transporter Consortium et al., 2010; Watanabe 2010) Part of this work was presented previously: Ménochet K, Kenworthy KE, Houston JB, and Galetin A (2010) Contribution of active uptake to the hepatic clearance of seven OATP substrates in rat and human plated hepatocytes, at the 9th International Society for the Study of Xenobiotics Meeting, 2010 Sept 4–8; Istanbul, Turkey. International Society for the Study of Xenobiotics, Washington, DC.

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ABBREVIATIONS: NCE, new chemical entity; OATP, organic anion-transporting protein; ABT, 1-aminobenzotriazole; M1, 2-desipiperidyl-2-amino repaglinide; M2, 2-desipiperidyl-2(S-carboxypentylamine) repaglinide; M4, 3’-hydroxy repaglinide; fu_med, unbound fraction in the media; fu_cell, intracellular unbound fraction; Pdiff passive diffusion clearance; Pdiff,u unbound passive diffusion clearance; Kmd,u unbound affinity constant; Vmax,u maximum uptake rate; CLactive,u unbound active uptake clearance; CL uptake,u unbound total uptake clearance; CLmet,u unbound metabolic clearance; Vmax,gluc,u unbound metabolic clearance caused by the formation of repaglinide glucuronide; CLmet,M2,u unbound metabolic clearance caused by the formation of M2; LogD7.4, distribution coefficient between octanol and water at pH 7.4; CV, coefficient of variation; PBPK, physiologically based pharmacokinetic; DPBS, Dulbecco’s phosphate-buffered saline; DMSO, dimethyl sulfoxide; LC, liquid chromatography; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; S_cell, total cell concentration; Smed,u, unbound media concentration; rmse, root mean square error; gmfe, geometric mean fold error.
et al., 2010). Activity of OATPs can be modulated as a result of polymorphisms and drug-drug interactions with cyclosporine, rifampicin, or gemfibrozil glucuronide, resulting in increased systemic exposure of OATP substrates linked to potentially severe toxicities (Simonson et al., 2004; Kajosaari et al., 2005; van Giersbergen et al., 2007; Kalliokoski et al., 2008; Ieiri et al., 2009). From the regulatory perspective, a thorough understanding of the active uptake of NCES in vitro is vital for assessing any potential clinical risk associated with this mechanism, either in particular patient population or with certain comedication (European Medicines Agency, 2010; International Transporter Consortium et al., 2010; Zhang et al., 2011).

Over the past decade, both the quality and availability of cryopreserved human hepatocytes have improved (Badolo et al., 2011), making this cellular model the tool of choice for predicting human hepatic disposition in vitro (Brown et al., 2007; Soars et al., 2007). Nevertheless, the cost of human hepatocytes still limits their extensive use. Rat hepatocytes can easily be isolated and used as a substitute when developing new assays. Although the expression of transporters differs between the two species (Hagenbuch and Gui, 2008), physiologically based pharmacokinetic (PBPK) models developed in the rat have provided a sound basis for the prediction of human hepatic disposition (Poirier et al., 2008; Watanabe et al., 2009).

Drug uptake into cells may consist of a saturable active component and the nonsaturable passive permeation driven by the physicochemical properties of the drug investigated. Several studies have demonstrated that the relation between these two processes is a key factor for understanding the hepatic disposition of compounds undergoing active uptake (Webborn et al., 2007; Paine et al., 2008; Poirier et al., 2008; Watanabe et al., 2009). Many uptake studies regard passive permeation as a unidirectional process occurring from the media into the cell during the short incubation time (often ≤2 min). Parker and Houston (2008) demonstrated that even after 2-min incubation the cell-to-media ratio of nelfinavir could be as high as 3000, suggesting that the drug would permeate back from the cell into the media in in vitro systems (Baker and Parton, 2007; Parker and Houston, 2008; Poirier et al., 2008). In addition, only a few studies have reported full uptake kinetics in hepatocytes (Poirier et al., 2008, 2009; Yabe et al., 2011) or investigated drugs with differential transporter-metabolism involvement. A thorough understanding of the saturable uptake process with appropriate passive permeation and intracellular binding considerations is critical for accurate estimation of any subsequent metabolism and prediction of liver and systemic concentrations by using a PBPK modeling approach. To that aim, a mechanistic two-compartment model was developed to simultaneously fit all of the concentration-time points during uptake experiments performed over a range of initial concentrations, extending the model described previously by Poirier et al. (2008). The model presented here allows the estimation of intracellular binding and characterization of uptake kinetics either in isolation or conjunction with metabolism.

The present study focuses on the characterization of seven drugs (rosuvastatin, pravastatin, pitavastatin, bosentan, valsartan, telmisartan, and repaglinide); all have been identified as OATP substrates in different in vitro systems. In addition, there is an increasing body of clinical evidence on the important role of OATP1B1 in the pharmacokinetics of these drugs based either on studies performed in subjects expressing different allelic variants of this transporter (SEARCH Collaborative Group et al., 2008; Ieiri et al., 2009) or clinical drug-drug interactions reported with OATP inhibitors (Stangier et al., 2000; Kajosaari et al., 2005; International Transporter Consortium et al., 2010).

The aim of the present study was to assess the ability of a mechanistic two-compartment model to describe active uptake, bidirectional passive diffusion, and intracellular binding in plated rat hepatocytes over a range of substrate concentrations. Parameter estimates obtained by the mechanistic two-compartment and the conventional two-step approaches were compared. Unlike the mechanistic model, the conventional two-step approach requires prior data transformation to delineate passive from active uptake into the cell. The impact of extended incubation times (up to 45–90 min to allow steady state between cell and the media concentrations to be achieved) on the uptake and binding parameter estimates was analyzed. The mechanistic model was expanded to simultaneously describe uptake and metabolism for telmisartan and repaglinide within the same experimental system. The impact of the incorporation of metabolism in the mechanistic model on uptake and intracellular binding parameter estimates was investigated.

**Materials and Methods**

**Chemicals.** Bosentan, pitavastatin, pravastatin, rosuvastatin, telmisartan, and valsartan were purchased from Sequoia Research Products (Pangbourne, UK). Telmisartan acetyl-β-D-glucuronide, 2-desipiperidyl-2-amino repaglinide (M1), 2-desipiperidyl-2(5-carboxypentylamine) repaglinide (M2), 3’-hydroxy repaglinide (M4), and repaglinide acetyl-β-D-glucuronide were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Insulin (4 mg/ml) was purchased from Invitrogen (Paisley, UK). Repaglinide, mibebradil, verapamil, and indomethacin were purchased from Sigma-Aldrich (Poole, UK). All other chemicals and reagents were obtained from Sigma-Aldrich and were of the highest grade available.

**Isolation of the Rat Hepatocytes.** Rat hepatocytes were isolated from 250 to 300 g Sprague-Dawley rat livers (Charles River, Margate, Kent, UK), following the two-step collagenase perfusion method described previously (Berry and Friend, 1969). Hepatocytes were suspended in William’s medium E supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.01% insulin at pH 7.4. Cells were counted under a microscope using a hemocytometer, and viability was assessed by using the trypan blue exclusion method. Only preparations with a viability >80% were used. Cell monolayers were checked before each experiment to ensure coverage of >80% of the well surface. The cell suspension was diluted to 400,000 cells/ml in supplemented William’s medium E. Hepatocytes were plated in 24-well collagen I-coated plates (BD Biosciences, Oxford, UK) at a density of 240,000 viable cells per well. Plates were incubated for 2 h at 37°C in an atmosphere containing 5% CO₂ to allow adherence to the collagen.

**Measurement of Uptake in Rat Hepatocytes.** Uptake was measured over a range of 10 concentrations between 0.1 and 300 μM (0.1, 0.3, 1, 3, 10, 20, 30, 60, 100, and 300 μM). The maximum concentration used for telmisartan was 100 μM, because of its limited solubility in aqueous buffer. This wide range was chosen because the affinity of the seven drugs of interest toward uptake transporters in rat hepatocytes was mostly unknown (Ishigami et al., 1995; Nezasa et al., 2003; Poirier et al., 2009). The medium was removed after plating. Monolayers were rinsed twice with prewarmed serum-free Dulbecco’s phosphate-buffered saline (DPBS). Substrate was dis-
OATP inhibitors (10 substrates through the cellular membrane. At 4°C, a single well was also carried out at 4°C to assess the passive diffusion of the substrate on top of the monolayers. Incubations were also carried out at 4°C to assess passive permeation at 37°C in the absence of active uptake. However, substrate-dependent inhibition was observed (data not shown); therefore, the use of control incubations at 4°C was selected as an initial measurement of passive permeation. Incubations were also extended to 45–90 min to reach saturation of the active uptake. In that case, four additional time points were added between 2 and 45–90 min for at least five concentrations. To inhibit phase I metabolism of repaglinide and bosentan, a nonspecific cytochrome P450 pan-inhibitor 1-aminobenzotriazole (ABT) was added to the incubations at a concentration of 1 mM (Mico et al., 1988; Yabe et al., 2011). Previous studies in human embryonic kidney-293 cells expressing OATP1B1 have shown that ABT has no effect on the activity of uptake transporters (Plise et al., 2010). In the current study, uptake of 1 μM rosuvastatin was measured over 2 min as a control of the uptake activity of each hepatocyte preparation. The mean uptake of the 1 μM rosuvastatin control across all experiments performed in this study (n = 22) was 54.8 ± 19.6 μM/min/10^6 cells.

Depletion Assay. As part of a preliminary analysis, depletion of 0.1 μM mefloquine and telmisartan was measured in rat hepatocytes plated as described above; the medium was removed after plating. Monolayers were rinsed twice with prewarmed serum-free DPBS. Substrate was dissolved in DMSO and diluted in DPBS (maximum 1% DMSO). Incubation was started by the addition of 400 μl of substrate on top of the monolayers. Reactions were stopped after 0.5, 1, 2, 5, 10, 30, and 45 min for repaglinide, and 0.5, 1, 2, 5, 10, 30, and 45 min for telmisartan, by the addition of 400 μl of the appropriate internal standard.

Sample Preparation and LC-MS/MS Analysis. Cell lysates and substrate samples from the uptake experiments were thawed and quenched with an equal volume of methanol containing 1 μM of internal standard. Samples were placed for at least 1 h at −20°C before being centrifuged for 10 min at 6720g. Likewise, samples generated in the depletion assay were centrifuged in a similar manner. In both cases, 20 μl of supernatant was analyzed by LC-MS/MS as described below. All samples were analyzed on either a Waters 2795 HPLC system coupled with a Micromass Quattro Ultima mass spectrometer (Waters, Milford, MA) or a Waters 2695 HPLC system coupled with a Micromass Quattro Micro mass spectrometer. The analyte and associated internal standard were separated on a Luna C18 column (3 μm, 50 × 4.6 mm) (Phenomenex, Torrance, CA). The flow through the HPLC system was 1 ml/min and split to 0.25 ml/min before entering the mass spectrometer. All analytes were ionized by positive electrospray. LC-MS/MS analysis of each analyte is described in detail in Table 1. Samples were quantified against a standard curve, and only standards within 30% of the nominal concentration were included in the standard curve. In addition to telmisartan, the appearance of telmisartan glucuronide was monitored for modeling purposes. In the case of repaglinide, M1, M2, M4, and repaglinide glucuronide were also monitored in the cell.

**Determination of Unbound Fraction in the Incubation Media.** The y intercept (t_0) of the linear regression of the media concentration over time plot was calculated at each incubation concentration. The media were assumed to be free of proteins because all hepatocytes were attached to the collagen support and the cell monolayers were rinsed thoroughly before each incubation as stated above. Therefore, fit_t0 was expressed as the slope of the linear regression of the unbound concentration extrapolated at t_0 versus the initial incubation concentration plot. A representative example of the fit_estimation is illustrated for pitavastatin in Supplemental Fig. S1.

**Initial Determination of Uptake Kinetic Parameters using a Conventional Two-Step Approach.** Uptake rate was calculated over 2 min at 37 and 4°C and expressed as the slope of the linear regression of the cell concentrations versus time plot. Passive diffusion (P_{diff}) was calculated from the uptake rates measured at 4°C as shown in eq. 1. P_{diff} was then inserted in eq. 2 as a constant, and the active uptake kinetic parameters were estimated by using Grafit version 6 (Erithacus Software Ltd, Horley, Surrey, UK).

\[
v = P_{diff} \times S
\]

\[
v = \frac{V_{max} \times S}{K_m + S} + P_{diff} \times S
\]

where v is the uptake rate; V_{max} is the maximum uptake rate; K_m is the affinity constant; P_{diff} is the passive diffusion clearance, and S is the substrate concentration. All kinetic parameters were corrected for nonspecific binding of the substrate to the incubation environment (fit_estimation). CL_{active,u} the unbound active uptake clearance, was expressed as the ratio of V_{max} over the unbound K_m (K_m,u). The total unbound uptake clearance (CL_{uptake,u}) included both the active component (CL_{active,u}) and clearance via passive diffusion (P_{diff,u}).

**Determination of Uptake Kinetic Parameters Using a Mechanistic Modeling Approach.** A two-compartment model,
based on the work by Poirier et al. (2008), was implemented in Matlab version 7.10 (2010) (The MathWorks, Inc., Natick, MA). Unlike the conventional two-step approach, this model allows simultaneous fitting of all concentration-time points during the experiment and relies only on measurements made at 37°C. It allows the assessment of multiple processes, namely active uptake of drugs into the hepatocytes, bidirectional passive diffusion, and intracellular binding. The uptake experiments were extended up to 45–90 min to reach steady state between concentrations in the media and intracellular compartment; analysis of uptake profiles after short and extended incubation times was performed. The scheme of steps taken to implement this model is presented in Fig. 1. The mechanistic model for the assessment of active uptake in hepatocytes is illustrated in Fig. 2A. Differential eqs. 3 and 4 show the change in cell and media concentrations over time, respectively. All parameters are expressed per well and therefore are normalized for the number of cells per well.

\[
\frac{dS_{\text{cell}}}{dt} = \frac{V_{\text{max}} \times S_{\text{med,u}} + P_{\text{diff,u}} \times S_{\text{med,u}} - P_{\text{diff,u}} \times S_{\text{cell}} \times f_{u_{\text{cell}}}}{V_{\text{cell}}} \quad (3)
\]

\[
\frac{dS_{\text{med,u}}}{dt} = \frac{-V_{\text{max}} \times S_{\text{med,u}} - P_{\text{diff,u}} \times S_{\text{med,u}} + P_{\text{diff,u}} \times S_{\text{cell}} \times f_{u_{\text{cell}}}}{V_{\text{med}}} \quad (4)
\]

where \( S_{\text{cell}} \) is the total cell concentration and \( S_{\text{med,u}} \) is the unbound media concentration expressed as nanomolar. 

\( f_{u_{\text{cell}}} \) as a parameter reflects both nonspecific intracellular binding and active processes for transport. Hence, there is an advantage of the modeling in comparison with the traditional binding assays that rely on the use of dead cells (i.e., no active transport present), providing an estimate of the nonspecific cellular binding in separation to the active process.

An intracellular volume (\( V_{\text{cell}} \)) in each well of 3.9 \( \mu \)l/10^6 cells was used for rat hepatocytes (Reinoso et al., 2001), and the volume of media (\( V_{\text{med}} \)) was 400 \( \mu \)l and expressed in liters. \( V_{\text{max}} \) is expressed in nanomoles/minutes, \( K_{m,u} \) is expressed in nanomoles, and \( P_{\text{diff,u}} \) is expressed in liters/minute. \( C_{\text{Lactive,u}} \) and \( C_{\text{Luptake,u}} \) were calculated as described above. Considering the saturable nature of the active transport with increasing substrate concentrations, the maximal contribution of the active process to the total uptake was estimated and expressed as the ratio of \( C_{\text{Lactive,u}} \) over \( C_{\text{Luptake,u}} \).

The nominal concentrations corrected for \( f_{u_{\text{med}}} \) were used as initial media concentrations. Initial cell concentrations were obtained by extrapolating the first four time points for each of the concentrations to time 0. The rationale was that not all of the drug could be washed from the cell membranes or experimental plates with DPBS during the washing steps. However, because of the differences in volumes between the cell and media compartments, the largest amount of drug found in the cells at time 0 represented <2% of the total amount of compound present in the incubation.

The solving of the rate equations was performed in Matlab version 7.10 (2010) using the ODE45 solver. Values for \( K_{m,u} \), \( V_{\text{max}} \), and \( P_{\text{diff,u}} \) obtained from the initial analysis based on the conventional two-step approach were used as a priori information in the model because of availability of the data. However, the mechanistic model could still converge to a low objective function even when uninformed priors were used, due to the richness of the data in the experimental set. A value of 0.1 was used as a priori information for \( f_{u_{\text{cell}}} \) for all drugs. In a first instance, the model was allowed to optimize the four parameters with open boundaries; the greatest uncertainty was associated with the determination of \( f_{u_{\text{cell}}} \). Therefore, secondary optimization was performed by using results from the initial optimization with set boundaries, as follows: \( K_{m,u} \leq 20\% \), \( V_{\text{max}} \leq 50\% \), \( P_{\text{diff,u}} \leq 100\% \), and \( f_{u_{\text{cell}}} \) between 0 and 1. These boundaries were used as estimates of \( K_{m,u} \) and \( V_{\text{max}} \) associated with low standard errors after the first round of optimization. Boundaries were greater for \( V_{\text{max}} \) than \( K_{m,u} \) to allow \( P_{\text{diff,u}} \) to be optimized, because these two parameters

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**Mechanistic Modeling of Uptake and Metabolism in Hepatocytes**

Fig. 1. Schematic representation of the input data and steps required for the simultaneous assessment of uptake and metabolism in hepatocytes by using a mechanistic two-compartment model. \( C_0 \) represents concentration at time 0.
are closely linked. The use of cell and media data, individually and in combination, was investigated. However, because of the small amount of drug being taken up into the cells relative to the total amount of drug present in the incubation, depletion of parent drug from the media caused by active uptake was lower than could be accurately quantified. The use of the media concentrations as an input into the model did not improve confidence in the kinetic parameters, resulting in increased standard errors associated with the parameter estimates (data not shown). Therefore, kinetic estimates in rat hepatocytes were based on the measurements of the cell concentrations alone. Nevertheless, a minimum of 64 data points were available to estimate $K_{m,u}$, $V_{max}$, $P_{diff,u}$, and $f_{u,cell}$ from the rat hepatocyte experiments carried out over 2 min. This number was increased to up to 160 when experiments were extended to 45–90 min.

Unlike most studies published so far (Soars et al., 2007; Watanabe et al., 2009; Yabe et al., 2011), the model presented here takes into account the efflux of the drug from the cell into the media via passive diffusion. Comparable with previous reports, efflux caused by active transport was not included in the model, because the rat hepatocytes were plated over a short period of time, during which repolarization of the cells was limited (Hewitt et al., 2007). Internalization of efflux transporters was assumed as reported previously (Bow et al., 2008), where efflux transporters P-glycoprotein and MDR2 were absent from the canalicular membrane. In addition, these transporters were not colocalized on the basolateral membrane alongside Oatp1b1. In the present study, repaglinide and telmisartan metabolites were not found in the media samples, and mass balance of the system was verified. These arguments led us to the conclusion that efflux transporters were unlikely to have a significant effect on the experimental system used here.

**Determination of Uptake Kinetic Parameters and Metabolic Clearance of Telmisartan using a Mechanistic Modeling Approach.** In humans, telmisartan is transformed to a single glucuronide metabolite (Stangier et al., 2000). Because of a lack of nonspecific UDP-glucuronosyltransferase inhibitor, changes of both parent drug and glucuronide metabolite concentrations in the cell and media were monitored during the uptake experiment. To estimate the uptake kinetic parameters and the metabolic clearance of this drug simultaneously, a cell subcompartment was added in the two-compartment model, as illustrated in Fig. 2B. Changes in parent drug cell and media concentrations over time are defined by eq. 5.

$$
\frac{dS_{cell}}{dt} = \frac{V_{max} \times S_{med,u}}{K_{m,u} + S_{med,u}} p_{diff,u} \times S_{med,u} - S_{cell} \times f_{u,cell} \times (P_{diff,u} + CL_{met1,u}) V_{cell}
$$
where \( CL_{\text{met},u} \) is the unbound metabolic clearance expressed in liters/minute. Modeling was performed in a stepwise manner as described previously (Fig. 1). \( CL_{\text{met}} \) obtained in the depletion assay (2.2 \( \mu \)M/min/10\(^6\) cells) was used as a priori information in this model. Only measurements made in the cell compartment (parent and metabolite) were used in the analysis; 128 data points were available to estimate \( \frac{V_{\text{max}} \times S_{\text{med},u}}{K_{m,u} + S_{\text{med},u}} - P_{\text{diff},u} \times S_{\text{med},u} + P_{\text{diff},u} \times S_{\text{cell}} \times f_{u\text{cell}} \) \( V_{\text{med}} \).

**Determinant of Uptake Kinetic Parameters and Metabolic Clearance of Repaglinide using a Mechanistic Modeling Approach.** Repaglinide is a CYP2C8 and CYP3A4 substrate, and to date six phase I and one phase II metabolites have been identified (Bidstrup et al., 2003; Gan et al., 2010). To identify the major metabolites generated in rat hepatocytes, formation of M1, M2, M4, and repaglinide glucuronide was monitored in the cells during uptake studies. M2 and repaglinide glucuronide were identified as the two major metabolites in rat hepatocytes (Fig. 3A) and considered for subsequent modeling purposes. Two cell sub-compartments were added to the mechanistic two-compartment model to simultaneously describe uptake, passive diffusion, and formation of these two metabolites. Equation 8 defines the changes in repaglinide cell concentrations, whereas changes in media concentrations are as described in the case of telmisartan.

\[
\frac{dS_{\text{med},u}}{dt} = \frac{V_{\text{max}} \times S_{\text{med},u}}{K_{m,u} + S_{\text{med},u}} - P_{\text{diff},u} \times S_{\text{med},u} + P_{\text{diff},u} \times S_{\text{cell}} \times f_{u\text{cell}} \frac{V_{\text{med}}}{V_{\text{cell}}} \quad (6)
\]

\[
\frac{dS_{\text{met}}}{dt} = \frac{CL_{\text{met},u} \times S_{\text{cell}} \times f_{u\text{cell}}}{V_{\text{cell}}} \quad (7)
\]

\[
\frac{dS_{\text{met},\text{gluc}}}{dt} = \frac{CL_{\text{met},\text{gluc},u} \times S_{\text{cell}} \times f_{u\text{cell}}}{V_{\text{cell}}} \quad (9)
\]

\[
\frac{dS_{\text{met},M2}}{dt} = \frac{CL_{\text{met},M2,u} \times S_{\text{cell}} \times f_{u\text{cell}}}{V_{\text{cell}}} \quad (10)
\]

where \( S_{\text{met,gluc}} \) is repaglinide glucuronide cell concentrations; \( S_{\text{met,M2}} \) is the M2 cell concentrations; \( CL_{\text{met,gluc},u} \) is the unbound metabolic clearance caused by the formation of repaglinide glucuronide; and \( CL_{\text{met,M2},u} \) is the unbound metabolic clearance caused by the formation of M2. Initial media, cell, and metabolite concentrations were calculated as explained previously. This model was tested with three different experimental scenarios to identify its limitations.

Uptake of repaglinide and formation of repaglinide glucuronide were estimated from experiments conducted in the presence of ABT (scenario 1). Then, \( CL_{\text{met,gluc},u} \) and \( CL_{\text{met,M2},u} \) were estimated simultaneously from an incubation carried out without ABT, where both metabolites were monitored in addition to repaglinide (scenario 2). Finally, \( CL_{\text{met,gluc},u} \) and \( CL_{\text{met,M2},u} \) were replaced in eqs. 9 and 10 by Michaelis-Menten kinetic parameters \( (K_{m,\text{M2},u}, V_{\text{max,M2}}) \) and \( (K_{m,\text{gluc},u}, V_{\text{max,gluc}}) \) respectively to describe the potential saturation of the metabolic activity (scenario 3).

**Fig. 3.** Formation of M1 (○), M2 (●), M4 (□), and repaglinide glucuronide (RPGG) ■ in plated rat hepatocytes over a range of concentrations (A) and at 100 \( \mu \)M in the presence (empty bar) or absence (filled bar) of 1 mM ABT (B). Metabolite concentrations were monitored in the cells over 2 min. M1, M2, and M4 formation rates are represented as mean ± S.D. of three experiments in the absence of ABT. All other data are results from a single experiment carried out in duplicate.
Uptake kinetic parameters were obtained by using the mechanistic two-compartment model (Fig. 2A) based on incubations in rat hepatocytes over either 2 or 45–90 min, as summarized in Table 3. Representative cellular uptake profiles for rosvuastatin after 2 and 45 min are shown in Fig. 4, A and B, respectively; the profiles for the remaining drugs are included in Supplemental Fig. S2. Analysis of the 2-min incubation data showed an 11-fold range in \( K_{\text{un}} \) values, from 3.41 to 37.0 \( \mu M \) for telmisartan and pravastatin, respectively. This difference in \( K_{\text{un}} \) was reflected in an 8-fold range in \( \text{CL}_{\text{active,un}} \) for this set of seven compounds; consistent with the initial analysis, pravastatin and telmisartan were the extremes. The range of \( P_{\text{diff,un}} \) values obtained by the mechanistic model was greater compared with the conventional two-step approach. \( P_{\text{diff,un}} \) estimates were increased for some of the most permeable drugs such as telmisartan or bosentan up to 2-fold, but not for repaglinide (4.49 \( \mu M/\text{min} \times \text{10}^{6} \text{cells} \)). In the case of hydrophilic compounds such as valsartan, estimate of \( P_{\text{diff,un}} \) was low (<0.2 \( \mu M/\text{min} \times \text{10}^{6} \text{cells} \)), consistent with estimates obtained from the conventional two-step approach. \( P_{\text{diff,un}} \) was positively correlated with \( \log D_{7.4} \) for the set of drugs investigated \( (R^2 = 0.986) \), as illustrated in Fig. 5A. The \( f_{\text{u,cell}} \) estimated from the rat hepatocyte incubations over 2 min ranged from 0.01 for telmisartan to up to 1 for valsartan. A negative linear correlation was observed between \( \log f_{\text{u,cell}} \) and \( \log D_{7.4} \) \( (R^2 = 0.889) \), as illustrated in Fig. 5B.

**TABLE 2**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( K_{\text{un}} ) ( \mu M )</th>
<th>( V_{\text{max}} ) pmol/min/10^6 cells</th>
<th>( P_{\text{diff,un}} ) ( \mu M/\text{min} \times \text{10}^{6} \text{cells} )</th>
<th>( f_{\text{u,med}} )</th>
<th>( \text{CL}_{\text{active,un}} ) ( \mu M/\text{min} \times \text{10}^{6} \text{cells} )</th>
<th>Maximal Proportion of Active Transport %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>6.49 ± 2.57</td>
<td>471 ± 112</td>
<td>3.39 ± 0.47</td>
<td>0.84 ± 0.13</td>
<td>77.8 ± 15.0</td>
<td>95.7 ± 1.2</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>21.0 ± 6.6</td>
<td>1380 ± 202</td>
<td>4.12 ± 2.00</td>
<td>0.86 ± 0.05</td>
<td>69.7 ± 21.0</td>
<td>93.6 ± 4.7</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>37.0 ± 20.5</td>
<td>448 ± 329</td>
<td>0.323 ± 0.071</td>
<td>0.96</td>
<td>11.3 ± 2.8</td>
<td>97.2 ± 0.5</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>18.0 ± 3.7</td>
<td>806 ± 308</td>
<td>6.62 ± 5.11</td>
<td>0.94 ± 0.02</td>
<td>44.1 ± 13.3</td>
<td>85.9 ± 13.1</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>13.5 ± 3.9</td>
<td>1119 ± 218</td>
<td>0.345 ± 0.166</td>
<td>0.83 ± 0.04</td>
<td>84.6 ± 9.2</td>
<td>99.6 ± 0.2</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>6.89 ± 3.72</td>
<td>548 ± 141</td>
<td>13.2 ± 8.4</td>
<td>0.77 ± 0.05</td>
<td>89.5 ± 31.7</td>
<td>86.8 ± 6.8</td>
</tr>
<tr>
<td>Valsartan</td>
<td>4.38 ± 1.40</td>
<td>128 ± 68</td>
<td>0.192 ± 0.035</td>
<td>1.0 ± 0.06</td>
<td>31.5 ± 16.6</td>
<td>99.2 ± 0.4</td>
</tr>
</tbody>
</table>

\( ^* \) Parameters are expressed relative to unbound media drug concentration.
TABLE 3

Uptake kinetic parameters of seven OATP substrates estimated in rat hepatocytes by using a two-compartment mechanistic model

Uptake kinetics was measured in freshly isolated rat hepatocytes plated for 2 h over 2 or 45–90 min at 10 concentrations (0.1–300 µM). Data represent mean of three experiments ± S.D.

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_{m,u}$</th>
<th>$V_{max}$</th>
<th>$P_{diff,u}$</th>
<th>$P_{cell}$</th>
<th>$\text{CL}_{active,u}$</th>
<th>Maximal Proportion of Active Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>pmol/min/10⁶ cells</td>
<td>μl/min/10⁶ cells</td>
<td></td>
<td>μl/min/10⁶ cells</td>
<td></td>
</tr>
<tr>
<td>Bosentan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>14.2 ± 1.1</td>
<td>883 ± 108</td>
<td>6.75 ± 0.64</td>
<td>0.104 ± 0.022</td>
<td>62.1 ± 3.8</td>
<td>90.2 ± 0.5</td>
</tr>
<tr>
<td>60 min</td>
<td>6.4</td>
<td>488</td>
<td>13.5</td>
<td>0.096</td>
<td>76.8</td>
<td>85.0</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>11.6 ± 2.1</td>
<td>1055 ± 153</td>
<td>8.12 ± 2.42</td>
<td>0.031 ± 0.033</td>
<td>95.4 ± 34.4</td>
<td>91.4 ± 4.0</td>
</tr>
<tr>
<td>90 min</td>
<td>6.3</td>
<td>747</td>
<td>10.9</td>
<td>0.053</td>
<td>119</td>
<td>91.6</td>
</tr>
<tr>
<td>Pravastatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>37.0 ± 20.1</td>
<td>500 ± 412</td>
<td>0.358 ± 0.298</td>
<td>1.0 ± 0.0</td>
<td>12.5 ± 4.1</td>
<td>97.4 ± 1.4</td>
</tr>
<tr>
<td>45 min</td>
<td>23.4</td>
<td>223</td>
<td>1.29</td>
<td>0.778</td>
<td>9.5</td>
<td>88.0</td>
</tr>
<tr>
<td>Repaglinide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>28.0 ± 17.2</td>
<td>1393 ± 934</td>
<td>4.49 ± 3.82</td>
<td>0.386 ± 0.538</td>
<td>48.8 ± 2.8</td>
<td>92.0 ± 6.8</td>
</tr>
<tr>
<td>45 min</td>
<td>8.8</td>
<td>586</td>
<td>24.0</td>
<td>0.050</td>
<td>66.3</td>
<td>73.4</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>14.6 ± 1.8</td>
<td>1218 ± 97</td>
<td>0.855 ± 0.459</td>
<td>0.507 ± 0.430</td>
<td>83.8 ± 6.8</td>
<td>99.0 ± 0.6</td>
</tr>
<tr>
<td>45 min</td>
<td>11.2</td>
<td>978</td>
<td>1.01</td>
<td>0.477</td>
<td>87.6</td>
<td>98.9</td>
</tr>
<tr>
<td>Telmisartan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>3.41 ± 1.95</td>
<td>337 ± 202</td>
<td>21.4 ± 16.0</td>
<td>0.010 ± 0.009</td>
<td>98.8 ± 43.5</td>
<td>80.9 ± 12.4</td>
</tr>
<tr>
<td>90 min</td>
<td>2.32</td>
<td>160</td>
<td>22.5</td>
<td>0.024</td>
<td>68.7</td>
<td>75.3</td>
</tr>
<tr>
<td>Valsartan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>6.38 ± 0.95</td>
<td>159 ± 70</td>
<td>0.184 ± 0.118</td>
<td>1.0 ± 0.0</td>
<td>26.4 ± 15.2</td>
<td>99.2 ± 0.5</td>
</tr>
<tr>
<td>90 min</td>
<td>2.7</td>
<td>117</td>
<td>0.37</td>
<td>1.0</td>
<td>43.3</td>
<td>99.1</td>
</tr>
</tbody>
</table>

* Parameters are expressed relative to unbound media drug concentration.

Fig. 4. Representative kinetic profile of rosuvastatin uptake in plated rat hepatocytes at 10 concentrations (0.1–300 µM) over 2-min incubation (A) and 45-min incubation (B). Lines represent the predicted uptake profile based on a mechanistic two-compartmental model describing the changes in drug concentrations in both the cells and the incubation media over time (eqs. 3 and 4). Data points are mean of duplicate measurements.

Using a mechanistic model to fit the data over 2 min, a larger interexperiment variation in active uptake parameter estimates was observed compared with the conventional two-step approach. In the case of rosuvastatin, repaglinide, and bosentan, variation in $\text{CL}_{active,u}$ ($n = 3$) was within the variability seen for the rosuvastatin control across all experiments (CV 35%). Moreover, when data from 2-min incubations were used, large standard errors (>50%) were associated with estimations of $P_{cell}$ and/or $P_{diff,u}$ for all drugs with the exception of bosentan. This resulted in high interexperiment variability in both parameters, with CV values as large as 106 and 139% for $P_{cell}$ of pitavastatin and repaglinide, respectively. To improve the estimation of these two parameters, incubations were extended to 45–90 min to allow a steady state between the media and intracellular compartments to be achieved, as illustrated in Fig. 4B for rosuvastatin. Extended uptake studies resulted in on average 2-fold lower $K_{m,u}$ estimates across the dataset ($p = 0.02$). Differences in $V_{max}$ estimates between short and extended incubations were negligible, resulting in a 30% increase in $\text{CL}_{active,u}$ when extended time points were used (Table 3). However, the use of longer incubations changed $P_{diff,u}$ and $P_{cell}$ estimates by, on average, 2.3-fold for this dataset. These changes were led mainly by pronounced differences in the estimates of these parameters obtained for repaglinide (4- to 8-fold; Table 3). When repaglinide was omitted from the dataset, the difference in $P_{diff,u}$ and $P_{cell}$ parameters between shorter and longer incubation was <2-fold. However, the use of extended incubations resulted in a de-
crease in standard errors associated with $P_{\text{diff, u}}$ and $f_{\text{u,cell}}$ to <50% for all seven drugs investigated. Comparison of uptake parameters estimates obtained in the current study in plated hepatocytes and data obtained previously by our group in suspended hepatocytes (Yabe et al., 2011) showed good agreement in $K_m$ values between the two sets of data (all within 2-fold). Only valsartan showed a greater $K_m$ in suspended compared with plated hepatocytes. $V_{\text{max}}$ estimates were also within 2-fold, with the exception of bosentan and telmisartan. Bosentan was the only drug that exhibited a greater $V_{\text{max}}$ in plated compared with suspended hepatocytes.

One of the advantages of the mechanistic two-compartment uptake model is that it accounts for bidirectional passive diffusion. Whereas the conventional two-step approach assumes that passive diffusion has an additive effect on total cellular uptake (Fig. 6, A and C), the use of the mechanistic model illustrates the differences in the rates of passive diffusion of the drug in and out of the cell over the range of concentrations studied. Passive diffusion from the cell back into the media can in some instances be more pronounced than the flux in the opposite direction (when the unbound cellular concentration is greater relative to the concentration in the media), as shown in Fig. 6, B and D for rosuvastatin and pitavastatin, respectively. The profiles for the remaining drugs are shown in Supplemental Fig. S3. The extent of this effect on total uptake is generally observed at low substrate concentrations, as illustrated in the case of pitavastatin in Fig. 6D. However, in the case of rosuvastatin, which displays comparable uptake $K_{m,u}$ to pitavastatin, but lower $P_{\text{diff, u}}$ and less pronounced intracellular binding, this effect is observed at substrate concentrations as high as >100 µM (Fig. 6B).

**Simultaneous Estimation of Uptake and Metabolism of Telmisartan in Plated Rat Hepatocytes.** No broad-spectrum chemical inhibitors of UDP-glucuronosyltransferases are currently available, hence both parent compound and telmisartan glucuronide were measured to estimate the extent of uptake and metabolism. In this case, the mechanistic two-compartment model was extended to allow the simultaneous estimation of uptake kinetic parameters and metabolic clearance of telmisartan, as illustrated in Fig. 2B. Incorporation of metabolism in the model decreased the estimate for telmisartan uptake $K_{m,u}$ by 1.7-fold, whereas a 2.6-fold increase in $f_{\text{u,cell}}$ was observed (0.026 versus 0.010) (Table 4); however, the $f_{\text{u,cell}}$ estimate was associated with a large variation (CV >70%). $P_{\text{diff, u}}$ and $CL_{\text{active, u}}$ were increased by 94 and 56%, respectively. $CL_{\text{met, u}}$ was estimated to be 38.9 µl/min/10⁶ cell, 15-fold greater than the clearance obtained from the depletion assay. Incorporation of metabolism in the modeling of telmisartan data improved the precision of fitting of telmisartan cell concentrations; rmse decreased from 52 to 46 when metabolism was included. No bias was observed in either case (gmfe <1.1), as illustrated in Supplemental Fig. S4. Extension of the incubation time to 90 min ($n = 1$) had minimal impact on $CL_{\text{met, u}}$ and uptake $K_{m,u}$ values. In contrast, $P_{\text{diff, u}}$ and $V_{\text{max}}$ estimates were decreased by 2.4- and 1.6-fold, respectively, leading to a 2.8-fold decrease in $CL_{\text{active, u}}$. However, the maximal proportion of total uptake caused by active transport was not affected compared with the use of shorter incubation time points (76 versus 79%, respectively).

**Estimation of Uptake and Metabolism of Repaglinide in Plated Rat Hepatocytes.** Uptake of repaglinide was assessed in the presence and absence of ABT in either a mechanistic or extended mechanistic two-compartment model. Analogous to telmisartan, the extended mechanistic model was required to account for repaglinide glucuronidation occurring during the uptake experiment performed in the presence of ABT. The addition of 1 mM ABT had no effect
on repaglinide glucuronidation, as illustrated in Fig. 3B; at the same time, formation of M2 metabolite was reduced by 99.6% compared with incubations in the absence of ABT. This refined modeling was performed by using the extended incubation up to 45 min to characterize the uptake and metabolism of repaglinide simultaneously; therefore, all parameters in this section for repaglinide are based on 45-min incubations (Table 5). Consideration of repaglinide glucuronide formation in the model (data in the presence of ABT) resulted in an uptake $K_{m,u}$ of 10.0 $\mu$M and $f_{u_{cell}}$ of 0.051, consistent with the estimates obtained from the mechanistic two-compartment uptake model when glucuronidation was not taken into account. However, $P_{diff,u}$ decreased by 45%, whereas $CL_{active,u}$ increased by 30%, resulting in a significant increase in the maximal proportion of repaglinide uptake caused by active transport from 73 to 87%.

### Table 4

**Uptake and metabolism kinetic parameters of telmisartan estimated in rat hepatocytes by using an extended two-compartment mechanistic model**

Uptake kinetics was measured in freshly isolated rat hepatocytes plated for 2 h over 2 min or 90 min at nine concentrations (0.1–100 $\mu$M). Data represent mean of three experiments $\pm$ S.D.

<table>
<thead>
<tr>
<th></th>
<th>Extended Two-Compartment Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 Min ($n = 3$)</td>
</tr>
<tr>
<td>$K_{m,u}$, $\mu$M</td>
<td>5.96 ± 3.23</td>
</tr>
<tr>
<td>$V_{max}$, pmol/min/10^6 cells</td>
<td>774 ± 208</td>
</tr>
<tr>
<td>$E_{diff,u}$, $\mu$M/10^6 cells</td>
<td>41.6 ± 12.9</td>
</tr>
<tr>
<td>$f_{u_{cell}}$</td>
<td>0.028 ± 0.019</td>
</tr>
<tr>
<td>$CL_{active,u}$, $\mu$M/10^6 cells</td>
<td>154 ± 68.8</td>
</tr>
<tr>
<td>$CL_{met,gluc,u}$, $\mu$M/10^6 cells</td>
<td>38.9 ± 38.8</td>
</tr>
<tr>
<td>Maximal proportion of active transport, %</td>
<td>78.7 ± 13.8</td>
</tr>
</tbody>
</table>

**Fig. 6.** Representative uptake kinetic profiles after 2-min incubation for rosuvastatin and pitavastatin over a range of substrate concentrations based on the conventional two-step approach (eqs. 1 and 2) (A and C) or the mechanistic modeling approach (eq. 3) (B and D). In A and C, total and passive uptake rates were obtained from measurements at 37 and 4°C, respectively. Active uptake was expressed as the difference between total uptake and passive diffusion. In B and D, only measurements at 37°C were used to estimate uptake kinetics; active transport and passive diffusion were delineated by using the mechanistic model (eq. 3). ○ and solid lines represent measured and predicted total uptake estimated from the kinetic parameters obtained with each approach, respectively. Dashed and dotted lines represent cellular uptake caused by active transport and passive diffusion, respectively. ● represent measurements obtained at 4°C. Data points are mean of duplicate measurements.
(0.90 μl/min/10⁶ cells) was 95-fold lower than CLactive,u and was lower than the metabolic clearance estimated from the depletion assay (11.3 μl/min/10⁶ cells). It is noteworthy that Km,u, Vmax, Pdiff,u, fH,c, and CLmet,gluc,u could be estimated only with confidence (CV <50%) when both repaglinide and repaglinide glucuronide cell concentrations were incorporated in the model.

After initial assessment of the uptake in the presence of ABT, uptake studies were performed in the absence of ABT. In this case, cell concentrations of the repaglinide, M2, and glucuronide metabolite were monitored, and models with different levels of complexity were applied for data analysis. In addition to parameters estimated in scenario 1, M2 and repaglinide glucuronide formation were described either by single clearance parameters or full kinetics of these pathways (scenarios 2 and 3, respectively). Any differences in parameter estimates and associated error depending on whether uptake and metabolic processes were considered in isolation or combined were investigated. All uptake parameters estimated from the data in the presence of ABT were consistent with the values obtained in the first scenario, with the exception of Vmax and Pdiff,u, which increased by 1.6- and 2.7-fold, respectively. Estimated uptake of Km,u and fH,c were low (Table 5), leading to a CLactive,u of 119 μl/min/10⁶ cells. CLmet,gluc,u was comparable with the value obtained from the experiment with ABT (0.63 compared with 0.90 μl/min/10⁶ cells). CLmet,M2,u was estimated to be 274-fold lower than CLactive,u and the overall unbound metabolic clearance was 112-fold lower than CLactive,u.

Finally, an attempt was made to estimate the Michaelis-Menten parameters describing the metabolism of repaglinide into M2 and repaglinide glucuronide (scenario 3). The experiment where repaglinide, M2, and repaglinide glucuronide cell concentrations were measured over time was used for this purpose. The model was able to fit repaglinide, M2, and repaglinide glucuronide cell concentration data accurately, as illustrated in Fig. 7. Estimated Km,M2,u was greater than the highest repaglinide cell concentration studied in this experiment (data not shown). Hence, a single clearance parameter was judged sufficient to describe accurately the kinetics of M2 formation. Both Km,gluc,u and Vmax,gluc were low (12 μM and 17 pmol/min/10⁶ cells, respectively), leading to a CLmet,gluc,u of 1.41 μl/min/10⁶ cells, which was approximately 2-fold greater than results obtained by using a single clearance parameter. Repaglinide glucuronide cell concentrations were better described by full kinetics than when CLmet,gluc,u alone was used (Fig. 7B). All uptake parameters were estimated with confidence (CV <54%) and were within 2-fold of the estimates obtained in the previous scenarios; however, the CV associated with uptake parameters was increased compared with scenario 2.

Extension of the mechanistic model and incorporation of various processes occurring during the uptake experiment improved the fitting of repaglinide cell concentrations. Accounting for metabolism occurring in the incubations carried out in the presence of ABT decreased the rmse from 216 to 176 when metabolism was incorporated in the mechanistic model (scenario 1; Supplemental Fig. S5). This value decreased further to 128 when incubations were performed without ABT and corresponding metabolite formation was described by clearance parameters (scenario 2). When the full kinetic of repaglinide glucuronide was estimated (scenario 3), rmse increased to 188. No bias was observed in the fitting in any of the scenarios investigated (gmfe <1.2).

### Discussion

In the present study, the suitability of a mechanistic model to characterize the interplay between active uptake, bidirectional passive diffusion, and intracellular binding was comprehensively investigated by using seven OATP substrates in plated rat hepatocytes. In contrast to previous studies, the use of a mechanistic model allowed the simultaneous assessment of both uptake and metabolism, as illustrated with telmisartan and repaglinide and, as such, represented substantial improvement over the conventional two-step approach. The impact of modeling of either single or multiple metabolic pathways on the parameter estimates describing uptake and intracellular binding was also investigated.

Hepatic uptake often consists of an active, saturable component driven by transporters and a passive, nonsaturable component determined by the ability of a drug to cross cellular membranes. Currently, the most common technique to estimate passive diffusion in the absence of active uptake is based on incubations at 4°C run in parallel with the experiment at 37°C (conventional two-step approach). However, at 4°C, fluidity of the cellular membrane is known to be disturbed, leading to temperature-dependent passive diffusion (Frézard and Garnier-Suillerot, 1998; Poirier et al., 2008). Alternatively, a cocktail of OATP inhibitors can be used to measure passive diffusion at 37°C in the absence of active uptake and metabolism at 4°C.

### Table 5

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Scenario 1, +ABT</th>
<th>Scenario 2, −ABT</th>
<th>Scenario 3, −ABT</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM</td>
<td>15.0</td>
<td>27.2</td>
<td>68.9</td>
</tr>
<tr>
<td>5 μM</td>
<td>11.0</td>
<td>25.7</td>
<td>41.2</td>
</tr>
<tr>
<td>10 μM</td>
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<td>27.2</td>
<td>68.9</td>
</tr>
<tr>
<td>5 μM</td>
<td>11.0</td>
<td>25.7</td>
<td>41.2</td>
</tr>
</tbody>
</table>

---, no data.
uptake. Both cyclosporine and rifampicin are potent inhibitors of the main OATPs expressed in the liver, i.e., OATP1B1 and OATP1B3 (IC\textsubscript{50} \(0.5\) M) (Tirona et al., 2003; Amundsen et al., 2010), but also a number of other transporters. In the present study, simultaneous use of 10 M cyclosporine and 20 M rifampicin resulted in the inhibition of uptake only in the case of pravastatin, rosuvastatin, and valsartan. These findings were in agreement with substrate-dependent inhibition seen in the transfected cell lines (Noé et al., 2007), but could also be a result of the differences in substrate/inhibitor specificities between rat and human (Hagenbuch and Gui, 2008). Therefore, one of the advantages of the mechanistic two-compartment model applied here was the ability to estimate \(P\text{\textsubscript{diff,\text{u}}}\) solely from measurements obtained at 37°C. In addition, unlike the conventional two-step approach, the mechanistic model allowed the dynamic assessment of passive diffusion by taking into account the bidirectional nature of this process between the cellular and media compartments. The importance of accounting for the passage of drug out of the cells back into the media is illustrated in Fig. 6 for pitavastatin and rosuvastatin. Both drugs show high affinity for uptake (\(K\text{\textsubscript{m,u}} \leq 10\) M), but an approximately 10-fold difference in their \(P\text{\textsubscript{diff,\text{u}}}\) and intracellular binding leads to a different unbound cellular concentration relative to the media, and consequently, total uptake of these drugs across the range of substrate concentrations differs.

The seven drugs included in the dataset showed different physicochemical properties, with LogD\textsubscript{7.4} values ranging from –1 to 2.85 for valsartan and rosuvastatin, respectively. Maximal contribution of the active transport to the total uptake was >80% for the drugs investigated based on the mechanistic two-compartment model (Tables 3–5). Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in \(K\text{\textsubscript{m,u}}\) for the drugs investigated. Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in \(K\text{\textsubscript{m,u}}\) for the drugs investigated. Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in \(K\text{\textsubscript{m,u}}\) for the drugs investigated. Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in \(K\text{\textsubscript{m,u}}\) for the drugs investigated. Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in \(K\text{\textsubscript{m,u}}\) for the drugs investigated. Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in \(K\text{\textsubscript{m,u}}\) for the drugs investigated.
influenced by lipophilicity (Fig. 5), whereas uptake $K_{m,u}$ was not driven by $\log D_{7.4}$, consistent with findings from studies in suspended hepatocytes (Yabe et al., 2011). In addition, no correlation was observed between $K_{m,u}$ and these two parameters. $P_{\text{diff},u}$ was the most sensitive parameter to the use of the mechanistic model, especially in the case of telmisartan and bosentan, the most lipophilic drugs studied here. Both $P_{\text{diff},u}$ and $f_{\text{u,cell}}$ required extended incubation times to allow the system to reach steady state and be estimated precisely. $K_{m}$ and $V_{\text{max}}$ values were generally consistent between plated and suspended hepatocytes (Yabe et al., 2011); however, $P_{\text{diff},u}$ estimates were on average 6-fold greater in suspended hepatocytes compared with plated hepatocytes. This difference is particularly noticeable for drugs with limited passive diffusion such as valsartan and rosuvastatin, which exhibited 21- and 8-fold differences between the two systems, respectively. Greater surface area being exposed to the drug in suspended hepatocytes in comparison to plated systems may account for the differences observed. The $f_{\text{u,cell}}$ estimates for pitavastatin, repaglinide, rosuvastatin, and telmisartan were in good agreement in both experimental settings, in contrast to the other three drugs (pravastatin, valsartan, and bosentan) where $f_{\text{u,cell}}$ values obtained in plated hepatocytes were generally higher. It is noteworthy that these results were generated in different experimental systems and were subject to different modeling approaches. Parameters obtained in the current study accounted for the nonspecific binding, in contrast to previous studies (Ishigami et al., 1995; Nezasa et al., 2003; Yabe et al., 2011). The extent of nonspecific binding for the drugs in the current dataset was not extensive (<25%) and would not be expected to contribute substantially to the differences observed.

Accounting for the loss of telmisartan caused by glucuronidation during the uptake experiment in the model (Fig. 2B) resulted in an approximately 2-fold increase in $K_{m,u}$, $P_{\text{diff},u}$, and $f_{\text{u,cell}}$ estimates. A low uptake $K_{m,u}$, as well as high intracellular binding obtained by the extended mechanistic two-compartment model (Table 4) led to the highest estimate of active uptake clearance observed in the present study. However, active uptake of telmisartan was only 4-fold greater than passive diffusion and the metabolic clearance. Thus active uptake is not the rate-limiting process of telmisartan hepatic disposition in rat, in contrast with other actively transported drugs (e.g., saquinavir and nelfinavir; Parker and Houston, 2008), which clearly show uptake rate limited hepatocellular kinetics.

In addition to telmisartan, repaglinide was used to explore the performance and limitations of the mechanistic and extended mechanistic two-compartment models. In the first scenario, ABT was used to delineate phase I and phase II metabolism; in that case, only repaglinide glucuronide was formed. Modeling demonstrated the need to monitor the cell concentrations of both parent and metabolite to estimate uptake and metabolism with confidence. In the absence of ABT (scenario 2), we were able to estimate repaglinide uptake kinetics together with the metabolic clearances by using the cell concentrations of repaglinide and the two major metabolites. Finally, in scenario 3, we showed that the measurement of both parent and metabolite cell concentrations (Fig. 7) allowed the model to be extended to estimate the kinetics of the metabolite formation, in addition to drug uptake (Table 5). To date this level of delineation has been challenging for uptake transporter substrates in hepatocyte assays, because the unbound intracellular concentration at the enzyme site is unknown. Repaglinide passive diffusion through the cellular membrane represented only 23% of the total uptake (consistent with its moderate lipophilicity; $\log D_{7.4} = 2.6$). Active uptake was 3.3-fold greater than passive diffusion and more importantly 112-fold greater than the overall metabolic clearance. Considering this significant contribution of active uptake of repaglinide to total uptake, metabolism can be identified as the slowest, rate-limiting step in the disposition of this drug in rat hepatocytes. Although the metabolic pathways differ in humans, if a similar pattern was observed in human hepatocytes, this would explain the strong drug-drug interactions reported with cyclosporine and gemfibrozil (and its glucuronide), which are both modulators of P450 enzymes and OATPs (Niemi et al., 2003; Kajosaari et al., 2005).

An additional advantage of the mechanistic approach is in the model-driven design of the uptake experiments, which is essential for defining various processes occurring and determining the rate-limiting process. The length of incubation required to estimate uptake and metabolism simultaneously could be predicted successfully from uptake kinetic parameters obtained from the two-compartment model and the metabolic clearance determined in the depletion assay. This showed that, although 2-min incubations were sufficient for the study of telmisartan uptake and metabolism, 45-min incubations were required for repaglinide. The present study clearly illustrates the need for uptake studies to be performed over a range of substrate concentrations (10 points) and multiple time points (eight points used here) to allow detailed delineation of uptake kinetics. Extended incubation times allowed steady state between cell and the media concentrations to be reached and resulted in corresponding improvements in the estimation of intracellular binding in particular. It is important to note that for drugs that do not undergo metabolism uptake parameter estimates were comparable between shorter (2 min) and extended (45–90 min) incubation times.

In the case of drugs undergoing both uptake and metabolism, the major metabolites need to be identified and the linearity of their formation confirmed. A stepwise approach ($\geq$ABT) can be implemented to differentiate phase I and phase II metabolic pathways and assess their impact on the estimation of uptake parameters, as shown in the case of repaglinide. This analysis has also shown that when the formation of all major metabolites cannot be monitored metabolite clearance value can be fixed to that obtained in a depletion assay. However, the use of this approach resulted in an underestimation of $CL_{\text{active},u}$ $P_{\text{diff},u}$ and $f_{\text{u,cell}}$, but not uptake $K_{m,u}$. The main limitation of the modeling approach presented here is the requirement for a large number of cells. In the present study, nearly 20 million hepatocytes were used for each incubation carried out over 2 min, 30 million for longer incubations. Moreover, a thorough knowledge of the metabolism of the drug of interest is of prime importance if metabolic clearances are to be estimated. However, the detailed characterization of uptake kinetics in rat hepatocytes presented here should facilitate the optimization of subsequent uptake studies in human hepatocytes.

In conclusion, a comprehensive analysis of uptake kinetics of seven OATP substrates was performed in rat hepatocytes.
The proposed mechanistic model allowed the simultaneous assessment of uptake, passive diffusion, intracellular binding, and metabolism, making it a tool for choice for investigating the interplay between multiple processes. Thorough understanding of these processes is crucial for identifying the rate-limiting step in hepatic disposition of a drug, and coupled with a whole-body PBPK approach it can aid with the understanding of the potential consequences of multiple disposition mechanisms in vivo and assist in the design of appropriate clinical drug–drug interaction studies.

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Authorship Contributions

Participated in research design: Ménochet, Kenworthy, Houston, and Galetin.
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References


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