Predicting Metabolic Clearance for Drugs that are Actively Transported into Hepatocytes: Incubational Binding as a Consequence of *in vitro* Hepatocyte Concentration is a Key Factor

Pär Nordell, Petter Svanberg, James Bird and Ken Grime

Respiratory & Inflammation DMPK, AstraZeneca R&D, Mölndal, Sweden
Running title:
A method to accurately assay CL_{int} of actively transported drugs

Corresponding author
Pär Nordell
E-mail address: par.nordell@astrazeneca.com
Address: AstraZeneca R&D Mölndal, Pepparedsleden, SE-431 83 MÖLNDAL, Sweden
Telephone: +46 (0)317761509
Fax: +46 (0)317763743

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Abbreviations
afe average fold error
CL_{int} intrinsic clearance from hepatocyte incubation
CL_{int,diff} intrinsic diffusion clearance in compartmental model
CL_{int,met} intrinsic metabolic clearance in compartmental model
CL_{int,u} unbound intrinsic clearance from hepatocyte incubation
CL_{int,up} intrinsic active uptake clearance in compartmental model
D_{cell,b} amount of bound drug in the cellular compartment
D_{cell,u} amount of unbound drug in the cellular compartment
D_{med,u} amount of unbound drug in the medium compartment
D_{mem} amount of drug in the outer membrane compartment
D_{tot} total amount of drug in the incubation
D_{u} unbound drug in the incubation
f_{u,c} intra-cellular fraction unbound
f_{u,inc} fraction unbound in the incubation
IVIVC in vitro-in vivo correlation
IVIVE in vitro-in vivo extrapolation
K_{cell} intra-cellular compartment binding constant
K_{med} outer membrane binding constant
\( K_{pu} \) unbound cell-to-media concentration ratio

NCE new chemical entity

\( V_{cell} \) volume of the cellular compartment

\( V_{med} \) volume of the medium compartment
Abstract

Incubational binding or the fraction of drug unbound in an *in vitro* incubation, $f_{u_{inc}}$, is an important parameter to predict or measure in the pursuit of accurate clearance predictions from *in vitro* data. Here we describe a method for $f_{u_{inc}}$ determination directly in the hepatocyte intrinsic clearance ($CL_{int}$) assay with emphasis on compounds that are actively transported into hepatocytes, hypothesizing that for such compounds the typical protocol of 1 million hepatocytes/mL systematically under-estimates the maximum attainable unbound intra-cellular drug concentration. Using the transporter substrate atorvastatin as a test compound, incubations were performed and a mathematical model applied to describe metabolism, distribution and binding at different hepatocyte concentrations. From these investigations it was evident that, since binding is more extensive intra-cellularly than in the medium, increased partitioning into the cellular volume, due to active uptake, increases the total amount of atorvastatin bound in the incubation. Consequently, a significant lowering of the hepatocyte concentration impacts the free drug concentration in the incubation and increases the observed rate of metabolism and therefore observed $CL_{int}$ (that is, when viewed from the media drug concentration). The applicability of the findings was tested for a series of 11 actively transported zwitterions for which standard rat hepatocyte metabolic $CL_{int}$ data (1 million cells/mL incubation) poorly predicted *in vivo* clearance (average fold error of 5.4). Using metabolic $CL_{int}$ determined at a lower hepatocyte concentration (0.125 million cells/mL) considerably improved clearance predictions (average fold error of 2.3).
Introduction

Arguably one of the most critical tasks within DMPK is the accurate prediction of \textit{in vivo} clearance (CL) from \textit{in vitro} data. For hepatic metabolic CL, it is now widely accepted that the use of \textit{in vitro} data can and should be relied on to make accurate predictions (\textit{in vitro-in vivo} extrapolation, IVIVE) (Sohlenius-Sternbeck, et al., 2012; Lavé, et al., 2009; Grime and Riley, 2006; Ito and Houston, 2005; Houston, 1994). In a drug discovery setting, hepatocyte intrinsic metabolic clearance (CL$_\text{int}$) and \textit{in vitro} incubational drug binding (fu$_\text{inc}$) are used to estimate the unbound intrinsic metabolic clearance (CL$_\text{int,u}$), which together with plasma protein binding and estimated blood/plasma partitioning facilitates a first line approach. Poor predictions of clearance should spark detailed investigations to ascertain the reasons. Since it is usually used as a predicted parameter from simple lipophilicity terms (Kilford, et al., 2008; Austin, et al., 2005), the accuracy of fu$_\text{inc}$ is in such situations of immediate interest. However, even when measured, the method for experimentally determining fu$_\text{inc}$ typically involves equilibrium dialysis analogous to plasma protein binding experiments using metabolically inactivated cells (perhaps with low or limited drug transporter functionality due to the incubation period taken to reach equilibration) and therefore does not replicate the conditions under which the drug experiences the possibility to be unavailable for metabolism during the assay for metabolic intrinsic clearance.

In the work presented here we investigated an alternative method for estimating the unbound drug fraction available for metabolism through determining the metabolic CL$_\text{int}$ at several different rat hepatocyte concentrations. This direct method for fu$_\text{inc}$ determination is of particular interest with compounds for which the rate of metabolism is limited by poor passive permeability into the hepatocytes and also for compounds that are actively transported into hepatocytes. In the first case, the rate of drug metabolism is likely to be rapid but limited by the permeability into the cells and therefore
the intra-cellular unbound drug concentration may be considerably lower than the extra-cellular unbound concentration (Lu, et al., 2006), whereas the second situation may be true when the active uptake increases the intra-cellular unbound drug concentration significantly above extra-cellular unbound concentration (Webborn, et al., 2007).

Whilst a method for predicting the in vivo hepatic clearance of compounds that are actively transported into hepatocytes exists (Soars, et al., 2007a), there are certain circumstances when this ‘media loss’ approach may be inappropriate or challenging to deliver high quality data from. Therefore we explored the possibility that a modified approach, assaying the metabolic CL_{int} at hepatocyte concentrations considerably lower than the typical protocol of 1 million hepatocytes per mL, could improve the prediction of the in vivo clearance. The ideas described above were examined in experiments performed with the uptake transporter substrate atorvastatin and an AstraZeneca NCE, AZ151, also a moderately lipophilic acid but devoid of any active hepatic uptake. A mathematical model, written to describe and aid understanding of the processes of active and passive uptake, extra-cellular and intra-cellular binding and metabolism of atorvastatin and AZ151 at the different hepatocyte concentrations incubated is also described. The mechanistic information allowed consideration of a simplified expression with the aim of clarifying and explaining the incubational binding phenomenon in situations where active hepatocyte uptake is a dominant process. Finally, the utility of the modified CL_{int} assay in a real drug discovery project environment was investigated. All the findings are discussed with regard to in vivo hepatic clearance predictions.
Materials and Methods

Materials

Atorvastatin (logD_{7.4} of 1.1; molecular weight of 559 g/mol) was obtained from Sequoia Research Products Ltd. (Oxford, UK). AZ151 (logD_{7.4} of 2.4; molecular weight of 421 g/mol) was obtained as a 10 mM DMSO stock solution from the AstraZeneca Compound Management Team in Mölndal, Sweden. Hepatocyte suspension medium (HSM) was prepared by supplementing William’s Medium E (Sigma-Aldrich Research St. Louis, MO, USA) with 25 mM HEPES and 2 mM L-glutamine (pH 7.4).

Measurement of logD_{7.4}

Partitioning of compounds (40 µM) between 1-octanol and 0.02 M phosphate buffer, pH 7.4, at 20°C was determined using a standard shake flask method (Leo, et al., 1971).

Hepatocyte preparation

Hepatocytes were isolated from adult male Sprague-Dawley rats (250-300 g) using a procedure based on the method of Seglen (1976) as described previously (Kenny and Grime, 2006). The rats were obtained from Harlan Nederland (Horst, The Netherlands). Hepatocytes had greater than 80% viability, assessed using the trypan blue exclusion method. Hepatocytes were kept in HSM on ice until pre-incubation and all incubations were started within 2 hours after isolation.
In vitro metabolic CL_int determined at different hepatocyte concentrations

Metabolic intrinsic clearance (CL_int) was assayed for as detailed previously (Soars et al., 2007a) using atorvastatin and AZ151 solutions diluted from the DMSO stock in HSM. Compound concentrations were 1 µM and incubation DMSO concentration was 0.05% v/v. However, the following modifications were made: Several cell concentrations were used (0.125, 0.1875, 0.25, 0.375, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0 million cells/mL) and each CL_int determination used ten time points (10, 20, 30, 45, 67, 80, 90, 100, 120 and 130 min). Incubations were performed in triplicate.

Determination of in vitro unbound metabolic CL_int (CL_int,u)

In vitro CL_int,u was estimated using two different methods. Firstly from measured CL_int / fu_inc at 1 million cells/mL where fu_inc was predicted from logD_{7.4} using the equation

$$\log \left( \frac{1 - f_{u_{inc}}}{f_{u_{inc}}} \right) = 0.40 \cdot \log D_{7.4} - 1.38 \quad \text{eq. 1}$$

as described previously (Austin et al., 2005). Secondly, CL_int,u and fu_inc at 1 million cells/mL were estimated from a simultaneous fit of all the CL_int data from each hepatocyte concentration to the expression
adapted from Grime and Riley (2006), where [hepatocyte] represents the hepatocyte concentration in units of 1 million cells/mL.

**Assessment of atorvastatin and AZ151 uptake into rat hepatocytes using an ‘oil-spin procedure’**

Mineral and silicon oil (Sigma-Aldrich Research St. Louis, MO, USA) were mixed to give a final density of 1.015 g / mL. Beckman 0.5 mL microtubes (Fisher Scientific GTF AB, Sweden) were prepared ahead of incubation start by addition of 15 µL 4% caesium chloride (CsCl) and 140 µL of the oil-mixture, followed by spinning at 4000g for 2 min. The AZ151 and atorvastatin DMSO stocks were diluted in HSM to 2 µM (0.1% DMSO) and the rat hepatocyte suspension (same preparation as for the hepatocytes used in the CL_int assay) was diluted to 2 million cells / mL. Oil-spin experiments were based on the centrifugal filtration method (Paine, et al., 2008; Petzinger and Fückel, 1992). In short, equal volumes of pre-warmed compound and cell solutions were mixed to give 1 µM drug and 1.0 million cells/mL in the final incubation. The temperature was controlled using a water bath set to 50 rpm (linear shaking) at 37°C. At selected time points (15, 30, 45 sec, 1, 2, 3, 5, 15, 30, 60, 90 and 120 min) a 100 µL aliquot was removed from each incubation, dispensed into a microtube and immediately centrifuged at 7000g for 15 sec to separate cells from media using a benchtop Eppendorf MiniSpin centrifuge. Medium samples were directly taken from the supernatant above the oil-layer after which the tubes containing the cell pellets were placed on dry ice. Cell samples were prepared for analysis by cutting frozen tube tips containing the cell pellet below the oil/CsCl interface into a 96-well plate. 200 µL of methanol containing a volume marker was added to each well and the plate was mixed at room temperature.
temperature using a plate shaker for 1h. After 100 µL of de-ionized water had been added to each well, the plate was centrifuged at 4000g at 4°C for 20 min. The supernatants were, together with samples from the medium fraction (diluted to attain the same methanol concentration), analysed by LC-MS/MS and the drug concentration determined from appropriate standard curves. Incubations were run in duplicates with reference incubations performed at 4°C.

**LC-MS/MS analysis**

Mass spectrometry was performed on an Acquity TQD triple quadrupole mass spectrometer (Waters, Milford, MA, USA) using multiple reaction monitoring in negative ion mode with chromatographic separation being performed using an Acquity UPLC sample and solvent manager (Waters, Milford, MA, USA). Chromatographic separation was achieved with an Acquity UPLC BEH C18 1.7 µm 2.1 × 30 mm column (Waters, Milford, MA, USA) using 10 µL of sample. The mobile phase consisted of water with 0.2% (v/v) formic acid (A) and acetonitrile containing 0.2% (v/v) formic acid (B). The gradient was as follows: 96% A (0–0.2min), 96% to 5% A (0.2-1.0min), 4%A (1–1.2 min). The flow rate was 1.0 mL/min and the column temperature was 40°C. Instrument control and data processing were performed using MassLynx 4.1 software, including QuanOptimize and QuanLynx (Waters, Milford, MA, USA).

**Mechanistic mathematical model for elucidating the underlying processes of drug distribution, binding and metabolism in the in vitro incubations**

The sets of kinetic data were analysed using a mechanistic model, comprising a medium, a cellular and an outer membrane compartment (figure 1), similar to the approach applied by Paine et al. (2008). In the model, transport between the medium and the cellular compartments is described by un-saturable active
uptake (CL_{int,up}) and bi-directional passive diffusion (CL_{int,diff}). While active biliary excretion can be a primary mechanism of elimination \textit{in vivo}, it is unclear to what extent the relevant drug efflux transporter functionality is retained shortly after hepatocyte isolation (Bow, et al., 2008; Li, et al., 2008). Active efflux, as well as transport associated with bi-directionality of uptake transporters, were therefore assumed to be limited in the present analysis. Elimination from the cellular compartment is described by the parameter CL_{int,met}.

Extracellular binding was assumed to be dominated by drug associating with the outer surface of the cellular membrane, described as a rapid equilibrium established between unbound drug in the medium (D_{med,u}) and drug in the membrane compartment (D_{mem}):

\begin{equation}
K_{med} = \frac{[D]_{mem}}{[D]_{med,u} \times [M]} \quad \text{eq. 3}
\end{equation}

where $K_{med}$ is the equilibrium constant and [M] represents the concentration of membrane in units of 1 million cells/mL. Intracellular binding was instead defined as a rapid equilibrium established between unbound (D_{cell,u}) and bound (D_{cell,b}) drug within the cellular compartment:

\begin{equation}
K_{cell} = \frac{[D]_{cell,b}}{[D]_{cell,u}} = \frac{1 - f_{u_{cell}}}{f_{u_{cell}}} \quad \text{eq. 4}
\end{equation}
where \( K_{\text{cell}} \) is the equilibrium constant. Since \( K_{\text{cell}} \) is independent of the hepatocyte concentration it may alternatively be described in terms of the intracellular fraction unbound (\( f_{\text{cell}} \)). Equations 5 and 6 define the change of the unbound drug concentration in the cell (of volume \( V_{\text{cell}} \)) and the medium (of volume \( V_{\text{med}} \)) compartments, respectively, with time when active efflux is assumed to be limited:

\[
\frac{d[D]_{\text{cell},\mu}}{dt} = (CL_{\text{int,up}} + CL_{\text{int,diff}}) \times \frac{[D]_{\text{med},\mu}}{V_{\text{cell}}} - (CL_{\text{int,diff}} + CL_{\text{int,met}}) \times \frac{[D]_{\text{cell},\mu}}{V_{\text{cell}}} \quad \text{eq. 5}
\]

\[
\frac{d[D]_{\text{med},\mu}}{dt} = -(CL_{\text{int,up}} + CL_{\text{int,diff}}) \times \frac{[D]_{\text{med},\mu}}{V_{\text{med}}} + CL_{\text{int,diff}} \times \frac{[D]_{\text{cell},\mu}}{V_{\text{med}}} \quad \text{eq. 6}
\]

Taking into account extracellular (equation 3) and intracellular (equation 4) binding, numerical integration of equation 5 and 6 give the concentration profiles of the cellular and medium compartment, respectively. Estimates of the adjustable clearance parameters \( CL_{\text{int,up}} \), \( CL_{\text{int,diff}} \) and \( CL_{\text{int,met}} \) as well as binding descriptors \( K_{\text{med}} \) and \( f_{\text{cell}} \) were obtained by simultaneous fit of the simulated total hepatocyte (cell + membrane compartments) amount to experimentally obtained cell fraction data at 37°C and 4°C using the non-linear least squares solver of the commercial software package Matlab 7.12 (The MathWorks Inc., Natick, MA, 2011). In the analysis only 4°C data reflecting approximate steady-state conditions (samples taken 120 min after incubation start) were used. \( CL_{\text{int,up}} \) and \( CL_{\text{int,met}} \), processes that are mediated by specific transporter or enzymatic activity, were furthermore considered inactivated at 4°C. Concentration profiles were calculated based on the assumption of a cellular volume \( V_{\text{cell}} \) of 4.0
µL/million hepatocytes (Reinoso, et al., 2001). The standard errors were calculated from the variances obtained from the Jacobian evaluated at the point estimates of the parameters (Bonate, 2005).

At pseudo steady state conditions, when \( \frac{d[D]_{cell,u}}{dt} = 0 \), the unbound cell-to-media concentration ratio \((K_{p,uu})\) is described by the ratio of clearances associated with drug entering and leaving the cellular compartment (Iwatsubo, et al., 1999; Shitara and Sugiyama, 2006). With the current parameterization \( K_{p,uu} \) is then calculated from

\[
K_{p,uu} = \frac{[D]_{cell,u}}{[D]_{med,u}} = \frac{CL_{int,up} + CL_{int,diff}}{CL_{int,diff} + CL_{int,met}} \quad \text{eq. 7}
\]

**Approximate description of \( fu_{inc} \) as a function of hepatocyte concentration upon variation of the unbound cell-to-media concentration ratio**

The following section outlines the derivation of an approximate description of how \( fu_{inc} \) (\( CL_{inc}/CL_{int,u} \)) is expected to vary, as the hepatocyte concentration and/or the unbound cell-to-media concentration ratio is changed, solely from consideration of the two separate binding equilibriums established in the media and inside the cell (equations 3 and 4, respectively). Equation 7 defines \( K_{p,uu} \) as the ratio of unbound drug in the cell to that of the medium (of volume \( V_{cell} \) and \( V_{med} \), respectively). The unbound concentration of drug in the media ([D]_{med,u}) and cell ([D]_{cell,u}) compartments can then be expressed in terms of the total unbound amount \( D_u \) in the incubation:

\[
D_u = [D]_{med,u} \times V_{med} + [D]_{cell,u} \times V_{cell} \quad \text{eq. 8}
\]

\[
[D]_{med,u} = \frac{D_u}{V_{med} + K_{p,uu} \times V_{cell}} \quad \text{eq. 9}
\]
Equation 9 is obtained by rearrangement of equation 8 after division by \([D]_{\text{med},u}\) and use of equation 7 for incorporation of \(K_{p,uu}\). Equation 9 can, at moderate \(K_{p,uu}\) levels, be approximated by equation 10 since the cellular volume \(V_{\text{cell}} \ll V_{\text{med}}\). Equation 11 is the corresponding expression for \([D]_{\text{cell},u}\):

\[
[D]_{\text{med},u} \approx \frac{D_u}{V_{\text{med}}} \quad \text{eq. 10}
\]

\[
[D]_{\text{cell},u} \approx \frac{K_{p,uu} \times D_u}{V_{\text{med}}} \quad \text{eq. 11}
\]

The unbound fraction in the incubation \((f_{u,\text{inc}} = D_u/D_{\text{tot}})\), where \(D_{\text{tot}}\) is the total amount of drug in the incubation) is related to the fraction bound in the medium \((f_{b,\text{med}})\) and the cells \((f_{b,\text{cell}})\):
\[ f_{u_{inc}} = \frac{D_u}{D_{tot}} = 1 - f_{B_{med}} - f_{B_{cell}} = \]

\[ = 1 - \frac{D_{mem}}{D_{tot}} - \frac{D_{cell,b}}{D_{tot}} = \]

\[ = 1 - \frac{1}{D_{tot}} \left( [D]_{mem} \times V_{med} + [D]_{cell,b} \times V_{cell} \right) = \]

\[ = 1 - \frac{1}{D_{tot}} \left( K_{med} \times [M] \times [D]_{med,\mu} \times V_{med} + [D]_{cell,\mu} \times K_{cell} \times V_{cell} \right) = \]

\[ = 1 - \frac{D_u}{D_{tot}} \left( K_{med} \times [M] + \frac{K_{p,\mu} \times K_{cell} \times V_{cell}}{V_{med}} \right) \]

in which the equations 3 and 4 are used to incorporate binding constants \( K_{med} \) and \( K_{cell} \), respectively. In the final substitution the unbound concentrations of drug in the media and the cell are replaced using equation 10 and equation 11, respectively. Rearrangement gives a final expression for the fraction unbound in the incubation

\[ f_{u_{inc}} = \frac{1}{1 + K_{med} \times [M] + K_{p,\mu} \times K_{cell} \times \frac{V_{cell}}{V_{med}} } \]

Equation 13 describes how the non-specific binding of drug in the incubation is expected to change as \([M]\) and \(V_{cell}/V_{med}\) changes. \( K_{p,\mu} \) is the unbound cell-to-media concentration ratio, which rapid metabolism or active uptake can drive considerably below or above 1. In either scenario there is a clear deviation from normal partitioning relationships \((K_{p,\mu} = 1)\) at which \(f_{u_{inc}}\) may usually be expected to be adequately described from \(\log D_{7.4}\). As \(f_{u_{inc}}\) relates the observed intrinsic metabolic clearance to the unbound metabolic clearance \((CL_{int,u} = CL_{int}/f_{u_{inc}})\) and since both \((K_{med} \times [M])\) and
(K_{cell} \times \frac{V_{cell}}{V_{med}} \approx K_{cell} \times \frac{V_{cell}}{V_{tot}}) are approximately proportional to the hepatocyte concentration, the ratio of the observed and unbound metabolic clearance can be approximated to

\[ \frac{CL_{int}}{CL_{int,u}} \approx \frac{1}{1 + \left( K_{med} + K_{p,uu} \times R_V \times \frac{1 - f_{u,cell}}{f_{u,cell}} \right) \times [Hepatocyte]} \quad \text{eq. 14} \]

Where \( K_{cell} \) is expressed using \( f_{u,cell} \) (equation 4), \( R_V \) denotes the cell-to-incubation volume ratio at 1 million cells / mL (4.0/1000) and \([Hepatocyte]\) is the hepatocyte concentration of unit 1 million cells / mL. The three constants included in the final expression can all be estimated from a fit of the mechanistic model (equation 3-6): \( K_{med} \) and \( f_{u,cell} \) are constitute model parameters, whereas \( K_{p,uu} \) can be indirectly assessed from estimated values of \( CL_{int,u}, CL_{int,diff} \) and \( CL_{int,met} \) using equation 7.
Predictions of in vivo hepatic clearance

*In vivo* clearance predictions were made using measured rat plasma protein binding and blood to plasma data (as described previously, Gardiner and Paine, 2011). The predictions used $f_{u,\text{inc}}$ predicted from measured logD$_{7.4}$ (equation 1) or estimated using the multiple hepatocyte concentration metabolic CL$_{\text{int}}$ data (equation 2). All this data was used to make the clearance predictions, essentially as described previously (Sohlenius-Sternbeck et al., 2012; Grime and Riley, 2006). Rat plasma clearance was determined after the administration of an intravenous dose (1 mg/kg) to male Sprague-Dawley rats, as described previously (Weaver and Riley, 2006). *In vivo* (observed) unbound CL$_{\text{int}}$ (CL$_{\text{int,u, in vivo}}$) was calculated by rearrangement of the Well Stirred Liver Model, as described previously (Soars et al., 2007a). Predicted *in vivo* unbound CL$_{\text{int}}$ values were calculated in the same way using the predicted *in vivo* hepatic CL and *in vivo* (observed) / predicted unbound CL$_{\text{int}}$ ratios were calculated.

A project example to determine the utility of the revised metabolic CL$_{\text{int}}$ method

For a chemical series of zwitterions (molecular weights ranging from 383 – 506 g/mol, logD$_{7.4}$ values ranging from 0.7 to 2.6) metabolic CL$_{\text{int}}$ values were determined using incubations containing 1 and 0.125 million rat hepatocytes/mL, as described above. Clearance predictions and *in vivo* rat plasma clearance values were also performed as above.
Results

**In vitro metabolic CL\textsubscript{int} determined at different hepatocyte concentrations**

AZ151 and atorvastatin were incubated with rat hepatocytes at eight cell concentrations in the interval 0.125 to 4.0 million cells / mL to determine the CL\textsubscript{int} values. The measured values increased with decreasing cell concentration, but despite the similar physico-chemical properties of the two compounds, the rate of increase of CL\textsubscript{int} with respect to cell concentration was considerably larger for atorvastatin (figure 2).

**Determination of in vitro unbound CL\textsubscript{int} (CL\textsubscript{int,u})**

Unbound CL\textsubscript{int} (CL\textsubscript{int,u}) values for both compounds were estimated from CL\textsubscript{int} / f\textsubscript{u\textsubscript{inc}} where f\textsubscript{u\textsubscript{inc}} was predicted from logD\textsubscript{7.4} using equation 1. Additionally, CL\textsubscript{int,u} was estimated from a simultaneous fit of all the CL\textsubscript{int} data from each hepatocyte concentration incubation to equation 2. Both methods for estimating CL\textsubscript{int,u} are detailed in Materials and Methods. Obtained estimates for CL\textsubscript{int,u} and f\textsubscript{u\textsubscript{inc}} are given in Table 1, with resulting simulations in figure 2.

**Assessment of atorvastatin and AZ151 uptake into rat hepatocytes using an ‘oil-spin procedure’**

Incubations with rat hepatocytes were performed in duplicate at 37°C at 1 million cells/mL and post sampling, the cellular fractions were separated from the media. To resolve passive events expected to be less dependent on temperature (diffusion and non-specific binding) from those mediated by specific transporter or enzymatic activity, reference incubations were performed at 4°C. Obtained cell fraction
kinetic data for AZ151 and atorvastatin are shown in the figure 3A and C, respectively (medium fraction data in Supplemental figure 1).

**Mechanistic mathematical model for elucidating the underlying processes of drug distribution, binding and metabolism in the in vitro incubations**

To elucidate the mechanism underlying the in vitro turnover of the two test compounds, the cellular concentration profiles obtained from the oil-spin experiments were analyzed by a mechanistic mathematical model (figure 1). Simultaneous fit to 37 and 4°C cell data gave estimates of the five model descriptors: active uptake (CL\textsubscript{int,up}), bi-directional diffusion (CL\textsubscript{int,diff}), metabolism (CL\textsubscript{int,met}), membrane binding (K\textsubscript{med}) and intra-cellular binding (fu\textsubscript{cell}). In contrast to the atorvastatin time-course data, the AZ151 data was well described without contribution of an active component to the uptake (CL\textsubscript{int,up} = 0). Model best-fit estimates are summarized in Table 2, with simulations included in figure 3A and C (the simulated amount, based on the fit to cell data, in the medium compartment is included in Supplemental figure 1).

Figure 3B and D shows the mechanistic model simulated drug concentration-time profile in the cell and the media compartments when the cell concentration was 1 million cells/mL. Over the course of the simulation, AZ151 and atorvastatin distribute quite differently between the two fractions. The unbound cell-to-media concentration ratio attained at pseudo steady state conditions (K\textsubscript{p,uu}) can be obtained by comparing the concentrations in the two compartments graphically or alternatively be calculated from the clearance parameters using equation 7. For AZ151 the calculated ratio was 0.7, whereas for atorvastatin the ratio was 27, implying that the maximum unbound atorvastatin concentration reached inside the hepatocytes is much greater than that of AZ151. Simulations of unbound intracellular
concentration were in addition performed at selected hepatocyte concentrations between 0.125 and 4 million cells/mL (figure 4). For AZ151 the maximum unbound intra-cellular concentration increases 1.25 times as the hepatocyte concentration is lowered from 1.0 to 0.125 million cells/mL whereas for atorvastatin the corresponding change of hepatocyte concentration results in 3.6 times higher unbound drug concentration.

**Approximate description of fu_{inc} as a function of hepatocyte concentration upon variation of the unbound cell-to-media concentration ratio**

From consideration of drug binding in the media and inside the hepatocytes, equation 14 was derived to understand how fu_{inc} (CL_{int}/CL_{int,u}) changes as the hepatocyte concentration is varied. The relationship also accounts for situations where the unbound cell-to-media concentration ratio (K_{p,uu}) deviates significantly from 1, here in particular observed for atorvastatin. In fitting of the mechanistic model, descriptors of binding, K_{med} and fu_{cell}, in equation 14, were estimated (Table 2). By insertion of the values associated with AZ151 and atorvastatin together with their estimated K_{p,uu} ratios (0.7 and 27, respectively) into the equation, the CL_{int}/CL_{int,u} profiles shown in figure 5 were calculated. The distinct CL_{int}/CL_{int,u} profiles experimentally observed for AZ151 and atorvastatin were both well described by the derived expression.

**Predictions of rat in vivo hepatic clearance of atorvastatin and a project example of the application of the revised metabolic CL_{int} method**

Atorvastatin observed plasma clearance in vivo in the rat was 46 mL/min/kg (and therefore calculated blood clearance was 33 mL/min/kg, using the measured blood to plasma ratio of 1.4). The in vitro measured value for plasma protein binding was 95.8%. Applying equation 1 for estimating fu_{inc} the
predicted blood clearance was 4.5 mL/min/kg, using the ‘regression corrected’ approach for predicting clearance (Grime and Riley, 2006; Soars et al., 2007a). The in vivo (observed) / predicted unbound CL_{int} ratio was 12.4. Using CL_{int,u} derived by fitting equation 2 to CL_{int} data from several hepatocyte concentrations the predicted blood clearance was 11 mL/min/kg and the in vivo (observed) / predicted unbound CL_{int} ratio was 4.6.

Initial predictions of rat clearance from standard rat hepatocyte in vitro metabolic CL_{int} data (1 million hepatocytes/mL) and fu_{inc} predicted from logD_{7.4} for a novel series of zwitterions were not acceptable, since the in vivo (observed) / predicted unbound CL_{int} ratio ranged from 2 to 13 and the average fold error (afe) for the predictions was 5.4 (figure 6). Metabolic CL_{int} was subsequently determined for the compounds at the lower cell concentration of 0.125 million hepatocytes/mL. The CL_{int} data from both hepatocyte concentrations were used to estimate CL_{int,u} from fit to equation 2, values that thereafter were used to predict in vivo rat CL. This resulted in a much improved IVIVC (figure 6), with the in vivo (observed) / predicted unbound CL_{int} ratio ranging from 1 to 3.8 and an afe of 2.3.
Discussion

Over the last twenty years, *in vitro* drug metabolism and kinetic data have been used with increasing refinement to predict *in vivo* clearance such that there is common belief that *in vitro* data is highly predictive of clinically relevant situations, provided that the underlying assumptions are understood (Soars, et al., 2007b). The importance and utility of incorporating drug binding terms has been previously reviewed in detail (Grime and Riley, 2006) and detailed *in vitro* methods to facilitate the accurate prediction of hepatic clearance and drug-drug interactions for drugs that are actively transported into hepatocytes have been published (Jones, et al., 2012; Bi, et al., 2012; Paine, et al., 2008; Grime, et al., 2008; Soars, et al., 2007a). Nevertheless, it continues to be worthwhile for DMPK scientists to critique experimental conditions in the search for further refinement and mechanistic understanding (Hallifax and Houston, 2012).

In this present work we considered the concept that a commonly used *in vitro* hepatocyte incubation protocol for determining unbound metabolic intrinsic clearance (CL\textsubscript{int} / \textit{fu}\textsubscript{inc} either predicted from logD\textsubscript{7.4} (equation 1) or measured using equilibrium dialysis) may be inappropriately employed for situations where active uptake is an important factor in drug disposition. We considered whether an alternative approach for estimating \textit{fu}\textsubscript{inc} directly in the CL\textsubscript{int} assay through assessing the impact of changing hepatocyte concentration on CL\textsubscript{int} (equation 2) may be more generally applicable. To establish if this is the case, experiments were performed with atorvastatin at several hepatocyte concentrations and a mathematical model was written to describe the findings. As a control we chose an AstraZeneca new chemical entity (AZ151) since it was also a moderately lipophilic acid but critically and in contrast to atorvastatin, AZ151 is not subject to active hepatic transport.
For AZ151, the prediction of $fu_{inc}$ from logD$_{7.4}$ (equation 1) matched the $fu_{inc}$ estimated from a simultaneous fit of CL$_{int}$ data at several hepatocyte concentrations (equation 2) with values being 0.72 and 0.78 respectively. Accordingly CL$_{int,u}$ values estimated using both methods also agree (37.5 µL/min/million cells observed and 40 µL/min/million cells, figure 2). For atorvastatin, the increase of CL$_{int}$ had a considerably stronger dependency on the decreasing cell concentration and as a result the $fu_{inc}$ calculated using equation 2 (0.33) was much lower than the logD$_{7.4}$ calculated $fu_{inc}$ value of 0.90. This results in a considerable under-prediction of CL$_{int,u}$ for atorvastatin when using the logD$_{7.4}$ prediction method (25 compared to 68 µL/min/million cells).

The mechanism underlying this observed discrepancy was explored through a separate study undertaken to quantitatively characterize how distribution, binding and metabolism of the two test compounds differed in the hepatocyte incubation. Experiments were performed in which the concentration of atorvastatin and AZ151 in the cell and media fractions during a 120 minute incubation was assessed by rapidly separating the two matrices using the ‘oil-spin’ procedure described in the Methods section. The obtained kinetic cell data sets were analyzed using a compartmental model. Several reports have recently described the application of physiologically inspired models for the mechanistic description of hepatocyte uptake kinetics (Poirier, et al., 2008; Watanabe, et al., 2009; Yabe, et al., 2011; Ménochet, et al., 2012a; Ménochet, et al., 2012b). We here used an un-saturable model, based on the model of Paine and co-workers (2008). Best-fit simulated profiles match well over the full 120 minute incubation to cell (figure 3A and C) and medium (Supplemental figure 1) data. Importantly, AZ151 data is described without contribution of active uptake (CL$_{int,up} = 0$). This is contrary to atorvastatin, for which active uptake accounts for 97% of the total uptake (CL$_{int,up}$ plus CL$_{int,diff}$), in agreement with literature data (Paine, et al., 2008). The distribution of unbound drug between the cell and the medium (Figure 3B and
D) was calculated from obtained model clearances (equation 7). For AZ151 and atorvastatin, obtained values were slightly below ($K_{p,uu} = 0.7$) and substantially higher ($K_{p,uu} = 27$) respectively, the expected value of 1 in absence of active uptake and metabolism.

A general description of $fu_{inc}$ ($CL_{int}/CL_{int,u}$) at the conditions of the in vitro incubation may take into account the two separate binding equilibriums established in the medium and inside the hepatocyte. A simplified expression was formulated to describe the influence of hepatocyte concentration ([hepatocyte]) on $CL_{int}/CL_{int,u}$ also in situations when $K_{p,uu}$ deviates from 1 (equation 14). The expression is helpful for conceptualizing the effect of active uptake on the observed intrinsic metabolic clearance as the hepatocyte concentration changes. Insertion of the mechanistic model binding descriptors $K_{med}$, $fu_{cell}$ and the estimated $K_{p,uu}$ ratio into equation 14 gave the $CL_{int}/CL_{int,u}$ profiles in figure 5A and B. The decrease in $CL_{int}/CL_{int,u}$ with increasing hepatocyte concentration experimentally observed for AZ151 and atorvastatin are essentially reproduced by the expression using the model derived parameters, validating the approach. The figure shows that the deviating $fu_{inc}$ profile of actively transported drugs can be understood by separating the total binding into a media and cellular part and accounting for the distribution of drug between them. Since drug is more extensively bound intracellularly than in the media, increased partitioning into the cellular volume due to active uptake will increase the total amount of drug bound. As a direct consequence, a variation of the hepatocyte concentration will have a stronger impact on the unbound amount of drug in the incubation (figure 4 and 5). In this case, the intracellular matrix acts as a sink sequestering the major part of drug under the standard conditions of 1 million cells/mL. Of course following the greater rise in the intracellular unbound concentration upon decreasing the hepatocyte concentration below 1 million cells/mL, the
greater is the increase in observed rate of metabolism (and therefore CL_{int} when viewed from the media drug concentration) for the actively transported drug.

Using CL_{int} values measured at several hepatocyte concentrations to obtain an accurate estimate of in vitro metabolic CL_{int,u} (equation 2, Table 1) considerably improved the prediction of the in vivo clearance for atorvastatin, verified by the fact that the in vivo (observed) / predicted unbound CL_{int} ratio decreased from 12.4 using the standard CL_{int} assay to 4.6. Of course a ratio of unity indicates a perfect prediction, but it is worth noting that following intravenous administration, a 50% recovery unchanged atorvastatin in rat bile has been reported (Watanabe, et al. 2010) and therefore the in vivo / in vitro metabolic CL_{int} discrepancy calculated here could in fact be considerably less than 4.6. Additionally, in the same report, the analysis indicated a 1.8 fold higher in vivo uptake clearance for atorvastatin compared to in vitro, indicating compromised hepatic uptake after cell isolation, which may be attributed to decrease in OATP expression after isolation (Kimoto, et al., 2012). This is supported by close consideration of the Soars dataset (2007a) in which 36 compounds thought to be substrates for hepatic uptake transporters gave a rat observed/predicted in vivo CL_{int,u} afe of 3-fold. Additonally, human in vivo CL_{int,u} predictions for atorvastatin, bosentan and montelukast showed an offset of two to three-fold. Taking these two factors (atorvastatin biliary clearance and possible lower in vitro than in vivo organic anion transporting polypeptide activity) into account, it can be claimed that the methods described may improve in vivo metabolic clearance predictions.

For the series of 11 zwitterionic NCEs described in this article, standard rat hepatocyte metabolic CL_{int} data at 1.0 million cells/mL did not afford good predictions of in vivo clearance when the fu_{inc} was
predicted from equation 1 (afe of 5.4, figure 6). Further experiments performed to investigate uptake into the rat hepatocytes indicated that all of the zwitterions assayed were subject to active uptake (using ‘oil spin’ experiments, data not shown). On basis of the findings presented, $CL_{\text{int}}$ was also measured at the lower hepatocyte concentration of 0.125 million cells/mL, which allowed $CL_{\text{int,u}}$ to be determined from fit of equation 2 (measurements of $CL_{\text{int}}$ at two hepatocyte concentrations are required to estimate $CL_{\text{int,u}}$, while data from additional concentrations allows for assessment of the quality of the fit). Use of the estimated $CL_{\text{int,u}}$ considerably improved the prediction (afe = 2.3, figure 6). This slight under-prediction is consistent with the discussion point above, that expression of involved drug transporters is down-regulated in vitro compared to the liver tissue (Kimoto, et al., 2012).

This work provides a practical in vitro method and a mechanistic background to facilitate the accurate prediction of hepatic metabolic clearance for drugs that are actively transported into the liver. Whilst the method is clearly not suitable for drugs that are extremely metabolically stable, the approach, based on determining $CL_{\text{int}}$ at a hepatocyte concentration considerably lower than the typical concentration of 1 million cells/mL, complements the ‘media loss’ assay (detailed in the publication by Soars and co-workers, 2007a), which essentially probes the distribution into the hepatocytes. Additionally the method described is more applicable to automated assay design and in comparison to the ‘media loss’ assay allows a greater mechanistic understanding of some of the processes that a drug encounters and therefore facilitates the accurate prediction of hepatic clearance and effective design of drugs with optimal pharmacokinetic characteristics.
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Authorship Contributions

Participated in research design: Nordell, Svanberg, Bird and Grime

Conducted experiments: Nordell, Svanberg and Bird

Performed data analysis: Nordell, Svanberg and Grime

Wrote or contributed to the writing of the manuscript: Nordell and Grime.
References


Figure legends

**Figure 1.** Schematic representation of the proposed mechanistic model comprising medium, cellular and outer membrane compartments. Adjustable parameters define active uptake (CL\text{int,up}), bi-directional diffusion clearance (CL\text{int,diff}), metabolic intrinsic clearance (CL\text{int,met}) as well as intra-cellular (fu\text{cell}) and membrane binding (K\text{med}). The cellular fractions collected experimentally include the cellular and the membrane model compartments (shaded area).

**Figure 2.** Effect of hepatocyte concentration on observed metabolic intrinsic clearance (CL\text{int}) obtained from rat hepatocyte incubations with AZ151 (A) and atorvastatin (B). The dashed line represents the dependence expected using the CL\text{int} obtained at 1 million cells / mL and fu\text{inc} predicted from logD\text{7.4} (equation 1). The solid line represents the dependence based on the best fit of the observed CL\text{int} data to equation 2. The resulting unbound metabolic clearance CL\text{int,u} from each of the two approaches are given from the y-axis intercept.

**Figure 3.** Cellular amount (intra-cellular + membrane bound) of AZ151 (A) and atorvastatin (C) in the 100 µL samples collected from 1 µM incubations at 1.0 million cells/mL. Experimentally observed values at 4°C and 37°C are squares and circles, respectively, and simulated, using the mathematical mechanistic model, is the solid line (37°C) or dashed line (4°C at steady state). Simulated profiles for AZ151 (B) and atorvastatin (D) total (solid) and unbound (dashed) intra-cellular concentrations and incubation medium concentration (dotted) at 1.0 million cells/mL.
**Figure 4.** Influence of hepatocyte concentration on the unbound intra-cellular concentration of AZ151 (A) and atorvastatin (B) obtained using the mechanistic model. Profiles are simulated at (top-to-bottom) 0.125, 0.3, 0.5, 1.0 (bold line), 2.0, and 4.0 million cells / mL.

**Figure 5.** Comparison of experimentally assessed and calculated $fu_{inc} = CL_{int}/CL_{int,u}$ profiles for AZ151 (A) and atorvastatin (B). The dependency determined from observed $CL_{int}/CL_{int,u}$ data (circles) is the solid line and the dashed line represents the profile calculated from equation 14.

**Figure 6.** Prediction of $CL_{int,u}$ in vivo for 11 AZ new chemical entities using $CL_{int,u}$ from 1) standard rat hepatocyte metabolic $CL_{int}$ at 1.0 million cells/mL and $fu_{inc}$ determined from logD7.4 (open circles) and 2) metabolic $CL_{int}$ at 1.0 and 0.125 million cells/mL using equation 2 (filled circles). The dashed line represents the linear regression analysis of data obtained from method 1: $y = 1.011x + 0.639$, $R^2 = 0.597$, afe = 5.4, and the dotted line the analysis of data obtained from method 2: $y = 1.083x + 0.077$, $R^2 = 0.689$, afe = 2.3. The solid line represents the line of unity.
Tables

Table 1. Summary of estimated unbound metabolic clearance (CL\textsubscript{int,u}) values using A) the standard method with fu\textsubscript{inc} being predicted from lipophilicity, described by equation 1 (CL\textsubscript{int,u} = CL\textsubscript{int}/fu\textsubscript{inc}) and B) the fit to CL\textsubscript{int} data from several hepatocyte concentrations (equation 2, estimate standard error in parenthesis).

<table>
<thead>
<tr>
<th>Drug</th>
<th>LogD\textsubscript{7,4}</th>
<th>A</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CL\textsubscript{int,u}</td>
<td>fu\textsubscript{inc} at 1 million cells/mL</td>
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<tr>
<td></td>
<td></td>
<td>µL/min/1 million cells</td>
<td></td>
</tr>
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<td>AZ151</td>
<td>2.41</td>
<td>40</td>
<td>0.72</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>1.1</td>
<td>25</td>
<td>0.90</td>
</tr>
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Table 2. Model best-fit parameter estimates obtained from simultaneous fit to 37°C and 4°C ‘oil-spin’ data (see Methods section): active uptake (CL_{int,up}), bi-directional diffusion clearance (CL_{int,diff}), metabolic intrinsic clearance (CL_{int,met}), membrane binding ($K_{med}$) and intra-cellular binding ($fu_{cell}$). Estimate standard error given in parenthesis. The unbound cell-to-media concentration ratio $K_{p,uu}$ was calculated using equation 7.

<table>
<thead>
<tr>
<th>Drug</th>
<th>CL_{int,up}</th>
<th>CL_{int,diff}</th>
<th>CL_{int,met}</th>
<th>$fu_{cell}$</th>
<th>$K_{med}$</th>
<th>$K_{p,uu}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$L/min/1 million cells</td>
<td>$\mu$L/min/1 million cells</td>
<td>$\mu$L/min/1 million cells</td>
<td></td>
<td>mL/1 million cells</td>
<td>(eq. 7)</td>
</tr>
<tr>
<td>AZ151</td>
<td>-</td>
<td>160 (23)</td>
<td>84 (5.8)</td>
<td>0.021 (0.012)</td>
<td>0.066 (0.0091)</td>
<td>0.7</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>590 (76)</td>
<td>21 (17)</td>
<td>1.9 (1.2)</td>
<td>0.032 (0.021)</td>
<td>0.100 (0.064)</td>
<td>27</td>
</tr>
</tbody>
</table>
Figure 1

Incubation medium

Cell
- free drug
- bound drug
- fu_cell

Outer cell membrane

CL_{int,diff} → CL_{int,up} → CL_{int,met}

K_{med}
Figure 2

A

$CL_{\text{int}}$ (μL/min/1 million cells)

Hepatocyte concentration (million cells/mL)

B

$CL_{\text{int}}$ (μL/min/1 million cells)

Hepatocyte concentration (million cells/mL)
Figure 4

A

Unbound cell conc (μM)

0.0 0.2 0.4 0.6 0.8

0.1 1 10 100 1000

Time (min)

B

Unbound cell conc (μM)

20 15 10 5 0

0.1 1 10 100 1000

Time (min)
Figure 5

A

\[ f_{\text{inc}} \left( \frac{CL_{\text{int}}}{CL_{\text{int,u}}} \right) \]

Hepatocyte concentration (million cells/mL)

B

\[ f_{\text{inc}} \left( \frac{CL_{\text{int}}}{CL_{\text{int,u}}} \right) \]

Hepatocyte concentration (million cells/mL)
Figure 6

Observed $\text{CL}_{\text{int,u in vivo}}$ (mL/min/kg)

Predicted $\text{CL}_{\text{int,u in vivo}}$ (mL/min/kg)