Kinetic characterization of rat hepatic uptake of 16 actively transported drugs

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Abbreviations used are:

$CL_{\text{active}}$, clearance by active uptake; $CL_{\text{int}}$, intrinsic clearance; $CL_{\text{uptake}}$, total clearance by active and passive uptake; $f_{\text{cell}}$, intracellular fraction of unbound drug; $K_{\text{p,total}}$, tissue-to-medium total drug concentration ratio; $K_{\text{p,u}}$, hepatocyte-to-medium unbound drug concentration ratio; $K_{\text{p,i}}$, measure of total cellular drug concentration relative to cytosolic drug concentration; OATP, organic anion transporter polypeptide; $P_{\text{diff}}$, passive uptake clearance.
Abstract

In order to explore the determinants of hepatic uptake, 16 compounds were investigated with different physicochemical and disposition characteristics, including five statins, three sartans, saquinavir, ritonavir, erythromycin, clarithromycin, nateglinide, repaglinide, fexofenadine and bosentan. Freshly isolated rat hepatocytes in suspension were used with the oil-spin method to generate kinetic parameters. Clearances, via passive diffusion (P_{diff}) and active uptake (CL_{active}, characterized by maximum uptake rate and K_m), were estimated from the initial uptake rate data over a 0.01-100 µM concentration range. The K_m values ranged 15-fold, with 10 of the 16 drugs with K_m<10 µM (median 6 µM). Both CL_{active} and P_{diff} ranged over 100-fold (median 188 and 14 µL/min/10^6 cells). Assessment of the relative contribution of P_{diff} and CL_{active} indicated that at low concentrations (~0.1 µM) the active process contributes >80% to the overall uptake for 13 drugs. Although high P_{diff} values were obtained for ritonavir and repaglinide, active process contributed predominantly to uptake; in contrast, high passive permeability dominates over transporter-mediated uptake for saquinavir over the full concentration range. For bosentan and erythromycin, active and passive processes were equally important. Hepatocyte-to-medium unbound concentration ratio was >10 for nine of the 16 drugs, ranging from 2-494 for bosentan and atorvastatin, respectively. Some drugs showed extensive intracellular binding (fraction unbound ranging from 0.01-0.6) which was not correlated with active uptake. LogD_{7.4} correlated significantly with P_{diff} and the extent of intracellular binding but not with active uptake. This study provides systematic assessment of the role of active uptake relative to the passive process; implications of the findings are discussed.
Introduction

A variety of uptake transporters are expressed on the basolateral membrane of hepatocytes which are important for several classes of drugs (Hagenbuch and Gui, 2008; Giacomini et al., 2010). While hepatocytes have been used to estimate metabolic intrinsic clearance (CL\textsubscript{int}) for prediction of in vivo drug clearance, significant under-prediction is commonly observed (Ito and Houston, 2004; Riley et al., 2005; Hallifax et al., 2010). Recently, studies have demonstrated that transporter-mediated uptake can be the rate-limiting step in the overall hepatic clearance and contribute to the under-prediction observed when using conventional hepatocyte assays (Parker and Houston, 2008; Soars et al., 2009; Watanabe et al., 2010). It is widely accepted that the intracellular unbound drug concentration (assumed to be available to the enzyme) is important for clearance and drug-drug interaction predictions (Houston and Galetin, 2008; Xu et al., 2009). When modeling hepatic clearance it is often assumed that intracellular free concentration is equal to extracellular concentration. However, in reality, the intracellular free concentration may differ markedly as a result of transporter activity and hence impact on the assessment of clearance and drug-drug interactions (Shitara and Sugiyama, 2006; Webborn et al., 2007; Brown et al., 2010; Watanabe et al., 2010). Intracellular binding and enzyme transporter interplay are further sources of complexity in elucidating in vitro-in vivo relationships (Lam et al., 2006; Poirier et al., 2008).

Despite the increasing number of in vitro transporter studies, a lack of consistency in rat and human in vitro uptake methodology and data is apparent; in particular, in the assessment and modeling of passive permeation, drug efflux and nonspecific binding (Kitamura et al., 2008; Paine et al., 2008; Poirier et al., 2008; Watanabe et al., 2009a).
In many cases uptake is limited to use of a single low and high substrate concentration to estimate saturable and nonsaturable uptake, respectively. In addition, efforts have focused primarily on single or a small number of substrates and hence there is no comprehensive database of values. This contrasts with the amount of information available on hepatic metabolism where a wide range of clearance values both in vitro (hepatocytes and microsomes) and in vivo are documented (Ito and Houston, 2004; Gertz et al., 2010; Halifax et al., 2010).

The rat often provides a useful model for characterizing drug disposition prior to detailed human studies and for mechanistic studies (Watanabe et al., 2009a; Watanabe et al., 2010). This source of hepatic tissue for in vitro studies has the advantage of being much more reproducible than the human; of particular concern are tissue storage, inter-donor variability and deviations from standard protocols in cell isolation, all of which are minimal when animal tissues are used. Thus, notwithstanding the documented species differences in both drug metabolizing enzymes and hepatic transporters (Hagenbuch and Gui, 2008), the rat offers many attractions as a model system for humans.

There are a number of approaches to determining hepatic uptake in vitro, however the common principle involves separation of cells and media, and the monitoring of drug concentrations in either or both matrices (Petzinger and Fuckel, 1992; Halifax and Houston, 2006; Poirier et al., 2008). In the present study, drug associated with the hepatocytes was measured after separation from free drug by layering the suspension of drug and hepatocytes over silicone/mineral oil followed by rapid centrifugation – the oil-spin method (Petzinger and Fuckel, 1992); a method used by several groups
(Ishigami et al., 1995; Nakai et al., 2001; Nezasa et al., 2003; Shimada et al., 2003; Hallifax and Houston, 2006) to characterize hepatic uptake by a clearance term for active transport, CL\text{active}, together with $K_m$ and $V_{\text{max}}$ and a passive permeability parameter, $P_{\text{diff}}$. As enzyme-transporter interplay complicates the interpretation of many of these types of studies, the nonspecific P450 inhibitor, l-aminobenzotriazole (ABT) (Mico et al., 1988), has been used to eliminate any potential metabolism. Intracellular binding has also been determined to complete the characterization of hepatocellular uptake.

The present study focuses on a group of 16 drugs that are likely OATP substrates (Shitara and Sugiyama, 2006; Kalliokoski and Niemi, 2009; Giacomini et al., 2010) and show a range of properties in terms of their physicochemical nature and metabolism (Table 1). These include five HMG-CoA reductase inhibitors (rosuvastatin, atorvastatin, pravastatin, pitavastatin, cerivastatin), three sartans (telmisartan, valsartan, olmesartan), saquinavir, ritonavir, erythromycin, clarithromycin, nateglinide, repaglinide, fexofenadine and bosentan. For many of the drugs selected there are clinical data in subjects with polymorphic OATP1B1 to support the contributing role of uptake (Kalliokoski et al., 2008; Ieiri et al., 2009). In addition, many of these drugs are associated with clinical drug-drug interactions which are believed to be, at least partially, mediated by transporters (Kajosaari et al., 2005; Hirano et al., 2006). However, there is a lack of supporting in vitro uptake data to assess the contribution of the active uptake relative to the passive process and intracellular binding of these drugs.
Materials and Methods

Chemicals: [3H]Pravastatin sodium salt, [3H]rosuvastatin calcium, [3H]atorvastatin calcium, [3H]pitavastatin calcium, [3H]cerivastatin sodium salt, [3H]saquinavir, [3H]ritonavir, [3H]erythromycin, [3H]clarithromycin and [3H]telmisartan were obtained from American Radiolabeled Chemicals (St. Louis, MO); unlabeled pravastatin sodium, rosuvastatin calcium, atorvastatin calcium, pitavastatin calcium, cerivastatin sodium, saquinavir, ritonavir, telmisartan, olmesartan, valsartan and bosentan were from Sequioia Research Products (Pangbourne, UK); erythromycin, clarithromycin, repaglinide and fexofenadine were from Sigma-Aldrich (Buchs, Switzerland).

Hepatocyte studies: For hepatocyte preparations anaesthetized rats were sacrificed by cervical dislocation and hepatocytes were prepared using an adaptation of the collagenase perfusion method as described previously (Hayes et al., 1995). Hepatocyte viability was determined using the trypan blue exclusion test and only those hepatocyte preparations with viabilities greater than 85% were used. All kinetic and inhibition studies were performed in duplicate under initial rate conditions with respect to incubation time and hepatocyte density. All hepatocyte studies were performed using 3-5 independent hepatocyte preparations.

Animal source, housing and diet: Male Sprague-Dawley rats (240-260 g) were obtained from the Biological Sciences Unit, Medical School, University of Manchester (Manchester, UK). They were housed in groups of two to four, in opaque boxes on a bedding of sawdust in rooms maintained at a temperature of 20 ± 3°C, with a relative humidity of 40 to 70% and a 12-h light/dark cycle. The animals were
allowed free access to CRM diet and fresh drinking water. All animal protocols were approved by University of Manchester review committee.

**Experimental design:** In order to characterize the uptake kinetics and evaluate the contribution of transporter-mediated active uptake to the overall clearance, studies were performed in rat hepatocytes in suspension by oil-spin method (Hallifax and Houston, 2006) over a 0.01-100 µM (n=8-10) concentration range. Clearance via passive diffusion (P_{diff}), maximum uptake rate (V_{max}) and Michaelis constant (K_m) were estimated from the initial uptake rate data and the parameters were used for the subsequent analysis of the uptake characteristics of the compounds.

For the initial rate experiments used to determine the above kinetic parameters, three data points obtained over the first 1.5 minutes were used. In additional experiments, longer incubation times were used (90 min) to allow attainment of an equilibrium to obtain the hepatocyte/medium concentration partition coefficient (K_{P,\text{total}}).

For the five statins, saquinavir, ritonavir, clarithromycin, erythromycin and telmisartan, rat hepatocytes were suspended in Krebs Henseleit buffer (pH 7.4) to perform with the concentration of 2×10^6 cells/mL and pre-incubated at 37°C for 5 min. The uptake study was initiated by the addition of equal volume of buffer containing [3H]-labelled and unlabeled compounds at the substrate concentration range of 0.01 to 100 µM (final cell concentration: 1×10^6 cells/mL). Aliquots were taken and placed in the narrow tube containing silicone-mineral oil (density: 1.015, Sigma-Aldrich) on the top of 3 M potassium hydroxide solution, followed by centrifugation through the silicone-mineral oil layer at the designated time points (30,
60 and 90 s or 20, 60 and 100 s) in order to separate cells from media. The radioactivity in both of cells and media was determined by liquid scintillation counter. For nonradiolabelled drugs, olmesartan, valsartan, repaglinide, nateglinide, fexofenadine and bosentan, rat hepatocytes were suspended in buffer at a concentration of 4×10^6 cells/mL with an equal volume of buffer containing 4 mM ABT added 15 min prior to inhibit potential cytochrome P450 mediated metabolism - (Parker and Houston, 2008), followed by pre-incubated at 37°C for 5 min. The uptake study for non-radiolabelled compounds in the dataset (e.g., repaglinide) was performed as described for radiolabelled compounds with the exception that 5 M ammonium acetate solution was used as the bottom layer instead of alkaline solution. After separation of cells from media by centrifugation, the tubes were frozen in the liquid nitrogen. After thawing, an aliquot (50 µL) of media was taken and quenched in 100 µL methanol and the tubes were frozen again and the tube was cut into 1.5-mL centrifuge tube including 200 µL methanol and bottom layer was and thawed again, the substrate concentration of both media and cellular fraction were analyzed using LC/MS/MS.

**LC-MS/MS analysis:** The LC-MS/MS systems used consisted of either a Waters 2790 with a Micromass Quattro Ultima triple quadruple mass spectrometer (Waters, Milford, MA) or an Agilent 1100 (Agilent Technologies, Santa Clara, CA) with a Micromass Quattro-LC triple quadruple mass spectrometer. Samples were centrifuged at 2500 rpm for 10 min, and an aliquot of 10 µl of both the dialysate and buffer was analyzed by LC-MS/MS. Varying gradients of four mobile phases were used, the compositions of which were 1) 90% water and 0.05% formic acid with 10% acetonitrile, 2) 10% water and 0.05% formic acid with 90% acetonitrile, 3) 90% water
and 10 mM ammonium acetate with 10% acetonitrile, and 4) 10% water and 10 mM ammonium acetate with 90% acetonitrile.

For bosentan, repaglinide, valsartan, olmesartan, nateglinide and fexofenadine a Luna C18 column (3 µm, 50 × 4.6 mm) was used for chromatographic separation of analytes, (Phenomenex, Torrance, CA). The flow rate was set at 1 ml/min, and this was split to 0.25 ml/min before entering the mass spectrometer. Mass transitions were 552.4 > 202.2, 453.3 > 230.2, 436.5 > 235.3, 502.3>466.2, 447.4>207.3 and 318.15>166.15 for bosentan, repaglinide, valsartan, fexofenadine, olmesartan and nateglinide respectively. Mibefradil was used as an internal standard for bosentan, valsartan, olmesartan and nateglinide, whereas indomethacin was applied for the analysis of repaglinide samples and terfenadine for fexofenadine sample analysis.

Data Analysis: The hepatic uptake clearance (CL<sub>uptake</sub>) was determined from the slope of the plot of cell-to-media ratio of radioactivity (concentration) versus time and used to calculate initial uptake velocity by multiplying by substrate concentration. Initial uptake velocity (v) is expressed as the following equation:

\[
v = \frac{V_{\text{max}} \times S}{K_m + S} + P_{\text{diff}} \times S \quad \text{Eq. 1}
\]

where \(P_{\text{diff}}\) is the clearance via passive diffusion and \(K_m\) and \(V_{\text{max}}\) is the Michaelis constant and the maximum uptake rate for the saturable uptake, respectively. Kinetic parameters were estimated by simultaneous fitting of all time and concentration points using non-linear regression in WinNonlin<sup>TM</sup> (Pharsight, Palo Alto, CA) The active uptake clearance (CL<sub>active</sub>) was calculated from the ratio of \(V_{\text{max}}\) over \(K_m\), whereas the total uptake clearance (CL<sub>uptake</sub>) included both the active and passive component (CL<sub>active</sub> and \(P_{\text{diff}}\)). In addition, relative importance of the active hepatic uptake in
comparison to the passive process was estimated over the range of concentrations investigated.

The parameter $K_{p_{\text{total}}}$ which reflects intracellular binding in addition to active uptake processes was calculated from eq. 2.

$$K_{p_{\text{total}}} = \frac{C_{\text{cell}}}{C_M} \quad \text{Eq. 2}$$

where $C_{\text{cell}}$ and $C_M$ represent concentration in the cell and media, respectively.

The hepatocyte/medium partition coefficient for unbound drug concentration ($K_{p_u}$) which provides a measure of the cytosolic cellular concentration relative to the external medium and hence reflects active uptake was calculated from eq. 3.

$$K_{p_u} = \frac{CL_{\text{active}} + P_{\text{diff}}}{P_{\text{diff}}} \quad \text{Eq. 3}$$

These two partitioning parameters are related by intracellular binding, as measured by the third partitioning parameter $K_{p_i}$.

$$K_{p_{\text{total}}} = K_{p_u} \cdot K_{p_i} \quad \text{Eq. 4}$$

where $K_{p_i}$ can be regarded as the ratio of the total cellular concentration to cytosolic unbound drug concentration (Parker and Houston, 2008) and is reflective on the fraction unbound in the hepatocyte ($f_{u_{\text{cell}}}$).

Hence -

$$K_{p_i} = \frac{K_{p_{\text{total}}}}{K_{p_u}} = \frac{1}{f_{u_{\text{cell}}}} \quad \text{Eq. 5}$$
The parameter $f_{u\text{cell}}$ was calculated using eq 5 with the exception of pravastatin, cerivastatin and atorvastatin. For these drugs $f_{u\text{cell}}$ was obtained using the logD$_{7.4}$ data and the regression equation $\log f_{u\text{cell}} = 0.9161 - 0.2567 \log \text{D}_{7.4}$ based on the remaining drugs ($n=13$), as illustrated in Figure 3B. The logD$_{7.4}$ for the drugs investigated were determined experimentally and kindly provided by Drug Metabolism & Pharmacokinetics Research Laboratories, R&D Division, Daiichi-Sankyo Co., Ltd., Tokyo, Japan. Polar surface area for individual drugs was generated using Molinspiration Cheminformatics software (http://www.molinspiration.com) using chemical structure of the drugs and corresponding SMILES (Simplified Molecular Input Line Entry Specification).
Results

Uptake kinetics for 16 drugs was investigated in freshly isolated rat hepatocytes in suspension. A time-dependent increase in cell-to-media concentration ratio was observed for all the compounds investigated and the use of a wide concentration range (0.01 – 100 µM) allowed full characterization of the uptake process (Figure 1). Nonlinear regression was used to estimate the kinetic parameters for passive (P_{diff}) and active (K_m, V_{max}, and CL_{active}) processes for the 16 drugs investigated (Table 2).

Clearance by active transport is expressed as a percentage of the total uptake clearance to assess the importance of transporter activity for each of 16 compounds investigated in Table 2. For all the compounds investigated hepatic uptake showed >50% dependence on transporters at low (likely therapeutic) concentrations; for 13 of the 16 drugs investigated this contribution was >80%. Figure 1 illustrates three distinct types of active uptake in relation to the passive process – rosuvastatin, for which active processes dominate over the full concentration range studied (95% of uptake); pitavastatin for which active uptake is also substantial (88% of uptake), yet readily saturable and hence passive permeability shows increasing importance, as drug concentration increases; and, saquinavir, for which passive permeability is comparable to active uptake at low concentrations (48% of uptake), but dominates at high concentrations.

P_{diff} values (Figure 2A) covered two orders of magnitude ranging from approximately 1 (olmesartan and pravastatin) to over 100 (ritonavir and saquinavir) with a median of 14 µL/min/10^6 cells (Table 2). It is noteworthy that bosentan, saquinavir and erythromycin, the three drugs with the lowest percentage contribution (approximately
50%) to uptake from transporters, show relatively high $P_{\text{diff}} (>10 \mu\text{L/min}/10^6 \text{cells})$. There was a strong ($r^2=0.867$) and statistically significant (p<0.001) correlation between $P_{\text{diff}}$ and the logD$_{7.4}$ value (Figure 3A).

$K_m$ values for active transport have a median value of 6 µM (see Figure 2B). The lowest $K_m$ values were obtained for ritonavir and repaglinide (approximately 2 µM), in contrast to clarithromycin, erythromycin, saquinavir and olmesartan where values were greater than 50 µM (Table 2). The parameter $V_{\text{max}}$ appears more consistent across the 16 compounds showing a median value of 850 pmol/min/10$^6$cells; with saquinavir and bosentan at the higher and lower end, respectively (Table 2).

$CL_{\text{active}}$ is defined by both $K_m$ and $V_{\text{max}}$ and for the 16 compounds investigated the dominant parameter was drug dependent. The range of $CL_{\text{active}}$ covered two orders of magnitude from 10.6 to 1500 µL/min/10$^6$ cells for olmesartan and atorvastatin, respectively, with a median value of 188 µL/min/10$^6$ cells (Figure 2A). There was no statistical relationship between $CL_{\text{active}}$ (or $K_m$) and $P_{\text{diff}}$ values. However, it was useful to consider these relationships (Figures 2A and B), as certain trends can be identified. For 11 drugs, $CL_{\text{active}}$ was approximately 12-fold higher than the $P_{\text{diff}}$ value (range 5-19). The most pronounced outliers were rosuvastatin and atorvastatin where this ratio was approximately 60 and 280, respectively. In the case of saquinavir, erythromycin and bosentan, values for $CL_{\text{active}}$ and $P_{\text{diff}}$ were approximately equivalent. When $K_m$ values are considered in relation to $P_{\text{diff}}$ (Figure 3B) a clear trend was evident for 13 drugs where $K_m$ decreased as $P_{\text{diff}}$ increased; outliers represented drugs with high $K_m$ values, namely clarithromycin, erythromycin and saquinavir.
$K_{pu}$ (calculated from $CL_{uptake}$ and $P_{diff}$ and shown in Figure 4A) varied more than 200-fold, ranging from 2 in the cases of erythromycin, saquinavir and bosentan to 494 for atorvastatin (Table 3). The extremes were the same drugs as discussed above in terms of the relative magnitude of $P_{diff}$ and $CL_{active}$. For 56% of the drugs in the dataset the $K_{pu}$ value was 10 and above. For most drugs $K_{total}$ was substantially higher than $K_{pu}$ (10 to 130-fold) with median values of 232 and 13 respectively (Table 3); less so for rosuvastatin (2-fold) and valsartan (6-fold) and for olmesartan the two $K_p$ values were approximately equal (see Figure 4A). However, there was no statistically significant trend between these two parameters. The intracellular binding process, as indicated by $f_{cell}$ was related to Log $D_{7.4}$ ($p<0.001$, $r^2 = 0.735$), as shown in Figure 3B. Figure 4B supports the notion of the independence between transporter mediated uptake (as measured by $K_{pu}$ – median value of 13 indicated in figure) and intracellular binding ($f_{cell}$). Therefore, measurement of only one of these processes will limit the characterization of hepatocellular drug accumulation.
Discussion

The hepatic uptake characteristics of a series of 16 drugs were investigated to provide a dataset of parameters for comparative purposes. Currently, the kinetic information available on both active and passive uptake to provide a framework for evaluating new compounds is limited. To date studies have primarily focused on single compounds or were not carried out over a sufficiently wide concentration range to achieve the above objectives. In contrast, there are several studies documenting inhibitory properties of many drugs against various hepatic transporters, particularly OATP1B1 (Hirano et al., 2006; Noe et al., 2007; Gui et al., 2009; Sharma et al., 2009).

In the current study, the rat was selected as the source of hepatocytes for several reasons. These rat hepatocytes were freshly isolated to eliminate any concerns associated with tissue storage or inter-donor variability, commonly observed with human material, and hence had the advantage of being a more reproducible in vitro system. The rat has also proved to be a useful model for characterizing drug hepatic distribution in vivo where multiple indicator dilution studies have been carried out (Yamazaki et al., 1993; Watanabe et al., 2009a). Furthermore, the use of freshly isolated cells in suspension, where internalization of efflux transporters has been documented (Bow et al., 2008) combined with the treatment with ABT (Hallifax and Houston, 2006) to eliminate P450 metabolism, has provided a valuable system to focus primarily on uptake characteristics. Despite existing species differences in transporters (Hagenbuch and Gui, 2008), this strategy provides valuable basic information on hepatocellular drug uptake which can be used for a variety of
purposes, including initial optimization of hepatic uptake in physiologically-based pharmacokinetic models (Watanabe et al., 2009a).

Active processes were confirmed as important for the uptake of these 16 drugs at low (therapeutic) concentrations (<0.1 µM); for 13 drugs this process showed a greater than 80% contribution to total hepatic uptake. For the remaining three drugs – bosentan, erythromycin and saquinavir – the importance of passive and active uptake was equal. In the case of saquinavir both $P_{\text{diff}}$ and $CL_{\text{active}}$ were large, whereas both parameters had low values for bosentan and erythromycin.

A wide range of $K_m$ values was obtained for the uptake of the compounds investigated, with ten out of 16 compounds showing high affinity for the uptake transporters ($K_m < 10$ µM, Table 1). Although $K_m$ values for pravastatin, clarithromycin, erythromycin, olmesartan and saquinavir were relatively high (>30 µM), their hepatic uptake was predominantly due to transporter-dependent active uptake. Our data are consistent with literature reported $K_m$ values for pravastatin (Yamazaki et al., 1993; Ishigami et al., 1995), rosuvastatin (Nezasa et al., 2003) and pitavastatin (Shimada et al., 2003).

$V_{\text{max}}$ values were more consistent across the compound set (median 850 pmol/min/10^6 cells) but saquinavir, ritonavir, telmisartan and clarithromycin showed values >2,000 pmol/min/10^6 cells. Both $CL_{\text{active}}$ and $P_{\text{diff}}$ values ranged over more than two orders of magnitude. It is of interest that the range of $P_{\text{diff}}$ (1-200 µl/min/10^6 cells) and $CL_{\text{active}}$ values (10-1500 µl/min/10^6 cells) reported in these studies were comparable to that previously documented for metabolic intrinsic clearance (1-1800 µl/min/10^6 cells) (Ito and Houston, 2004) in fresh isolated rat hepatocytes. The rank
order for uptake clearance of pitavastatin, rosuvastatin, pravastatin, valsartan and olmesartan was in good agreement with previously published values determined at a single low concentration (Watanabe et al., 2009b). Although no statistical relationship between $CL_{\text{active}}$ and $P_{\text{diff}}$ was established with this set of drugs, the association between these two parameters for specific cases provides a useful framework for discussion. A saturable uptake mechanism was responsible for >88% of the hepatic uptake of pitavastatin, pravastatin, atorvastatin and rosuvastatin; the statins recommended as candidate probes for clinical transporter-mediated drug-drug interaction studies by the recent transporter consortium ‘white’ paper (Giacomini et al., 2010). Pitavastatin and cerivastatin showed a larger passive clearance (approximately 20 $\mu$L/min/10^6 cells) in comparison to atorvastatin, pravastatin and rosuvastatin (1-7 $\mu$L/min/10^6 cells); however, the saturable component was characterized by similar low $K_m$ values (Table 2). Fexofenadine, valsartan and olmesartan showed similar trends to the latter three statins, whereas uptake characteristics of telmisartan and nateglinide were comparable to pitavastatin and cerivastatin.

Both saquinavir and ritonavir have large passive clearances of 191 and 118 $\mu$L/min/10^6 cells, respectively (Table 2), resulting in the large uptake rates at higher concentration. However, considering the low $K_m$ value of 2.6 $\mu$M for ritonavir uptake, an active process is likely an important contributor to the uptake of ritonavir. The contribution of its active process is dominant over passive process at low substrate concentrations less than 10 $\mu$M. In contrast to ritonavir, uptake of saquinavir was characterized by a more pronounced passive uptake contributing > 50% at the lower substrate concentration. The $K_m$ value for the active uptake (52 $\mu$M) was consistent
with the previously reported value (Parker and Houston, 2008). Repaglinide uptake kinetics was comparable to ritonavir. The high affinity for uptake transporters seen for repaglinide was in agreement with a number of clinical studies indicating the importance of hepatic uptake for disposition of this drug (Kajosaari et al., 2005; Kalliokoski et al., 2008). For bosentan and erythromycin, active and passive processes were equally important. Clarithromycin, by virtue of its high \( V_{\text{max}} \), has a much greater dependence on active uptake.

The associations between each of the kinetic parameters and with physicochemical properties highlight our limited understanding of the determinants of hepatic uptake and emphasize the need for further experimentation with larger datasets. While the positive correlation between \( P_{\text{diff}} \) and \( \log D_{7.4} \) is of no surprise, any relationship between \( P_{\text{diff}} \) and \( CL_{\text{active}} \) was not prominent. There was a tendency (although not statistically significant) for these two parameters to be positively related (\( CL_{\text{active}} \) being approximately ten times \( P_{\text{diff}} \)), in particular for six drugs with the lowest and highest \( P_{\text{diff}} \). In contrast, ten drugs with intermediate \( P_{\text{diff}} \) values (approximately 10 \( \mu \text{L/min/10}^6\text{cells} \)) showed \( CL_{\text{active}} \) that ranged from 500-fold larger to equal to \( P_{\text{diff}} \). The relationship between \( K_m \) and \( P_{\text{diff}} \) was negative for 13 of the 16 drugs. The high \( K_m \) outliers represented, in the main, the drugs with comparable \( CL_{\text{active}} \) and \( P_{\text{diff}} \) parameter values.

Unbound hepatocyte-to-medium concentration ratio (\( K_{pu} \)) and the extent of intracellular binding were indirectly obtained from the parameter estimates defining active and passive uptake and therefore reflect any variability or uncertainty in these parameter estimates. However, as discussed earlier, the experimentally manipulated
lack of metabolic clearance and efflux transporters in these studies provided $K_{pu}$ values calculated solely from the clearances for active uptake and passive permeability and hence should represent pure distribution parameters (Brown et al., 2010). For nine drugs in the dataset $K_{pu} > 10$, whereas smaller (but >2) values were obtained for saquinavir, erythromycin, valsartan, ritonavir, repaglinide, clarithromycin and bosentan (Figure 4). Overall $K_{pu}$ ranged >200-fold between saquinavir and atorvastatin. The lack of correlation between $K_{pu}$ and intracellular binding is of importance as several investigators (for example, Yamano et al., 1999; Yamano et al., 2000) have assumed $K_{ptotal}$ (mainly driven by intracellular binding) to reflect an increase in cellular free concentration and hence the concentration available to enzymes (for metabolism or inhibition). In contrast, we have previously demonstrated similar inhibition potency in microsomes and hepatocytes after appropriate binding corrections for six inhibitors that showed a $K_{ptotal}$ range of 4-1200 (Brown et al., 2007).

The processes of active transport and cellular binding play distinct roles in defining $K_{ptotal}$, which for all drugs in the current dataset exceeds 40 with a range >100-fold (Figure 4B). For several drugs unbound intracellular concentrations are apparently equal to that in the plasma due to passive uptake and $K_{pu}$ values of unity are evident. In other cases $K_{pu}$ is substantially greater than one, as illustrated here for 16 substrates for hepatic transporters. Unlike $K_{ptotal}$, $K_{pu}$ requires determination of several parameters to describe both active and passive processes adequately. The current study emphasizes the need to understand the determinants of intracellular drug concentrations, to establish a more appropriate term than plasma concentration for in vitro-in vivo extrapolation and hence progress our mechanistic understanding of the
rate determining processes contributing to drug clearance and governing drug-drug interactions.
Authorship contribution

Participated in research design: Yabe, Galetin and Houston

Conducted experiments: Yabe

Contributed analytic tools: not applicable

Performed data analysis: Yabe

Wrote or contributed to the writing of the manuscript: Yabe, Galetin and Houston
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Footnotes

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Figure Legends

FIG. 1 Examples of concentration-dependent hepatic uptake rates and relative contribution of active and passive processes. Panels A, D rosuvastatin; B, E pitavastatin; C, F saquinavir. Symbols and the solid lines represent observed values and the fitted curves to the equation 1, respectively. Dashed and dotted lines represent active and passive uptake components, respectively.

FIG. 2 Hepatocellular uptake characteristics of 16 drugs in rat isolated hepatocytes. Relationship between $P_{\text{diff}}$ and $C_{L_{\text{active}}}$ (A) and $P_{\text{diff}}$ and $K_m$ (B). The solid line in panel A represents a 12-fold difference from the line of unity which is indicated as a dashed line. Eleven drugs consist with this trend are shown within the ellipse and outliers identified: atorvastatin (1), rosuvastatin (2), bosentan (3), erythromycin (4), saquinavir (5) and clarithromycin (6).

FIG. 3 Role of log $D_{7.4}$ in defining $P_{\text{diff}}$ (A) and $f_{u_{\text{cell}}}$ (B). Relationships between parameters were best described by the following equations: $\log P_{\text{diff}} = 0.3207 \log D_{7.4} + 0.7000$ and $\log f_{u_{\text{cell}}} = 0.9161-0.2567 \log D_{7.4}$. The latter equation (based on 13 drugs from the current dataset) was subsequently used to estimate $f_{u_{\text{cell}}}$ for atorvastatin, cerivastatin and pravastatin.

FIG. 4 Hepatocyte-to-medium drug concentration ratios for 16 drugs in rat isolated hepatocytes. Range of $K_{p_{\text{total}}}$ and $K_{p_{u}}$ values observed (A) and lack of relationship between $K_{p_{u}}$ and $f_{u_{\text{cell}}}$ (B). Dashed line represents a median $K_{p_{u}}$ of 13 and outliers identified: atorvastatin (1), rosuvastatin (2), bosentan (3), erythromycin (4) and
saquinavir (5).
TABLE 1

Physicochemical and metabolic characteristics of the 16 drugs studied

<table>
<thead>
<tr>
<th>Compound</th>
<th>LogD&lt;sub&gt;7.4&lt;/sub&gt;</th>
<th>Polar Surface Area</th>
<th>Metabolically Stable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>1.3</td>
<td>112</td>
<td>No</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1.25</td>
<td>146</td>
<td>No</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>1.9</td>
<td>100</td>
<td>No</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>1.8</td>
<td>183</td>
<td>No</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1.11</td>
<td>194</td>
<td>No</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>0.3</td>
<td>81</td>
<td>Yes</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>1.22</td>
<td>66</td>
<td>No</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>-1.56</td>
<td>130</td>
<td>Yes</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>1.2</td>
<td>91</td>
<td>Yes</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>-0.4</td>
<td>124</td>
<td>Yes</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>2.3</td>
<td>79</td>
<td>No</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>4.8</td>
<td>146</td>
<td>No</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>-0.33</td>
<td>141</td>
<td>Yes</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>4.8</td>
<td>167</td>
<td>No</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>2.5</td>
<td>73</td>
<td>Yes</td>
</tr>
<tr>
<td>Valsartan</td>
<td>-1.11</td>
<td>112</td>
<td>Yes</td>
</tr>
</tbody>
</table>
### TABLE 2

Kinetic parameters for the hepatic uptake of 16 drugs in rat hepatocytes in suspension

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/10^6 cells)</th>
<th>$P_{diff}$ (µL/min/10^6 cells)</th>
<th>$CL_{active}$ (µL/min/10^6 cells)</th>
<th>$CL_{active}$/ $CL_{uptake}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>4.03 ± 4.01</td>
<td>1340 ± 320</td>
<td>5.35 ± 3.79</td>
<td>1500 ± 1980</td>
<td>98.5</td>
</tr>
<tr>
<td>Bosentan</td>
<td>5.24 ± 1.56</td>
<td>105 ± 85</td>
<td>17.5 ± 4.4</td>
<td>18.7 ± 12.6</td>
<td>48.9</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>5.17 ± 4.78</td>
<td>804 ± 168</td>
<td>23.7 ± 6.4</td>
<td>285 ± 247</td>
<td>86.7</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>58.0 ± 47.9</td>
<td>2770 ± 2470</td>
<td>10.9 ± 4.1</td>
<td>50.7 ± 16.4</td>
<td>81.8</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>42.9 ± 13.9</td>
<td>520 ± 359</td>
<td>12.2 ± 4.4</td>
<td>11.4 ± 5.8</td>
<td>47.7</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>7.41 ± 439</td>
<td>885 ± 474</td>
<td>6.75 ± 2.48</td>
<td>167 ± 126</td>
<td>94.3</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>4.83 ± 1.27</td>
<td>834 ± 373</td>
<td>16.6 ± 7.9</td>
<td>179 ± 96</td>
<td>90.7</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>53.0 ± 28.8</td>
<td>514 ± 326</td>
<td>0.863 ± 0.664</td>
<td>10.6 ± 4.2</td>
<td>92.1</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>6.30 ± 5.06</td>
<td>1070 ± 960</td>
<td>18.8 ± 11.8</td>
<td>197 ± 121</td>
<td>87.9</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>30.5 ± 26.6</td>
<td>789 ± 385</td>
<td>1.67 ± 1.02</td>
<td>31.7 ± 10.4</td>
<td>95.1</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>2.98 ± 1.28</td>
<td>804 ± 346</td>
<td>58.2 ± 44.2</td>
<td>299 ± 122</td>
<td>84.6</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>2.62 ± 1.84</td>
<td>2160 ± 1530</td>
<td>118 ± 29</td>
<td>873 ± 216</td>
<td>87.5</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>6.05 ± 5.43</td>
<td>1250 ± 400</td>
<td>7.08 ± 3.23</td>
<td>418 ± 319</td>
<td>97.7</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>51.7 ± 21.0</td>
<td>12000 ± 7700</td>
<td>191 ± 24</td>
<td>239 ± 152</td>
<td>52.1</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>3.85 ± 1.18</td>
<td>2220 ± 670</td>
<td>28.1 ± 10.0</td>
<td>630 ± 286</td>
<td>94.5</td>
</tr>
<tr>
<td>Valsartan</td>
<td>12.3 ± 13.0</td>
<td>217 ± 220</td>
<td>3.91 ± 2.32</td>
<td>30.1 ± 32.4</td>
<td>79.4</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of 3 to 5 experiments.
TABLE 3

Parameters defining relative importance of active uptake and cellular binding in hepatic uptake of 16 drugs in rat hepatocytes in suspension

<table>
<thead>
<tr>
<th>Compound</th>
<th>CL_{active}/P_{diff}</th>
<th>K_{pu}^a</th>
<th>K_{p_{total}}</th>
<th>fu_{cell}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>493 ± 651</td>
<td>494 ± 651</td>
<td>8781^b</td>
<td>0.056</td>
</tr>
<tr>
<td>Bosentan</td>
<td>1.06 ± 0.56</td>
<td>2.06 ± 0.56</td>
<td>150 ± 37</td>
<td>0.013</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td>13.1 ± 11.0</td>
<td>14.1 ± 11.0</td>
<td>353^b</td>
<td>0.039</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>5.05 ± 2.29</td>
<td>6.05 ± 2.29</td>
<td>250 ± 22</td>
<td>0.024</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1.00 ± 0.49</td>
<td>2.00 ± 0.49</td>
<td>41 ± 9</td>
<td>0.05</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>24.5 ± 19.4</td>
<td>25.5 ± 19.4</td>
<td>245 ± 20</td>
<td>0.10</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>11.4 ± 5.4</td>
<td>12.4 ± 5.4</td>
<td>250 ± 51</td>
<td>0.05</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>23.1 ± 27.5</td>
<td>24.1 ± 27.5</td>
<td>38 ± 10</td>
<td>0.62</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>21.9 ± 29.2</td>
<td>22.9 ± 29.2</td>
<td>800 ± 59</td>
<td>0.028</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>21.9 ± 8.2</td>
<td>22.9 ± 8.2</td>
<td>149^b</td>
<td>0.15</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>6.94 ± 3.95</td>
<td>7.94 ± 3.95</td>
<td>220 ± 43</td>
<td>0.036</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>7.94 ± 3.37</td>
<td>8.94 ± 3.37</td>
<td>616^c</td>
<td>0.015</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>57.2 ± 11.9</td>
<td>58.2 ± 32.8</td>
<td>125 ± 16</td>
<td>0.48</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>1.34 ± 1.06</td>
<td>2.34 ± 1.06</td>
<td>306^c</td>
<td>0.0075</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>26.6 ± 16.1</td>
<td>27.6 ± 16.1</td>
<td>680 ± 26</td>
<td>0.04</td>
</tr>
<tr>
<td>Valsartan</td>
<td>7.69 ± 6.23</td>
<td>8.69 ± 6.23</td>
<td>50 ± 36</td>
<td>0.17</td>
</tr>
</tbody>
</table>

^a Calculated from CL_{active}/P_{diff}.  
^b Calculated using the regression equation logfu_{cell} = 0.9161-0.2567 logD_{7.4}  
^c From Parker and Houston (2008)
Figure 1

A. Rosuvastatin concentration (µM) vs. Uptake rate (pmol/min/10^6 cells)

B. Pitavastatin concentration (µM) vs. Uptake rate (pmol/min/10^6 cells)

C. Saquinavir concentration (µM) vs. Uptake rate (pmol/min/10^6 cells)

D. Rosuvastatin concentration (µM) vs. Contribution to total uptake (%)

E. Pitavastatin concentration (µM) vs. Contribution to total uptake (%)

F. Saquinavir concentration (µM) vs. Contribution to total uptake (%)
Figure 2

A

B

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Figure 3

A

\[ \log P_{\text{diff}} \]

B

\[ \log f_{\text{cell}} \]
Figure 4

A

B

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