Importance of the Unstirred Water Layer and Hepatocyte Membrane Integrity In Vitro for Quantification of Intrinsic Metabolic Clearance[§]

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Received October 12, 2017; accepted December 11, 2017

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 the measurement of intrinsic hepatic clearance mediated by active uptake transport and metabolism, the 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 nonviable by trypan blue staining showed undiminished metabolic activity for probe cytochrome P450 (P450) substrates midazolam and propranolol; supplementation with

NADPH enhanced these activities. Extensive permeabilization of the plasma membrane using saponin showed either full or minimal P450 activity, depending on the presence or absence of 1 mM NADPH, respectively. The 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). Permeabilization allowed static incubation metabolic rates to approach those of shaking for intact cells, indicating an artificially induced breakdown of the UWL. Permeabilization 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.

Introduction

There is some consensus that the prediction of hepatic clearance from in vitro systems is in urgent need of refinement because of a 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 the incubation of a drug with hepatocytes is thus implied and, with a lack of standardization among reported prediction studies, a fundamental reassessment of the assay procedure is needed.

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. International Society for the Study of Xenobiotics, Washington, DC.

This work was funded by the Centre for Applied Pharmacokinetic Research consortium membership, which included GSK, Janssen, Eli Lilly, and Pfizer.

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<https://doi.org/10.1124/dmd.117.078949>.

§ This article has supplemental material available at dmd.aspetjournals.org.

The potential causes of clearance-dependent underprediction from hepatocyte incubations appear to include cofactor 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 the saturation of metabolic enzymes (Klopf and Worboys, 2010) or a failure to distinguish unmetabolized drug cleared by uptake transport (Shitara et al., 2006; Soars et al., 2007; Watanabe et al., 2010). This report focuses 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 metabolized drugs could be limited by cofactor replenishment rate in vitro in a way that does not reflect the in vivo situation (Wood et al., 2017).

ABBREVIATIONS: BDDCS, Biopharmaceutics Drug Disposition Classification System; CL_{int} , intrinsic clearance; $CL_{int,app}$, apparent intrinsic clearance; $CL_{int,app,mem}$, apparent intrinsic clearance due to membrane barrier; $CL_{int,met}$, metabolic intrinsic clearance; $CL_{int,u}$, unbound intrinsic clearance; CL_{mem} , clearance through the membrane barrier; CL_{UWL} , clearance through the unstirred water layer; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; MS, mass spectrometer; P450, cytochrome P450; UWL, unstirred water layer; WME, Williams' Medium E.

In the parallel field of in vitro assessment of drug gut permeability, the involvement of the UWL is well recognized (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 a 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, a UWL can also exist, although generally at considerably lesser depths compared with some in vitro situations (Lennernas, 1998). Although the need to control the UWL phenomenon in vitro has been recognized for absorption prediction, for the prediction of metabolic clearance this has been much less so. The depth of a 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 the underprediction of clearance was examined in relation to passive permeability using reported parallel artificial membrane permeability assay, caco-2 assay, or empirical in silico methods, no relationship was apparent (Hallifax et al., 2012). However, considering the incidence of suboptimal 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, the examination of the roles of both the UWL and passive permeability on in vitro assay of intrinsic metabolic clearance focused on drugs predominantly cleared by cytochrome P450 (P450)-mediated metabolism in the relatively robust and reproducible rat suspended hepatocyte system. The effect of supplementation of the P450 cofactor 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 permeabilization and shaking. The plant glycoside saponin was used 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 ultrasonication to provide a system in which neither the plasma membrane permeability barrier nor an equivalent-sized UWL was present.

Materials and Methods

Chemicals. Chlorpromazine, dextromethorphan, diclofenac, metoprolol, naloxone, propafenone, ($\pm$)propranolol, saquinavir, tolbutamide, triazolam, verapamil, HEPES, EGTA, calcium chloride, trypsin inhibitor, reduced NADP, nicotinamide, and saponin were purchased from Sigma-Aldrich (Dorset, UK). Midazolam was supplied by F. Hoffmann-La Roche (Basel, Switzerland). Dulbecco's phosphate-buffered saline 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 Laboratory Supplies Ltd. (Nottingham, UK). All solvents were obtained from BDH Laboratory Supplies (VWR International, Lutterworth, 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 250–300 g were supplied by Charles River Laboratories (Margate, UK). Animals were culled by CO₂ 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, first with EBSS containing EGTA followed by a washout period (EBSS only) and second with EBSS containing calcium chloride, 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 μ m nylon mesh (Plastok Associates Ltd., Merseyside, UK) before washing via centrifugation (29g for 3 minutes followed by 16g 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 (supplied by Sekisui XenoTech LLC, Lenexa, KS) and stored in the vapor phase of liquid nitrogen. For each experiment, two vials of cryopreserved hepatocytes were thawed simultaneously; one was centrifuged at 100g for 5 minutes in DMEM containing isotonic Percoll (as per supplier instructions) (Percoll purified), and the other was centrifuged at 60g for 3 minutes in DMEM (unpurified).

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

Determination of Cell Viability. After centrifugation, all cell preparations were resuspended 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. Fifty microliters of cell suspension was diluted into 400 μ l of Dulbecco's phosphate-buffered saline plus 50 μ l of 0.4% trypan blue. Aliquots were loaded into a hemocytometer 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 $\geq 80\%$; the viability of cryopreserved rat hepatocytes, which were divided between unpurified and Percoll purified (as described in the preparation of cryopreserved hepatocyte suspensions), is given in Table 1; the viability of cryopreserved human hepatocytes was $\geq 91\%$.

Preparation of Permeabilized/Sonicated Hepatocyte Suspensions. Saponin-permeabilized hepatocytes were produced by preincubation of fresh rat hepatocytes (1×10^6 viable cells/ml (midazolam, propranolol, saquinavir incubations) or 2×10^6 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 predetermined as the minimum required to render $>95\%$ of cells nonviable according to trypan blue exclusion. Sonicated hepatocytes were prepared using an ultrasonic probe homogenizer (Omni Ruptor 400, CamLab Ltd., Cambridge, UK); 1×10^6 viable cells/ml were sonicated for 8×1 -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 P450 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 P450-metabolized substrate with permeation limitation, namely saquinavir (CYP3A) (Eagling et al., 2002; Usansky et al., 2008).

Substrate concentration (for depletion) was based upon either equivalent published experiments and/or reported values for Michaelis constant (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, was set as low as practicable to avoid enzyme saturation while allowing for quantitative determination of the depletion profile; in practice, this was considered to be achieved at 0.1 μ M.

Hepatocyte Incubations and Sample Analysis. Rat hepatocytes prepared as described above were diluted to twice the required incubation concentration

(Supplemental Table 1) based upon original estimates of cell density (including permeabilized 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 (Schwabach, Germany) Titramax 1000 Microtiterplate Shaker at 37°C, either unshaken or at an agitation rate of 900 rpm. This agitation rate was optimized based on the observed intrinsic clearance (CL_{int}) of midazolam in rat hepatocytes across different shaking speeds and the practical limitations of this method. Hepatocytes (60 μ l cell suspension/well) were preincubated for 5 minutes with 24 μ l of either WME (intact hepatocytes) or WME containing NADPH (permeabilized/sonicated hepatocytes); 36 μ l of substrate (WME solution) was added to initiate the reaction (total incubation volume, 120 μ l). 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, optimized 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 used 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 described above were diluted to twice the required final concentration (0.25, 0.4, and 1×10^6 cells/ml, midazolam, saquinavir, and propranolol, respectively). Incubations were conducted as described above at 0.1 μ M (midazolam, propranolol) or 1 μ M (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 1 hour before centrifugation at 1125g for 10 minutes; supernatant was removed for analysis by liquid chromatography tandem mass spectrometry. Parent drug or metabolite (tolbutamide only) was quantified by electrospray ionization multiple reaction monitoring using either a Waters Alliance 2795 (Waters, Watford, UK) or an Agilent 1100 (Agilent Technologies, Stockport, UK) high-performance liquid chromatography system coupled to either a Micromass Quattro Ultima or Quattro Micro (Waters) mass spectrometer (MS). With the exception of propranolol and metoprolol (plus internal standards), which were separated on a Luna Phenyl-Hexyl column (3 μ m, 50×4.6 mm), all compounds were separated on a Luna C18 column (3 μ m, 50×4.6 mm) (Phenomenex, Macclesfield, UK) prior to MS analysis. Mobile phases consisted of formic acid/methanol, ammonium acetate/methanol, or a mixture of both according to predetermined gradients for each analyte; the 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 analyzed alongside samples to allow quantification using QuanLynx software (MassLynx version 4.1; Waters).

Intrinsic Clearance of Total Drug. CL_{int} was determined by substrate depletion rate (except for tolbutamide; see below). Drug concentrations were fitted to incubation time using a single exponential function (eq. 1) in GraFit (version 7.0.03; Erithacus Software Ltd., Horley, UK):

$$C(t) = C_0 e^{-kt} \quad (1)$$

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, which is substituted into eq. 2 for the calculation of CL_{int} (in microliters per minute per 10^6 cells):

$$CL_{int} = \frac{V \cdot k}{No. \text{ of cells}} \quad (2)$$

where V is the incubation volume (in microliters) and $No. \text{ of cells}$ is the number of cells in the incubation/ 10^6 cells.

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 μ M) were chosen to verify the maximum rate below the reported K_m value of tolbutamide 4'-hydroxylation in rat hepatocytes (Ashforth et al., 1995;

Griffin and Houston, 2004) by linearity of metabolite formation rate with initial concentration. The CL_{int} was calculated as the slope determined by linear regression of the plot of reaction velocity against substrate concentration.

CL_{int} of Unbound Drug. As the viability and therefore the total cell number were significantly different between preparations of unpurified and Percoll-purified cryopreserved rat hepatocytes, unbound CL_{int} ($CL_{int,u}$) was calculated using eq. 3, for comparison of metabolic activity:

$$CL_{int,u} = \frac{CL_{int}}{fu_{heps}} \quad (3)$$

where fu_{heps} (eq. 4) is the fraction unbound in the hepatocyte incubation, based on total cell number:

$$fu_{heps} = \frac{1}{1 + 125 \cdot V_R \cdot 10^{0.072 \cdot \log P/D^2 + 0.067 \cdot \log P/D - 1.126}} \quad (4)$$

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

Physiologic Scaling of Intrinsic Clearance. For comparison with in vivo data, hepatocyte CL_{int} (in microliters per minute per 10^6 cells) was corrected for incubational binding (fu_{heps}) and scaled to predicted in vivo $CL_{int,u}$ (eq. 5):

$$\text{Predicted in vivo } CL_{int,u} = \frac{\text{in vitro } CL_{int} \cdot \text{hepatocellularity} \cdot LW}{fu_{heps}} \quad (5)$$

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 b.wt. for human and 40 g/kg b.wt. for rat (Davies and Morris, 1993).

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

Theoretical Considerations on Potentially Rate-Limiting Processes. For drugs that enter hepatocytes predominantly passively, the following 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 metabolic CL_{int} ($CL_{int,met}$). 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. 6:

$$CL_{int,app,mem} = CL_{int,met} \cdot \frac{CL_{mem}}{CL_{mem} + CL_{int,met}} \quad (6)$$

CL_{mem} can then be obtained by rearrangement of eq. 6 to eq. 7:

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

Any restriction of $CL_{int,met}$ by both CL_{mem} and CL_{UWL} ($CL_{int,app}$) can be represented by eq. 8:

$$CL_{int,app} = CL_{int,app,mem} \cdot \frac{CL_{UWL}}{CL_{UWL} + CL_{int,app,mem}} \quad (8)$$

CL_{UWL} can be obtained by rearrangement of eq. 8 to eq. 9:

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

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 of the following sections report on sequential investigations that progress from fundamental assessment of the metabolic activity of nonviable 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. Permeabilization 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 on the impact of optimized methods in human hepatocytes and on the 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 nonspecific incubational binding, $CL_{int,u}$ was used to compare metabolic activity using total cell concentration to estimate fu_{heps} values.

Although the population of nonviable cells is not expected to influence the $CL_{int,u}$, midazolam and propranolol $CL_{int,u}$, expressed per the number of viable cells (as is common practice), was considerably greater in unpurified hepatocytes compared with Percoll-purified hepatocytes; the apparent losses of activity in Percoll-purified preparations were 25% and 31% for midazolam and propranolol, respectively (without NADPH) (Table 1). Yet, when $CL_{int,u}$ was normalized to total cell number, the activity in unpurified and Percoll-purified hepatocytes was similar (Table 1), suggesting that those cells deemed nonviable (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 the number of nonviable cells, strongly suggesting that metabolic activity is approximately equivalent in viable and nonviable hepatocytes (rather than the result of 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 $\mu\text{l}/\text{min}$ per 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 $\mu\text{l}/\text{min}$ per 10^6 cells versus 212 ± 57 $\mu\text{l}/\text{min}$ per 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 $CL_{int,u}$ values for midazolam (Table 1), and this was reflected in a similar (41%) increase in propranolol $CL_{int,u}$ values 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 “nonviable” 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 the study by 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-Permeabilized Rat Hepatocyte CL_{int}

Fresh rat hepatocytes treated with 0.01% w/v saponin for 5 minutes of preincubation ubiquitously displayed trypan blue staining and hence were deemed entirely nonviable by this test. Saponin is a selective membrane permeabilization 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} values of midazolam, propranolol, and saquinavir (with potentially significant differences in permeability) were compared between fresh intact hepatocytes and saponin-permeabilized hepatocytes with and without exogenous NADPH supplementation. CL_{int} values were normalized to the initial total cell number.

Saponin treatment resulted in effectively complete inhibition of midazolam, propranolol, and saquinavir clearance (Fig. 1; Table 2). However, the addition of 1 mM NADPH to these preparations restored substrate metabolism to levels either consistent with (midazolam) or substantially greater than (propranolol, saquinavir) that of intact hepatocytes, demonstrating that the P450 enzymes responsible for the metabolism of each drug retained latent functionality under this treatment.

Effect of Shaking on CL_{int} in Intact Rat Hepatocytes

Twelve well-characterized substrates of multiple metabolic pathways, encompassing a wide range of in vivo CL_{int} 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-fold and 5.5-fold) in shaken incubations compared with static; this was statistically

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 the mean $\pm$ S.D. ($n \geq 3$).

Drug	Parameter	Unpurified		Percoll Purified	
		- NADPH	+ NADPH	- NADPH	+ NADPH
Midazolam	Initial viability (%)		62 $\pm$ 3		86 $\pm$ 1***
	Total cell concentration ($10^6/\text{ml}$)		0.81 $\pm$ 0.04		0.58 $\pm$ 0.01***
	$CL_{int,u}$ ($\mu\text{l}/\text{min}$ per 10^6 cells) normalized to viable cell number	262 $\pm$ 61.7	399 $\pm$ 58.5§§§	196 $\pm$ 34.7*	219 $\pm$ 33.4§§§
	$CL_{int,u}$ ($\mu\text{l}/\text{min}$ per 10^6 cells) normalized to total cell number	160 $\pm$ 31.3	245 $\pm$ 31.3§§§	169 $\pm$ 28.4	188 $\pm$ 27.5§§§
Propranolol	Initial viability (%)		67 $\pm$ 3		89 $\pm$ 3***
	Total cell concentration ($10^6/\text{ml}$)		0.37 $\pm$ 0.02		0.28 $\pm$ 0.01***
	$CL_{int,u}$ ($\mu\text{l}/\text{min}$ per 10^6 cells) normalized to viable cell number	262 $\pm$ 45.4	370 $\pm$ 54.2§§§§	182 $\pm$ 38.4	162 $\pm$ 35.8§
	$CL_{int,u}$ ($\mu\text{l}/\text{min}$ per 10^6 cells) normalized to total cell number	176 $\pm$ 26.4	249 $\pm$ 33.1§§§§	163 $\pm$ 32.9	144 $\pm$ 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.

Fig. 1. CL_{int} of midazolam (A), and propranolol and saquinavir (B) in intact hepatocytes (■) and permeabilized hepatocytes in the absence (□) and presence (■) of exogenous NADPH. Data are normalized to total cell number and represent the mean $\pm$ S.D. ($n \geq 3$). *No depletion detected.

significant for 10 of 11 remaining compounds (Table 3). There was an apparent tendency for a fold increase in CL_{int} values caused by shaking to increase with increasing CL_{int} (taken as the maximal shaken value) in an apparently nonlinear way; given the large range in CL_{int} examined, this suggests a maximum shaking effect of about 5-fold (Fig. 2).

Effect of Prior Shaking on Static Rat Hepatocyte CL_{int} . To verify a lack of membrane damage caused by the 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 after either a static or shaken (900 rpm) 15-minute preincubation period. There was no difference in midazolam CL_{int} values between static ($71 \pm 13 \mu\text{l}/\text{min}$ per 10^6 cells) and shaken ($62 \pm 4 \mu\text{l}/\text{min}$ per 10^6 cells) preincubations, 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 after preincubation (5 minutes) with trypan blue (according to viability assay), immediately after the addition of trypan blue, or without trypan blue addition until immediately after shaking. There was no significant difference in viability among the three treatments ($82.5\% \pm 3.8\%$, $85.2\% \pm 1.2\%$, $83.1\% \pm 4.1\%$, respectively), demonstrating a complete lack of plasma membrane leakage to the azo dye (mol. wt., 872) caused by shaking and thereby indicating a likely lack of leakage to other small molecules under the same condition.

Effect of Shaking on CL_{int} in Saponin-Permeabilized Rat Hepatocytes

Four substrates were selected for investigation of the impact of shaking on permeabilized hepatocytes (Table 4). To clarify trends otherwise obscured by experimental variability, individual incubation replicates (by cell preparation batch) are shown (Fig. 3). In intact cells, there was a clear tendency toward increased CL_{int} between static and shaken cells for midazolam, propranolol, and saquinavir (Fig. 3, A–C). However, for the same drugs in permeabilized cells, there was no clear trend of increased CL_{int} (with shaking) except for saquinavir (Fig. 3C). Also, there was little difference in CL_{int} between shaken intact cells and static permeabilized 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 permeabilized 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 permeabilized cells (Fig. 3D); conversely, there were indications of a decrease in tolbutamide CL_{int} with shaking (more pronounced for permeabilized 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 Fig. 3 to aid interpretation of the drug-dependent and condition-dependent effects.

Effect of Shaking on Saquinavir CL_{int} in Ultrasonicated Rat Hepatocytes

There was no significant difference in the CL_{int} of saquinavir between static incubations ($668 \pm 239 \mu\text{l}/\text{min}$ per 10^6 cells) and shaken incubations ($746 \pm 281 \mu\text{l}/\text{min}$ per 10^6 cells) of ultrasonicated rat hepatocytes. Therefore, in contrast to saponin-permeabilized 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 permeabilization) and its associated UWL by sonication.

A comparison of activity among intact, saponin-permeabilized, and sonicated hepatocytes is shown in Fig. 4.

TABLE 2

CL_{int} of midazolam, propranolol, and saquinavir in intact (un-supplemented) rat hepatocytes and saponin-permeabilized rat hepatocytes with and without NADPH supplementation

Data are normalized to total cell number and represent the mean $\pm$ S.D. ($n \geq 3$).

Drug	CL_{int}		
	Intact (un-supplemented)	Permeabilized	
		Un-supplemented	NADPH Supplemented
		$\mu\text{l}/\text{min}$ per 10^6 cells	
Midazolam	257 ± 21.5	$7.66 \pm 6.90^*$	$207 \pm 43.4\%$
Propranolol	1050 ± 367	ND	$1660 \pm 375\%$
Saquinavir	311 ± 118	ND	$778 \pm 236^*,\%$

ND, no depletion detected.

* $P < 0.01$ using paired Student's t test in comparison with intact hepatocytes.

$\%$ $P < 0.05$, $\%$ $P < 0.01$ using paired Student's t test in comparison with un-supplemented permeabilized hepatocytes.

TABLE 3
 CL_{int} of selected substrates in rat hepatocytes in static and shaken incubations

Data represent mean $\pm$ S.D. ($n \geq 3$).

Substrate	CL_{int}		Average Fold Increase in CL_{int}
	Static	Shaken	
	$\mu\text{l/min per } 10^6 \text{ cells}$		
Chlorpromazine	366 ± 40.1	$1940 \pm 509^{**}$	5.4
Dextromethorphan	168 ± 6.21	$920 \pm 222^{**}$	5.5
Diclofenac	59.9 ± 5.78	$192 \pm 16.5^{***}$	3.2
Midazolam	118 ± 27.9	$303 \pm 42.4^{****}$	2.8
Metoprolol	38.3 ± 11.7	$129 \pm 39.4^{**}$	3.4
Naloxone	238 ± 104	$1110 \pm 145^{**}$	5.5
Propafenone	487 ± 81.0	$2160 \pm 312^{***}$	4.4
Propranolol	520 ± 136	$1210 \pm 419^*$	2.8
Saquinavir	138 ± 73.5	$288 \pm 143^{**}$	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	$755 \pm 68.8^{***}$	4.0

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

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. After conducting experiments similar to those already described, the CL_{int} of midazolam ($1 \mu\text{M}$) was not affected by shaking ($36 \pm 1 \mu\text{l/min per } 10^6 \text{ cells}$) compared with static incubation ($38 \pm 15 \mu\text{l/min per } 10^6 \text{ cells}$) in human hepatocytes. However, CL_{int} of both propranolol at $1 \mu\text{M}$ (7 ± 2 versus $14 \pm 2 \mu\text{l/min per } 10^6 \text{ cells}$) and saquinavir at $1 \mu\text{M}$ (35 ± 4 versus $98 \pm 27 \mu\text{l/min per } 10^6 \text{ cells}$) was significantly ($p < 0.05$) increased in shaken human hepatocytes, indicating a critical, if drug-dependent, impact in humans. At the lower substrate concentration ($0.1 \mu\text{M}$), there was no difference in the CL_{int} for midazolam, but there was an approximately 2-fold increase for propranolol (10 ± 0.4 versus $21 \pm 3 \mu\text{l/min per } 10^6 \text{ cells}$; $P < 0.05$), with some indication of substrate concentration dependence (increased rate compared with higher concentration).

Effect of Shaking of Hepatocytes on Prediction of In Vivo CL_{int}

In the rat, scaled in vitro $CL_{int,u}$ values (in milliliters per minute per kilogram) were compared with in vivo $CL_{int,u}$ values (in milliliter per minute per kilogram) derived from observed hepatic clearance literature data (Table 5). In static incubations of rat hepatocytes, 5 of 12 drugs were predicted within 2-fold, 2 of 12 drugs were predicted above 2-fold, and 5 of 12 drugs were predicted below 2-fold. In shaken incubations, 6 of 12 drugs were within 2-fold, 5 of 12 drugs were above 2-fold, and 1 of 12 drugs were below 2-fold. Excluding tolbutamide (slowly cleared), the increase in CL_{int} due to shaking was between 3-fold and 5-fold. The in vivo CL_{int} values for these substrates ranged between about 250 and 3300 ml/min per kilogram, 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 and 1000 ml/min per kilogram (Wood et al., 2017)—representing a similar shift in CL_{int} and potential improvement in prediction. However, the reported ratio of in vitro $CL_{int,u}/\text{in vivo } CL_{int,u}$ for the drugs used here ranged between 0.1 and 5.1, and so this group of drugs included overpredictions as well as underpredictions of $CL_{int,u}$; the overpredicted drugs were chlorpromazine, naloxone, verapamil, propafenone, and saquinavir, the latter two designated as Biopharmaceutics Drug Disposition Classification System (BDDCS) Class 2 (all others, except tolbutamide are BDDCS Class 1). Overall, for rats, 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 an underprediction of 5.8-fold at $1 \mu\text{M}$ and of 3.7-fold at $0.1 \mu\text{M}$, compared with the average reported underprediction of 6.7-fold for this drug and the average underprediction of 12-fold for the $CL_{int,u}$ range of 100–1000 ml/min per kilogram (Wood et al., 2017). Saquinavir was underpredicted by 12-fold in shaken incubations compared with 34-fold in static incubations ($1 \mu\text{M}$); there is no previously reported prediction for this drug, but the average underprediction for the in vivo $CL_{int,u}$ range of 1000–10,000 ml/min per kilogram is 30-fold. The midazolam in vivo $CL_{int,u}$ was underpredicted by 3.9-fold and 3.1-fold in static and shaken incubations, respectively ($0.1 \mu\text{M}$), compared with a 9.3-fold underprediction reported in the literature (Wood et al., 2017), an apparent improvement but one that implies the involvement of other (unidentified) sources of variability.

Discussion

Prediction of clearance from in vitro systems has been recognized for many years as not mechanistically quantitative (Iwatsubo et al., 1997;

Fig. 2. Average fold increase in CL_{int} in rat hepatocytes with shaking versus CL_{int} determined in shaken incubations. Data represent the mean ($n \geq 3$); line fitted by nonlinear regression using $y = A \times x/(B + x)$, where A and B represent the maximal and half-saturation constants.

TABLE 4
CL_{int} in intact and saponin-permeabilized rat hepatocytes in static and shaken (900 rpm) incubations
Data are normalized to total cell number and represent mean ± S.D. (*n* ≥ 3).

Drug	<i>CL_{int}</i>			
	Intact Hepatocytes		Permeabilized Hepatocytes	
	Static	Shaken	Static	Shaken
μl/min per 10 ⁶ cells				
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).

Hallifax and Houston, 2009). Explanations for this have focused on physiologic scaling, liver dispersion models, plasma protein binding inaccuracies, as well as in vitro methodology, but without comprehensive validation. Among recent reappraisal of experimental methodology (Bowman and Benet, 2016; Wood et al., 2017) a tendency for clearance prediction to become increasingly inaccurate with increasing in vivo *CL_{int}*, independent of in vitro system and species, has been highlighted.

Hence, fundamental aspects of the *CL_{int}* assay—cell viability and potentially rate-limiting UWL and membrane barriers—have been examined using rat hepatocytes.

Hepatocytes deemed nonviable 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

Fig. 3. Individual replicates of *CL_{int}* of midazolam (A), propranolol (B), saquinavir (C), and tolbutamide (D) in unsupplemented intact and NADPH-supplemented saponin-permeabilized rat hepatocytes in static (□) and shaken (■) incubations. Data are normalized 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).

Fig. 4. Individual replicates of CL_{int} of saquinavir in unsupplemented intact hepatocytes and NADPH-supplemented permeabilized and ultrasonicated hepatocytes in static ($\square$) and shaken ($\blacksquare$) incubations. Data are normalized to total cell number. $\uparrow$ indicates a significant increase in CL_{int} with shaking; $\leftrightarrow$ indicates no significant overall change in CL_{int} with shaking.

of the nonviable 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} values for midazolam, propranolol, and saquinavir comparable to those of intact cells, verifying P450 enzyme functionality in hepatocytes with porous plasma membranes. For permeabilized 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 that exclude trypan blue (viable). The findings here, however, imply that such estimates of CL_{int} may be compromised by the metabolic viability of nonviable cells; in addition, a differential in the permeability barrier may exist between cells of a different condition, which could skew $CL_{int,app}$ 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, the shaking of incubates was employed; UWL effects, which, although well recognized in permeability assays (Avdeef et al., 2004), have not been fully addressed in the context of CL_{int} assays. Compared with static incubations, shaken rat hepatocytes gave up to 5-fold greater CL_{int} values, 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 $\mu\text{l}/\text{min}$ per 10^6 cells (equivalent to 430 ml/min per kilogram) but was constant at higher CL_{int} levels, indicating an upper limit to the impact of the UWL (possibly reflecting prevailing plasma membrane permeability limitation). The CL_{int} was dependent on shaking speed (optimized to 900 rpm for 96-well plates), and therefore previous studies employing shaking could still have incurred rate limitation if not optimized. 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 permeabilized with saponin, there was an increase in CL_{int} in static incubations (compared with intact cells), and hence the effect of shaking was reduced, depending on the drug, implying a reduced UWL associated with a permeabilized

membrane. In permeabilized hepatocytes, the CL_{int} of midazolam and propranolol was little affected by shaking, whereas the 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 the CL_{int} for saquinavir in permeabilized 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 permeabilized cells, providing evidence that the plasma membrane can be effective as a barrier in cells permeabilized with saponin, possibly by involvement of an accompanying and effective UWL. In contrast to the three highly cleared drugs, tolbutamide CL_{int} was

TABLE 5

Predicted $CL_{int,u}$ of selected substrates in rat hepatocytes in static and shaken incubations in comparison with observed in vivo $CL_{int,u}$

Data represent mean $\pm$ S.D., unless otherwise indicated.

Substrate	Predicted $CL_{int,u}$		Observed $CL_{int,u}$
	Static	Shaken	
		ml/min per kilogram	
Chlorpromazine	4780 $\pm$ 524	25,300 $\pm$ 6650	2260 ^a
Dextromethorphan	995 $\pm$ 36.8	5450 $\pm$ 1310	635 ^b
Diclofenac	297 $\pm$ 28.7	955 $\pm$ 81.9	687 ^b
Midazolam	591 $\pm$ 140	1520 $\pm$ 212	1930 ^b
Metoprolol	195 $\pm$ 59.8	656 $\pm$ 201	513 ^b
Naloxone	1160 $\pm$ 504	5390 $\pm$ 701	253 ^b
Propafenone	2440 $\pm$ 405	10,800 $\pm$ 1560	3230 ^b
Propranolol	2680 $\pm$ 699	6230 $\pm$ 2160	3300 ^b
Saquinavir	702 $\pm$ 375	1470 $\pm$ 728	911 ^b
Tolbutamide	1.10 $\pm$ 0.742	0.705 $\pm$ 0.722	6 ^b
Triazolam	368 $\pm$ 51.4	1950 $\pm$ 1140	1880 ^b
Verapamil	1110 $\pm$ 132	4450 $\pm$ 405	1060 ^b

^aSawada et al. (1985).

^bWood et al. (2017).

TABLE 6

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

Data represent mean $\pm$ S.D., unless otherwise indicated.

Drug	Predicted $CL_{int,u}$				Literature	Observed $CL_{int,u}$
	0.1 μ M		1 μ M			
	Static	Shaking	Static	Shaking		
	ml/min per kilogram					
Midazolam	100 $\pm$ 10.4	125 $\pm$ 53.1	92.1 $\pm$ 2.14	96.6 $\pm$ 39.2	42.2 ^a	390 ^a
Propranolol	42.9 $\pm$ 1.52	89.2 $\pm$ 13.2*	28.4 $\pm$ 7.64	57.5 $\pm$ 8.94*	50.3 ^a	333 ^a
Saquinavir			95.2 $\pm$ 12.4	263 $\pm$ 48.1*		3200 ^a

^aWood et al. (2017).

* $P < 0.05$

not increased by shaking or permeabilization, as would be expected for a slowly metabolized drug for which the overall clearance is limited solely by metabolic rate.

The foregoing provides experimental means to identify rate-limiting processes in vitro that would otherwise confound the accurate determination of CL_{int} . For drugs with predominantly passive hepatic uptake, the following three sequential mechanistic processes can be appropriately assumed (Fig. 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 permeabilized hepatocytes, minimizing the UWL surrounding the porous cell membrane and maximizing access of the 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 eqs. 6 and 7. The restriction of $CL_{int,met}$ by both CL_{mem} and CL_{UWL} ($CL_{int,app}$) is represented by the $CL_{int,app}$ observed in static incubations of intact hepatocytes and can be determined by eqs. 8 and 9. Midazolam, propranolol, and saquinavir are exemplary drugs for which the 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 optimization of the in vitro assay of CL_{int} to eliminate artifactual 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; a UWL of 10 μ m has been estimated (Bass and Pond, 1988), although this exceeds the diameter of the space itself (1–2 μ m, Barry and Diamond, 1984). But, despite this apparently minimal barrier in the liver, extraction of highly permeable drugs by the isolated perfused rat liver has been shown to be limited by a UWL (Ichikawa et al., 1992). In this study, the uptake of highly permeable drugs was relatively independent of free fraction (when albumin was added to the perfusate) compared with less permeable drugs, which is consistent with the “albumin-mediated transport” phenomenon but is explained by rate-limiting diffusion through the UWL. From a mechanistic perspective, extrapolation from in vitro to in vivo should be based on minimization of artifactual in vitro UWL effects, and so it is recommended that the shaking of hepatocyte incubations be optimized to maximize CL_{int} as described. This would allow scope, for example, to reappraise 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 to be highly permeable, such as propranolol. It is not known, however, whether 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 permeabilized shaken hepatocytes would offer an indication of this possibility.

As anticipated, the prediction of in vivo CL_{int} for the drugs in this study gave a mixed outcome. Predictions of in vivo CL_{int} for the rat, based on shaken hepatocyte values in this study, tended to overestimate 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 a relatively lower metabolic rate (than the rat) with respect to diffusion through the UWL, it is clear that the recommendation to optimize 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 the

Fig. 5. Sequential passive processes involved in in vitro apparent CL_{int} . P.M., plasma membrane.

Fig. 6. The effect of changes in CL_{UWL} and CL_{mem} on $CL_{int,app}$ induced by shaking and cell permeabilization. All clearances are expressed relative to $CL_{int,met}$; estimated experimental shift in $CL_{int,app}$ is shown for midazolam (MDZ), propranolol (PPL), and saquinavir (SQV). ● represents static incubation. CL_{mem} was determined from eq. 7 using $CL_{mem}/CL_{int,app}$ derived from permeabilized versus nonpermeabilized CL_{int} values (Table 4). CL_{UWL} was determined in an analogous way to that of CL_{mem} using $CL_{mem}/CL_{int,app}$ values derived from static intact versus shaken permeabilized CL_{int} (Table 4).

successful progress of prediction methodology. This strongly advocates a multitrack experimental approach for elucidating drug hepatic CL_{int} , allowing the disposition processes to be resolved. As a minimum, the assay of investigative compound disposition using isolated hepatocytes in parallel with microsomal CL_{int} assay is recommended for all drug types, including highly permeable/metabolized 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 permeabilized hepatocytes, as shown in this study, would allow the 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 toward drugs that 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.

Acknowledgments

We thank Sue Murby for expert guidance in the LCMS/MS analysis.

Authorship Contributions

Participated in research design: Wood, Houston, and Hallifax.

Conducted experiments: Wood.

Performed data analysis: Wood and Hallifax.

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

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