DMD#78949

Title page

Importance of the unstirred water layer and hepatocyte membrane integrity *in vitro* for quantification of intrinsic metabolic clearance

Wood FL, Houston JB and Hallifax D

Centre for Applied Pharmacokinetic Research, Division of Pharmacy and Optometry, School of Health Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, M13 9PT, Manchester, UK.

1

DMD#78949

Running title page

Running title

In vitro assay rate limitations for prediction of clearance

Corresponding author:

D Hallifax, Centre for Applied Pharmacokinetic Research, Division of Pharmacy and Optometry, School of Health Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PT, UK.

Telephone: +44 (0)161 275 8347

Fax: +44 (0)161 275 8349

Email: David.Hallifax@manchester.ac.uk

Number of text pages: 28; **Number of tables:** 6

Number of figures: 6; **Number of references:** 53

Number of words in Abstract: 241; **Number of words in Introduction:** 749

Number of words in Discussion: 1582

Non-standard abbreviations: CL_{int}, intrinsic clearance; $CL_{int,u}$, unbound intrinsic clearance; $CL_{int,met}$, metabolic intrinsic clearance; $CL_{int,app}$, apparent intrinsic clearance; $CL_{int,app,mem}$, apparent intrinsic clearance due to membrane barrier; CL_{UWL}, clearance through the unstirred water layer; CL_{mem}, clearance through the membrane barrier; P450, cytochrome P450; UWL, unstirred water layer; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EGTA, ethylene glycol tetraacetic acid; $CaCl_2$, calcium chloride; DPBS, Dulbecco's Phosphate Buffered Saline; EBSS, Earle's Balanced Salt Solution; DMEM, Dulbecco's Modified Eagle's Medium; WME, Williams' Medium E.

Abstract

Prediction of clearance – a vital component of drug discovery – remains in need of improvement and, in particular, requires more incisive assessment of mechanistic methodology *in vitro*, according to a number of recent reports. Although isolated hepatocytes have become an irreplaceable standard system for measurement of intrinsic hepatic clearance mediated by active uptake transport and metabolism, lack of prediction reliability appears to reflect a lack of methodological validation, especially for highly cleared drugs, as we have previously shown. Here, novel approaches were employed to explore fundamental experimental processes and associated potential limitations of *in vitro* predictions of clearance. Rat hepatocytes deemed non-viable by trypan blue staining showed undiminished metabolic activity for probe P450 substrates midazolam and propranolol; supplementation with NADPH enhanced these activities. Extensive permeabilisation of the plasma membrane using saponin showed either full or minimal P450 activity depending on the presence or absence of 1 mM NADPH, respectively. Shaking of incubations facilitated P450 metabolic rates up to 5-fold greater than static incubation, depending on intrinsic clearance, indicating the critical influence of the unstirred water layer (UWL). Permeabilisation allowed static incubation metabolic rates to approach those of shaking for intact cells, indicating an artificially induced breakdown of the UWL. Permeabilisation combined with shaking allowed an increased metabolic rate for saquinavir, resolving the membrane permeability limitation for this drug. These findings advance the interpretation of the rate limiting processes involved in intrinsic clearance measurements, and could be critical for successful *in vitro* prediction.

DMD#78949

Introduction

There is some consensus that prediction of hepatic clearance from *in vitro* systems is in urgent need of refinement because of several-fold average underprediction combined with high imprecision (Hallifax et al., 2010; Bowman and Benet, 2016). This bias is clearance-dependent, particularly for hepatocytes, regardless of human or rat origin, and is largely independent of drug type in terms of uptake mechanism (Wood et al., 2017). A methodological deficiency at the level of incubation of drug with hepatocytes is thus implied and, with a lack of standardisation among reported prediction studies, a fundamental re-assessment of assay procedure is needed.

Potential causes of clearance-dependent underprediction from hepatocyte incubations appear to include co-factor exhaustion (Swales and Utesch, 1998; Hengstler et al., 2000; Wang et al., 2005), rate-limiting membrane permeation (Hallifax et al., 2010; Foster et al., 2011) and rate-limiting diffusion through the unstirred water layer (UWL), although none of these putative factors has been conclusively investigated for this type of assay. Effective assessment could be complicated by other, simultaneous, sources of bias (but independent of clearance), adding to the imprecision. These could include excessive substrate concentration resulting in saturation of metabolic enzymes (Klopf and Worboys, 2010), or failure to distinguish un-metabolised drug cleared by uptake transport (Shitara et al., 2006; Soars et al., 2007; Watanabe et al., 2010). This report focusses on drugs predominantly undergoing passive hepatic uptake.

Cofactor depletion from hepatocytes has been evidenced by reduced metabolic activity in cryopreserved hepatocytes compared with fresh hepatocytes (Swales et al., 1996; Hewitt et al., 2000; Hewitt and Utesch, 2004). However, a recent analysis of published predictions demonstrated no bias in intrinsic clearance between fresh and cryopreserved human hepatocytes, implying a lack of cryopreservation impact. It is not clear, though, whether rapidly metabolised drugs could be limited by co-factor replenishment rate *in vitro* in a way that does not reflect the *in vivo* situation (Wood et al., 2017).

In the parallel field of *in vitro* assessment of drug gut permeability, the involvement of the UWL is well recognised (Naruhashi et al., 2003; Avdeef et al., 2004). The UWL is a solute-dependent region adjacent to a cellular membrane in which solvent movement is markedly slower than in the bulk medium, causing a diffusional barrier to passage both into and out of a cell (Korjamo et al., 2009).

The presence of an UWL has been demonstrated to substantially reduce the apparent permeability of highly permeable compounds (Naruhashi et al., 2003; Korjamo et al., 2009)). At gut lumen/enterocyte or extracellular fluid/cell interfaces *in vivo*, an UWL can also exist, although generally at considerably lesser depths compared with some *in vitro* situations (Lennernas, 1998). While the need to control the UWL phenomenon *in vitro* has been recognised for absorption prediction, for prediction of metabolic clearance this has been much less so. The depth of an UWL surrounding a membrane is greatest in an unstirred system and may be reduced by shaking or agitation (Williams et al., 1990; Avdeef et al., 2004; Shibayama et al., 2015). However, a widely held assumption persists that vigorous shaking has a detrimental effect on the structural integrity of isolated hepatocytes (Berry et al., 1991; Seglen, 2013) and consequently clearance assays are often performed under static or inadequately agitated conditions.

When underprediction of clearance was examined in relation to passive permeability using reported PAMPA, caco-2 assay or empirical *in silico* methods, no relationship was apparent (Hallifax et al., 2012). However, considering the incidence of sub-optimal protocols for permeability assays (Karlsson and Artursson, 1991; Avdeef et al., 2004), the lack of relationship may be false and further investigation into the potential limitation of passive permeation of drugs into hepatocytes is warranted.

In the present study, examination of the roles of both the UWL and passive permeability on *in vitro* assay of intrinsic metabolic clearance focussed on drugs predominantly cleared by P450-mediated metabolism in the relatively robust and reproducible rat suspended hepatocyte system. The effect of supplementation of the P450 co-factor NADPH and its metabolic precursor nicotinamide was examined in freshly isolated or cryopreserved hepatocytes with and without purification using Percoll. The influence of the UWL and permeability was investigated using a combination of controlled permeabilisation and shaking. The plant glycoside saponin was utilised to create pores in the plasma membrane permeable to drug molecules leaving internal membrane structures intact (Cook et al., 1983; Katz and Wals, 1985). Additionally, hepatocytes were subjected to gross membrane destruction by ultra-sonication to provide a system in which neither the plasma membrane permeability barrier nor an equivalent-sized UWL were present.

Materials and Methods

DMD#78949

Chemicals

Chlorpromazine, dextromethorphan, diclofenac, metoprolol, naloxone, propafenone, (±)propranolol, saquinavir, tolbutamide, triazolam, verapamil, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), ethylene glycol tetraacetic acid (EGTA), calcium chloride ($CaCl_2$), trypsin inhibitor, reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide and saponin were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). Midazolam was kindly supplied by F. Hoffmann-La Roche (Basel, Switzerland). Dulbecco's Phosphate Buffered Saline (DPBS) and Earle's Balanced Salt Solution (EBSS) were purchased from Life Technologies (Renfrewshire, UK). Collagenase H was purchased from Roche Applied Sciences (West Sussex, UK) and Williams' Medium E (WME) was purchased from Scientific Lab Supplies Ltd. (Nottingham, UK). All solvents were obtained from BDH Laboratory Supplies (VWR International (Leicestershire, UK)). Hepatocyte thawing kits containing two tubes of supplemental Dulbecco's Modified Eagle's medium (DMEM) with and without isotonic Percoll were purchased from Tebu-Bio Ltd (Cambridgeshire, UK). InVitroGRO CP medium was purchased from Bioreclamation IVT (Brussels, Belgium).

Preparation of fresh hepatocyte suspensions

Male Sprague Dawley rats weighing 250-300g were supplied by Charles River Laboratories (Margate, UK). Animals were culled by CO_2 asphyxiation and cervical dislocation. Hepatocytes were prepared using the collagenase perfusion method described by Griffin and Houston (2004). Briefly, this involves *ex vivo* perfusion of the liver lobes, firstly with EBSS containing EGTA followed by a wash-out period (EBSS only) and secondly with EBSS containing $CaCl_2$, trypsin inhibitor and collagenase to facilitate digestion of the tissue. Hepatocytes are dissociated by gentle agitation of the perfused liver lobe in ice cold WME, followed by filtration through 100 μm nylon mesh (Plastok Associates Ltd., Merseyside, UK) before washing via centrifugation (29 x *g* for 3 minutes followed by 16 x *g* for 3 minutes with removal of supernatant and resuspension of cell pellet in WME between each step).

Preparation of cryopreserved hepatocyte suspensions

Cryopreserved male Sprague Dawley rat hepatocytes (pool of eight animals) were purchased from Tebu-Bio Ltd. (supplied by Sekisui XenoTech LLC (Kansas, USA)) and stored in the vapour phase of liquid nitrogen. For each experiment two vials of cryopreserved hepatocytes were thawed simultaneously; one was centrifuged at 100 x *g* for 5 minutes in the DMEM containing isotonic Percoll

6

(as per the supplier's instructions) (Percoll-purified) and the other centrifuged at 60 x g for 3 minutes in DMEM (unpurified).

Cryopreserved human hepatocytes (pool of 20 donors) were purchased from Bioreclamation IVT (Brussels, Belgium). After thawing cells were centrifuged at 50 x g for 5 minutes in InVitroGRO CP medium.

Determination of cell viability

After centrifugation, all cell preparations were re-suspended in a small volume of WME supplemented with HEPES (final concentration 24 mM), pH 7.4. This solution was used as the incubation medium for all hepatocyte studies. Viability was measured by the proportion of cells excluding the azo dye trypan blue. 50 µl of cell suspension was diluted into 400 µl of DPBS plus 50 µl of 0.4% trypan blue. Aliquots were loaded into a haemocytometer and counted under a light microscope to determine total cell number and trypan blue-stained number. Hepatocytes were subsequently diluted to the required density in WME accordingly.

The viability of freshly isolated rat hepatocytes was ≥ 80%; the viability of cryopreserved rat hepatocytes, which were divided between unpurified and Percoll-purified (as described in preparation of cryopreserved hepatocyte suspensions), is given in Table 3; the viability of cryopreserved human hepatocytes was ≥ 91%.

Preparation of permeabilised/sonicated hepatocyte suspensions

Saponin-permeabilised hepatocytes were produced by pre-incubation of fresh rat hepatocytes (1 x 10^6 viable cells/ml (midazolam, propranolol, saquinavir incubations) or 2 x 10^6 viable cells/ml (tolbutamide incubations)) with 0.01% w/v saponin (0.5% WME solution) for 5 minutes; cell viability was checked before use. The concentration of saponin was pre-determined as the minimum required to render > 95% of cells non-viable according to trypan blue exclusion. Sonicated hepatocytes were prepared using an ultrasonic probe homogeniser (Omni Ruptor 400, CamLab Ltd., Cambridge, UK); 1 x 10^6 viable cells/ml were sonicated for 8 x 1 second pulses at 50% power and the resulting suspension was viewed under the microscope to confirm a lack of intact cells before use. Preliminary investigation showed that this treatment caused loss of cell structural integrity and complete loss of drug metabolic activity in the absence of supplemented NADPH.

Selection of substrate and concentration

To meet the overall aims of this study, the focus was on compounds with predominantly passive disposition including metabolism by CYP enzymes, primarily midazolam (CYP3A - Patki et al., 2003) and propranolol (CYP2D - McGinnity et al., 2000; Sten et al., 2006). Other drugs were selected to include acids and bases and a CYP-metabolised substrate with permeation limitation, namely saquinavir (CYP3A - Eagling et al., 2002; Usansky et al., 2008).

Substrate concentration (for depletion) was either based upon equivalent published experiments and/or reported K_m (midazolam - Jones and Houston, 2004; Brown et al., 2007a; tolbutamide - Ashforth et al., 1995; Griffin and Houston, 2004), or, where information was absent or variable, set as low as practicable to avoid enzyme saturation while allowing for quantitative determination of the depletion profile; in practice this was considered achieved at 0.1 µM.

Hepatocyte incubations and sample analysis

Rat hepatocytes prepared as above were diluted to twice the required incubation concentration (Supplemental Table 1) based upon original estimates of cell density (including permeabilised and sonicated cells). Incubations were conducted in Nunc™ round-bottomed 0.5 ml 96-well polypropylene plates (Thermo Fisher Scientific Ltd., Loughborough, UK), on a Heidolph Titramax 1000 microtiterplate shaker (Heidolph, Schwabach, Germany) at 37°C, either unshaken or at an agitation rate of 900 rpm. This agitation rate was optimised based on the observed CL_{int} of midazolam in rat hepatocytes across different shaking speeds and the practical limitations of this method. Hepatocytes (60 µl cell suspension/well) were pre-incubated for 5 minutes with 24 µl of either WME (intact hepatocytes) or WME containing NADPH (permeabilised/sonicated hepatocytes); 36 µl of substrate (WME solution) was added to initiate the reaction (total incubation volume, 120 µl). NADPH and a precursor, nicotinamide, were also added to some intact hepatocytes to assess their effect (Supplemental Table 1). Although some controversy exists, it appears likely that nicotinamide can be taken up by hepatocytes whereas NADPH cannot (Nikiforov et al., 2011; Pittelli et al., 2011). Substrate concentrations, optimised incubation times and treatments are given in Supplemental Table 1; the concentration of NADPH in the final incubation was 1 mM. Individual incubations were terminated at one of the selected time points by the addition of an equal volume of ice-cold methanol containing internal standard, providing data for a single time point. Eight time points in duplicate were utilised to produce a substrate depletion time profile, except where metabolite formation was

measured (tolbutamide). Assays were repeated to achieve at least three replicates of each condition; in some cases, where resources allowed, a fourth or fifth replicate was performed; all data obtained are presented.

Cryopreserved human hepatocytes prepared as above were diluted to twice the required final concentration (0.25, 0.4 and 1 x 10^6 cells/ml, midazolam, saquinavir and propranolol, respectively). Incubations were conducted as above at 0.1 µM (midazolam, propranolol) or 1 µM (midazolam, propranolol, saquinavir); at least six time points (duplicate) were included over 60 minutes to produce a substrate depletion time profile.

Terminated incubations were stored at -20°C for at least one hour before centrifugation at 1125 x *g* for 10 minutes; supernatant was removed for analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). Parent drug or metabolite (tolbutamide only) was quantified by electrospray ionisation multiple reaction monitoring using either a Waters Alliance 2795 (Waters, Watford, UK) or Agilent 1100 (Agilent Technologies, Stockport, UK) high-performance liquid chromatography system coupled to either a Micromass Quattro Ultima or Quattro Micro (Waters, Watford, UK) mass spectrometer. With the exception of propranolol and metoprolol (plus internal standards), which were separated on a Luna Phenyl-Hexyl column (3 µm, 50 x 4.6 mm), all compounds were separated on a Luna C18 column (3 µm, 50 x 4.6 mm) (Phenomenex, Macclesfield, UK) prior to MS analysis. Mobile phases consisted of either formic acid/methanol, ammonium acetate/methanol or a mixture of both according to pre-determined gradients for each analyte; flow rate was 1 ml/min with 0.25 ml directed into the MS. The MS conditions were standard for either positive or negative ion mode with mass transitions set according to manual tuning. Calibration curves were prepared using untreated hepatocytes/medium spiked with drug and analysed alongside samples to allow quantification using QuanLynx software (MassLynx v4.1, Waters, Watford, UK).

Intrinsic clearance of total drug

Intrinsic clearance was determined by substrate depletion rate (except tolbutamide – see below). Drug concentrations were fitted to incubation time using a single exponential function (Equation 1) in GraFit (v7.0.03, Erithacus Software Ltd., Horley, UK).

$$C_{(t)} = C_0 e^{-kt} \tag{1}$$

9

where C is the substrate concentration at any given time (t), C_0 is the substrate concentration in the incubation medium at time 0 and k is the elimination rate constant, substituted into Equation 2 for calculation of intrinsic clearance (CL_{int}) (μl/min/10^6 cells).

$$CL_{int} = \frac{V \cdot k}{No. \ of \ cells} \tag{2}$$

where V is the incubation volume (μl) and No. of cells is the number of cells in the incubation/10^6.

Tolbutamide CL_{int} was determined by measurement of the formation of 4'-hydroxy tolbutamide over time. Four low substrate concentrations (1, 2.5, 5 and 10 μM) were chosen to verify maximum rate below the reported K_m of tolbutamide 4'-hydroxylation in rat hepatocytes (Ashforth et al., 1995; Griffin and Houston, 2004) by linearity of metabolite formation rate with initial concentration. CL_{int} was calculated as the slope determined by linear regression of the plot of reaction velocity against substrate concentration.

Intrinsic clearance of unbound drug

As the viability and therefore total cell number was significantly different between preparations of unpurified and Percoll-purified cryopreserved rat hepatocytes, unbound intrinsic clearance ($CL_{int,u}$) was calculated using Equation 4, for comparison of metabolic activity.

$$CL_{int,u} = \frac{CL_{int}}{fu_{heps}} \tag{4}$$

where fu_{heps} (Equation 5) is the fraction unbound in the hepatocyte incubation, based on total cell number.

$$fu_{heps} = \frac{1}{1 + 125 \cdot V_R \cdot 10^{0.072 \cdot logP/D^2 + 0.067 \cdot logP/D - 1.126}} \tag{5}$$

where logP/D is the logP value for basic and neutral drugs and the logD value for acidic drugs and V_R is the volume ratio of hepatocytes to medium (0.005 for 1 x 10^6 cells/ml) (Kilford et al., 2008; Brown et al., 2007b).

Physiological scaling of intrinsic clearance

For comparison to *in vivo* data, hepatocyte CL_{int} (μl/min/10^6 cells) was corrected for incubational binding (fu_{heps}) and scaled to predicted *in vivo* $CL_{int,u}$ (Equation 6).

10

$$\text{Predicted } in\ vivo\ CL_{int,u} = \frac{in\ vitro\ CL_{int} \cdot hepatocellularity \cdot LW}{fu_{heps}} \qquad (6)$$

Hepatocellularity of 120×10^6 hepatocytes/g liver was used for both human (Hakooz et al., 2006) and rat (Bayliss et al., 1999) and liver weight relative to bodyweight (LW) was 21.4 g/kg bodyweight for human and 40 g/kg bodyweight for rat (Davies and Morris, 1993).

Observed *in vivo* $CL_{int,u}$ values are as reported in Wood et al. (2017).

Theoretical considerations on potentially rate limiting processes

For drugs which enter hepatocytes predominantly passively, three sequential, potentially rate limiting processes (and their apparent CL_{int} terms) can be envisaged, mechanistically: diffusion through the UWL (CL_{UWL}), diffusion through the plasma membrane (CL_{mem}) and intrinsic metabolic clearance ($CL_{int,met}$). Where there is no UWL restriction, any restriction of $CL_{int,met}$ by CL_{mem} will result in an apparent CL_{int} ($CL_{int,app,mem}$) which can be represented by eq. 7.

$$CL_{int,app,mem} = CL_{int,met} \cdot \frac{CL_{mem}}{CL_{mem} + CL_{int,met}} \qquad (7)$$

CL_{mem} can then be obtained by rearrangement of eq. 7 to eq. 8.

$$CL_{mem} = \frac{CL_{int,app,mem} \cdot CL_{int,met}}{CL_{int,met} - CL_{int,app,mem}} \qquad (8)$$

Any restriction of $CL_{int,met}$ by both CL_{mem} and CL_{UWL} ($CL_{int,app}$) can be represented by eq. 9.

$$CL_{int,app} = CL_{int,app,mem} \cdot \frac{CL_{UWL}}{CL_{UWL} + CL_{int,app,mem}} \qquad (9)$$

CL_{UWL} can be obtained by rearrangement of eq. 9 to eq. 10.

$$CL_{UWL} = \frac{CL_{int,app} \cdot CL_{int,app,mem}}{CL_{int,app,mem} - CL_{int,app}} \qquad (10)$$

The results of the experiments conducted were explored to assess how the hypothetical apparent CL_{int} values could be experimentally resolved.

Results

Overview of studies

The first six following sections report sequential investigations which progress from fundamental assessment of the metabolic activity of non-viable cells through attempted removal of progressive passive rate limiting barriers (UWL, plasma membrane) preceding drug metabolism in *in vitro* incubation using rat hepatocytes. The impact/necessity for cofactor (NADPH) supplementation was assessed. Permeabilisation of plasma membranes using saponin was employed to reduce/remove any cell permeation barrier for passively permeating drugs. Shaking of hepatocyte suspensions was used to reduce/remove any UWL barrier external to suspended hepatocytes and finally ultrasonication of hepatocytes was used where necessary to ensure comprehensive barrier removal.

Subsequent sections report the impact of optimised methods in human hepatocytes and on prediction of clearance *in vivo* from both species.

Effect of Percoll purification on rat hepatocyte CL$_{int}$

As anticipated, cryopreserved rat hepatocytes purified with Percoll had significantly ($p < 0.01$) greater viability as determined by trypan blue exclusion than unpurified hepatocytes (Table 1). Consequently, the total cell concentrations in purified preparations were substantially lower (24 - 28%) and to account for any resulting differences in non-specific incubational binding, CL$_{int,u}$ was used to compare metabolic activity, using total cell concentration to estimate fu$_{heps}$.

Although the population of non-viable cells is not expected to influence the CL$_{int,u}$, midazolam and propranolol CL$_{int,u}$, expressed per number of viable cells (as is common practice), was considerably greater in unpurified hepatocytes compared to Percoll-purified hepatocytes; the apparent loss of activity in Percoll-purified preparations was 25% and 31% for midazolam and propranolol respectively (without NADPH, Table 1). Yet when CL$_{int,u}$ was normalised to total cell number, the activity in unpurified and Percoll-purified hepatocytes was similar (Table 1), suggesting that those cells deemed non-viable (stained with trypan blue) significantly contributed to the metabolism of both drugs. Also, the difference in activity expressed per viable cell number between preparations was in proportion to the difference in number of non-viable cells, strongly suggesting that metabolic activity is approximately equivalent in viable and non-viable hepatocytes (rather than due to a loss of activity in purified viable cells).

Effect of cofactor supplementation on rat hepatocyte CL$_{int}$

In fresh hepatocytes, there was no difference in midazolam CL_{int} in the presence or absence of nicotinamide (208 +/- 49 vs. 212 +/- 73 µl/min/10^6 cells, respectively). However, there was an average increase in CL_{int} of midazolam of about 25% in the presence of NADPH (264 +/- 86 vs. 212 +/- 57 µl/min/10^6 cells, respectively), although again this was not statistically significant (data not tabulated). When NADPH was added to unpurified cryopreserved rat hepatocytes, there was a substantial (52%) increase in $CL_{int,u}$ of midazolam (Table 1) and this was reflected in a similar (41%) increase in propranolol $CL_{int,u}$ under the same conditions. For Percoll-purified cryopreserved hepatocytes however, there was no discernible change in $CL_{int,u}$ (Table 1). These findings not only indicate a substantial availability of NADPH to unpurified hepatocytes (evidencing a permeability barrier to this cofactor in intact cells), but also show substantial NADPH-limited enzyme activity in 'non-viable' cells. The concentration of the precursor of cytosolically produced NADPH, NADP+, has been estimated to be about 200 µM in rat hepatocyte cytosol (based on Reiss et al., 1984) and the ratio NADPH/NADP has been reported as 4.2 in rat hepatocytes (Fabregat et al., 1985); assuming normal concentrations of NADPH of this order, it can be appreciated that significant exhaustion of this cofactor might occur in leaky cells.

Comparison of intact and saponin-permeabilised rat hepatocyte CL_{int}

Fresh rat hepatocytes treated with 0.01% w/v saponin for 5 minutes pre-incubation ubiquitously displayed trypan blue staining and hence were deemed entirely non-viable by this test. Saponin is a selective membrane permeabilisation agent at this concentration, predominantly confining pore formation to the plasma membrane; the cells in question remained visibly intact, supporting such selective damage.

The CL_{int} of midazolam, propranolol and saquinavir (with potentially significant differences in permeability), was compared between fresh intact hepatocytes and saponin-permeabilised hepatocytes with and without exogenous NADPH supplementation. CL_{int} values were normalised to initial total cell number.

Saponin treatment resulted in effectively complete inhibition of midazolam, propranolol and saquinavir clearance (Table 2, Figure 1). However, addition of 1 mM NADPH to these preparations restored substrate metabolism to levels either consistent with (midazolam), or substantially greater than

DMD#78949

(propranolol, saquinavir), that of intact hepatocytes, demonstrating that the CYP enzymes responsible for metabolism of each drug retained latent functionality under this treatment.

Effect of shaking on CL$_{int}$ in intact rat hepatocytes

12 well-characterised substrates of multiple metabolic pathways, encompassing a wide range of *in vivo* intrinsic clearance and potentially rate-limiting permeability were selected for comparison of static and shaken clearance assays. With the exception of tolbutamide, the CL$_{int}$ of all drugs was considerably increased (between 2.8- and 5.5-fold) in shaken incubations compared to static; this was statistically significant for 10 of 11 remaining compounds (Table 3). There was an apparent tendency for fold increase in CL$_{int}$ caused by shaking to increase with increasing CL$_{int}$ (taken as the maximal, shaken value) in an apparently non-linear way; given the large range in CL$_{int}$ examined, this suggests a maximum shaking effect of about 5-fold (Figure 2).

Effect of prior shaking on static rat hepatocyte CL$_{int}$ To verify a lack of membrane damage caused by potential mechanical stress of shaking leading to enhanced metabolism of substrate, the CL$_{int}$ of midazolam was measured in static incubation of rat hepatocytes immediately following either a static or shaken (900 rpm) 15 minute pre-incubation. There was no difference in midazolam CL$_{int}$ between static (71 ± 13 µl/min/10^6 cells) and shaken (62 ± 4 µl/min/10^6 cells) pre-incubations, indicating that shaking does not permanently alter the membrane permeability of hepatocytes.

Absorption of trypan blue by rat hepatocytes during shaking To assess the possibility of reversible membrane leakage during incubation shaking, fresh rat hepatocytes were shaken for 5 minutes at 37°C either after pre-incubation (5 minutes) with trypan blue (according to viability assay), immediately after addition of trypan blue, or without trypan blue addition until immediately after shaking. There was no significant difference in viability between the three treatments (82.5 +/- 3.8%, 85.2 +/- 1.2%, 83.1 +/- 4.1%, respectively), demonstrating a complete lack of plasma membrane leakage to the azo dye (Mol. Wt. 872) caused by shaking and thereby indicating likely lack of leakage to other small molecules under the same condition.

Effect of shaking on CL$_{int}$ in saponin-permeabilised rat hepatocytes

Four substrates were selected for investigation of the impact of shaking on permeabilised hepatocytes (Table 4). To clarify trends otherwise obscured by experimental variability, individual incubation replicates (by cell preparation batch) are shown (Figure 3). In intact cells, there was a clear tendency

14

toward increased CL_{int} between static and shaken cells for midazolam, propranolol and saquinavir (Figure 3, A-C). However, for the same drugs in permeabilised cells, there was no clear trend of increased CL_{int} (with shaking) except for saquinavir (Figure 3, C). Also, there was little difference in CL_{int} between shaken intact cells and static permeabilised cells for any of these three compounds, indicating substantial removal of the apparent UWL. In the single case of saquinavir, the positive effect of shaking on permeabilised cells indicated removal of a significant UWL associated with a more severe membrane barrier for this compound. In contrast to the three other drugs, tolbutamide CL_{int} showed no increase during shaking in either intact or permeabilised cells (Figure 3, D); conversely, there were indications of a decrease in tolbutamide CL_{int} with shaking (more pronounced for permeabilised cells) but this lacked statistical significance for the number of replicates used and, at present, there would be no mechanistic explanation for this. The overall directional impact – where significant - is indicated in Figure 3 to aid interpretation of the drug-dependent and condition-dependent effects.

Effect of shaking on saquinavir CL_{int} in ultra-sonicated rat hepatocytes

There was no significant difference in the CL_{int} of saquinavir between static (668 ± 239 µl/min/10^6 cells) and shaken incubations (746 ± 281 µl/min/10^6 cells) of ultra-sonicated rat hepatocytes. Therefore, in contrast to saponin-permeabilised cells, the CL_{int} of saquinavir in sonicated cells was maximal in both static and shaking conditions, indicating complete removal of both the residual membrane barrier (after saponin permeabilisation) and its associated UWL, by sonication.

A comparison of activity between intact, saponin-permeabilised and sonicated hepatocytes is shown in Figure 4.

Effect of shaking on CL_{int} in human hepatocytes

Having established the critical effect of shaking on drug CL_{int} determinations in rat hepatocytes, it was pertinent to confirm the impact for human hepatocytes because of implications for clinical prediction. Following similar experiments to those already described, the CL_{int} of midazolam (1 µM) was not affected by shaking (36 +/- 1 µl/min/10^6 cells) compared with static incubation (38 +/- 15 µl/min/10^6 cells) in human hepatocytes. However, CL_{int} of both propranolol at 1 µM (7 +/- 2 µl/min/10^6 cells vs. 14 +/- 2 µl/min/10^6 cells) and saquinavir at 1 µM (35 +/- 4 µl/min/10^6 cells vs. 98 +/- 27 µl/min/10^6 cells)

was significantly ($p < 0.05$) increased in shaken human hepatocytes, indicating a critical, if drug-dependent, impact in human. At the lower substrate concentration (0.1 µM), there was no difference in CL_{int} for midazolam, but there was an approximately 2-fold increase for propranolol (10 +/- 0.4 µl/min/10^6 cells vs. 21 +/- 3 µl/min/10^6 cells, $p < 0.05$), with some indication of substrate concentration dependence (increased rate compared to higher concentration).

Effect of shaking of hepatocytes on prediction of *in vivo* CL_{int}

In rat, scaled *in vitro* $CL_{int,u}$ (ml/min/kg) was compared to *in vivo* $CL_{int,u}$ (ml/min/kg) derived from observed CL_h literature data (Table 5). In static incubations of rat hepatocytes, 5/12 drugs were predicted within 2-fold, 2/12 predicted above 2-fold and 5/12 predicted below 2-fold. In shaken incubations, 6/12 drugs were within 2-fold, 5/12 above, and 1/12 below 2-fold. Excluding tolbutamide (slowly cleared), the increase in CL_{int} due to shaking was between 3- and 5-fold. The *in vivo* CL_{int} for these substrates ranged between about 250 and 3300 ml/min/kg and in a recent analysis of published CL_{int} from rat hepatocytes (n = 128), the underprediction of *in vivo* CL_{int} was on average 5.9-fold for between 100-1000 ml/min/kg (Wood et al., 2017) – representing a similar shift in CL_{int} and potential improvement in prediction. However, the reported ratio of *in vitro* $CL_{int,u}$/ *in vivo* $CL_{int,u}$ for the drugs used here ranged between 0.1 and 5.1 and so this group of drugs included over- as well as underpredictions of $CL_{int,u}$; the overpredicted drugs were chlorpromazine, naloxone, verapamil, propafenone and saquinavir, the latter two designated BDDCS Class 2 (all others, except tolbutamide are Class 1). Overall, for rat, there was no clear relationship between increase in CL_{int} due to shaking and prediction accuracy.

In human hepatocytes, as indicated earlier, propranolol and saquinavir, although not midazolam, showed increased CL_{int} with shaking (Table 6). For propranolol, this resulted in underprediction of 5.8-fold at 1 µM and 3.7-fold at 0.1 µM, compared with the average reported prediction of 6.7-fold underprediction for this drug and the average underprediction of 12-fold for the $CL_{int,u}$ range of 100-1000 ml/min/kg (Wood et al., 2017). Saquinavir was underpredicted by 12-fold in shaken incubations compared to 34-fold in static incubations (1 µM); there is no previously reported prediction for this drug, but the average underprediction for the *in vivo* $CL_{int,u}$ range 1000-10000 ml/min/kg is 30-fold. Midazolam *in vivo* $CL_{int,u}$ was underpredicted by 3.9- and 3.1-fold in static and shaken incubations respectively (0.1 µM) compared with 9.3-fold underprediction reported in the literature (Wood et al.,

16

DMD#78949

2017), an apparent improvement but one which implies involvement of other (unidentified) sources of variability.

Discussion

Prediction of clearance from *in vitro* systems has been recognised for many years as not mechanistically quantitative (Iwatsubo et al., 1997; Hallifax and Houston, 2009). Explanations for this have focussed on physiological scaling, liver dispersion models, plasma protein binding inaccuracies as well as *in vitro* methodology, but without comprehensive validation. Among recent re-appraisal of experimental methodology (Bowman and Benet, 2016; Wood et al., 2017) a tendency for clearance prediction to become increasingly inaccurate with increasing *in vivo* intrinsic clearance, independent of *in vitro* system and species, has been highlighted. Hence fundamental aspects of the intrinsic clearance assay - cell viability and potentially rate limiting UWL and membrane barriers - have been examined, using rat hepatocytes.

Hepatocytes deemed non-viable by the standard trypan blue exclusion assay were metabolically active to an extent comparable with 'viable' cells (for P450 substrates midazolam and propranolol). Further, enhanced levels of metabolic capacity were evident upon supplementation of the 'non-viable' cells with the P450 cofactor NADPH. Hepatocytes treated with the pore forming agent saponin and incubated in the presence of 1 mM NADPH gave CL_{int} for midazolam, propranolol and saquinavir comparable with intact cells, verifying CYP enzyme functionality in hepatocytes with porous plasma membranes. For permeabilised cells, the external dispersion of NADPH appeared to be the single confounding impediment to clearance – lack of supplemented NADPH halted metabolism. Therefore, those normally-prepared hepatocytes which are susceptible to absorption of trypan blue, appear not to lose this cofactor to any significant extent. Protocols for CL_{int} assay typically preclude hepatocyte batches below a designated threshold of viability but otherwise express CL_{int} per number of cells which exclude trypan blue ('viable'). The findings here, however, imply that such estimates of intrinsic clearance may be compromised by the metabolic viability of 'non-viable' cells; in addition, a differential in permeability barrier may exist between cells of different condition which could skew apparent intrinsic clearance measurement in cases where this was rate limiting.

A lack of methodological consistency among CL_{int} assays in the literature has been highlighted recently (Wood et al., 2017), including to what extent, if any, shaking of incubates was employed;

UWL effects, which although well recognised in permeability assays (Avdeef et al., 2004) have not been fully addressed in the context of CL_{int} assays. Compared to static incubations, shaken rat hepatocytes gave up to 5-fold greater CL_{int}, for a range of metabolically cleared drugs indicating a substantial rate limitation due to the UWL; the increase appeared to depend on CL_{int} up to about 500 $\mu l/min/10^6$ cells (equivalent to 430 ml/min/kg) but was constant at higher CL_{int} levels, indicating an upper limit to the impact of the UWL (possible reflecting prevailing plasma membrane permeability limitation). The CL_{int} was dependent on shaking speed (optimised to 900 rpm for 96 well plates) and therefore previous studies employing shaking could still have incurred rate limitation if not optimised. It was experimentally verified that the impact of shaking on CL_{int} was not due to damage (leakage) to the plasma membrane.

When hepatocytes were permeabilised with saponin, there was an increase in CL_{int} in static incubations (compared to intact cells) and hence the effect of shaking was reduced, depending on the drug, implying a reduced UWL associated with a permeabilised membrane. In permeabilised hepatocytes, CL_{int} of midazolam and propranolol was little affected by shaking, whereas CL_{int} of saquinavir was substantially increased. Pores in membranes are reported to allow flux of water molecules which can lead to breakdown of the UWL (Korjamo et al., 2009). The observation that shaking clearly increased CL_{int} for saquinavir in permeabilised cells beyond that in shaken intact cells indicates, importantly, that the plasma membrane was contributing to rate limitation. This was expected for saquinavir whose CL_{int} has been demonstrated to be uptake rate limited in rat hepatocytes (Parker and Houston, 2008), with approximately equal contribution between active and passive uptake mechanisms (Yabe et al., 2011). Using ultrasonicated cells, where membranes were effectively destroyed, saquinavir CL_{int} was not affected by shaking and was equivalent to the (maximal) values obtained in shaken permeabilised cells, providing evidence that the plasma membrane can be effective as a barrier in cells permeabilised with saponin, possibly by involvement of an accompanying and effective UWL. In contrast to the three highly cleared drugs, tolbutamide CL_{int} was not increased by shaking or permeabilisation, as would be expected for a slowly metabolised drug, for which the overall clearance is limited solely by metabolic rate.

The foregoing provides experimental means to identify rate limiting processes *in vitro* which would otherwise confound accurate determination of CL_{int}. For drugs with predominantly passive hepatic

uptake, three sequential mechanistic processes can be appropriately assumed (Figure 5): diffusion through the apparent UWL (CL_{UWL}), diffusion through the plasma membrane (CL_{mem}) and intrinsic metabolic clearance ($CL_{int,met}$). $CL_{int,met}$ can be measured in shaken incubations of NADPH-supplemented permeabilised hepatocytes, minimising the UWL surrounding the porous cell membrane, maximising access of substrate. The restriction of $CL_{int,met}$ by CL_{mem} ($CL_{int,app,mem}$) - represented by the apparent CL_{int} observed in shaken incubations of intact hepatocytes - can be determined using equations 7 and 8. The restriction of $CL_{int,met}$ by both CL_{mem} and CL_{UWL} ($CL_{int,app}$) is represented by the apparent CL_{int} observed in static incubations of intact hepatocytes and can be determined by equations 9 and 10. Midazolam, propranolol and saquinavir are exemplary drugs for which rat CL_{int} is rate limited by the UWL in the absence of adequate shaking *in vitro*. Figure 6 illustrates how the $CL_{int,app}$ is influenced by changes to CL_{UWL} and CL_{mem} (all relative to $CL_{int,met}$). Notably, CL_{UWL} and CL_{mem} must be at least 10-fold greater than $CL_{int,met}$ for $CL_{int,app}$ to approach $CL_{int,met}$. A very low CL_{UWL} or CL_{mem} (relative to $CL_{int,met}$) severely restricts $CL_{int,app}$.

So far, the optimisation of *in vitro* assay of CL_{int} to eliminate artefactual and confounding dispositional factors has been discussed. Nevertheless, extrapolation to *in vivo* CL_{int} is likely to be confounded by the lack of comparability between hepatocytes and the intact liver. For example, a lack of equivalent UWL in the hepatic interstitial space (Space of Disse) might be assumed; an UWL of 10 μm has been estimated (Bass and Pond, 1987) although this exceeds the diameter of the space itself (1-2 μm, Barry and Diamond, 1984). But, despite this apparently minimal barrier in the liver, extraction of highly permeably drugs by the isolated perfused rat liver has been shown to be limited by an UWL (Ichikawa et al., 1992). In this study, uptake of highly permeable drugs was relatively independent of free fraction (when albumin was added to the perfusate) compared to less permeable drugs – consistent with the 'albumin mediated transport' phenomenon, but explained by rate limiting diffusion through the UWL. From a mechanistic perspective, extrapolation from *in vitro* to *in vivo* should be based on minimisation of artefactual *in vitro* UWL effects and so it is recommended that shaking of hepatocyte incubations be optimised to maximise CL_{int} as described. This would allow scope, for example, to re-appraise inclusion of plasma proteins in these assays. Beyond this, the hepatocyte plasma membrane would remain a potential barrier to highly cleared drugs both *in vitro* and *in vivo* and this could include drugs considered highly permeable, such as propranolol. It is not known, however, if quantitative differences in permeation between *in vitro* and *in vivo* exist. In cases where permeability may be

limiting, CL_{int} measured in microsomes alone might overestimate *in vivo* hepatic clearance but comparison of apparent CL_{int} between intact shaken- and permeabilised shaken hepatocytes would offer an indication of this possibility.

As anticipated, prediction of *in vivo* CL_{int} for the drugs in this study gave a mixed outcome. Predictions of *in vivo* CL_{int} for rat, based on shaken hepatocyte values in this study, tended to over-estimate *in vivo* values; however, static CL_{int} values were greater than those previously reported, indicating additional factors confounding these particular comparisons, such as potentially saturating substrate concentrations *in vivo*. The critical impact of shaking on CL_{int} determination demonstrated for rat hepatocytes was reflected in human hepatocytes for two of the three drugs tested, propranolol and saquinavir. And, although midazolam CL_{int} was not increased in shaken human hepatocytes – possibly due to relatively lower metabolic rate (than rat) with respect to diffusion through the UWL - it is clear that the recommendation to optimise shaking applies to human hepatocytes. From human hepatocytes, predictions of *in vivo* CL_{int} were substantially improved with shaking, for the two most highly cleared drugs (propranolol and saquinavir).

Together, these findings indicate the potentially critical importance of both the apparent UWL and the hepatocyte membrane *in vitro*, for successful progress of prediction methodology. This strongly advocates a multi-track experimental approach for elucidating drug hepatic CL_{int}, allowing the disposition processes to be resolved. As a minimum, assay of investigative compound disposition using isolated hepatocytes in parallel with microsomal CL_{int} assay is recommended for all drug types, including highly permeable/metabolised drugs (BDDCS Class 1 or 2); such approaches have been advocated previously for drugs dependent on active hepatic uptake for clearance (Umehara and Camenisch, 2012). A yet more direct comparison, using intact and permeabilised hepatocytes, as shown in this study, would allow estimation of clearance to be flagged when this is rate-limited by passive permeability (in the absence of knowing that this limitation is accurately matched *in vivo*). Given the trend towards drugs which depend on hepatic uptake transport, more investigation is required (particularly with human hepatocytes) to resolve the quantitative capabilities of *in vitro* systems and improve their methodology, particularly so that both active and passive permeation are sufficiently representative of *in vivo*.

DMD#78949

Author contributions

Participated in research design: Wood, Hallifax and Houston.

Performed experiments: Wood

Performed data analysis: Wood and Hallifax.

Wrote or contributed to the writing of the manuscript: Wood, Hallifax and Houston.

References

Ashforth EIL, Carlile DJ, Chenery R, and Houston JB (1995) Prediction of in-vivo disposition from in-vitro systems - clearance of phenytoin and tolbutamide using rat hepatic-microsomal and hepatocyte data. *J Pharmacol Exp Ther* **274:**761-766.

Avdeef A, Nielsen PE, and Tsinman O (2004) PAMPA - a drug absorption in vitro model 11. Matching the in vivo unstirred water layer thickness by individual-well stirring in microtitre plates. *Eur J Pharm Sci* **22:**365-374.

Barry PH and Diamond JM (1984) Effects of unstirred layers on membrane phenomena. *Physiol Rev* **64:**763-872.

Bass L and Pond SM (1988) In, Pecile, A and Rescino, A (Eds), Pharmacokinetics: mathematical and statistical approaches to metabolism and distribution of chemicals and drugs, Plenum, London. Pp. 241-265.

Bayliss MK, Bell JA, Jenner WN, Park GR, and Wilson K (1999) Utility of hepatocytes to model species differences in the metabolism of loxtidine and to predict pharmacokinetic parameters in rat, dog and man. *Xenobiotica* **29:**253-268.

Berry MN, Edwards AM, and Barritt GJ (1991) *Isolated Hepatocytes: Preparation, Properties and Applications*. Elsevier, Amsterdam.

Bowman CM and Benet LZ (2016) Hepatic Clearance Predictions from In Vitro-In Vivo Extrapolation and the Biopharmaceutics Drug Disposition Classification System. *Drug Metab Dispos* **44:**1731-1735.

Brown HS, Chadwick A, and Houston JB (2007a) Use of isolated hepatocyte preparations for cytochrome p450 inhibition studies: comparison with Microsomes for K-i determination. *Drug Metab Dispos* **35:**2119-2126.

Brown HS, Griffin M, and Houston JB (2007b) Evaluation of cryopreserved human hepatocytes as an alternative in vitro system to microsomes for the prediction of metabolic clearance. *Drug Metab Dispos* **35:**293-301.

Cook GA, Gattone VH, Evan AP, and Harris RA (1983) Structural-changes of isolated hepatocytes during treatment with digitonin. *Biochimica Et Biophysica Acta* **763:**356-367.

Davies B and Morris T (1993) Physiological-parameters in laboratory-animals and humans. *Pharmaceut Res* **10:**1093-1095.

Eagling VA, Wiltshire H, Whitcombe IWA, and Back DJ (2002) CYP3A4-mediated hepatic metabolism of the HIV-1 protease inhibitor saquinavir in vitro. *Xenobiotica* **32:**1-17.

Fabregat I, Vitorica J, Satrustegui J and Machado A (1985) The pentose phosphate cyle is regulated by NADPH/NADP ratio in rat liver. *Arch Bioch Biophys* **236:** 110-118.

Foster JA, Houston JB, and Hallifax D (2011) Comparison of intrinsic clearances in human liver microsomes and suspended hepatocytes from the same donor livers: clearance-dependent relationship and implications for prediction of in vivo clearance. *Xenobiotica* **41:**124-136.

Griffin SJ and Houston JB (2004) Comparison of fresh and cryopreserved rat hepatocyte suspensions for the prediction of in vitro intrinsic clearance. *Drug Metab Dispos* **32:**552-558.

Hakooz N, Ito K, Rawden H, Gill H, Lemmers L, Boobis AR, Edwards RJ, Carlile DJ, Lake BG, and Houston JB (2006) Determination of a human hepatic microsomal scaling factor for predicting in vivo drug clearance. *Pharmaceut Res* **23:**533-539.

Hallifax D, Foster JA, and Houston JB (2010) Prediction of human metabolic clearance from in vitro systems: Retrospective analysis and prospective view. *Pharmaceut Res* **27:**2150-2161.

Hallifax D and Houston JB (2009) Methodological uncertainty in quantitative prediction of human hepatic clearance from in vitro experimental systems. *Curr Drug Metab* **10:**307-321.

Hallifax D and Houston JB (2012) Evaluation of hepatic clearance prediction using in vitro data: Emphasis on fraction unbound in plasma and drug ionisation using a database of 107 drugs. *J Pharm Sci* **101:**2645-2652.

Hengstler JG, Utesch D, Steinberg P, Platt K, Diener B, Ringel M, Swales N, Fischer T, Biefang K, Gerl M, Bottger T, and Oesch F (2000) Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction. *Drug Metab Rev* **32:**81-118.

Hewitt NJ, Fischer T, Zuehlke U, Oesch F, and Utesch D (2000) Metabolic activity of fresh and cryopreserved cynomolgus monkey (Macaca fascicularis) hepatocytes. *Xenobiotica* **30:**665-681.

Hewitt NJ and Utesch D (2004) Cryopreserved rat, dog and monkey hepatocytes: measurement of drug metabolizing enzymes in suspensions and cultures. *Hum Exp Toxicol* **23:**307-316.

Ichikawa M, Tsao SC, Lin TH, Miyauchi S, Sawada Y, Iga T, Hanano M, and Sugiyama Y (1992) Albumin-mediated transport phenomenon observed for ligands with high membrane-

permeability - effect of the unstirred water layer in the disse space of rat-liver. *Journal of Hepatology* **16:**38-49.

Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, and Sugiyama Y (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* **73:**147-171.

Jones HM and Houston JB (2004) Substrate depletion approach for determining in vitro metabolic clearance: Time dependencies in hepatocyte and microsomal incubations. *Drug Metab Dispos* **32:**973-982.

Karlsson J and Artursson P (1991) A Method for the Determination of Cellular Permeability Coefficients and Aqueous Boundary-Layer Thickness in Monolayers of Intestinal Epithelial (Caco-2) Cells Grown in Permeable Filter Chambers. *Int J Pharm* **71:**55-64.

Katz J and Wals PA (1985) Studies with digitonin-treated rat hepatocytes (nude cells). *J Cell Biochem* **28:**207-228.

Kilford PJ, Gertz M, Houston JB and Galetin A (2008) Hepatocellular binding of drugs: correction for unbound fraction in hepatocyte incubations using microsomal binding or drug lipophilicity data. *Drug Met Dispos* **36:** 1194-1197.

Klopf W and Worboys P (2010) Scaling in vivo pharmacokinetics from in vitro metabolic stability data in drug discovery. *Combinatorial Chem High Throughput Screening* **13:**159-169.

Korjamo T, Heikkinen AT, and Monkkonen J (2009) Analysis of unstirred water layer in in vitro permeability experiments. *J Pharm Sci* **98:**4469-4479.

Lennernas H (1998) Human intestinal permeability. *J Pharm Sci* **87:**403-410.

McGinnity DF, Parker AJ, Soars M, and Riley RJ (2000) Automated definition of the enzymology of drug oxidation by the major human drug metabolizing cytochrome P450s. *Drug Metab Dispos* **28:**1327-1334.

Naruhashi K, Tamai I, Li Q, Sai Y, and Tsujii A (2003) Experimental demonstration of the unstirred water layer effect on drug transport in Caco-2 cells. *J Pharm Sci* **92:**1502-1508.

Nikiforov A, Dolle C, Niere M, and Ziegler M (2011) Pathways and subcellular compartmentation of NAD biosynthesis in human cells from entry of extracellular precursors to mitochondrial NAD generation. *J Biol Chem* **286:**21767-21778.

Parker AJ and Houston JB (2008) Rate-limiting steps in hepatic drug clearance: Comparison of hepatocellular uptake and metabolism with microsomal metabolism of saquinavir, nelfinavir, and ritonavir. *Drug Metab Dispos* **36:**1375-1384.

Patki KC, von Moltke LL, and Greenblatt DJ (2003) In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes P450: Role of CYP3A4 and CYP3A5. *Drug Metab Dispos* **31:**938-944.

Pittelli M, Felici R, Pitozzi V, Giovannelli L, Bigagli E, Cialdai F, Romano G, Moroni F, and Chiarugi A (2011) Pharmacological effects of exogenous NAD on mitochondrial bioenergetics, DNA repair, and apoptosis. *Mol Pharmacol* **80:**1136-1146.

Reiss PD, Zuurendonk PF and Veech RL (1984) Measurement of tissue purine, pyrimidine, and other nucleotides by radial compression high-performance liquid chromatography. *Analyt Biochem* **140:** 162-171.

Sawada Y, Hanano M, Sugiyama Y, and Iga T (1985) Prediction of the disposition of 9 weakly acidic and 6 weakly basic drugs in humans from pharmacokinetic parameters in rats. *J Pharmacokinet Biop* **13:**477-492.

Seglen PO (2013) Isolation of hepatocytes by collagenase perfusion, in: *In Vitro Biological Systems: Methods in Toxicology* (Tyson CA and Frazier JM eds), Academic Press, Inc.

Shibayama T, Morales M, Zhang X, Martinez-Guerrero LJ, Berteloot A, Secomb TW, and Wright SH (2015) Unstirred water layers and the kinetics of organic cation transport. *Pharmaceut Res* **32:**2937-2949.

Shitara Y, Horie T, and Sugiyama Y (2006) Transporters as a determinant of drug clearance and tissue distribution. *Eur J Pharm Sci* **27:**425-446.

Soars MG, McGinnity DF, Grime K, and Riley RJ (2007) The pivotal role of hepatocytes in drug discovery. *Chem-Biol Interact* **168:**2-15.

Sten T, Qvisen S, Uutela P, Luukkanen L, Kostiainen R, and Finel M (2006) Prominent but reverse stereoselectivity in propranolol glucuronidation by human UDP-glucuronosyltransferases 1A9 and 1A10. *Drug Metab Dispos* **34:**1488-1494.

Swales NJ, Johnson T, and Caldwell J (1996) Cryopreservation of rat and mouse hepatocytes: 2. Assessment of metabolic capacity using testosterone metabolism. *Drug Metab Dispos* **24:**1224-1230.

Swales NJ and Utesch D (1998) Metabolic activity of fresh and cryopreserved dog hepatocyte suspensions. *Xenobiotica* **28:**937-948.

Umehara KI and Camenisch G (2012) Novel in vitro-in vivo extrapolation (IVIVE) method to predict hepatic organ clearance in rat. *Pharmaceut Res* **29:**603-617.

Usansky HH, Hu P, and Sinko PJ (2008) Differential roles of P-glycoprotein, multidrug resistance-associated protein 2, and CYP3A on Saquinavir oral absorption in Sprague-Dawley rats. *Drug Metab Dispos* **36:**863-869.

Wang Q, Jia R, Ye C, Garcia M, Li JB, and Hidalgo IJ (2005) Glucuronidation and sulfation of 7-hydroxycoumarin in liver matrices from human, dog, monkey, rat, and mouse. *In Vitro Cellular & Developmental Biology-Animal* **41:**97-103.

Watanabe T, Kusuhara H, Maeda K, Kanamaru H, Saito Y, Hu Z, and Sugiyama Y (2010) Investigation of the rate-determining process in the hepatic elimination of HMG-CoA reductase inhibitors in rats and humans. *Drug Metab Dispos* **38:**215-222.

Williams KJ, Brocia RW, and Fisher EA (1990) The unstirred water layer as a site of control of apolipoprotein-B secretion. *J Biol Chem* **265:**16741-16744.

Wood FL, Houston JB and Hallifax (2017) Clearance prediction methodology needs fundamental improvement: trends common to rat and human heptocytes/microsomes and implications for experimental methodology. *Drug Metab Dispos* DOI: 101124dmd.117.077040.

Yabe Y, Galetin A, and Houston JB (2011) Kinetic characterization of rat hepatic uptake of 16 actively transported drugs. *Drug Metab Dispos* **39:**1808-1814.

DMD#78949

Footnote

Part of this work was previously presented as a poster communication: Wood FL, Hallifax D, and Houston JB (2015) Assessing Metabolic Competence in Isolated Hepatocytes: Exploring the Relationship Between Enzyme Function and Plasma Membrane Integrity via Saponin Treatment. 13th European ISSX Meeting, Glasgow, UK.

F Wood current affiliation: The Institute of Cancer Research, Sutton, United Kingdom.

Figure legends

Figure 1. CL_{int} of midazolam (A), propranolol and saquinavir (B) in intact hepatocytes (■) and permeabilised hepatocytes in the absence (■) and presence (■) of exogenous NADPH. Data are normalised to total cell number and represent the mean ± SD (n ≥ 3); * no depletion detected.

Figure 2. Average fold-increase in CL_{int} in rat hepatocytes with shaking vs. CL_{int} determined in shaken incubations. Data represent mean (n ≥ 3); line fitted by non-linear regression using $y=A*x/(B+x)$, where A and B represent maximal and half saturation constants.

Figure 3. Individual replicates of CL_{int} of midazolam (A), propranolol (B), saquinavir (C) and tolbutamide (D) in unsupplemented intact and NADPH-supplemented saponin-permeabilised rat hepatocytes in static (□) and shaken (■) incubations. Data are normalised to total cell number. ↑ indicates a significant increase in CL_{int} with shaking; ↔ indicates no significant overall change in CL_{int} with shaking. ↓ indicates an apparent decrease in CL_{int} with shaking (not statistically significant).

Figure 4. Individual replicates of CL_{int} of saquinavir in unsupplemented intact hepatocytes and NADPH-supplemented permeabilised and ultra-sonicated hepatocytes in static (□) and shaken (■) incubations. Data are normalised to total cell number. ↑ indicates a significant increase in CL_{int} with shaking; ↔ indicates no significant overall change in CL_{int} with shaking.

Figure 5. Sequential passive processes involved in *in vitro* apparent $CL_{int;}$ P.M: plasma membrane.

Figure 6. The effect of changes in CL_{UWL} and CL_{mem} on $CL_{int,app}$ induced by shaking and cell permeabilisation; all clearances are expressed relative to $CL_{int,met}$; estimated experimental shift in $CL_{int,app}$ shown for midazolam (MDZ), propranolol (PPL) and saquinavir (SQV); (●) represents static incubation. [CL_{mem} determined from eqn 8 using $CL_{met}/CL_{int,app}$ derived from permeabiised v non-permeabilised CL_{int} (Table 4); CL_{UWL} determined analogous to CL_{mem} using $CL_{met}/CL_{int,app}$ derived from static intact v shaken permeabilised CL_{int} (Table 4)].

Table 1. Viability (as determined by trypan blue exclusion) and CL$_{int,u}$ of midazolam and propranolol in unpurified and Percoll-purified cryopreserved rat hepatocytes +/- NADPH. Data represent mean ± SD (n ≥ 3).

Drug	Parameter	Unpurified		Percoll-purified	
		- NADPH	+ NADPH	- NADPH	+ NADPH
Midazolam	Initial viability (%)	62 ± 3		86 ± 1***	
	Total cell concentration (10^6/ml)	0.81 ± 0.04		0.58 ± 0.01***	
	CL$_{int,u}$ (µl/min/10^6 cells) normalised to <u>viable</u> cell number	262 ± 61.7	399 ± 58.5[§§]	196 ± 34.7*	219 ± 33.4[§§]
	CL$_{int,u}$ (µl/min/10^6 cells) normalised to <u>total</u> cell number	160 ± 31.3	245 ± 31.3[§§]	169 ± 28.4	188 ± 27.5[§§]
Propranolol	Initial viability (%)	67 ± 3		89 ± 3***	
	Total cell concentration (10^6/ml)	0.37 ± 0.02		0.28 ± 0.01***	
	CL$_{int,u}$ (µl/min/10^6 cells) normalised to <u>viable</u> cell number	262 ± 45.4	370 ± 54.2[§§§]	182 ± 38.4	162 ± 35.8[§]
	CL$_{int,u}$ (µl/min/10^6 cells) normalised to <u>total</u> cell number	176 ± 26.4	249 ± 33.1[§§§]	163 ± 32.9	144 ± 31.5[§]

* p < 0.1, *** p < 0.01 using Student's paired *t*-test to compare unpurified and Percoll-purified preparations; [§] p < 0.1, [§§] p < 0.05, [§§§] p < 0.01 using Student's paired *t*-test to compare CL$_{int,u}$ in preparations +/- NADPH.

Table 2. CL_{int} of midazolam, propranolol and saquinavir in intact (unsupplemented) rat hepatocytes and saponin-permeabilised rat hepatocytes with and without NADPH supplementation. Data are normalised to total cell number and represent the mean ± SD (n ≥ 3).

Drug	CL_{int} (µl/min/10^6 cells)		
	Intact (unsupplemented)	Permeabilised	
		Unsupplemented	NADPH-supplemented
Midazolam	257 ± 21.5	7.66 ± 6.90*	207 ± 43.4[§]
Propranolol	1050 ± 367	ND	1660 ± 375[§]
Saquinavir	311 ± 118	ND	778 ± 236** [§§]

ND: no depletion detected. * p < 0.01 using Student's paired *t*-test in comparison to intact hepatocytes. [§] p < 0.05, [§§] p < 0.01 using Student's paired *t*-test in comparison to unsupplemented permeabilised hepatocytes.

Table 3. CL_{int} of selected substrates in rat hepatocytes in static and shaken incubations. Data represent mean ± SD (n ≥ 3).

Substrate	CL_{int} (µl/min/10^6 cells)		Average fold increase in CL_{int}
	Static	Shaken	
Chlorpromazine	366 ± 40.1	1940 ± 509**	5.4
Dextromethorphan	168 ± 6.21	920 ± 222**	5.5
Diclofenac	59.9 ± 5.78	192 ± 16.5***	3.2
Midazolam	118 ± 27.9	303 ± 42.4****	2.8
Metoprolol	38.3 ± 11.7	129 ± 39.4**	3.4
Naloxone	238 ± 104	1110 ± 145**	5.5
Propafenone	487 ± 81.0	2160 ± 312***	4.4
Propranolol	520 ± 136	1210 ± 419*	2.8
Saquinavir	138 ± 73.5	288 ± 143**	2.8
Tolbutamide	0.191 ± 0.104	0.123 ± 0.108	0.6
Triazolam	73.2 ± 10.2	387 ± 227	5.2
Verapamil	188 ± 22.4	755 ± 68.8***	4.0

* $p < 0.1$ ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$ using Student's paired *t*-test.

31

Table 4. CL_{int} in intact and saponin-permeabilised rat hepatocytes in static and shaken (900 rpm) incubations. Data are normalised to total cell number and represent mean ± SD (n ≥ 3).

Drug	CL_{int} (µl/min/10^6 cells)			
	Intact Hepatocytes		Permeabilised Hepatocytes	
	Static	Shaken	Static	Shaken
Midazolam	81.4 ± 15.3	257 ± 21.5**	178 ± 51.4[§]	207 ± 43.4
Propranolol	454 ± 121	1050 ± 367*	1320 ± 409[§]	1660 ± 375
Saquinavir	126 ± 36.5	311 ± 118**	407 ± 70.8[§§]	778 ± 236*[§§]
Tolbutamide	0.163 ± 0.088	0.105 ± 0.091	0.331 ± 0.311	0.105 ± 0.080

* $p < 0.1$ ** $p < 0.05$ using Student's paired *t*-test between static and shaken incubations of the same type of hepatocyte preparation (intact/permeabilised).

[§] $p < 0.1$ [§§] $p < 0.01$ using Student's paired *t*-test between intact and permeabilised hepatocytes in the same incubation condition (static/shaken).

Table 5. Predicted $CL_{int,u}$ of selected substrates in rat hepatocytes in static and shaken incubations in comparison to observed *in vivo* $CL_{int,u}$.

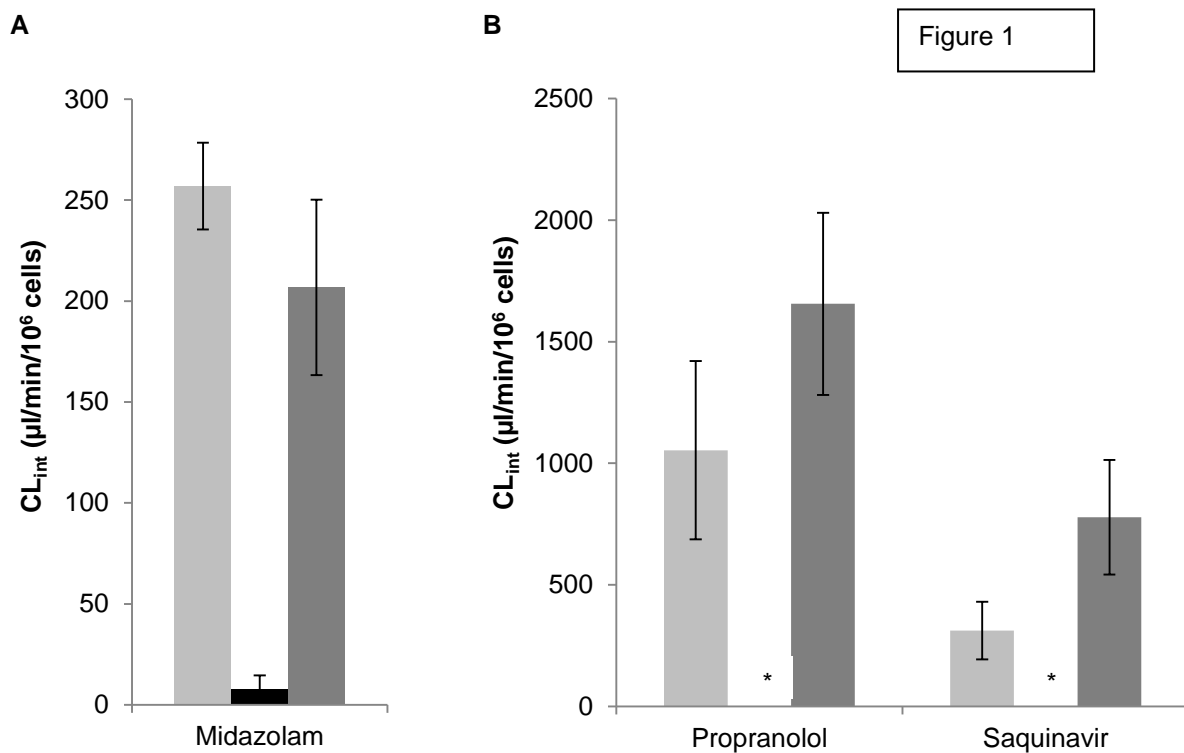| Substrate	Predicted $CL_{int,u}$ (ml/min/kg)		Observed $CL_{int,u}$ (ml/min/kg)
	Static	Shaken	
Chlorpromazine	4780 ± 524	25300 ± 6650	2260[1]
Dextromethorphan	995 ± 36.8	5450 ± 1310	635[2]
Diclofenac	297 ± 28.7	955 ± 81.9	687[2]
Midazolam	591 ± 140	1520 ± 212	1930[2]
Metoprolol	195 ± 59.8	656 ± 201	513[2]
Naloxone	1160 ± 504	5390 ± 701	253[2]
Propafenone	2440 ± 405	10800 ± 1560	3230[2]
Propranolol	2680 ± 699	6230 ± 2160	3300[2]
Saquinavir	702 ± 375	1470 ± 728	911[2]
Tolbutamide	1.10 ± 0.742	0.705 ± 0.722	6[2]
Triazolam	368 ± 51.4	1950 ± 1140	1880[2]
Verapamil	1110 ± 132	4450 ± 405	1060[2]

[1] Sawada et al. (1985), [2] Wood et al. (2017)

DMD#78949

Table 6. Predicted $CL_{int,u}$ of midazolam, propranolol and saquinavir in human hepatocytes in static and shaken incubations in comparison to predicted values from *in vitro* literature data and observed *in vivo* $CL_{int,u.}$

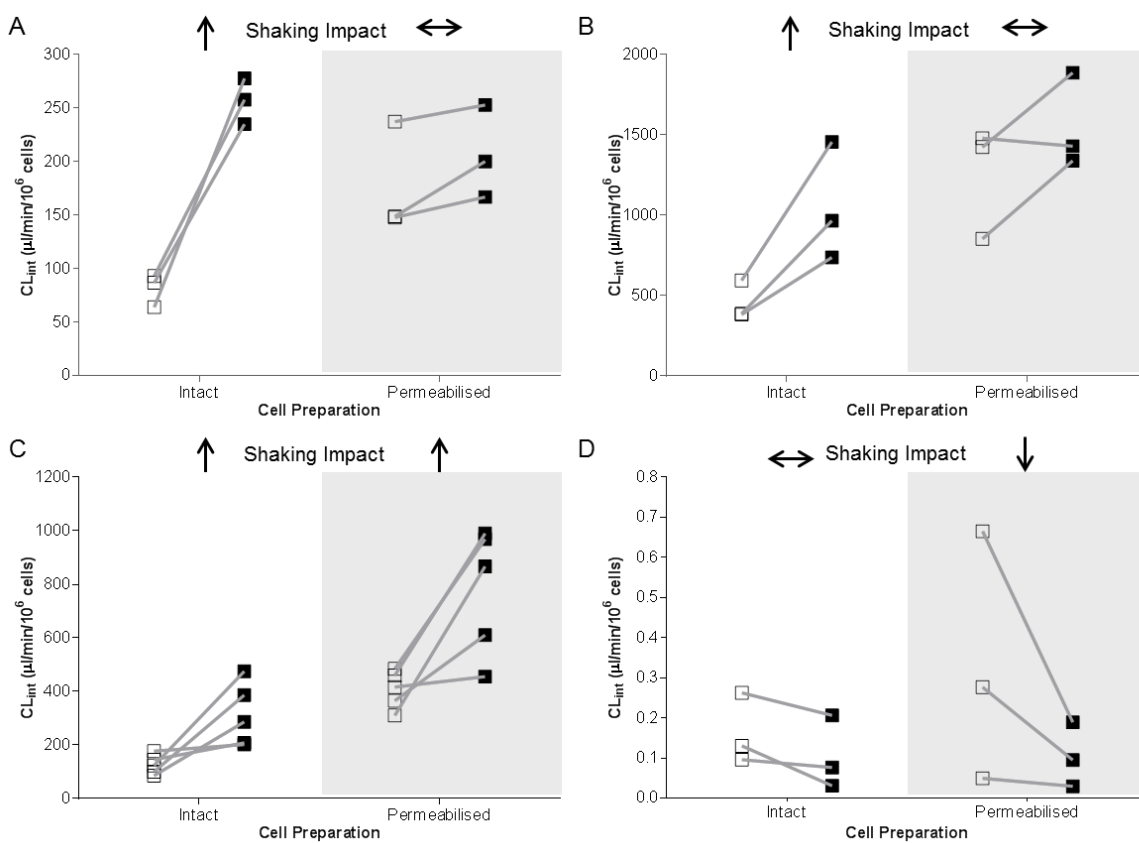| Drug	Predicted $CL_{int,u}$ (ml/min/kg)					Observed $CL_{int,u}$ (ml/min/kg)
	0.1 µM		1 µM			
	Static	Shaking	Static	Shaking	Literature	
Midazolam	100 ± 10.4	125 ± 53.1	92.1 ± 2.14	96.6 ± 39.2	42.2[1]	390[1]
Propranolol	42.9 ± 1.52	89.2 ± 13.2*	28.4 ± 7.64	57.5 ± 8.94*	50.3[1]	333[1]
Saquinavir			95.2 ± 12.4	263 ± 48.1*	-	3200[1]

[1] Wood et al. (2017).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6