Exploring the boundaries for in vitro-in vivo extrapolation: Use of isolated rat hepatocytes in co-culture and impact of albumin binding properties in the prediction of clearance of various drug types

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Running title page

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Abbreviations
Alb.CL_{act}: albumin enhanced active clearance, Alb.CL_{pass}: albumin enhanced passive clearance, AMU: albumin mediated uptake, AUC: area under the curve, BSA: bovine serum albumin, CL: clearance, CL_{int,AUC}: CL_{int} calculated using AUC intracellular profiles, CL_{int,in vitro}: in vitro clearance, CL_{int,u in vitro}: unbound CL_{int, in vitro}, CL_{met}: metabolic clearance, CL_{uptake}: uptake clearance, DPBS: Dulbecco's phosphate-buffered saline. FDH: free-drug hypothesis, f_{unb}: fraction unbound in the medium, f_{unb}: fraction unbound due to non-specific binding, GMFE: geometric mean fold error, IVIVE: In vitro-in vivo extrapolation, K_{a}: binding association equilibrium constant, Kp: intracellular-medium drug concentration ratio, Kp_{stromal}: Stromal cell partitioning, RMSE: root mean squared error, UWL: unstirred water layer, V_{Exp}: Experimental incubation volume, WME: Williams’ Medium E
Abstract

Prediction of hepatic clearance of drugs (via uptake or metabolism) from in vitro systems continues to be problematic, particularly when plasma protein binding is high. The following work explores simultaneous assessment of both clearance processes, focusing on a commercial hepatocyte-fibroblast co-culture system (HµREL), over a 24 hour period, using six probe drugs (ranging in metabolic and transporter clearance and low-to-high plasma protein binding). A rat hepatocyte co-culture assay was established using drug depletion (measuring both medium and total concentrations) and cell uptake kinetic analysis, both in the presence and absence of plasma protein (1% BSA). Secretion of endogenous albumin was monitored as a marker of viability and this reached 0.004% in incubations (at a rate similar to in vivo synthesis). Binding to stromal cells was substantial and required appropriate correction factors. Drug concentration-time courses were analysed both by conventional methods and a mechanistic cell model, prior to in vivo extrapolation. Clearance assayed by drug depletion in conventional suspended rat hepatocytes provided a benchmark to evaluate co-culture value. Addition of albumin appeared to improve predictions for some compounds (where fu_{med}<0.1); however, for high binding drugs, albumin significantly limited quantification and thus predictions. Overall, these results highlight ongoing challenges concerning in vitro hepatocyte system complexity and limitations of practical expediency. Considering this, more reliable measurement of hepatically cleared compounds seems possible through judicious use of available hepatocyte systems, including co-culture systems, as described herein; this would include those compounds with low metabolic turnover but high active uptake clearance.
Significance statement

Co-culture systems, combining hepatocytes with stromal cells, offer a more advanced tool than standard hepatocytes, with the ability to be cultured for longer periods of time, yet their potential as an in vitro tool has not been extensively assessed. We evaluate the strengths and limitations of the HµREL system using six drugs representing various metabolic and transporter-mediated clearance pathways with various degrees of albumin binding. Studies in the presence/absence of albumin allow IVIVE and a framework to maximise their utility.
Introduction

Predicting human hepatic in vivo clearance (CL) for candidate drugs based on in vitro systems remains a key pharmaceutical challenge. Commonly used in vitro assays have demonstrated a systematic underprediction of high clearance drugs and uptake transporter substrates, as well as poor detection of low clearance compounds, due to the short-lived nature of industry standard in vitro systems (Varma et al., 2015; Wood et al., 2017). In order to improve current in vitro – in vivo extrapolation (IVIVE), various approaches have been explored, including optimising in vitro assays to determine the rate-limiting step in hepatic clearance, using alternative in vitro models that allow for maintenance of hepatocytes for longer periods of time, as well as mathematical approaches (such as empirical scaling factors) to improve predictions (de Bruyn et al., 2018; Umehara et al., 2020; Wood et al., 2018; Yadav et al., 2021). Whilst such mathematical approaches have their utility, the clearance determined from an in vitro assay is a fundamental parameter used in all IVIVE approaches, and thus ensuring the intrinsic clearance measured in vitro (CL$_{int, \text{ in vitro}}$) is a relevant representation of the drug’s CL in vivo is vital.

Isolated hepatocytes have long been viewed as the gold standard for estimations of in vivo CL (due to their full complement of enzymes and transporters); however, what remains less well defined is the optimum assay design to maintain the key hepatocyte characteristics such as transporter expression and metabolic activity (den Braver-Sewradj et al., 2017; Di et al., 2012; Soldatow et al., 2013). Commonly used in vitro systems such as suspended hepatocytes and microsomes have the advantage of being high throughput, but experimentally can only capture the metabolic clearance (CL$_{met}$) of drugs for up to 4 hours. The “medium loss” (Harrison et al., 2018) and “oil-spin” (Yabe et al., 2011) assays are marginally more labour-intensive, but owing to the separation of cellular material from extracellular medium, can capture uptake clearance (CL$_{uptake}$) within the first few minutes of drug incubation, as well as CL$_{met}$, up to a maximum of 4 hours (due to the suspended nature of hepatocytes). In a monolayer format, hepatocytes assays can also capture CL$_{uptake}$ and CL$_{met}$, for up to 24 hours, but such assays are again more labour intensive with lower throughput (Heslop et al., 2017; Soldatow et al., 2013). Sandwich-culture hepatocytes, which are maintained for up to 5 days, can estimate biliary clearance due to their export transporter orientation, but typically regress in both
uptake transporter and metabolic enzyme expression, meaning they poorly predict CL\textsubscript{uptake} and CL\textsubscript{met} (Cantrill & Houston, 2017; de Bruyn et al., 2013). In recent years more advanced hepatocyte models have been developed, focused on maintaining hepatic function for longer time periods, and increasing similarity to in vivo conditions. Co-culture of hepatocytes with fibroblasts, such as in the H\textsubscript{μ}REL\textsuperscript{TM} or HepatoPac\textsuperscript{TM} systems, enables the hepatocytes to maintain metabolic and transporter activity for up to 7 days, in particular, allowing the study of low clearance compounds, which are typically cleared by more passive/metabolic routes (Bonn et al., 2016; Chan et al., 2013; Da-silva et al., 2018; Docci et al., 2020; Hultman et al., 2016; Kamel et al., 2021). The cell number is a key consideration for CL studies (Louisse et al., 2020), as whilst measured CL\textsubscript{int, in vitro} values are normalised to cellular number/protein, the number of hepatocytes present will ultimately determine the extent of depletion, recognising that sufficient drug CL (typically at least 20% reduction in drug concentration) must be detected to reliably quantify CL\textsubscript{int, in vitro} (above the limit of quantification). Experiments performed using a low number of hepatocytes, such as in co-culture systems may struggle to reach this 20% threshold.

To this end, the current work intends to mechanistically evaluate the rat H\textsubscript{μ}REL\textsuperscript{TM} hepatocyte co-culture system. Six drugs representing various metabolic and transporter-mediated CL pathways with various degrees of CL and albumin binding (in human, consistent with the ECCS, Varma et al. (2015)) were selected for study (Table 1). CL\textsubscript{int, in vitro} values in co-culture were compared to suspended hepatocytes using medium depletion (after cell removal) and total depletion (media and cells combined) to capture the different clearance mechanisms. The impact of albumin (in response to recent publications) on CL\textsubscript{int, in vitro} was investigated by addition of bovine serum albumin (BSA) to assay incubations, and direct measurement of native albumin secreted in the case of co-culture. A similar extent of binding to BSA between rat and human was assumed; physiological concentration (4%) or lower (1%) was used, depending on detectability of unbound drug. Finally, IVIVE across all systems was performed, comparing predictions based on albumin-free and BSA conditions in hepatocyte assays, including testing the free-drug hypothesis (FDH) using a mechanistic model for albumin-mediated uptake (AMU) in the H\textsubscript{μ}REL co-culture system. This work is intended to improve our understanding of the strengths and limitations of in vitro hepatocyte systems typically used in IVIVE, and therefore how such systems should be interpreted and used in IVIVE predictions.
Materials and Methods

Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (Merck) unless otherwise stated.

Rat HµREL co-culture systems

HµREL co-culture 96-well plates were used to investigate the CL under protein-free and 1% BSA conditions, examining both medium depletion and intracellular accumulation. 1% BSA was selected to provide a range of fraction unbound drug concentration in the medium (f_{u_{med}}) values, especially for highly bound drugs. Co-culture plates were maintained in HµREL maintenance medium on arrival for a maximum of 4 days at 37°C, 5% CO₂ and 95% humidity, as per manufacturer’s instructions. Prior to initiation of experiments, HµREL maintenance medium was removed, cells were washed twice in 50 µL of HµREL dosing medium containing appropriate BSA concentrations (0% or 1%) and left in a third wash for 4 hours at 37°C, 5% CO₂ and 95% humidity. Experiments were performed in reverse time order, initiating the longest timepoint (24 hours) first. Based on pilot incubations (data not shown), 24 hours was estimated to be sufficient to calculate CL_{int \text{ in vitro}} for the 6 probe drugs tested. Prior to initiation of experiments (at appropriate time points), the final wash was removed, and wells were washed twice with HµREL dosing medium. 25µL of 2X BSA dosing medium was then added to each well, and 25µL of 2µM drug (protein-free) medium was added to initiate time points, yielding a final 1µM, 1X BSA dosing medium in the experiment. At designated timepoints (0.5, 1, 15, 30, 60 minutes, 4 and 24 hours) 40µL of dosing medium was removed and quenched directly in methanol containing appropriate internal standard. The remaining medium was aliquoted into a 96-well plate and stored at -20°C until analysis by rat albumin ELISA assay (ab108789, Promega), performed as per manufacturer’s instructions. Cells were washed thrice with 40µL ice-cold Dulbecco's phosphate-buffered saline (DPBS) and left dry until all time points were collected. In order to minimise metabolism whilst the remaining time points were collected, experiments were performed in reverse time order, so that all time points could finish within a similar time frame and that the earliest time points were exposed to the shortest drying time (the maximum drying time elapsed was 40 minutes.
for the 24 hour sample (3% deviation) whereas the 0.5 minute timepoint was immediately lysed and frozen upon completion. Despite best efforts, it is possible that some minor residual metabolism could have occurred within this time period. After completion of all timepoints, 50µL of distilled water was added to each well, and plates were stored at -20°C overnight. All experiments were performed in duplicate on three separate occasions.

**Rat hepatocyte studies**

Cryopreserved pooled mixed gender Sprague-Dawley rat hepatocytes (BioIVT, Sussex UK, lots FVN, XVJ and FNH; minimum donor pool size was 24) were removed from liquid nitrogen and immediately thawed at 37°C for approximately 2 minutes according to manufacturer’s instructions. The hepatocytes were transferred into pre-warmed INVITROGRO HT thawing medium (BioIVT) and centrifuged at 50g for 5 minutes in an Eppendorf 5804 centrifuge (Stevenage, UK), then re-suspended in a small volume of Williams’ Medium E (WME, SLS, Nottingham UK), supplemented with 24mM HEPES, pH 7.4. Cell viability was determined by Trypan Blue exclusion (cell viability was ≥80%), and cells were re-suspended to a viable cell concentration of $4 \times 10^6$ hepatocytes/mL. 96-well plates were loaded with 62.5µL of 4X (4 times concentrated) BSA supplemented WME, and 62.5µL of $4 \times 10^6$ hepatocytes/mL. At appropriate time points (0.5, 1, 1.5, 2, 15, 30, 45 and 60 minutes), 125µL of 2X drug solution was added, giving a final concentration of 1µM drug in 1X BSA medium to $1 \times 10^6$ hepatocytes/mL. 4% BSA was selected to mimic physiological conditions, and 1% BSA was selected to provide a range of $f_{unmed}$ values, especially for highly bound drugs. The conventional (total drug) depletion assay and the medium depletion assay were performed as described previously by (Harrison et al., 2018). The Bradford protein assay was performed as per manufacturer’s instructions on cell lysates from 0% BSA conditions, using the mean values to determine protein concentration per well. The same protein concentration was assumed for the 1% and 4% conditions for each experimental day. Each experiment was performed in duplicate on three separate occasions. Non-specific binding experiments were performed in parallel to the assays above (performed identically to the conventional total drug depletion assay but in the absence of hepatocytes), to determine non-specific binding to
plastics. For the six probe drugs used here non-specific binding was low and thus was not included in calculations (Supplement 1).

Sample preparation for LC-MS/MS analysis

For medium depletion samples (both rat hepatocyte studies and HµREL), aliquots were quenched directly in methanol (VWR) containing appropriate internal standard and stored at -20°C until analysis by LC-MS/MS. Medium from cell only control wells were used to generate matrix samples for the calibration curve. Calibration curves were prepared in duplicate for each of the protein concentrations tested. In the HµREL co-culture systems, the top and bottom rows were used for this (as they are not recommended to be used for experimental wells in low clearance studies due to evaporation issues). To examine cellular concentrations in the HµREL co-culture system, the cell lysate was thawed at room temperature, and 40 µL of cell lysate was added to methanol containing internal standard, and again, the top and bottom rows of the HµREL co-culture plate were used to generate blank matrix for the calibration curve. Plates were stored at -20°C until LC-MS/MS analysis, performed as described in Supplement 2.

HµREL data analysis

To minimise experimental variability, the applied medium concentration of drug in each experiment was also measured, and data was normalised to the nominal medium concentration by the nominal/applied ratio (1 µM). The HµREL system is a co-culture of hepatocytes and stromal cells, at approximately a 1:1 ratio. To account for background non-specific binding and stromal cell drug binding/accumulation from intracellular measurements (and therefore obtain hepatocyte-only concentrations), additional experiments were performed in the same manner as above in 0% BSA stromal cell only plates. Medium and intracellular concentrations were determined, assuming a hepatocyte volume of 3.9 µL/10⁶ hepatocytes (Cantrill & Houston, 2017), 25,000 hepatocytes and 25,000 stromal cells per well in the HµREL co-culture plates, 25,000 stromal cells per well in the stromal cell only plates, and stromal cells having equivalent cellular volume to hepatocytes. The intracellular-medium ratio (Kp, the concentration of drug in the cell divided by the concentration of drug in the medium) at each time point was determined from each experiment (n=3), and the mean of
each of these timepoints was used to observe how Kp changed over time in the stromal cell only plates at 0% BSA (Supplement 3), the HµREL co-culture plates at 0% BSA, and HµREL co-culture plates at 1% BSA. The relationship between the change in cellular Kp from the 0% BSA stromal only and the 0% BSA HµREL co-culture plates was assumed to be equivalent to what would be observed under 1% BSA conditions, and thus this relationship was used to predict the expected Kp of a 1% BSA stromal only plate (not experimentally performed). In most cases Kp from the 0% stromal cell only plate reached equilibrium within 1 hour; however, for midazolam, saquinavir and repaglinide, accumulation continued up to the 24-hour time point, and therefore corrections were performed per time point.

Kp of the HµREL co-culture plate from 0% and 1% BSA conditions (KpHµREL,0% and KpHµREL,1%, respectively) were used to calculate the fraction unbound in the HµREL co-culture plates based on Eq. 1 or Eq. 2, fuHµREL,0% and fuHµREL,1% respectively, (Hallifax & Houston, 2007).

\[
fu_{H\mu REL,0\%} = \frac{1}{1 + (Kp_{H\mu REL,0\%} \times \frac{V_{H\mu REL}}{V_{Exp}})}
\]

Eq. 1

\[
fu_{H\mu REL,1\%} = \frac{1}{1 + (Kp_{H\mu REL,1\%} \times \frac{V_{H\mu REL}}{V_{Exp}})}
\]

Eq. 2

Where \(V_{H\mu REL}/V_{Exp}\) represents the fractional co-culture cellular volume of the experiment (0.0039), where \(V_{H\mu REL}\) represents the cellular volume per well of the HµREL system (assuming 50,000 cells per well with equivalent volume to hepatocytes, 3.9 µL/10^6 hepatocytes (Cantrill & Houston, 2017)), and \(V_{Exp}\) represents the total volume of the incubation per well (50µL).

These equations can be written to include both cell types present within the system, using the calculated Kp values of the stromal cell only plates at 0% or 1% BSA conditions (KpStromal, 0% and
KpStromal, 1%, respectively), to determine the Kp of the hepatocytes at 0% and 1% BSA conditions
(KpHepatocyte, 0% and KpHepatocyte, 1%, Eq. 3 and Eq. 4, respectively).

\[ f_{uHREL, 0\%} = \frac{1}{1 + (Kp_{Stromal, 0\%} \times \frac{V_{Stromal}}{V_{Exp}}) + (Kp_{Hepatocyte, 0\%} \times \frac{V_{Hepatocyte}}{V_{Exp}})} \]

Eq. 3

\[ f_{uHREL, 1\%} = \frac{1}{1 + (Kp_{Stromal, 1\%} \times \frac{V_{Stromal}}{V_{Exp}}) + (Kp_{Hepatocyte, 1\%} \times \frac{V_{Hepatocyte}}{V_{Exp}})} \]

Eq. 4

Where \( V_{Stromal}/V_{Exp} \) and \( V_{Hepatocyte}/V_{Exp} \) represents the fractional stromal cellular volume of the experiment and the fractional hepatocyte cellular volume of the experiment, respectively (0.00195).

Finally, the determined KpHepatocyte values and the measured medium values were used to calculate intracellular hepatocyte concentrations at each experimental time point.

**Calculation of CL\textsubscript{int, in vitro}**

For rat hepatocyte studies, 1-phase and 2-phase exponential decay models were utilised to fit the data as described in Harrison et al. (2018), using the solver function in Excel\textsuperscript{TM}. A 1-phase or 2-phase model was selected for each compound based on the lowest sum of the residuals squared, and the ability of the model to consistently calculate CL\textsubscript{int, in vitro} on every experimental day. CL\textsubscript{int, in vitro} was calculated based on Eq. 5 or Eq. 6, using a 1-phase or 2-phase model, respectively.

\[ CL_{int, in vitro} = \frac{V \times k}{P} \]

Eq. 5

Where V represents the experimental volume per well (50 µL), k represents the elimination rate constant and P represents the amount of protein per well.

\[ CL_{int, in vitro} = \left( \frac{C_0 \times V}{\frac{A}{K_1} + \frac{B}{K_2}} \right)/P \]
Eq. 6

And \( C_0 \) represents the initial medium substrate concentration, \( A \) and \( k_1 \) represent the back-calculated drug concentration and the elimination rate constant in the first phase, and \( B \) and \( k_2 \) represent the back-calculated drug concentration and the elimination rate constant in the second phase.

Unbound \( \text{CL}_{\text{int, in vitro}} \) (\( \text{CL}_{\text{int,u in vitro}} \)) was calculated by dividing \( \text{CL}_{\text{int, in vitro}} \) by the \( f_u_{\text{med}} \), which was predicted based on the binding association equilibrium constant (\( K_a \)) of the drug to albumin. The \( K_a \) was back calculated using the fraction unbound in plasma (\( f_u_p \), as reported by Drugbank, Wishart et al. (2018)), and assuming an albumin protein concentration ([P]) of 4% at physiological levels, using Eq. 7. Then, for 1% BSA conditions, a protein concentration of 1% was applied to the calculated \( K_a \) in Eq. 7 to derive the \( f_u_{\text{med}} \) under 1% BSA conditions. Under 0% BSA conditions, \( f_u_{\text{med}} \) was assumed to be 1.

\[
f_u_{\text{med}} = \frac{1}{(1 + K_a \times [P])}
\]

Eq. 7

For H\( \mu \)REL co-culture, in vitro CL was determined using a range of conventional calculation approaches:

\( \text{CL}_{\text{met}} \): Calculated using medium depletion data, with a 1-phase exponential decay model between 1-24 hours for all drugs and BSA conditions, assuming \( Y_0 \) is 1000nM due to data normalisation and a plateau of 0. To correct to unbound in BSA conditions, \( \text{CL}_{\text{met}} \) was divided by \( f_u_{\text{med}} \).

\( \text{CL}_{\text{uptake}} \): Calculated using intracellular uptake profiles between 0.5-2 minutes for 0% BSA conditions and 2 - 15 minutes for 1% BSA conditions, as described in Cantrill & Houston (2017). To correct to unbound concentration in BSA conditions, \( \text{CL}_{\text{uptake}} \) was divided by \( f_u_{\text{med}} \).

\( \text{CL}_{\text{int,AUC}} \): Calculated (Eq. 8) using intracellular concentration profiles to calculate the area under the curve (AUC).

\[
\text{CL}_{\text{int,AUC}} = \frac{d \times f_u_{\text{med}}}{\text{AUC}}
\]

Eq. 8
Where \( d \) represents the dose, calculated based on the mass of drug present in 50\( \mu \)L of 1\( \mu \)M solution, and \( f_u_{med} \) is calculated based on the predicted binding under 0\% and 1\% BSA conditions, as described above.

Additionally, mechanistic models (implemented in NONMEM, Version 7.5) were designed and used to determine CL in the H\( \mu \)REL system, by simultaneously fitting the data from the medium and intracellular measurements, aiming to simultaneously estimate parameter values for the separate CL processes (active uptake, \( CL_{active,u} \); passive uptake, \( CL_{passive,u} \); metabolic CL, \( CL_{metabolism,u} \)). This approach could not be used with the suspended rat hepatocyte assays as intracellular measurements were not obtained. Data from both albumin free and 1\% albumin conditions were applied to both a conventional FDH and an AMU model (Figure 1). In the FDH model, the parameter \( f_u_{med} \) was estimated (based on the observed differences in CL between the 0\% BSA and 1\% BSA conditions). In the AMU model \( f_u_{med} \) was fixed based on predicted binding (Eq. 7), and the additional albumin enhanced uptake parameters (Alb.\( CL_{pass} \) and Alb.\( CL_{act} \), passive and active clearance, respectively) were incorporated to account for the differences in CL between the 0\% BSA and 1\% BSA conditions. Based on in vitro measurements, native albumin secreted by the hepatocytes in the H\( \mu \)REL system were not included as they were deemed negligible. Non-specific binding (\( f_u_{ub} \)) was also incorporated into the AMU model (representing binding to plastics). Predicted CL values were subsequently scaled per mg protein to allow for IVIVE.

**In vitro-in vivo extrapolation (IVIVE)**

IVIVE of CL\(_{int,u \text{ in vitro}} \) values was performed using physiological rat scaling factors of 200 mg protein/g liver and 40 g liver/kg bodyweight (Davies & Morris, 1993; Seglen, 1976), and compared to previously reported mean in vivo values. Geometric mean fold error (GMFE, Eq. 9) and root mean squared error (RMSE, Eq. 10) was calculated for each in vitro method to assess bias and precision, respectively.

\[
GMFE = 10^{\frac{\sum \log_{10} \left( \frac{CL_{int,u \text{ in vitro}}}{CL_{int,in \text{ vivo}}} \right)}{n}}
\]

Eq. 9
\[ \text{RMSE} = \sqrt{\frac{1}{n} \sum (\text{predicted} - \text{observed})^2} \]

Eq. 10

Results

Secretion of native albumin in Rat HµREL co-culture

The possible impact of native rat albumin secretion in the HµREL co-culture system was investigated under albumin free and 1% BSA conditions, as performed experimentally. Albumin synthesis in the HµREL co-culture system was monitored during the experiments (n=3), displayed in Figure 2. Stromal only control plates (with no hepatocytes) produced no quantifiable albumin. Under 0% and 1% BSA conditions the average albumin secretion rate was 2,900 ± 530 ng/hour/10^6 hepatocytes and 3,800 ± 730 ng/hour/10^6 hepatocytes, respectively – marginally lower than reported in vivo values (3,900 – 4,700 ng/hour/10^6 hepatocytes, Peters (1995)), but still within reported variability. Albumin secretion after 24 hours reached 0.004% in the medium, thus native albumin secreted by the hepatocytes was deemed negligible and unlikely to have an impact on the 0% BSA medium conditions.

Drug clearance studies in HµREL co-culture

The utility of the HµREL co-culture system was investigated in drug clearance studies, and control experiments in stromal cell only plates under 0% BSA conditions were additionally performed. The extent of drug accumulation in stromal cells varied dependent on drug, with rosuvastatin, pitavastatin and alprazolam showing minimal accumulation (maximum \(K_{p,\text{Stromal}}\) values were 1.5, 4, and 6, respectively), whilst repaglinide, midazolam and saquinavir showed more extensive accumulation that increased over time (maximum \(K_{p,\text{Stromal}}\) values were 23, 33, and 51, respectively, see Supplement 3 for full \(K_{p,\text{Stromal}}\) curves). The rank order of maximum \(K_{p,\text{Stromal}}\) values appear to correlate with LogD_{7.4}.
values (Supplement 4). The extent of stromal binding was accounted for, using Eq. 1-4, to obtain purely hepatic intracellular concentrations from the HμREL system.

Both medium and cellular concentrations were measured in the HμREL system (Figure 3). Under protein free conditions, transporter compounds such as pitavastatin and rosuvastatin showed little depletion in the medium. However, clear uptake profiles were observed from the intracellular measurements with a tendency to plateau, as expected for transporter compounds. For metabolically cleared compounds (alprazolam and midazolam), or compounds that undergo both metabolic and transporter CL (saquinavir and repaglinide), medium depletion profiles were observed, as well as intracellular measurements that showed a clear accumulation and depletion phase.

In the presence of 1% BSA, medium and intracellular profiles remained similar for low binding drugs (alprazolam and rosuvastatin). For pitavastatin (a high binding transported compound), a clear decrease in the rate of intracellular accumulation was observed. For midazolam (a high binding metabolised compound), medium depletion appeared slower (but quantifiable); however very little drug could be measured in the hepatocytes. Further investigation showed that despite the low levels of intracellular midazolam, both major midazolam metabolites (1- and 4-hydroxy-) were present in the medium, at equal concentrations, between the 0% and 1% BSA conditions after 24 hours (Supplement 5). Saquinavir and repaglinide were also observed to bind extensively to the stromal cells which precluded quantification of intracellular measurements in the presence of BSA (and thus calculation on CL_{int} based on intracellular measurements).

Various approaches were used to estimate CL from the rat HμREL studies (Table 2). Under 0% BSA conditions CL estimates could be achieved using all three conventional approaches; however, under 1% BSA conditions CL could not always be determined, as anticipated, based on observed medium and intracellular profiles. CL_{met,\text{fu}_\text{med}} was the only method that could consistently be calculated, however this method only captures metabolism, and thus is not appropriate for transported compounds, as exemplified by rosuvastatin and pitavastatin (CL_{met,\text{fu}_\text{med}} values of 0.30 and 0.76, μL/min/mg cellular protein, respectively, under 0% BSA conditions). CL_{uptake,\text{fu}_\text{med}} produced higher values for pitavastatin and rosuvastatin (14.9 and 10.2 μL/min/mg cellular protein, respectively), but the highest CL values were observed using the AUC method (ranging from 18.0 to
92.6 μL/min/mg cellular protein) – likely as this method captured multiple processes. In the presence of 1% BSA, CL\text{met, fum} values were generally higher than for 0% BSA conditions; however, both CL\text{uptake, fum} and CL\text{int, AUC} decreased in the presence of BSA.

**Drug clearance in hepatocyte suspensions**

Standard (total) depletion and medium depletion assays were investigated in suspended rat hepatocytes to compare with and evaluate the utility of the HµREL system (Table 3). In the absence of BSA, alprazolam showed consistent CL\text{int, u in vitro} values across both assay formats (31 μL/min/mg cellular protein). However, for compounds that undergo transporter-mediated clearance (saquinavir, rosuvastatin and pitavastatin), CL\text{int, u in vitro} was higher in the medium depletion assay (53, 8, and 35 μL/min/mg cellular protein, respectively) than the total depletion (33, 2.8, and 9 μL/min/mg cellular protein, respectively), as expected and in agreement with previous work (Harrison et al., 2018). Midazolam, a metabolically cleared compound, appeared to have lower clearance by medium depletion rather than by total depletion (84 compared to 122 μL/min/mg cellular protein), but this is likely to be a reflection of the larger standard deviation observed. Whilst the CL of most drugs was comparable to previous studies, midazolam CL\text{int, u in vitro} was lower here than in some reports (Harrison et al., 2018; Wood et al., 2018), but within the high variability of midazolam CL observed in rat (Lundquist et al., 2014).

Suspended rat hepatocyte studies were also performed in the presence of 1% and 4% BSA (Table 3). For all drugs, the presence of albumin in the incubation decreased the total CL\text{int in vitro} in both the medium depletion and total depletion assays, and generally increased variability. As previously observed, the medium depletion assay produced higher CL\text{int, u in vitro} values than the total depletion assay – a consequence of accounting for both metabolism and transporter-mediated CL. The CL\text{int, u in vitro} values for alprazolam (a low albumin binder) were consistent across all albumin concentrations tested, whereas most other drugs showed an increase in CL\text{int, u in vitro} values in the presence of at least 4% BSA. Generally, fold-changes in CL\text{int, u in vitro} >2 were only observed when the f\text{u, med} was <0.1 (Supplement 6), with the exception of Midazolam which appeared to show a decrease in CL\text{int, u in vitro} in the presence of BSA. To further explore the decrease in midazolam CL\text{int, u in vitro}...
values, additional 0.1% and 0.2% BSA conditions were tested, under both static and shaken conditions, based on previous work that suggested a possible mechanism of albumin-enhanced uptake by overcoming rate-limiting diffusion through the unstirred water layer (UWL) (Ichikawa et al., 1992). Under shaken conditions, \( \text{CL}_{\text{int,u in vitro}} \) decreased with increasing concentration of BSA, confirming the above observations. Under static conditions in the absence of BSA, midazolam \( \text{CL}_{\text{int,u in vitro}} \) decreased to approximately a third of the \( \text{CL}_{\text{int,u in vitro}} \) observed under shaken conditions, in agreement with previous studies by Wood et al. (2018) (Supplement 7). However, the presence of BSA in static conditions appeared to further reduce \( \text{CL}_{\text{int,u in vitro}} \) values, thus challenging the proposition that BSA could overcome rate-limiting diffusion through the UWL for midazolam.

Comparison of the HµREL assay to the standard suspended hepatocyte assay format demonstrated that the HµREL system generally produced lower CL values, particularly under 1% BSA conditions. However, under 0% BSA conditions, \( \text{CL}_{\text{int,AUC}} \) values in the HµREL system were quantitatively similar to the suspended medium depletion assay, particularly for alprazolam. The addition of BSA generally increased inter-assay variability and was problematic for quantification, but increased \( \text{CL}_{\text{int,u in vitro}} \) or \( \text{CL}_{\text{med,\text{fu med}}} \) values. Transporter substrates could be identified by the differences between the medium depletion and intracellular profiles in the HµREL system as well as by the different profiles (and subsequent \( \text{CL}_{\text{int,u in vitro}} \) values) in the total depletion and medium depletion suspended hepatocytes assay.

**Mechanistic Modelling**

Mechanistic cell models (Figure 1) were implemented to simultaneously fit the cellular and medium measurements for the HµREL system, in the presence and absence of BSA. Under the FDH model, where \( \text{fu med} \) was fitted based on the observed differences between 0% and 1% BSA conditions, the unbound passive, unbound active and metabolic clearance parameter (\( \text{CL}_{\text{pass,u}} \), \( \text{CL}_{\text{act,u}} \) and \( \text{CL}_{\text{met}} \), respectively) values could be evaluated for each compound. In the albumin-mediated uptake model, where \( \text{fu med} \) was fixed at a predicted 1% BSA concentration (calculated from Eq. 7), differences between 0% and 1% BSA conditions were accounted for by incorporating additional unbound albumin-enhanced passive and unbound albumin-enhanced active CL parameters (Alb.\( \text{CL}_{\text{pass,u}} \) and
Alb.CL_{act,u}, respectively). The fraction unbound due to non-specific binding was also incorporated into the albumin-mediated uptake model. Data are shown in Table 4.

For all drugs except repaglinide, the model-determined $f_u_{med}$ was higher in the FDH model than (fixed) in the AMU model, indicating that for the FDH to be valid, the effective $f_u_{med}$ values must be higher than those conventionally calculated, to account for the observed differences between the 0% and 1% BSA conditions. Saquinavir and repaglinide were observed to have the highest binding to plastics in the AMU model ($f_u_{unb}$ values of 0.64 and 0.68, respectively), in line with the high binding/accumulation observed in the stromal cell only controls (Supplement 3). Whilst in both the FDH and AMU models the active, passive and metabolic components to CL were separately identified, the clear predominant CL pathway was the active uptake component, across all drugs. Therefore the CL_{act,u} parameter was considered as not necessarily representative of the actual individual CL pathways for all compounds, and likely to represent a combination of active and metabolic pathways. This is exemplified by the apparent CL_{act,u} and CL_{met} values of the non-transported and highly metabolised drug, midazolam as 64 and 0.17 μL/min/mg cellular protein, in the FDH model, respectively. The individual CL component values produced by the model should therefore be interpreted with caution, and calculations to determine total CL_{int,u} based on sequential individual CL values, such as used by Jigorel & Houston (2012), are not appropriate for these model outputs. Consequently, the active uptake parameter (both unbound and albumin mediated, in the AMU) was deemed representative of total CL_{int,u in vitro}.

The AMU model predicted higher CL_{int,u in vitro} values for all drugs compared to the FDH model, with CL_{int,u in vitro} values ranging from 8.7 to 74 μL/min/mg cellular protein in the FDH model and 18.9 – 599 μL/min/mg cellular protein in the AMU model. Alprazolam CL_{int,u in vitro} values from the FDH mechanistic model (38.4 μL/min/mg cellular protein) were comparable to CL_{int,AUC} values from the 0% BSA HµREL studies (37 μL/min/mg cellular protein) and comparable to the suspended hepatocyte medium depletion studies (31 and 35 μL/min/mg cellular protein for 0% and 1% BSA, respectively, Table 3), with <2 fold higher values in the AMU model, as expected for this low binding compound. Saquinavir CL_{int,u in vitro} in the FDH and AMU model (30 and 81 μL/min/mg cellular protein) were closer to those observed in the suspended rat hepatocyte total depletion assay (33 and
117 μL/min/mg cellular protein under 0% and 1% BSA conditions, respectively) than those from the medium depletion assay (53 and 192 μL/min/mg cellular protein under 0% and 1% BSA conditions, respectively). Pitavastatin CL_{int,u in vitro} values were considerably higher in the HμREL system (74 and 599 μL/min/mg cellular protein in the FDH and AMU, respectively) in comparison to the suspended hepatocyte medium depletion and total depletion assays, and showed the greatest fold difference between the FDH and AMU models (8.1). Midazolam showed similar CL_{int,u in vitro} values between the 0% BSA suspended hepatocyte medium depletion assay and FDH HμREL model (84 and 64 μL/min/mg cellular protein, respectively), but much higher values were observed in the AMU model in comparison to the suspended hepatocyte medium depletion assay under 1% BSA conditions (207 and 57 μL/min/mg cellular protein, respectively). Rosuvastatin displayed the same characteristics, with similar FDH and 0% BSA medium depletion assay values (8.7 and 8.0 μL/min/mg cellular protein, respectively), and higher AMU values in comparison to the suspended hepatocyte medium depletion assay under 1% BSA conditions (19 and 6.6 μL/min/mg cellular protein, respectively).

In vitro in vivo extrapolation

IVIVE was performed for all data generated using the rat suspended hepatocyte and HμREL assays (Table 5). In the rat suspended hepatocyte studies, the medium depletion routinely showed less bias than the total depletion assay (GMFE values of 3.7 to 4.4 compared to 5.3 to 7.4), although no clear improvements in precision were seen, based on RMSE values. The reduced bias was largely attributed to the higher predictions of rosuvastatin and pitavastatin (transporter-mediated CL compounds), which were poorly predicted in the suspended hepatocyte total depletion assay. With increasing concentrations of BSA in the hepatocyte studies for both medium depletion and total depletion assay formats, reduced bias but decreased precision was observed. This was most notably observed in the medium depletion assay under 4% BSA conditions, with a GMFE of 3.70 and RMSE of 1636.

In the HμREL system, the addition of BSA also reduced bias but decreased precision when CL was calculated based on the medium depletion (method 1, CL_{med,\text{fu}_{\text{med}}}). However, when CL was calculated based on the uptake rate, or by the AUC profiles, predictions were better in the absence rather than the presence of BSA. The addition of 1% BSA to the assays were frequently problematic, depending on
Discussion

Due to their increased cellular longevity (Umehara et al., 2020), hepatic co-culture systems have mostly been employed for drugs with low metabolic turnover and, consequently, comprehensive assessment (to capture all mechanisms of CL) has yet to be achieved. Most previous co-culture studies have simply focused on either measuring drug depletion profiles in total incubation to characterise CL_{met} (Bonn et al., 2016; Chan et al., 2013; Chang et al., 2019; Da-silva et al., 2018; Hultman et al., 2016), or metabolite identification (Burton et al., 2018; Cassidy & Yi, 2018). The present study simultaneously measured drug medium depletion and intracellular concentration profiles, as an enhanced approach for the co-culture format in terms of the potential identification of transporter dependent and non-transporter dependent compounds, based on the observed concentration-time profiles. The addition of albumin to the medium and its potential impact on uptake was also investigated, as part of a comprehensive experimental approach.

Given the experimental scope of this work, multiple data analysis approaches were explored to quantify CL_{int in vitro} in the H\(\mu\)REL co-culture system. In addition to the procedures conventionally used for suspended hepatocytes, drug concentrations in hepatocyte co-culture required correction for stromal cell drug accumulation/binding; this was achieved by parallel monitoring of stromal cell drug uptake (\(K_{p\text{stromal}}\)) with time, in the absence of hepatocytes. The extent of drug accumulation in the stromal cells was drug-specific and correlated with Log \(D_{7.4}\), a property which may prove valuable in future applications (Supplement 4). For some drugs, this accumulation was considerable, leading to additional experimental uncertainty, particularly in the presence of BSA. Nevertheless, this correction...
procedure enabled direct quantification of drug uptake into hepatocytes, maximising potential to
distinguish between the predominant hepatocyte CL mechanisms.

The CL characteristics of the 6 drugs selected for study were well described in the 24h HµREL
incubation. Interestingly, there was good quantitative comparability between CL by hepatocytes in
suspensions and in the HµREL system. This contrasts with previous comparisons between hepatocyte
formats that demonstrated a marked decrease in clearance from hepatocytes in monolayer culture
relative to suspensions (Cantrill & Houston, 2017). This enforces the value of co-culture in not only
allowing prolonged incubation but also maintaining higher activity than standard short term
monolayer culture.

The role of plasma protein, albumin in particular, on drug uptake is being increasingly investigated, as
evidence emerges that the presence of albumin in vitro can increase unbound drug uptake, particularly
for OATP transporter substrates (Bowman et al., 2019, 2020; Chang et al., 2019; Kim et al., 2019;
Francis et al., 2021; Miyauchi et al., 2022). An intrinsic property of co-culture systems is their ability
to secrete native albumin and this has been attributed to improved CL predictions in these systems,
due to albumin-mediated uptake (Da-silva et al., 2018). Therefore, in the present study, albumin
secretion was measured, showing that the HµREL system did indeed secrete rat albumin at a rate
comparable to that observed in vivo; however, at a concentration of only 0.004% reached by 24 hours,
this would have had an insignificant effect on CL measurement (with or without the addition of BSA).

For future reference, the use of co-culture systems for longer time periods (i.e., up to 7 days, as
recommended for lower CL compounds (Cassidy & Yi, 2018)), may be susceptible to consequences
from native albumin secretion.

The role of albumin on CL_{int,u in vitro} values was explored across both systems by the addition of 4%
and/or 1% BSA to drug incubations, depending upon the extent of binding and quantitation of
unbound drug. In the suspended hepatocyte systems, the addition of BSA reduced the total CL_{int in vitro}
measured and increased variability. However, once normalised to unbound values, CL_{int,u in vitro} was
generally higher, and typically showed fold changes greater than 2 when f_{med} was less than 0.1, in
agreement with previous observations; the high binding transporter substrates pitavastatin,
rosuvastatin and saquinavir showed the greatest increases in CL_{int,u in vitro} in the presence of BSA, in
agreement with previous studies (Bowman et al., 2019, 2020; Miyachi et al., 2018). Alprazolam, a low-binding non-transporter substrate served as a negative control in showing no clear difference in $\text{CL}_{\text{int,u in vitro}}$ values in the absence or presence of BSA in the total or medium depletion suspension assays, thus supporting previous evidence that AMU is only relevant for highly bound drugs. Midazolam was selected as a high-binding, non-transporter substrate to assess whether albumin-enhanced uptake would also occur via passive uptake. Based on the suspended hepatocyte assays, midazolam $\text{CL}_{\text{int,u in vitro}}$ values decreased in the presence of BSA, suggesting that albumin-enhanced uptake may be more relevant for less permeable substrates where hepatic uptake is dominated by $\text{CL}_{\text{active}}$ routes. The alternative hypothesis that albumin could increase midazolam $\text{CL}_{\text{int}}$ values via enhanced diffusion through the UWL was also explored, but evidence in support of this was not obtained in this study. However, the UWL within the space of Disse may still play a role in vivo (Miyachi et al., 2022).

In the HμREL system, 1% BSA conditions were explored, as measurement of $\text{CL}_{\text{int,in vitro}}$ under 4% BSA conditions was anticipated to be poorly captured. The impact of albumin on total uptake, as observed by the intracellular accumulation profiles, typically reflected the extent of binding: the low binding drugs such as alprazolam and rosuvastatin showed minimal differences in intracellular uptake profiles between 0% and 1% BSA conditions, whereas for the highly bound drugs, clear differences in uptake were observed, with reduced accumulation in the presence of albumin. Midazolam and repaglinide accumulated extensively within stromal cells and due to this high background binding/accumulation, the intracellular hepatocyte concentration under 1% BSA conditions could not be quantified. Thus, quantification of uptake of some drugs (with high logD) in the presence of moderate concentrations of albumin is likely to be experimentally challenging. $\text{CL}_{\text{int,u in vitro}}$ based on the medium depletion profiles ($\text{CL}_{\text{met, fmed}}$) were higher in the presence of albumin, as observed in the cell suspension assays. Values calculated using intracellular concentrations, however, did not show this trend. Even when normalised to the $\text{fu}_{\text{med}}$ $\text{CL}_{\text{uptake, fmed}}$ and $\text{CL}_{\text{int,AUC}}$ values were lower than their 0% BSA counterparts, and lower than expected when based on the FDH.

To further analyse the effect of albumin on the HμREL system and circumvent the noted limitations of the conventional analysis, mechanistic models based on either the FDH or AMU were formulated.
and implemented. The observed differences in $\text{CL}_{\text{int, in vitro}}$ were accounted for using the AMU model. For the FDH to be valid, however, higher $f_u_{\text{med}}$ values than those reported would be necessary to invoke this model. Hence, this simultaneous mechanistic approach succeeded in confirming that the higher binding drugs showed the greatest increase in $\text{CL}_{\text{int, in vitro}}$ values in the presence of 1% BSA, in line with previous observations and hypotheses. It is also apparent that enhanced uptake is more significant for active as opposed to passive routes, in agreement with Liang et al. (2020). Whilst the AMU model provides a potential framework for evaluating the role of albumin in the HμREL system, it must be acknowledged that the complexity involved would lead to difficulties in general use; nevertheless, this exercise has served to elucidate some of the properties of co-culture systems in relation to AMU and highlight some of the inherent uncertainties. Therefore, conventional methods of calculating $\text{CL}_{\text{int, in vitro}}$, such as based on drug depletion from the medium or based on AUC, may offer optimum practicality, when co-culture characteristics and limitations are fully considered. Following this, it is clear that the addition of albumin to hepatocyte systems should be carefully considered; to anticipate the extent of binding for any particular drug, use of a theoretical estimation (Francis et al., 2021) of the AMU effect on $\text{CL}_{\text{int}}$ may be a viable option.

Following completion of the various analytical methods, IVIVE analysis was performed to assess the predictive capability of the co-culture system in comparison to the suspended hepatocyte system and between the analysis methods. Using suspended hepatocytes, the medium depletion assay showed greater accuracy in IVIVE predictions than the total depletion assay, again highlighting the medium depletion assay’s superiority. The presence of albumin in both the total drug depletion and medium depletion assays reduced the GMFE, mainly due to improvements in transporter-substrate predictions. In the HμREL system, the presence of albumin improved predictions when CL was calculated based on medium depletion. However, the most accurate predictions overall for this system resulted from the mechanistic AMU model, supporting the utility of the simultaneous medium depletion and cell uptake approach, as highlighted above. For the HμREL system, accurate predictions based on AUC were dependent on predominant CL mechanism, for reasons already given above. Whilst conventional suspended hepatocyte assays have previously yielded improved predictions with the addition of albumin (Bi et al., 2021; Blanchard et al., 2004; Koyanagi et al., 2019; N. Li et al., 2020;
Shibata et al., 2000), reliability seems to be drug dependent and are therefore not a good general approach.

In the present study, we have further defined uncertainties that can arise in hepatocyte-based methods, with particular focus on the co-culture system. The practical limitations that are inherent in these methodologies, whilst perhaps not surprising retrospectively, may not be well recognised. Further to this, different methods may be more or less accurate depending on the properties of individual drugs including predominant CL pathways (uptake versus metabolism) – a situation which confounds the establishment of a single optimum in vitro method. We have also shown that methodological uncertainty can be exacerbated by the use of albumin in vitro. All of the above points stress the need for a multi-step approach to attain more reliable predictions of CL\textsubscript{int}. Albumin-free incubations of suspended hepatocytes analysed by medium depletion appears to be a valuable approach, particularly to help identify CL by active uptake. However, it will be important to quantify metabolic turnover (and metabolites) even when this is low and masked (in suspended hepatocytes) by high active uptake which would dominate CL. Quantifying low metabolic turnover requires a longer term system such as co-culture and because in these systems any concomitant active uptake will not significantly impact the medium depletion profile, metabolic rates can be reliably determined. Importantly, significant active uptake CL can be simultaneously identified by monitoring cell accumulation (as a possible flag for suspended hepatocyte assay), as shown in this study. Future studies to extend the number and range of probe drugs analysed in the co-culture format are recommended.
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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Author contributions

Participated in research design: de Bruyn, Francis, Houston, and Hallifax

Conducted experiments: Francis

Contributed new reagents or analytical tools: Ogungbenro

Performed data analysis: Francis, Houston, Hallifax, Ogungbenro,

Wrote or contributed to the writing of the manuscript: de Bruyn, Francis, Houston, Hallifax
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*Archives of Toxicology, 91*(1), 439–452.


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Footnotes

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Figure Legends

Figure 1: HµREL data was assessed using a mechanistic model based on either the free-drug hypothesis, or an albumin-mediated uptake model.

Figure 2: Albumin secretion of the Rat HµREL co-culture system

Figure 3: Medium depletion (left panel, open circles, A, C, E, G, I, K) and hepatocyte uptake (right panel, closed circles, B, D, F, H, J, L) profiles of the six drugs investigated in the HµREL studies under 0% BSA (black) and 1% BSA (red) conditions. Data represent mean ± SD, n = 3.

Figure 4: IVIVE of the suspended rat hepatocyte assays (A-C) and the rat HµREL system (D-F), under 0% (black), 1% (red), and 4% (blue) BSA conditions. In vivo values are represented in grey bars, with error bars representing 2-fold error of the in vivo values. Open circles represent scaled values from the medium depletion assay, closed circles represent scaled values from the total depletion assay (A - C). Open squares represent scaled values from calculated CL_{med}/fU_{med}, closed squares represent scaled values from calculated CL_{uptake}/fU_{med}, and closed triangles represent scaled values from calculated CL_{int,AUC} (D and E). Black crosses represent scaled values based on the FDH model of the HµREL data, red crosses represent scaled values based on the AMU model of the HµREL data (F).
### Tables

Table 1: Pharmacokinetic properties of the drugs selected for study

<table>
<thead>
<tr>
<th>Drug</th>
<th>fu_p</th>
<th>ECCS</th>
<th>LogD_{7.4}</th>
<th>Rat CL_{int,u} (mL/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
<td>0.2</td>
<td>2</td>
<td>1.26</td>
<td>67</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.03</td>
<td>2</td>
<td>3.8</td>
<td>1628</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>0.01</td>
<td>1b</td>
<td>1.2</td>
<td>1165</td>
</tr>
<tr>
<td>Repaglinide</td>
<td>0.02</td>
<td>1b</td>
<td>2.3</td>
<td>440</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>0.12</td>
<td>3b</td>
<td>-0.33</td>
<td>1320</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>0.02</td>
<td>2</td>
<td>4.8</td>
<td>911</td>
</tr>
</tbody>
</table>

Table 2: Conventional approaches to calculate CL from the rat HμREL studies. Values in brackets represent the fold difference compared to 0% BSA conditions.

<table>
<thead>
<tr>
<th>Method</th>
<th>Intrinsic clearance (μL/min/mg cellular protein)</th>
<th>0% BSA</th>
<th>1% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alprazolam</td>
<td>Midazolam</td>
</tr>
<tr>
<td>fumed CLmet</td>
<td>4.62</td>
<td>9.04</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>CLuptake</td>
<td>6.10</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td>CLint, AUC</td>
<td>37.0</td>
<td>23.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method</th>
<th>Intrinsic clearance (μL/min/mg cellular protein)</th>
<th>0% BSA</th>
<th>1% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alprazolam</td>
<td>Midazolam</td>
</tr>
<tr>
<td>fumed CLmet</td>
<td>6.23 (1.35)</td>
<td>18.4 (2.03)</td>
<td>14.5 (19.1)</td>
</tr>
<tr>
<td></td>
<td>CLuptake</td>
<td>0.89 (0.15)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CLint, AUC</td>
<td>28.4 (0.77)</td>
<td>ND</td>
</tr>
</tbody>
</table>

1. CLmet,fumed calculated based on the medium depletion profile between 1-24 hours.
2. CLuptake,fumed based on intracellular uptake profiles between 0.5-2 minutes for 0% BSA conditions and 2 - 15 minutes for 1% BSA conditions, calculating CLuptake as described in (Cantrill & Houston, 2017).
3. CLint, AUC calculated based on the AUC of intracellular measurements, CLint,AUC = (fumed*d)/AUC, where d represents drug amount applied.
ND: Not determined due to unreliable fit.
Table 3: CL_{int, in vitro} and CL_{int,u, in vitro} using the standard total depletion (DEP) and medium depletion (MD) assay in suspended rat hepatocytes under various protein conditions. n=3, data represent mean ± SD, values in brackets represent the fold-change compared to 0% BSA concentrations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Assay</th>
<th>BSA concentration</th>
<th></th>
<th>CL_{int, in vitro} (µL/min/mg cellular protein)</th>
<th>CL_{int,u, in vitro} (µL/min/mg cellular protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0%</td>
<td>1%</td>
<td>4%</td>
<td>0%</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>MD</td>
<td>30.9 ± 10.9</td>
<td>17.5 ± 5.34</td>
<td>6.28 ± 5.83</td>
<td>30.9 ± 10.9</td>
</tr>
<tr>
<td></td>
<td>DEP</td>
<td>31.7 ± 6.20</td>
<td>7.48 ± 6.63</td>
<td>10.9 ± 6.64</td>
<td>31.7 ± 6.20</td>
</tr>
<tr>
<td></td>
<td>f_{med}</td>
<td>1</td>
<td>0.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Midazolam</td>
<td>MD</td>
<td>83.9 ± 17.5</td>
<td>6.32 ± 1.49</td>
<td>1.42 ± 0.59</td>
<td>83.9 ± 17.5</td>
</tr>
<tr>
<td></td>
<td>DEP</td>
<td>122 ± 20.8</td>
<td>6.32 ± 2.26</td>
<td>0.95 ± 0.84</td>
<td>122 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>f_{med}</td>
<td>1</td>
<td>0.11</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>MD</td>
<td>34.6 ± 10.4</td>
<td>3.66 ± 2.15</td>
<td>2.38 ± 0.28</td>
<td>34.6 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>DEP</td>
<td>8.66 ± 3.22</td>
<td>0.23 ± 0.40</td>
<td>0.68 ± 1.18</td>
<td>8.66 ± 3.22</td>
</tr>
<tr>
<td></td>
<td>f_{med}</td>
<td>1</td>
<td>0.04</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>MD</td>
<td>8.02 ± 0.59</td>
<td>2.32 ± 1.70</td>
<td>3.37 ± 1.45</td>
<td>8.02 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>DEP</td>
<td>2.81 ± 1.95</td>
<td>1.05 ± 0.52</td>
<td>0.83 ± 1.35</td>
<td>2.81 ± 1.95</td>
</tr>
<tr>
<td></td>
<td>f_{med}</td>
<td>1</td>
<td>0.35</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Saquinavir</td>
<td>MD</td>
<td>52.9 ± 5.07</td>
<td>14.5 ± 2.72</td>
<td>10.2 ± 5.05</td>
<td>52.9 ± 5.07</td>
</tr>
<tr>
<td></td>
<td>DEP</td>
<td>33.0 ± 1.26</td>
<td>8.87 ± 1.10</td>
<td>4.47 ± 0.20</td>
<td>33.0 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>f_{med}</td>
<td>1</td>
<td>0.08</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Data was assessed by 1-phase or 2-phase depletion models, depending on best fit.
Clearance values below 2µL/min/mg cellular protein should be considered semi-quantitative as <20% depletion observed.

Pilot experiments performed with repaglinide were poorly quantifiable, and therefore was excluded from suspension studies.
Table 4: CL predictions from the HµREL data generated in a mechanistic cell incubation model under the FDH or AMU assumption.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Model</th>
<th>CL_{pass,u} (µL/min/mg cellular protein)</th>
<th>CL_{act,u} (µL/min/mg cellular protein)</th>
<th>CL_{met} (µL/min/mg cellular protein)</th>
<th>fu_{med}</th>
<th>fu_{lab} (^1) (µL/min/mg cellular protein)</th>
<th>Alb.CL_{pass} (^2) (µL/min/mg cellular protein)</th>
<th>Alb.CL_{act} (^3) (µL/min/mg cellular protein)</th>
<th>CL_{int,u} (^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alprazolam</td>
<td>FDH</td>
<td>1.84</td>
<td>38.4</td>
<td>0.18</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
<td>38.4</td>
</tr>
<tr>
<td></td>
<td>AMU</td>
<td>1.98</td>
<td>48.8</td>
<td>0.17</td>
<td>0.5 FIXED</td>
<td>0.87</td>
<td>0.2</td>
<td>0.2</td>
<td>50 (1.3)</td>
</tr>
<tr>
<td>Midazolam</td>
<td>FDH</td>
<td>1.13</td>
<td>64</td>
<td>0.17</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>AMU</td>
<td>0.81</td>
<td>64.4</td>
<td>0.06</td>
<td>0.11 FIXED</td>
<td>0.73</td>
<td>8.52</td>
<td>143</td>
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<td>74</td>
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<td>63.2</td>
<td>0.01</td>
<td>0.04 FIXED</td>
<td>0.85</td>
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<td>53.6</td>
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<tr>
<td></td>
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<td>0.78</td>
<td>80.4</td>
<td>0.04</td>
<td>0.08 FIXED</td>
<td>0.68</td>
<td>1.54</td>
<td>0.08</td>
<td>80.5 (1.5)</td>
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<tr>
<td>Rosuvastatin</td>
<td>FDH</td>
<td>0.81</td>
<td>8.68</td>
<td>0.02</td>
<td>0.71</td>
<td></td>
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<tr>
<td></td>
<td>AMU</td>
<td>0.74</td>
<td>8.52</td>
<td>0.03</td>
<td>0.35 FIXED</td>
<td>1.00</td>
<td>0.08</td>
<td>10.4</td>
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<td>0.88</td>
<td>30.3</td>
<td>0.12</td>
<td>0.19</td>
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<td>0.88</td>
<td>68.8</td>
<td>0.05</td>
<td>0.08 FIXED</td>
<td>0.64</td>
<td>0.04</td>
<td>11.8</td>
<td>80.6 (2.7)</td>
</tr>
</tbody>
</table>

1. fu_{lab} values were calculated on a logit scale for the model, and transformed to a normal scale (0-1), as shown in the table.
2. Alb.CL_{pass}: albumin enhanced passive clearance
3. Alb.CL_{act}: albumin enhanced active clearance
4. $CL_{int,u}$ values based on active uptake for the unbound, and unbound plus albumin enhanced active uptake, in the FDH and AMU model, respectively. Values in brackets represent fold change between the AMU and FDH model.
Table 5: IVIVE of $\text{CL}_{\text{int,u}}$ values from rat hepatocyte and HμREL studies compared to previously reported in vivo values.

<table>
<thead>
<tr>
<th></th>
<th>Rat Suspended Hepatocyte Studies</th>
<th>Rat HμREL Studies</th>
<th>Calculated Values</th>
<th>Model Derived</th>
<th>In vivo 1</th>
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<tbody>
<tr>
<td></td>
<td>Medium Depletion</td>
<td>Total Depletion</td>
<td>1. $\text{CL}<em>{\text{met,fum}}/\text{fum}</em>\text{med}$</td>
<td>2. $\text{CL}<em>\text{uptake,fum}/\text{fum}</em>\text{med}$</td>
<td>3. $\text{CL}_{\text{int,AUC}}$</td>
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<tr>
<td></td>
<td>0%</td>
<td>1%</td>
<td>4%</td>
<td>0%</td>
<td>1%</td>
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<td>Alprazolam</td>
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<td>280</td>
<td>251</td>
<td>254</td>
<td>120</td>
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<td>1,536</td>
<td>4,080</td>
<td>264</td>
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<td>671</td>
<td>459</td>
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<td>976</td>
<td>240</td>
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<td>Repaglinide</td>
<td>$\text{NP}$</td>
<td>$\text{NP}$</td>
<td>$\text{NP}$</td>
<td>$\text{NP}$</td>
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<td>1,636</td>
<td>867</td>
<td>986</td>
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</tbody>
</table>

1. For in vivo references, see Table 1.

$\text{NP}$: Not performed. $\text{NQ}$: Not Quantifiable
Free drug hypothesis

Medium $\stackrel{f_{med}}{\longrightarrow}$ Albumin

$\text{CL}_{\text{active},u}$ $\text{CL}_{\text{passive},u}$

Cell

$\text{CL}_{\text{metabolism},u}$

Albumin mediated uptake

Medium $\stackrel{f_{med}}{\longrightarrow}$ Albumin

$\text{CL}_{\text{active},u}$ $\text{CL}_{\text{passive},u}$ $\text{Alb}.\text{CL}_{\text{active},u}$ $\text{Alb}.\text{CL}_{\text{passive},u}$

Cell

$\text{CL}_{\text{metabolism},u}$

Figure 1
Figure 2
Figure 3

Graphs showing concentration over time for various substances:

A. Alprazolam
B. Midazolam
C. Pitavastatin
D. Rosuvastatin
E. Repaglinide
F. Saquinavir

Axes:
- X-axis: Time (mins)
- Y-axis: Concentration (nM)
Figure 4