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

Karelle Ménochet, Kathryn E. Kenworthy, J. Brian Houston and Aleksandra Galetin

Centre for Applied Pharmacokinetic Research, School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, M13 9PT, United Kingdom (K.M., J.B.H. and A.G.)

Department of Drug Metabolism and Pharmacokinetics, GlaxoSmithKline, Ware, United Kingdom (K.E.K.)
Running title: Mechanistic modeling of uptake and metabolism in hepatocytes

Corresponding author: Dr Aleksandra Galetin
School of Pharmacy and Pharmaceutical Sciences,
University of Manchester, Stopford Building
Oxford Road,
Manchester, M13 9PT, UK
Tel: (+) 44 161 275 6886
Fax: (+) 44 161 275 8349
Email: Aleksandra.Galetin@manchester.ac.uk

Tables: 5
Figures: 7
Abstract: 249
Introduction: 808
Discussion: 1655
References: 41

Recommended section assignment: Metabolism, Transport, and Pharmacogenomics

Abbreviations: OATP, Organic Anion Transporting Protein; ABT, 1-aminobenzotriazole; M1, 2-despiperidyl-2-amino repaglinide; M2, 2-despiperidyl-2(5-carboxypentylamine) repaglinide; M4, 3’-hydroxy repaglinide; fu med, unbound fraction in the media; fu cell, intracellular unbound fraction; Pdiff, unbound passive diffusion clearance; K m,u, unbound affinity constant; V max, maximum transport rate; CL active,u, unbound active uptake clearance;
$CL_{\text{uptake,un}}$, unbound total uptake clearance; $CL_{\text{met,un}}$, unbound metabolic clearance; $\text{LogD}_{7.4}$, distribution coefficient between octanol and water at pH 7.4; CV, coefficient of variation; NCE, new chemical entity; PBPK, physiologically-based pharmacokinetic models.
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 in order to describe all the processes occurring during the in vitro uptake experiments performed in freshly isolated rat hepatocytes plated for 2h. Uptake of rosuvastatin, pravastatin, pitavastatin, valsartan, bosentan, telmisartan and repaglinide was investigated over a 0.1-300 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 using a mechanistic two-compartment model describing uptake kinetics (Km,u, Vmax, CLactive,u), passive diffusion (Pdiff,u) and intracellular binding (fu cell). When required (telmisartan and repaglinide), the model was extended to account for the metabolism (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 LogD7.4 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 (OATP) are expressed on the sinusoidal membrane of hepatocytes and have been identified as important contributors to the disposition of the statins, angiotensin II receptor inhibitors and anti-diabetics (Hagenbuch and Gui, 2008; Giacomini et al., 2010; Watanabe 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 active uptake of NCEs in vitro is vital to assess any potential clinical risk associated with this mechanism, either in particular patient population or with certain co-medication (EMA, 2010; Giacomini 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 to predict human hepatic disposition from 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 of 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 drug would permeate back from the cell into the media 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; Poirier et al., 2009; Yabe et al., 2011) or have 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 to estimate accurately any subsequent metabolism and predict liver and systemic concentrations using a PBPK modeling approach. To that aim, a mechanistic two-compartment model was developed to simultaneously fit all the concentration-time points during uptake experiments performed over a range of initial concentrations, extending the model previously described by Poirier et al (2008). The model presented here allows the estimation of intracellular binding and characterization of uptake kinetics either in isolation or in conjunction with metabolism.

The present study focuses on the characterization of seven drugs, namely 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 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 (Link et al., 2008; Ieiri et al., 2009) or clinical drug-drug interactions reported with OATP inhibitors (Stangier et al., 2000; Kajosaari et al., 2005; Giacomini 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 approach were compared. Unlike the mechanistic model, conventional two-step approach requires prior data transformation to delineate passive from the 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.
Material and Methods

Chemicals. Bosentan, pitavastatin, pravastatin, rosuvastatin, telmisartan and valsartan were purchased from Sequoia Research Products (Pangbourne, UK). Telmisartan acyl-β-D-glucuronide, 2-despiperidyl-2-amino repaglinide (M1), 2-despiperidyl-2(5-carboxypentylamine) repaglinide (M2), 3’-hydroxy repaglinide (M4) and repaglinide acyl-β-D-glucuronide were obtained from Toronto Research Chemicals (Toronto, 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 (Poole, UK) and of the highest grade available.

Isolation of the rat hepatocytes. Rat hepatocytes were isolated from 250-300 g Sprague Dawley rat livers (Charles River, Margate, UK), following the two-step collagenase perfusion method described previously (Berry and Friend, 1969). Hepatocytes were suspended in William’s medium E (WME) 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 haemocytometer and the viability was assessed 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 WME. 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 2h at 37°C in an atmosphere containing 5% CO₂ to allow adhesion to the collagen.

Measurement of uptake in rat hepatocytes. Uptake was measured over a range of 10 concentrations comprised 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, due to its limited solubility in aqueous buffer. This wide range was chosen as affinity of the seven drugs of interest towards 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 pre-warmed serum-free Dulbecco’s Phosphate Buffered Saline (DPBS). Substrate was dissolved in dimethylsulfoxide (DMSO), and diluted in DPBS (maximum 1% DMSO). Incubation was started by the addition of 400 µL of substrate on top of the monolayers. After incubation for 30, 60, 90 or 120 seconds at 37°C, the substrate was collected for analysis. Cells were washed three times with ice-cold DPBS. 200 µL of water was added to each monolayer. Samples were stored at -20°C until analysis. In each experiment, incubations were carried out in duplicate at 37°C. For each drug, experiments were performed in rat hepatocytes prepared from 3 separate isolations. Incubations were also carried out at 4°C in order to assess passive diffusion of the substrates through the cellular membrane. At 4°C, a single well was used per time-point. In preliminary studies, the use of a cocktail of OATP inhibitors (10 µM cyclosporine and 20 µM rifampicin) was investigated to assess passive permeation at 37°C in the absence of active uptake. However, substrate-dependent inhibition was observed (data not shown) and 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 in order 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 5 concentrations. In order to inhibit phase I metabolism of repaglinide and bosentan, a non-specific P450 pan-inhibitor 1-aminobenzotriazole (ABT) was added to the incubations at concentration of 1 mM (Mico et al., 1988; Yabe et al., 2011). Previous studies in HEK-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 ± 19.5 µL/min/10^6 cells.

**Depletion assay.** As part of a preliminary analysis, depletion of 0.1 µM repaglinide and telmisartan was measured in rat hepatocytes plated as described above; the medium was removed after plating. Monolayers were rinsed twice with pre-warmed 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, 60 and 90 min for telmisartan and 0.5, 1, 2, 5, 10, 30 and 45 min for repaglinide, by the addition of 400 µL ice-cold methanol containing 1 µM of the appropriate internal standard. Samples were stored at -20°C until analysis.

**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 6720 g. Likewise, samples generated in the depletion assay were centrifuged in a similar manner. In both cases, 20 µL of supernatant was analysed by LC-MS/MS as described below. All samples were analysed 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 (Waters, Milford, MA). The analyte and associated internal standard were separated on a Luna C18 column (3 µm, 50 x 4.6 mm) (Phenomenex, Torrance, CA). The flow through the HPLC was 1 mL/min and was 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 (fu\textsubscript{med}).** The y intercept (t\textsubscript{0}) of the linear regression of the media concentration over time plot was calculated at each incubation concentration. The media was assumed to be free of proteins as all hepatocytes were attached to the collagen support and the cell monolayers were rinsed thoroughly before each incubation, as stated above. Therefore, fu\textsubscript{med} was expressed as the slope of the linear regression of the unbound concentration extrapolated at t\textsubscript{0} vs. the initial incubation concentration plot. Representative example of the fu\textsubscript{med} estimation is illustrated for pitavastatin in the supplementary data (Figure S1).

**Initial determination of uptake kinetic parameters using 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 vs. time plot. Passive diffusion (P\textsubscript{diff}) was calculated from the uptake rates measured at 4°C as shown in equation 1. P\textsubscript{diff} was then inserted in equation 2 as a constant and the active uptake kinetic parameters were estimated using Grafit v6 (Erithacus Software Ltd, Horley, Surrey, UK).

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

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{diff}} \times S \]
where \( v \) is the uptake rate and \( V_{\text{max}} \) the maximum uptake rate. \( K_m \) is the affinity constant, \( P_{\text{diff}} \) the passive diffusion clearance and \( S \) the substrate concentration. All kinetic parameters were corrected for nonspecific binding of the substrate to the incubation environment (\( f_u \text{med} \)). \( CL_{\text{active,u}} \), the unbound active uptake clearance, was expressed as the ratio of \( V_{\text{max}} \) over the unbound \( K_m \) (\( K_{m,u} \)). The total unbound uptake clearance (\( CL_{\text{uptake,u}} \)) included both the active component (\( CL_{\text{active,u}} \)) and clearance via passive diffusion (\( P_{\text{diff,u}} \)).

### 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 v7.10® (2010) (Mathworks, Natick, MA, USA). Unlike the conventional two-step approach, this model allows simultaneous fitting of all concentration-time points during the experiment and only relies on measurements made at 37°C. It allows 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; the analysis of uptake profiles after short and extended incubation times was performed. The scheme of steps taken to implement this model is presented in Figure 1. The mechanistic model for the assessment of active uptake in hepatocytes is illustrated in Figure 2A. Differential equations 3 and 4 describe the change in cell and media concentrations over time, respectively. All parameters are expressed per well and therefore normalized for the number of cells per well.

\[
\frac{dS_{\text{cell}}}{dt} = \frac{V_{\text{max}} S_{\text{med,u}}}{K_{m,u} + S_{\text{med,u}}} + P_{\text{diff,u}} S_{\text{med,u}} - P_{\text{diff,u}} S_{\text{cell}} f_u \text{cell} \tag{3}
\]

\[
\frac{dS_{\text{med,u}}}{dt} = -\frac{V_{\text{max}} S_{\text{med,u}}}{K_{m,u} + S_{\text{med,u}}} - P_{\text{diff,u}} S_{\text{med,u}} + P_{\text{diff,u}} S_{\text{cell}} f_u \text{cell} \tag{4}
\]
where $S_{\text{cell}}$ is the total cell concentration and $S_{\text{med},u}$ the unbound media concentration expressed in nM. $f_{u,\text{cell}}$ is the unbound fraction in the cell.

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

$V_{\text{cell}}$ is the intracellular volume in each well and $V_{\text{med}}$ the volume of media. $V_{\text{cell}}$ of 3.9 µL/10⁶ cells was used for rat hepatocytes (Reinoso et al., 2001). $V_{\text{med}}$ was 400 µL and expressed in L. $V_{\text{max}}$ is expressed in nmol/min, $K_{\text{m},u}$ in nM and $P_{\text{diff},u}$ in L/min. $CL_{\text{active},u}$ and $CL_{\text{uptake},u}$ were calculated as described previously. Considering saturable nature of the active transport with increasing substrate concentrations, maximal contribution of the active process to the total uptake was estimated and expressed as the ratio of $CL_{\text{active},u}$ over $CL_{\text{uptake},u}$.

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 concentration to time 0. The rationale was that not all the drug could be washed from the cell membranes or experimental plates with DPBS during the washing steps. However, due to 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 v. 7.10® (2010) using the ODE45 solver. Values for $K_{\text{m}}$, $V_{\text{max}}$ and $P_{\text{diff}}$ obtained from the initial analysis based on the conventional two-step approach were used as a priori information in the model due to availability of the data. However, 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 4 parameters with open boundaries; the greatest uncertainty was associated with the determination of $f_{u,\text{cell}}$. Therefore, secondary optimization was performed using results from the initial optimization with set boundaries, as follows: $K_{m,a} \pm 20\%$, $V_{\max} \pm 50\%$, $P_{\text{diff},a} \pm 100\%$ and $f_{u,\text{cell}}$ between 0 and 1. These boundaries were used as $K_{m,a}$ and $V_{\max}$ estimates were associated with low standard errors after the first round of optimization. Boundaries were greater for $V_{\max}$ than $K_{m,a}$ to allow $P_{\text{diff},a}$ to be optimized, as these two parameters are closely linked. The use of cell and media data, individually and in combination, was investigated. However, due to 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 due to active uptake was lower than could be accurately quantified. The use of the media concentrations as an input into the model did not improve the 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 $K_{m,a}$, $V_{\max}$, $P_{\text{diff},a}$ and $f_{u,\text{cell}}$ 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 to previous reports, efflux due to active transport was not included in the model, as the rat hepatocytes were plated over a short period of time, during which repolarization of the cells is limited (Hewitt et al., 2007). Internalization of efflux transporters was assumed as reported previously (Bow et al., 2008), where efflux transporters P-gp and MRP2 were absent from the canalicular membrane. In addition, these
transporters were not co-localized 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 in the experimental system used here.

**Determination of uptake kinetic parameters and metabolic clearance of telmisartan using a mechanistic modeling approach.** In human, telmisartan is transformed to a single glucuronide metabolite (Stangier et al., 2000). Due to a lack of nonspecific UGT inhibitor, changes of both parent drug and glucuronide metabolite concentrations in the cell and media were monitored during the uptake experiment. In order to estimate the uptake kinetic parameters and the metabolic clearance of this drug simultaneously, a cell sub-compartment was added in the two-compartment model, as illustrated in Figure 2B. Changes in parent drug cell and media concentrations are described by differential equations 5 and 6, respectively, whereas changes in metabolite cell concentrations over time are defined by equation 7.

\[
\frac{dS_{\text{cell}}}{dt} = \frac{V_{\text{max}} \times S_{\text{med},u} + P_{\text{diff},u} \times S_{\text{med},u} - S_{\text{cell}} \times f_u \times (P_{\text{diff},u} + CL_{\text{met},u})}{K_{m,u} + S_{\text{med},u}} \tag{5}
\]

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

\[
\frac{dS_{\text{met}}}{dt} = \frac{CL_{\text{met},u} \times S_{\text{cell}} \times f_u}{V_{\text{cell}}} \tag{7}
\]

where \(CL_{\text{met},u}\) is the unbound metabolic clearance, expressed in L/min. Modeling was performed in a stepwise manner as described previously (Figure 1). \(CL_{\text{met}}\) obtained in the
depletion assay (2.2 μL/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 K_{m,u}, V_{max}, P_{diff,u}, f_{u,cell} and CL_{met,u}. Initial media and cell concentrations were measured as described previously. Initial metabolite cell concentrations were obtained by extrapolating concentrations obtained for the first four time points to time 0. Initial metabolite concentrations were not null, but the amount found in the cell at time 0 represented <0.01% of the total amount of drug present in the incubation. Linearity of telmisartan glucuronide formation over the length of the incubations (90 min) was confirmed. Telmisartan glucuronide was also monitored in the media, but concentrations were found to be below the limit of quantification.

**Determination 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). In order 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 2 major metabolites in rat hepatocytes (Figure 2A) and were considered for subsequent modeling purposes. Two cell sub-compartments were added to the mechanistic two-compartment model in order 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 (Equation 6). Repaglinide glucuronide and M2 cell concentrations over time are described by equations 9 and 10, respectively.
\[
\frac{dS_{\text{cell}}}{dt} = \frac{V_{\text{max}} \times S_{\text{med,u}}}{K_{\text{m,u}} + S_{\text{med,u}}} + P_{\text{diff,u}} \times S_{\text{med,u}} - S_{\text{cell}} \times f_u \times \left( P_{\text{diff,u}} + CL_{\text{met,gluc,u}} + CL_{\text{met,M2,u}} \right) - S_{\text{cell}} \times f_u \times \left( P_{\text{diff,u}} + CL_{\text{met,gluc,u}} + CL_{\text{met,M2,u}} \right) \]

(8)

\[
\frac{dS_{\text{met,gluc}}}{dt} = \frac{CL_{\text{met,gluc,u}} \times S_{\text{cell}} \times f_u}{V_{\text{cell}}}
\]

(9)

\[
\frac{dS_{\text{met,M2}}}{dt} = \frac{CL_{\text{met,M2,u}} \times S_{\text{cell}} \times f_u}{V_{\text{cell}}}
\]

(10)

where \( S_{\text{met,gluc}} \) is repaglinide glucuronide cell concentrations and \( S_{\text{met,M2}} \) the M2 cell concentrations. \( CL_{\text{met,gluc,u}} \) is the unbound metabolic clearance due to the formation of repaglinide glucuronide and \( CL_{\text{met,M2,u}} \) is the unbound metabolic clearance due to the formation of M2. Initial media, cell and metabolite concentrations were calculated as explained before. This model was tested with three different experimental scenarios in order 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 equations 9 and 10 by Michaelis-Menten kinetic parameters \( (K_{m,M2,u} \) and \( V_{\text{max,M2}} \) and \( K_{m,gluc,u} \) and \( V_{\text{max,gluc}} \), respectively) in order to describe the potential saturation of the metabolic activity (Scenario 3). \( CL_{\text{met}} \) obtained in the depletion assay was used as a priori information in this model. In all the cases, only measurements made in the cell compartments (parent and metabolites) were used in the analysis. Formation of all metabolites was linear over the length of the incubation (45 min). Depending on the scenario investigated, between 240 and 360 data points were available to estimate \( K_{m,u} \) \( V_{\text{max}, P_{\text{diff}}, u} \)
Any differences in parameter estimates and associated error depending on whether uptake and metabolic processes were considered in isolation or combined were investigated.

**Identifiability of the kinetic parameters estimated from the various models.**

Identifiability of the kinetic parameters was verified using Differential Algebra for Identifiability of SYstems software (DAISY) (Bellu et al., 2007). Estimates from all the models described previously were found to be only locally identifiable, when only observations made in the cell compartment were used. However, one of the two potential sets of kinetic estimates was predicted to always contain a nonsensical negative $K_{m,u}$ value. By setting boundaries to ensure only positive values of the kinetic parameters were taken into account, a unique set of estimates could be defined.

**Statistical analysis.** Standard error associated with each parameter estimate was computed in Matlab v7.10® (2010) using a Jacobian approach reported previously (Landaw and DiStefano, 1984). Coefficients of variation (CV) for each estimate were subsequently calculated and expressed as percentages to assess the quality of the parameter estimates. The arithmetic mean, standard error and CV were calculated from experiments conducted using rat hepatocytes isolated on three different occasions. Goodness of fit was assessed by visual inspection of the data and minimum objective function value. In order to quantify the improvement of the fitting when metabolism was added to the model, the root mean square error (rmse) and the geometric mean fold error (gmfe) were calculated using equations 11 and 12 for results obtained with telmisartan when incubations were performed over 2 min and for repaglinide when incubations were carried out over 45 min.
\[ \text{rmse} = \sqrt{\frac{1}{N} \sum (\text{Predicted cell concentration} - \text{Observed cell concentration})^2} \]  

(11)

\[ \text{grmfe} = 10 \left( \frac{1}{N} \sum \left( \frac{\text{Predicted cell concentration}}{\text{Observed cell concentration}} \right) \right) \]  

(12)

**Collation of LogD~7.4~.** LogD~7.4~ values determined experimentally were collated from the literature for each drug investigated. Values used in this study and corresponding references are presented in the Supplementary Material (Supplementary Table S1).
Results

Initial analysis of uptake kinetics based on the conventional two-step approach. Uptake of seven selected compounds was assessed in plated rat hepatocytes; kinetic parameters obtained in the initial analysis based on the conventional two-step approach are summarized in Table 2. Parameter estimates were corrected for nonspecific binding in the media, which was <25% for all the drugs investigated. The \( f_{\text{med}} \) ranged from 0.77 (telmisartan) to 1 (valsartan). Nonspecific binding had only a marginal impact on the kinetic parameters, in particular for pravastatin and repaglinide where \( f_{\text{med}} > 0.9 \). The \( K_{m,u} \) ranged from 4.38 to 37.0 \( \mu M \) for valsartan and pravastatin, respectively. Pravastatin showed the lowest extent of active uptake with \( CL_{\text{active,u}} \) of 11.3 \( \mu L/min/10^6 \) cells, whereas telmisartan had the highest \( CL_{\text{active,u}} \) in rat hepatocytes (89.5 \( \mu L/min/10^6 \) cells). Variation in uptake activity observed between hepatocyte isolations for all drugs in the dataset was assessed relative to rosuvastatin (included in all incubation sets as a control) and was comparable for all drugs (CV within 35%), with the exception of valsartan (CV of 53% due to variations in \( V_{\text{max}} \)). Passive diffusion clearances through the cellular membrane estimated from 4°C data were low relative to the active uptake clearances for all compounds investigated, ranging from 0.192 to 13.2 \( \mu L/min/10^6 \) cells for valsartan and telmisartan, respectively.

Estimation of uptake kinetic parameters based on mechanistic modeling approach. Following initial analysis, uptake kinetic parameters were obtained using the mechanistic two-compartment model (Figure 2A) based on incubations in rat hepatocytes over either 2 or 45-90 min, as summarized in Table 3. Representative cellular uptake profiles for rosuvastatin after 2 and 45 min are shown in Figure 4A and B, respectively; the profiles for the remaining drugs are included in Supplementary Material, Figure S2. Analysis of the 2 min incubation data showed an 11-fold range in \( K_{m,u} \) values, from 3.41 to 37.0 \( \mu M \) for telmisartan and
pravastatin, respectively. This difference in \( K_{m,u} \) was reflected in an 8-fold range in \( CL_{active,u} \) for this set of seven compounds; consistent with the initial analysis, pravastatin and telmisartan were the extremes. The range of \( P_{diff,u} \) values obtained by the mechanistic model was greater compared to the conventional two-step approach. \( P_{diff,u} \) 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 \( \mu L/min/10^6 \) cells). In the case of hydrophilic compounds such as valsartan, estimate of \( P_{diff,u} \) was low (<0.2 \( \mu L/min/10^6 \) cells), consistent with estimates obtained from the conventional two-step approach. \( P_{diff,u} \) was positively correlated with LogD_{7.4} for the set of drugs investigated (\( R^2 = 0.986 \)), as illustrated in Figure 5A. The \( f_{cell} \) 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 \( f_{cell} \) and LogD_{7.4} (\( R^2 = 0.889 \)), as illustrated in Figure 5B.

Using a mechanistic model to fit the data over 2 min, a larger inter-experiment variation in active uptake parameter estimates was observed compared to the conventional two-step approach. In the case of rosuvastatin, repaglinide and bosentan, variation in \( CL_{active,u} \) (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 \( f_{cell} \) and/or \( P_{diff,u} \) for all drugs with the exception of bosentan. This resulted in high inter-experiment variability in both parameters, with CV values as large as 106 and 139% for \( f_{cell} \) of pitavastatin and repaglinide, respectively. In order 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 Figure 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 30% increase in \( CL_{active,u} \) when extended
time-points were used (Table 3). However, the use of longer incubations changed $P_{\text{diff,u}}$ and $f_{\text{u,cell}}$ estimates by, on average, 2.3-fold for this dataset. These changes were mainly led by pronounced differences in estimates of these parameters obtained for repaglinide (4- to 8-fold, Table 3). When repaglinide was omitted from the dataset, difference in $P_{\text{diff,u}}$ and $f_{\text{u,cell}}$ parameters between shorter and longer incubation was <2-fold. However, use of extended incubations resulted in decrease in standard errors associated with the $P_{\text{diff,u}}$ 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 in 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 to plated hepatocytes. $V_{\text{max}}$ estimates were also within two-fold, with the exception of bosentan and telmisartan. Bosentan was the only drug that exhibited a greater $V_{\text{max}}$ in plated compared to suspended hepatocytes.

One of the advantages of the mechanistic two-compartment uptake model is that it accounts for the bidirectional passive diffusion. While the conventional two-step approach assumes that passive diffusion has additive effect on total cellular uptake (Figure 6A and C), the use of the mechanistic model illustrates 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 Figures 6B and D for rosuvastatin and pitavastatin, respectively. The profiles for the remaining drugs are shown in Supplementary Material, Figure S3. The extent of this effect on total uptake is generally observed at low substrate concentrations, as illustrated in the case of pitavastatin in Figure 6D. However, in the case of rosuvastatin, which displays comparable uptake $K_{\text{m,u}}$ 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 (Figure 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 in order to estimate the extent of uptake and metabolism. In this case, the mechanistic two-compartment model was extended to allow simultaneous estimation of uptake kinetic parameters and metabolic clearance of telmisartan, as illustrated in Figure 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 $f_{u,cell}$ was observed (0.026 vs. 0.010) (Table 4); however, the $f_{u,cell}$ estimate was associated with large variation (CV > 70%). $P_{diff,u}$ and $CL_{active,u}$ were both increased by 94 and 56%, respectively. $CL_{met,u}$ 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 Figure S4 - Supplementary material. Extension of the incubation time to 90 min (n=1) had minimal impact on $CL_{met,u}$ and uptake $K_{m,u}$ values. In contrast, $P_{diff,u}$ and $V_{max}$ estimates were decreased by 2.4- and 1.6-fold, respectively, leading to a 2.8-fold decrease in $CL_{active,u}$. However, the maximal proportion of total uptake due to active transport was not affected compared to the use of shorter incubation time points (76% vs. 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, applying 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. Addition of 1 mM ABT had no effect on repaglinide glucuronidation, as illustrated in Figure 3B; at the same time, formation of M2 metabolite was reduced by 99.6% compared to incubations in the absence of ABT. This refined modeling was performed using the extended incubation up to 45 min in order to characterize 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 a uptake $K_m,u$ of 10.0 µM and $f_{u,\text{cell}}$ of 0.051, consistent with the estimates obtained from the mechanistic two-compartment uptake model when glucuronidation was not taken into account. However, $P_{\text{diff},u}$ decreased by 45% while $CL_{\text{active},u}$ increased by 30%, resulting in a significant increase in the maximal proportion of repaglinide uptake due to active transport from 73 to 87%. $CL_{\text{met,gluc},u}$ (0.90 µL/min/10^6 cells) was 95-fold lower than $CL_{\text{active},u}$ and was lower than the metabolic clearance estimated from the depletion assay (11.3 µL/min/10^6 cells). It is noteworthy that $K_m,u$, $V_{\text{max}}$, $P_{\text{diff},u}$, $f_{u,\text{cell}}$ and $CL_{\text{met,gluc},u}$ could only be estimated with confidence (CV<50%) when both repaglinide and repaglinide glucuronide cell concentrations were incorporated in the model.

Following 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 $V_{\text{max}}$ and $P_{\text{diff},u}$ which were increased by 1.6- and 2.7-fold, respectively. Estimated uptake $K_{\text{m},u}$ and $f_{u\text{cell}}$ were low (Table 5), leading to a $\text{CL}_{\text{active},u}$ of 119 µL/min/10^6 cells. $\text{CL}_{\text{met,gluc},u}$ was comparable to the value obtained from the experiment with ABT (0.63 compared to 0.90 µL/min/10^6 cells). $\text{CL}_{\text{met,M2},u}$ was estimated to be 274-fold lower than $\text{CL}_{\text{active},u}$ and the overall unbound metabolic clearance was 112-lower than $\text{CL}_{\text{active},u}$.

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 Figure 7A-C. Estimated $K_{\text{m},M2,u}$ 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 $K_{\text{m,gluc},u}$ and $V_{\text{max,gluc}}$ were low (12 µM and 17 pmol/min/10^6 cells, respectively), leading to a $\text{CL}_{\text{met,gluc},u}$ of 1.41 µL/min/10^6 cells, which was approximately 2-fold greater than results obtained using a single clearance parameter. Repaglinide glucuronide cell concentrations were better described by full kinetics than when $\text{CL}_{\text{met,gluc},u}$ alone was used (Figure 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 to the 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, Figure S5 - Supplementary material). 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 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 (Frezard 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. Both cyclosporine and rifampicin are potent inhibitors of the main OATPs expressed in the liver, i.e., OATP1B1 and OATP1B3 (IC_{50} ≤ 0.5 µM) (Tirona et al., 2003; Amundsen et al., 2010), but also a number of other transporters. In the present study, simultaneous use of 10 µM cyclosporine and 20 µM 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 (Noe 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 $P_{\text{diff,}u}$ solely from measurements obtained at 37°C. In addition, unlike the conventional two-step approach, the mechanistic model allowed dynamic assessment of passive diffusion 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 Figure 6 for pitavastatin and rosvastatin. Both drugs show high affinity for uptake ($K_{m,u} \leq 10 \mu M$), but an approximately 10-fold difference in their $P_{\text{diff,}u}$ 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 Log$D_{7.4}$ values ranging from -1 to 2.85 for valsartan and rosvastatin, respectively. Maximal contribution of the active transport to the total uptake was > 80% for the drugs investigated based on mechanistic two-compartment model (Tables 3-5). Analysis of uptake kinetic data by the mechanistic model resulted in more than 10-fold range in $K_{m,u}$ with telmisartan and valsartan showing the highest uptake affinity ($K_{m,u} < 10 \mu M$). Both passive diffusion and $f_{u\text{cell}}$ parameters were highly influenced by lipophilicity (Figure 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 $f_{u\text{cell}}$ required extended incubation times to allow system to reach steady-state in order to be estimated precisely.
Km and Vmax values were generally consistent between plated and suspended hepatocytes (Yabe et al., 2011); however, Pdiff estimates were on average 6-fold greater in suspended compared to plated hepatocytes. This difference is particularly noticeable for drugs with limited passive diffusion such as valsartan and rosuvastatin, which exhibited 21- and 8-fold difference between the two systems, respectively. Greater surface area being exposed to the drug in suspended hepatocytes in comparison to plated system may rationalize differences observed. The fucell estimates for pitavastatin, repaglinide, rosuvastatin and telmisartan were in good agreement in both experimental settings, in contrast to remaining three drugs (pravastatin, valsartan and bosentan) where fucell 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., 2001; Nezasa et al., 2003; Yabe et al., 2010). 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 due to glucuronidation during the uptake experiment in the model (Figure 2B), resulted in an approximately 2-fold increase in Km,u, Pdiff,u and fucell estimates. A low uptake Km,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 in order 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 using the cell concentrations of repaglinide and the two major metabolites. Finally, in scenario 3, we showed that measurement of both parent and metabolite cell concentrations (Figure 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, as 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, i.e., rate limiting step in the disposition of this drug in rat hepatocytes. Although the metabolic pathways differ in human, 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), both modulators of CYPs 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 to define various processes occurring and determine 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 (8 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 (+/- ABT) can be implemented to differentiate phase I and phase II metabolic pathways and assess their impact on the estimation of uptake parameters, as illustrated in the case of repaglinide. This analysis has also shown that when the formation of all major metabolites cannot be monitored, metabolic clearance value can be fixed to that obtained in a depletion assay. However, the use of this approach resulted in an under-estimation 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, detailed characterization of uptake kinetics in rat hepatocytes presented here should facilitate 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 simultaneous assessment of uptake, passive diffusion, intracellular binding and metabolism, making it a tool of choice to investigate 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 can aid the understanding of the potential consequences of multiple ADME mechanisms in vivo and assist in the design of appropriate clinical drug-drug interaction studies.
Acknowledgements

We acknowledge the assistance of Dr. David Hallifax and Sue Murby with the LC-MS/MS analysis and of Drs Michael Gertz, Henry Pertinez and Kayode Ogungbenro (all University of Manchester) with the modeling of uptake data.
Authorship contribution

Participated in research design: Ménochet, Galetin, Houston, Kenworthy

Conducted experiments: Ménochet

Performed data analysis: Ménochet, Galetin

Wrote or contributed to the writing of the manuscript: Ménochet, Galetin, Houston, Kenworthy
References


Ieiri I, Higuchi S, and Sugiyama Y (2009) Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for inter-individual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. Expert Opin Drug Metab Toxicol 5:703-729.


Niemi M, Backman JT, Neuvonen M, and Neuvonen PJ (2003) Effects of gemfibrozil, itraconazole, and their combination on the pharmacokinetics and pharmacodynamics of
repaglinide: potentially hazardous interaction between gemfibrozil and repaglinide.

*Diabetologia* **46**:347-351.


Footnotes

K.M. was supported by a PhD studentship from GlaxoSmithKline, Ware, UK and the Biotechnology and Biological Sciences Research Council, UK.

Part of this work was presented at the 9th International ISSX meeting in Istanbul, Turkey, September 4-8, 2010.
Figure legends

**Figure 1:** Schematic representation of the input data and steps required for the simultaneous assessment of uptake and metabolism in hepatocytes using mechanistic two-compartment model. $C_0$ represents concentration at time zero.

**Figure 2:** A. Two-compartment model (Equations 3 and 4) describing the change in cell and media concentration of the parent drug over time due to active uptake ($K_{m,u}$, $V_{max}$), passive diffusion ($P_{diff,u}$) and intracellular binding ($f_{u\text{cell}}$) in a plated rat hepatocyte assay. $S_{\text{cell}}$ and $S_{\text{med,u}}$, represent the total cell and unbound media concentrations, respectively. B. Extended mechanistic two-compartment model (Equations 8-10) describing the interplay of active uptake ($K_{m,u}$, $V_{max}$), passive diffusion ($P_{diff,u}$), intracellular binding ($f_{u\text{cell}}$) and metabolism ($CL_{\text{met1,u}}$ and $CL_{\text{met2,u}}$) in plated rat hepatocyte assay. $S_{\text{med,u}}$, $S_{\text{cell}}$, $S_{\text{Met1,cell}}$ and $S_{\text{Met2,cell}}$ represent the unbound concentration of the parent drug in the media, the total concentration of drug in the cell and the total concentration of the metabolites in the cell, respectively. In the case of telmisartan, a single metabolite ($\text{met1}$) was taken into account for the modeling of its uptake and metabolism.

**Figure 3:** Formation of M1 (○), M2 (●), M4 (□) and repaglinide glucuronide RPGG (■) in plated rat hepatocytes over a range of concentrations (A) and at 100 µM in the presence (white bar) or absence (black bar) of 1 mM 1-aminobenzotriazole (ABT) (B). Metabolite concentrations were monitored in the cells over 2 min. M1, M2 and M4 formation rates are represented as mean ± SD of 3 experiments in absence of ABT. All other data are results from a single experiment carried out in duplicate.
**Figure 4:** Representative kinetic profile of rosuvastatin uptake in plated rat hepatocytes at 10 concentrations (0.1 – 300 µM) over 2 min (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 (Equations 3 and 4). Data points are mean of duplicate measurements.

**Figure 5:** Relationship between LogD_{7.4} and P_{diff,u} (A) and f_{u,cell} (B) in rat hepatocytes for seven OATP substrates. Estimates were obtained using a two-compartment model describing the change of drug concentrations in both the cell and the incubation media over 2 min incubations for non-metabolized drugs. Estimates for repaglinide and telmisartan (identified as 6 and 7) were generated using an extended two-compartment model incorporating metabolism. Data are represented as mean ± SD of 3 experiments with the exception of repaglinide (n=1). 1: Valsartan; 2: Pravastatin; 3: Rosuvastatin; 4: Bosentan; 5: Pitavastatin; 6: Repaglinide; 7: Telmisartan.

**Figure 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 (Equations 1 and 2) (A, C) or the mechanistic modeling approach (Equation 3) (B, D). In panels 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 panels B and D, only measurements at 37°C were used to estimate uptake kinetics, active transport and passive diffusion were delineated using the mechanistic model (Equation 3). Closed symbols and solid lines represent measured and predicted total uptake estimated from kinetic parameters obtained with each approach, respectively. Dashed and dotted lines represent cellular uptake due to active transport and passive diffusion.
respectively. Open symbols represent measurement obtained at 4°C. Data points are mean of duplicate measurements.

**Figure 7:** Representative cellular concentrations of repaglinide (A), repaglinide glucuronide (B) and M2 (C) obtained when measured in plated rat hepatocytes at 10 concentrations (0.1 – 300 µM) over 45 min incubations without 1-aminobenzotriazole. Lines represent the predicted uptake profile based on the extended mechanistic two-compartmental model (Figure 2B) describing the changes in drug and metabolite concentrations in both the cells and the incubation media over time. Data points are mean of duplicate measurements.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>LC gradient</th>
<th>m/z</th>
<th>CE (eV)</th>
<th>Internal standard</th>
<th>m/z</th>
<th>CE (eV)</th>
<th>Limit of quantification (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan C, D</td>
<td>C, D</td>
<td>552.40 &gt; 202.20</td>
<td>35</td>
<td>Mibefradil</td>
<td>496.20 &gt; 202.20</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>Pitavastatin A, B, C, D</td>
<td>A, B, C, D</td>
<td>422.25 &gt; 290.25</td>
<td>40</td>
<td>Mibefradil</td>
<td>496.20 &gt; 202.20</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Repaglinide C, D</td>
<td>C, D</td>
<td>453.25 &gt; 230.20</td>
<td>25</td>
<td>Indomethacin</td>
<td>358.10 &gt; 139.10</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>M1 C, D</td>
<td>C, D</td>
<td>385.35 &gt; 162.15</td>
<td>9</td>
<td>Indomethacin</td>
<td>358.10 &gt; 139.10</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>M2 C, D</td>
<td>C, D</td>
<td>485.50 &gt; 194.20</td>
<td>15</td>
<td>Indomethacin</td>
<td>358.10 &gt; 139.10</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>M4 C, D</td>
<td>C, D</td>
<td>469.45 &gt; 246.30</td>
<td>25</td>
<td>Indomethacin</td>
<td>358.10 &gt; 139.10</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>Repaglinide glucuronide</td>
<td>C, D</td>
<td>629.40 &gt; 230.40</td>
<td>60</td>
<td>Indomethacin</td>
<td>358.10 &gt; 139.10</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>Rosuvastatin A, B, C, D</td>
<td>A, B, C, D</td>
<td>482.30 &gt; 258.20</td>
<td>30</td>
<td>Mibefradil</td>
<td>496.20 &gt; 202.20</td>
<td>20</td>
<td>0.1</td>
</tr>
<tr>
<td>Telmisartan A, B, D</td>
<td>A, B, D</td>
<td>515.15 &gt; 276.15</td>
<td>60</td>
<td>Verapamil</td>
<td>455.10 &gt; 165.00</td>
<td>35</td>
<td>0.1</td>
</tr>
<tr>
<td>Telmisartan glucuronide</td>
<td>A, B, D</td>
<td>691.10 &gt; 515.05</td>
<td>33</td>
<td>Verapamil</td>
<td>455.10 &gt; 165.00</td>
<td>35</td>
<td>0.1</td>
</tr>
<tr>
<td>Valsartan C, D</td>
<td>C, D</td>
<td>436.45 &gt; 235.25</td>
<td>15</td>
<td>Mibefradil</td>
<td>496.20 &gt; 202.20</td>
<td>20</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^a\): A: Water:Methanol (90:10) containing 0.05% (v/v) formic acid, B: Water:Methanol (10:90) containing 0.05% (v/v) formic acid, C: Water:Methanol (90:10) containing 10 mM ammonium acetate, D: Water:Methanol (10:90) containing 10 mM ammonium acetate
TABLE 2

Uptake kinetic parameters of seven OATP substrates estimated in rat hepatocytes using a conventional two-step approach. Uptake kinetics was measured in freshly isolated rat hepatocytes plated for 2h over 2 min at 10 concentrations (0.1 – 300 µM). Data represent mean of 3 experiments ± SD, with the exception of fu_{med} of pravastatin (n=1)

<table>
<thead>
<tr>
<th>Drug</th>
<th>K_{m,u}a (µM)</th>
<th>V_{max} (pmol/min/10^6 cells)</th>
<th>P_{diff,u}a (µL/min/10^6 cells)</th>
<th>fu_{med} (µL/min/10^6 cells)</th>
<th>CI_{active,u}a (µL/min/10^6 cells)</th>
<th>Maximal proportion of active transport (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>6.39 ± 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>6.99 ± 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>

*a Parameters are expressed relative to unbound media drug concentration
TABLE 3

Uptake kinetic parameters of 7 OATP substrates estimated in rat hepatocytes using a two-compartment mechanistic model. Uptake kinetics was measured in freshly isolated rat hepatocytes plated for 2h over 2 or 45-90 min at 10 concentrations (0.1 – 300 µM). Data represent mean of 3 experiments ± SD

<table>
<thead>
<tr>
<th>Drug</th>
<th>2 min</th>
<th>60 min</th>
<th>2 min</th>
<th>90 min</th>
<th>2 min</th>
<th>45 min</th>
<th>2 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_m,u</td>
<td>V_max</td>
<td>P_dif,u</td>
<td>f_u cell</td>
<td>CL_active,u</td>
<td>Maximal proportion of active transport (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bosentan</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Pitavastatin</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Pravastatin</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Repaglinide</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Telmisartan</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>
<td></td>
<td></td>
</tr>
<tr>
<td>Valsartan</td>
<td>2 min</td>
<td>90 min</td>
<td>6.38 ± 0.95</td>
<td>2.7</td>
<td>159 ± 70</td>
<td>117</td>
<td>0.184 ± 0.118</td>
<td>0.37</td>
</tr>
</tbody>
</table>
TABLE 4

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

Uptake kinetics was measured in freshly isolated rat hepatocytes plated for 2h over 2 min or 90 min at 9 concentrations (0.1 – 100 µM). Data represent mean of 3 experiments ± SD

<table>
<thead>
<tr>
<th></th>
<th>Extended 2-compartment model</th>
<th>Extended 2-compartment model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 min (n=3)</td>
<td>90 min (n=1)</td>
</tr>
<tr>
<td>$K_{m,u}$ (µM)</td>
<td>5.96 ± 3.23</td>
<td>8.65</td>
</tr>
<tr>
<td>$V_{max}$ (pmol/min/10^6 cells)</td>
<td>774 ± 208</td>
<td>482</td>
</tr>
<tr>
<td>$P_{diff,u}$ (µL/min/10^6 cells)</td>
<td>41.6 ± 12.9</td>
<td>17.2</td>
</tr>
<tr>
<td>$f_{u\text{cell}}$</td>
<td>0.026 ± 0.019</td>
<td>0.002</td>
</tr>
<tr>
<td>$CL_{active,u}$ (µL/min/10^6 cells)</td>
<td>154 ± 68.8</td>
<td>55.7</td>
</tr>
<tr>
<td>$CL_{met,gluc,u}$ (µL/min/10^6 cells)</td>
<td>38.9 ± 38.8</td>
<td>37.5</td>
</tr>
<tr>
<td>Maximal proportion of active transport (%)</td>
<td>78.7 ± 13.8</td>
<td>76.4</td>
</tr>
</tbody>
</table>

$K_{m,u}$: Unbound affinity constant; $V_{max}$: Maximum uptake rate; $P_{diff,u}$: Unbound passive diffusion clearance; $f_{u\text{cell}}$: Intracellular unbound fraction; $CL_{active,u}$: Unbound active uptake; $CL_{met,gluc,u}$: Unbound metabolic clearance due to telmisartan glucuronide formation.
**TABLE 5**

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

Uptake kinetics was measured in freshly isolated rat hepatocytes plated for 2h over 45 min at 10 concentrations (0.1 – 300 µM)

<table>
<thead>
<tr>
<th>Scenario</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model</strong></td>
<td><strong>Extended 2-compartment model 45 min</strong></td>
<td><strong>Extended 2-compartment model 45 min</strong></td>
<td><strong>Extended 2-compartment model 45 min</strong></td>
</tr>
<tr>
<td><strong>Condition</strong></td>
<td>+ ABT</td>
<td>- ABT</td>
<td>- ABT</td>
</tr>
<tr>
<td><strong>Input data</strong></td>
<td>Repaglinide and repaglinide glucuronide cell concentrations (n=1)</td>
<td>Repaglinide, repaglinide glucuronide and M2 cell concentrations (n=1)</td>
<td>Repaglinide, repaglinide glucuronide and M2 cell concentrations (n=1)</td>
</tr>
<tr>
<td><strong>Km,u (µM)</strong></td>
<td>10.0</td>
<td>11.0</td>
<td>15.0</td>
</tr>
<tr>
<td><strong>Vmax (pmol/min/10^6 cells)</strong></td>
<td>857</td>
<td>1315</td>
<td>903</td>
</tr>
<tr>
<td><strong>Pdiff,u (µL/min/10^6 cells)</strong></td>
<td>13.1</td>
<td>35.7</td>
<td>27.2</td>
</tr>
<tr>
<td><strong>fu_cell</strong></td>
<td>0.051</td>
<td>0.074</td>
<td>0.058</td>
</tr>
<tr>
<td><strong>CLactive,u (µL/min/10^6 cells)</strong></td>
<td>85.9</td>
<td>119</td>
<td>60.2</td>
</tr>
<tr>
<td><strong>CLmet,M2,u (µL/min/10^6 cells)</strong></td>
<td>-</td>
<td>0.434</td>
<td>0.546</td>
</tr>
<tr>
<td><strong>Km,gluc,u (µM)</strong></td>
<td>-</td>
<td>-</td>
<td>12.0</td>
</tr>
<tr>
<td><strong>Vmax,gluc (pmol/min/10^6 cells)</strong></td>
<td>-</td>
<td>-</td>
<td>17.0</td>
</tr>
<tr>
<td>Parameter</td>
<td>CL_{met,gluc,u} (\mu L/min/10^6 cells)</td>
<td>Maximal proportion of active transport (%)</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------</td>
<td>------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.901</td>
<td>86.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.630</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.412</td>
<td>68.9</td>
<td></td>
</tr>
</tbody>
</table>

*K_{m,u}*: Unbound affinity constant; \( V_{\text{max}} \): Maximum uptake rate; \( P_{\text{diff},u} \): Unbound passive diffusion clearance; \( f_{u,\text{cell}} \): Intracellular unbound fraction; \( \text{CL}_{\text{active},u} \): Unbound active uptake; \( \text{CL}_{\text{met,gluc,u}} \): Unbound metabolic clearance due to telmisartan glucuronide formation; \( \text{CL}_{\text{met,M2,u}} \): Unbound metabolic clearance due to formation of M2; \( K_{m,\text{gluc,u}} \): Unbound affinity constant towards UGT responsible for formation of repaglinide glucuronide; \( V_{\text{max,gluc}} \): Maximum formation rate of repaglinide glucuronide; \( \text{CL}_{\text{met,gluc,u}} \): Unbound metabolic clearance due to formation of repaglinide glucuronide.
Media samples

- Extrapolation of C0 for each concentration (time points 0.5 – 2 min)
- $f_{\text{med}}$
- Nominal substrate concentrations corrected for $f_{\text{med}}$
- Initial unbound media concentrations

Cell samples

- Parent drug
- Extrapolation of C0 for each concentration (time points 0.5 – 2 min)
- Initial total cell concentrations
- Total cell concentrations $S_{\text{cell}}$ (all time points)

- Metabolite (s)
- Extrapolation of C0 for each concentration (time points 0.5 – 2 min)
- Initial total metabolite cell concentrations
- Total cell metabolite concentrations $S_{\text{M,cell}}$ (all time points)

Mechanistic two-compartment model

- $K_{m,u}, V_{\text{max}}, P_{\text{diff, u}}, f_{\text{cell}}, CL_{\text{met,u}} (K_{m,\text{met,u}}, V_{\text{max,met}})$
Figure 2A

\[
\frac{V_{\text{max}} \times S_{\text{med},u}}{K_{\text{m},u} + S_{\text{med},u}}
\]

\[
P_{\text{diff},u} \times S_{\text{med},u}
\]

\[
P_{\text{diff},u} \times S_{\text{cell}} \times f_{\text{cell}}
\]

$S_{\text{cell}}$

Parent drug in cell

$S_{\text{med},u}$

Parent drug in media
Figure 2B

\[ S_{M1,cell} \]

Metabolite 1 in cell

\[ S_{\text{cell}} \]

Parent drug in cell

\[ \frac{V_{\text{max}} \times S_{\text{med,cell}}}{K_m,\text{cell} + S_{\text{med,cell}}} \]

\[ P_{\text{diff,cell}} \times S_{\text{med,cell}} \]

\[ P_{\text{diff,cell}} \times S_{\text{cell}} \times f_{\text{cell}} \]

\[ S_{\text{med,cell}} \]

Parent drug in media

\[ S_{M2,cell} \]

Metabolite 2 in cell

\[ CL_{\text{met1,cell}} \times S_{\text{cell}} \times f_{\text{cell}} \]

\[ CL_{\text{met2,cell}} \times S_{\text{cell}} \times f_{\text{cell}} \]
Figure 3

A

Metabolite formation (pmol/min/10^6 cells)

[Repaglinide] (µM)

B

Production rate at 100 µM (pmol/min/10^6 cells)

M1  M2  M4  RPGG

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4

A

B

Rosuvastatin cell concentration (µM)

Time (min)

Smed = 0.1 µM

Smed = 1 µM

Smed = 10 µM

Smed = 30 µM

Smed = 60 µM

Smed = 300 µM

Smed = 0.3 µM

Smed = 3 µM

Smed = 20 µM

Smed = 100 µM

Smed = 60 µM

Smed = 300 µM

Smed = 20 µM

Smed = 10 µM

Smed = 3 µM

Smed = 0.3 µM

Smed = 0.1 µM

Smed = 1 µM

Smed = 10 µM

Smed = 30 µM

Smed = 60 µM

Smed = 300 µM
A

Log $P_{\text{diff},u}$ = 0.5977 x Log$D_{7.4}$ - 0.0437
$R^2 = 0.986$

B

Log $f_{\text{u,cell}}$ = -0.4129 x Log$D_{7.4}$ - 0.4379
$R^2 = 0.889$
Figure 7

A

**Repaglinide cell concentration (µM)**

- Smed = 0.1 µM
- Smed = 0.3 µM
- Smed = 1 µM
- Smed = 3 µM
- Smed = 10 µM
- Smed = 20 µM
- Smed = 30 µM
- Smed = 60 µM
- Smed = 100 µM
- Smed = 300 µM

B

**Repaglinide glucuronide cell concentration (µM)**

C

**M2 metabolite cell concentration (µM)**
Supplementary Data

Simultaneous Assessment of Uptake and Metabolism in Rat Hepatocytes: A Comprehensive Mechanistic Model – The Journal of Pharmacology and Experimental Therapeutics (JPET #187112)

K. Ménochet, K.E. Kenworthy, J.B. Houston and A. Galetin

Table S1: Measured Log$_{D_{7.4}}$ values collated from the literature for each of the 7 investigated OATP substrates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log$<em>{D</em>{7.4}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valsartan</td>
<td>-1.00</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>-0.49</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>-0.33</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1.30</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>1.49</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>2.60</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>2.85</td>
</tr>
</tbody>
</table>

References:


**Figure S1:** Representative profile of the estimation of the unbound fraction in the media ($f_{u\text{med}}$) for pitavastatin in rat hepatocytes. Data point represent the mean of 3 experiments ± SD. Solid line is the line of best fit. Dashed line represents the line of unity. $f_{u\text{med}}$ equals to the slope of the line of best fit.
Figure S2: Representative uptake kinetic profile of pitavastatin (A), pravastatin (B), bosentan (C), valsartan (D), repaglinide (E) and telmisartan (F) in plated rat hepatocytes at 10 concentrations (0.1 – 300 µM) over 2 min incubations. 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. Data points are mean of duplicate measurements.
**Figure S3:** Representative uptake kinetic profiles after 2 min incubation for pravastatin, valsartan, telmisartan, bosentan and repaglinide over a range of substrate concentrations based on the Michaelis-Menten approach (Equations 1 and 2) (A, C, E, G, I) or the mechanistic modeling approach (Equation 3) (B, D, F, H, J). Closed symbols and solid lines represent measured and predicted total uptake estimated from kinetic parameters obtained with each approach, respectively. Dashed and dotted lines represent cellular uptake due to active transport and passive diffusion, respectively. Open symbols represent passive diffusion measured at 4°C. Data points are mean of duplicate measurements.
Figure S4: Predicted and observed telmisartan cell concentrations when data were analyzed using a mechanistic two-compartment (A) or an extended two-compartment model, incorporating metabolism (B). Each plot represents cell concentrations measured in three experiments, over a range of concentrations (0.1 - 100 µM), in incubations carried out over 2 min.
**Figure S5:** Predicted and observed repaglinide cell concentrations when data were analyzed using a mechanistic two-compartment (A) or an extended two-compartment model, incorporating phase 2 metabolism (Scenario 1) (B), phase 1 and phase 2 metabolism (Scenario 2 - clearance approach) (C) or phase 1 and phase 2 metabolism (Scenario 3 - full kinetic approach) (D). Each plot represents cell concentrations measured in one experiment, over a range of concentrations (0.1 - 300 µM), in incubations carried out over 45 min.