Evaluation of the Disconnect between Hepatocyte and Microsome Intrinsic Clearance and In Vitro In Vivo Extrapolation Performance[§]

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ABSTRACT

The use of *in vitro in vivo* extrapolation (IVIVE) from human hepatocyte (HH) and human liver microsome (HLM) stability assays is a widely accepted predictive methodology for human metabolic clearance (CL_{met}). However, a systematic underprediction of CL_{met} from both matrices appears to be universally apparent, which can be corrected for via an empirical regression offset. After physiological scaling, intrinsic clearance (CL_{int}) for compounds metabolized via the same enzymatic pathway should be equivalent for both matrices. Compounds demonstrating significantly higher HLM CL_{int} relative to HH CL_{int} have been encountered, raising questions regarding how to predict CL_{met} for such compounds. Here, we determined the HLM:HH CL_{int} ratio for 140 marketed drugs/compounds, compared this ratio as a function of physicochemical properties and drug metabolism enzyme dependence, and examined methodologies to predict CL_{met} from both matrices. The majority (78%) of compounds displaying a high HLM:HH CL_{int} ratio were CYP3A substrates. Using HH CL_{int} for CYP3A substrates, the current IVIVE regression offset approach remains an appropriate strategy to predict CL_{met} (% compounds overpredicted/correctly predicted/underpredicted 27/62/11, respectively).

However, using the same approach for HLM significantly overpredicts CL_{met} for CYP3A substrates (% compounds overpredicted/correctly predicted/underpredicted 56/33/11, respectively), highlighting that a different IVIVE offset is required for CYP3A substrates using HLM. This work furthers the understanding of compound properties associated with a disproportionately high HLM:HH CL_{int} ratio and outlines a successful IVIVE approach for such compounds.

SIGNIFICANCE STATEMENT

Oral drug discovery programs typically strive for low clearance compounds to ensure sufficient target engagement. Human liver microsomes and isolated human hepatocytes are used to optimize and predict human hepatic metabolic clearance. After physiological scaling, intrinsic clearance for compounds of the same metabolic pathway should be equivalent between matrices. However, a disconnect in intrinsic clearance is sometimes apparent. The work described attempts to further understand this phenomenon, and by achieving a mechanistic understanding, improvements in clearance predictions may be realized.

Introduction

For oral drug discovery programs, there is typically a requirement to design and develop low clearance (CL) compounds to ensure sufficient extent and duration of target engagement. Optimization of CL is usually one of the more significant challenges in drug discovery. With hepatic metabolic elimination remaining the predominant CL pathway for drugs (Cerny, 2016), the use of *in vitro in vivo* extrapolation (IVIVE) from metabolic stability assays is a widely accepted predictive methodology for human metabolic CL (CL_{met}) (Riley et al., 2005; Bowman and Benet, 2019a,b; Williamson et al., 2020). IVIVE comparisons of measured *in vivo* CL in animal species can assist with developing a cross-species mechanistic understanding of compound disposition.

Moreover, for candidate drugs, successful prediction of an acceptable human *in vivo* CL is important to enable testing of the pharmacological hypothesis in the clinic and thus in reducing drug attrition for pharmacokinetics (PK) reasons (Hay et al., 2014; Davies et al., 2020).

Within drug discovery, two hepatic *in vitro* matrices are primarily used for metabolic stability assays to optimize and predict CL_{met}: human liver microsomes (HLM) and isolated human hepatocytes (HH). HLM offer the ability for enhanced-throughput intrinsic clearance (CL_{int}) screening at relatively low cost and therefore can be used to triage suitable compounds into more expensive hepatocyte incubations. HH are regarded as the most predictive *in vitro* system since they contain the full complement of enzymes and transporters that a compound may encounter during first-pass metabolism, hence they often form the basis of IVIVE for CL_{met}. HLM and HH CL_{int}, corrected for unbound fraction, can be scaled to *in vivo* CL_{int} (ml/min/kg) using physiological parameters (Table 1). Therefore, with the reasonable assumption of

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ABBREVIATIONS: A-B, apical to basolateral; AAFE, absolute AFE; AFE, average fold error; B-A, basolateral to apical; CL, clearance; CL_{int}, intrinsic CL; CL_{int,u}, scaled unbound CL_{int}; CL_{met}, metabolic CL; ER, efflux ratio; fu_{inc}, fraction unbound in the incubation; HH, human hepatocyte; HLM, human liver microsome; ISEF, intersystem extrapolation factor; IVIVE, *in vitro-in vivo* extrapolation; LC, liquid chromatography; LogD, partition coefficient of a compound between octanol and water at pH7.4; MDCK, Madin-Darby Canine Kidney; MDR, multidrug resistance-1; MS, mass spectrometry; MW, molecular weight; Pgp, P-glycoprotein; PK, pharmacokinetics; Qh, hepatic blood flow; rCYP, recombinant CYP; RED, rapid equilibrium device; RH, rat hepatocyte; UGT, uridine 5'-diphospho-glucuronosyltransferase; V_d, volume of distribution; WSM, "well stirred model".

TABLE 1
Human physiologic scaling factors

Species	Liver Blood Flow (Qh) (ml/min/kg)	Liver Weight (g)	Body Weight (kg)	Microsomal Protein (mg/g Liver)	Hepatocellularity/g Liver
Human	20.7	1500	70	40	120 × 10 ⁶

similar binding in the HLM and HH incubations and assuming the same metabolic pathways in both systems, the scaled HLM:HH CLint ratio should approximate 1. Indeed, an indicator of the presence of significant additional metabolic pathways, such as glucuronidation, which is present in HH and not HLM (using only NADPH as a cofactor), would be a scaled HLM:HH CLint ratio significantly less than 1.

The commonly accepted approach for IVIVE involves inputting the CLint from HLM and HH into a mathematical model of liver perfusion, typically the “well stirred model” (WSM) (Rowland et al., 1973; Yang et al., 2007), to predict CLmet. However, this approach leads to a systematic underprediction of CLmet for reasons not presently understood (Riley et al., 2005; Foster et al., 2011; Bowman and Benet, 2019a). Many hypotheses to account for this underprediction have been proposed (Bowman and Benet, 2019a; Williamson et al., 2020), all of which should be considered during IVIVE for key compounds. However, none of these explanations solely satisfy the systematic nature of the CLmet underprediction. To account for this underprediction, an empirical correction can be applied to the *in vitro* data from hepatocytes or microsomes. This offset can be derived using a regression approach, in which the derived unbound *in vitro* and *in vivo* CLint values (ml/min/kg) form a correlation line from which future predictions of unbound *in vivo* CLint values for new compounds can be made (Riley et al., 2005; Sohlenius-Sternbeck et al., 2012). Notably, this offset appears to be relatively consistent between liver matrices, species, and laboratories and is independent of compound (Riley et al., 2005; Bowman and Benet, 2016; Wood et al., 2017).

Importantly, for CYP-mediated disposition, which remains the most prevalent CL pathway for drug-like compounds, there appears to be an overall comparable IVIVE predictive performance between HLM and HH (Riley et al., 2005; Chiba et al., 2009; Bowman and Benet, 2016). More recently, it has been postulated that underprediction from both matrices is increased as CL increases (Bowman and Benet, 2019a), and HLM are more accurate for predicting CL of CYP3A substrates (Bowman and Benet, 2019b).

Several reports have also noted a disconnect between HLM and HH CLint (Stringer et al., 2008; Foster et al., 2011; Bowman and Benet, 2019b), whereby a scaled HLM:HH CLint ratio significantly greater than 1 has been determined for specific compounds. Considerably larger CLint values in HLM relative to HH have been observed for CYP3A substrates yet, intriguingly, not for substrates of other drug metabolism enzymes (Bowman and Benet, 2019b). The mechanistic basis for this phenomenon has yet to be elucidated, although it has been hypothesized that this observation could be due to the overlapping substrate specificity between CYP3A and the efflux transporter, P-glycoprotein (Pgp), that is located on the hepatocyte membrane, restricting compound access to drug metabolism enzymes in hepatocytes relative to unhindered access to the same enzymes in microsomes (Bowman and Benet, 2019b), thus resulting in disproportionately lower CLint determined in HH relative to HLM.

Recently, Lombardo et al. (2018) published a large data set of compounds to investigate the relationship between physicochemical properties and human PK. This data set was used to investigate factors contributing to the phenomena, which we have observed in our

laboratory, of the apparent disconnect between HLM and HH CLint and the predictive performance of IVIVE between matrices for such compounds. The data and analyses presented herein provide further insight regarding the HLM:HH CLint disconnect with a significant data set of 140 marketed drugs/compounds. By achieving a mechanistic understanding, improvements in IVIVE accuracy and CL predictions may be realized.

Materials and Methods

Compound Selection. The Lombardo data set (Lombardo et al., 2018) comprised 1352 compounds with measured human PK parameters and physicochemical properties. The data set was cross-referenced with compounds available in the AstraZeneca compound bank and filtered according to the following criteria:

- Compounds with a measured human CL approaching or less than liver blood flow (Qh 20.7 ml/min/kg) were included to disregard compounds with extraordinarily high CL that may have a significant extrahepatic component to their elimination.
- Only compounds with a molecular weight (MW) 150–800 and octanol: water partition coefficient LogD at pH 7.4 (LogD) 0.5–4 were included to broadly represent the typical small-molecule physicochemical property space encountered in oral drug discovery.
- HLM CLint, HH CLint, human plasma protein binding, and incubational binding (fuinc) values were determined. Compounds with limits on measured values (< and >) were subsequently excluded to avoid bias.

The resulting data set (*n* = 140 compounds) mainly comprised biopharmaceuticals class 1 compounds predominantly cleared by hepatic metabolism, with human CL ranging 0.1–20 ml/min/kg. When required, additional data were generated, including Caco-2 permeability (Papp), Caco-2 efflux, Madin-Darby Canine Kidney (MDCK) multidrug resistance mutation 1 (MDR1) efflux, and CYP phenotyping (if the main route of elimination was via CYP, but the contribution of each isoform was unknown from literature sources). Given the reasonable assumption of consistencies in fuinc between species and matrices (Winiwarter et al., 2019), fuinc in HLM or rat hepatocytes (RH) was used. For LogD and fuinc, experimental values were supplemented with the use of *in-house in silico* models generated from machine learning methods. When possible, the main hepatic metabolic route of elimination was obtained from literature references or using databases, including the University of Washington Drug Interaction Database (this information was based on or an extract from Drug Interaction Database Copyright University of Washington, accessed: April 2020) (Supplemental Table 1). It was assumed that the clinical disposition pathway obtained from the literature reflects the main metabolic pathway in HH and HLM. Only in absence of such references were additional *in vitro* reaction phenotyping data obtained to define the main contributing enzyme (Supplemental Table 1).

For all *in vitro* assays detailed below, the experimental work was conducted at the Contract Research Organization, Pharmaron, China.

Materials. HLM (150 donors; Lot QQY and Lot 38289) were purchased from Corning or BioIVT (Shanghai, China), respectively. HH (10 donors; Lot LYB and Lot IRK) were purchased from BioIVT. Human plasma was purchased from BioIVT (mixed donors, with a minimum of two males and two females). Recombinant human CYP enzymes were purchased from CYPDEX (Shanghai, China). EDTA-K2 was purchased from Beijing Chemical Reagents Company (Beijing, China). Bovine serum albumin was purchased from Beijing Xingjike Biotechnology Co., Ltd (Beijing, China). FBS, Hank's balanced salt solution, nonessential amino acids, and the rapid equilibrium device (RED) were purchased from Gibco by Thermo Fisher Scientific (Shanghai, China). Dulbecco's modified Eagle's medium was purchased from Corning. 2-(N-morpholino)ethanesulfonic acid was purchased from Sigma (Shanghai, China). The 96-well equilibrium dialysis plate was purchased from LLC (CT). All other chemicals and materials were purchased from Solarbio S&T Co., LTD (Beijing, China).

Determination of Human Hepatocyte CLint. Test compound was prepared to 10 mM in 100% DMSO and further diluted to 100 μM in 100% acetonitrile. The hepatocyte incubations were prepared in Leibovitz's L-15 Medium (pH 7.4) containing 1 million hepatocytes/ml and a final compound concentration of 1 μM. Cell viability was determined using a Cellometer Vision, and >80% cell viability

was required to proceed with the compound incubation. The compound/cell solution (250 μ l) was incubated for 2 hours at 37°C and shaken at 900 rpm on an Eppendorf Thermomixer Comfort plate shaker. Samples (20 μ l) were taken at 0.5, 5, 15, 30, 45, 60, 80, 100, and 120 minutes and quenched with 100 μ l of 100% ice-cold acetonitrile. Samples were shaken at 800 rpm for 2 minutes and centrifuged at 4000 rpm for 20 minutes at 4°C to pellet precipitated protein. The supernatant fraction was diluted 1:5 with deionized water, shaken at 1000 rpm for 2 minutes, and further diluted 1:1 with deionized water. Samples were analyzed by liquid chromatography (LC)–mass spectrometry (MS)/MS.

As described previously (Williamson et al., 2020), submicromolar K_m values (lower than compound assay concentrations) can impact CL_{int}, but occurrence is infrequent, and determination of K_m was beyond the scope of this work. Hence, 1 μ M was selected as an appropriate concentration in the CL_{int} assays.

Determination of Human Microsome CL_{int}. Test compound was prepared to 10 mM in 100% DMSO and further diluted to 100 μ M in 100% acetonitrile. The microsomal incubations were prepared in phosphate buffered solution (pH 7.4) containing 1 mg/ml microsomal protein, 1 mM NADPH, and a final compound concentration of 1 μ M. After a preincubation with NADPH for 8 minutes, reactions were initiated through the addition of the test compound (final volume 250 μ l) and incubated at 37°C in a water bath for 30 minutes. At each time point (0.5, 5, 10, 15, 20, 30 minutes), 20 μ l of incubation mixture was quenched with 100 μ l of 100% ice-cold acetonitrile. Samples were shaken at 800 rpm for 2 minutes and centrifuged at 4000 rpm for 20 minutes at 4°C to pellet precipitated protein. The supernatant fraction was diluted 1:5 with deionized water, shaken at 1000 rpm for 2 minutes, and further diluted 1:1 with deionized water. Samples were analyzed by LC-MS/MS.

Determination of Human Plasma Protein Binding. Plasma protein binding was completed using an RED device. Test compound was prepared to 1 mM in 100% DMSO and further diluted in plasma to achieve a final compound concentration of 5 μ M in the incubation. Immediately, 50 μ l of the spiked plasma was aliquoted as a control T = 0 sample. The T = 0 sample was matrix-matched with 50 μ l of blank phosphate buffered solution (pH 7.4) and quenched with 400 μ l of 100% ice-cold acetonitrile. Phosphate buffered solution (pH 7.4; 500 μ l) was added to the receiver chamber of the RED device, and spiked plasma (300 μ l) was added to the donor chamber. The plate was covered with a gas-permeable lid and incubated for 18 hours at 37°C with 5% CO₂ on an orbital shaker at 300 rpm. Remaining spiked plasma was incubated in a plastic plate for 18 hours at 37°C with 5% CO₂ on an orbital shaker at 300 rpm, representing an T = 18 hours sample. At the end of incubation, 50 μ l of post dialysis sample from the donor or T = 18 sample and receiver wells were aliquoted into separate wells and matrix matched with 50 μ l of phosphate buffered solution (pH 7.4) or blank plasma, respectively. The samples were subsequently quenched separately in 400 μ l of ice-cold 100% acetonitrile. Quenched samples, including T = 0, were shaken at 1000 rpm for 10 minutes and centrifuged for 30 minutes at 4000 rpm to pellet precipitated protein. The supernatant fraction was further diluted 1:1 with deionized water for analysis by LC-MS/MS. An eight-point calibration curve (1–7500 nM) matrix matched with plasma or phosphate buffered solution and quenched with 400 μ l of ice-cold 100% acetonitrile was used to determine the concentration in the donor and receiver wells. Compound recovery and stability in the plasma were determined using the T = 0 and T = 18 samples.

Determination of fu_{inc}. HLM or RH binding was completed using a 96-well equilibrium dialysis plate. Test compound was prepared to 100 μ M in 100% DMSO and further diluted in 1 mg/ml HLM/phosphate buffer or 1 X 10⁶ inactivated (1 hour incubation with 1 mM 1-Aminobenzotriazole and 1.5 mM salicylamide) RH/phosphate buffer to achieve a final compound concentration of 1 μ M in the incubation. Immediately, 50 μ l of the spiked RH or HLM/phosphate buffered solution was aliquoted as a control T = 0 sample. The T = 0 sample was matrix matched with 50 μ l of blank phosphate buffered solution (pH 7.4) and quenched with 400 μ l of 100% ice-cold acetonitrile. Phosphate buffered solution (pH 7.4; 150 μ l) was added to the receiver chamber of the dialysis block and spiked HLM/RH suspension (150 μ l) was added to the donor chamber. The plate was covered with a gas-permeable lid and incubated for 4 hours at 37°C with 5% CO₂ on an orbital shaker at 350 rpm. Remaining spiked matrix was incubated in a plastic plate for 18 hours at 37°C with 5% CO₂ on an orbital shaker at 300 rpm, representing an T = 18 hours sample. At the end of incubation, 50 μ l of postdialysis sample from the donor or T = 18 sample and receiver wells were matrix-matched with 50 μ l of phosphate buffered solution (pH 7.4) or blank RH or HLM/phosphate buffer, respectively. The samples were subsequently

quenched separately in 400 μ l of ice-cold 100% acetonitrile. Quenched samples were shaken at 1000 rpm for 10 minutes and centrifuged for 30 minutes at 4000 rpm to pellet precipitated protein. The supernatant fraction was further diluted 1:1 with deionized water for analysis by LC-MS/MS. For RH fu_{inc}, a five-point calibration curve (1–2000 nM) was generated, and for HLM fu_{inc}, a six-point calibration curve (1–2000 nM) was generated. Each standard was matrix matched with RH or HLM/phosphate buffer and phosphate buffered solution and quenched with 400 μ l of ice-cold 100% acetonitrile. The calibration curve was used to determine the concentration in the donor and receiver wells. Compound recovery and stability in the matrix were determined using the T = 0 and T = 18 samples.

Determination of Caco-2 or MDCK-MDR1 Permeability and Cell Efflux Ratio. Test compound was prepared to 10 mM in 100% DMSO and further diluted to 200 μ M in 100% acetonitrile. Caco-2 and MDCK-MDR1 cells were diluted in Dulbecco's modified Eagle's medium (culture medium) at a density of 6.86 X 10⁵ and 1.56 X 10⁶ cells/ml, respectively. Fifty microliters of cell suspension was added to a 96-well transwell insert plate containing 50 μ l of culture medium. The base plate contained 25 ml of culture medium. The Caco-2 cells were cultured for 14–18 days, and MDCK cells were cultured for 4–8 days, with culture medium replaced every other day. Once confluent (as determined by lucifer yellow) and electrical resistance was >230 Ω .cm² for Caco-2 or >42 Ω .cm² for MDCK, the cells were used in the assay. The cells were washed and incubated with Hank's balanced salt solution containing 25 mM HEPES (pH 7.4) (transport buffer) for 30 minutes. Compounds were diluted to 2 mM in 100% DMSO and then further diluted to 10 μ M in transport buffer. To determine the rate of compound transport in the apical to basolateral (A-B) direction, 108 μ l of the 10 μ M compound solution was added to the transwell (apical), and 300 μ l of transport buffer was added to the receiver well (basolateral). Immediately, 8 μ l from the apical compound solution was diluted in 72 μ l of transport buffer and quenched with 240 μ l of ice-cold 100% acetonitrile; this sample represented a control T = 0 sample. Similarly, to determine the rate of compound transport in the basolateral to apical (B-A) direction, 308 μ l of the 10 μ M compound solution was added to the receiver well (basolateral), and 100 μ l of transport buffer was added to the transwell (apical). A T = 0 sample was immediately prepared by diluting 8 μ l from the basolateral compound solution in 72 μ l of transport buffer and quenching with 240 μ l of ice-cold 100% acetonitrile. The plates were then incubated at 37°C for 2 hours. At the end of the 2 hour incubation, 8 μ l of sample was aliquot from the donor side (apical for A-B and basolateral for B-A) and added to 72 μ l of transport buffer and 240 μ l of ice-cold 100% acetonitrile. For the receiver compartments (basolateral for A-B and apical for B-A), 72 μ l was aliquoted and added to 240 μ l of ice-cold 100% acetonitrile. Quenched samples were shaken at 1000 rpm for 5 minutes and centrifuged for 20 minutes at 4000 rpm to pellet precipitated protein. The supernatant fraction was further diluted 1:1 with deionized water for analysis by LC-MS/MS.

CYP Reaction Phenotyping. Test compound was prepared to 10 mM in 100% DMSO and further diluted to 200 μ M in 100% acetonitrile. Recombinant human CYP (rCYP: CYP3A4, CYP3A5, CYP2C9, CYP2C19, CYP2C8, CYP1A2, CYP2D6, CYP2E1, CYP2B6) were diluted with phosphate buffered solution (pH 7.4) to achieve a final concentration of 112 pmol/ml. The diluted CYP solution was preincubated with test compound (final concentration 2 μ M) at 37°C for 15 minutes. The reaction was initiated through the addition of the 10 mM NADPH and incubated at 37°C on a plate shaker at 100 rpm for 30 minutes. At each time point (2.5, 5, 10, 15, 20, 30 minutes), 30 μ l of incubation mixture was quenched with 120 μ l of 100% ice-cold acetonitrile. Samples were shaken at 1000 rpm for 10 minutes and centrifuged at 4000 rpm for 20 minutes. The quench plate was further incubated at 4°C for 30 minutes and recentrifuged at 4000 rpm for 20 minutes to pellet precipitated protein. The supernatant fraction was diluted 1:1 with deionized water, shaken at 1000 rpm for 2 minutes, and analyzed by LC-MS/MS.

LC-MS/MS Analysis. The MS/MS instrument used was either a Waters XEVO TQ-S, Waters XEVO TQ-D, or API 4000 (AB Sciex). The ultra-mass spectrometer used for sample analysis was completed in the multiple reaction monitoring mode (MS/MS). Reverse-phase high performance liquid chromatography with a C18 column was used to separate the analytes. A mobile phase of 99% water/0.1% formic acid (solvent A) and a solvent phase of 99% acetonitrile/0.1% formic acid (solvent B) was used. A generic LC gradient elution was used at a flow rate of 0.5 ml/min with 95% solvent A and 5% solvent B for 0.3 minutes, after which the concentration of solvent B was increased to 95% over 0.9 minutes

before restoring it back to 5% for the remaining 0.5 minutes. Mass-spectrometer methods were optimized for each compound.

For all assays, the 100% ice-cold acetonitrile used to quench the samples contained internal standards to ensure efficient extraction of sample, confirm injection into the mass spectrometer, and allow assessment of ionization variability. Data were accepted if the internal standard peak area coefficient of variation was <20%.

Data Analysis. The $t_{1/2}$ and, subsequently, the CLint of the compounds incubated in HH or HLM were calculated according to eqs. 1 and 2.

$$t_{1/2}(\text{min}) = \frac{\text{Ln}(2)}{-\text{slope}}. \quad (1)$$

$$\text{CL}_{\text{int}}(\mu\text{L}/\text{min} / \times 10^6 \text{ cells or mg protein}) = \frac{\text{Ln}(2) \times V}{t_{1/2}}. \quad (2)$$

In which V ($\mu\text{L}/\times 10^6$ cells or mg protein) is the incubation volume (μL) divided by the number of cells ($\times 10^6$) or microsomal protein content (mg) in the incubation.

The unbound fraction (fu) of the compounds in human plasma, HLM, or RH was calculated according to eq. 3:

$$\text{Fraction unbound (fu)} = \frac{[\text{Compound}]_{\text{Receiver}}}{[\text{Compound}]_{\text{Donor}}}. \quad (3)$$

Compound recovery and stability in the relevant matrix were determined according to eqs. 4 and 5:

$$\text{Compound recovery (\%)} = \frac{[\text{Compound}]_{\text{Receiver}} + [\text{Compound}]_{\text{Donor}}}{[\text{Compound}]_{T=0}} \times 100. \quad (4)$$

$$\text{Compound remaining at 18 h (\%)} = \frac{[\text{Compound}]_{T=18}}{[\text{Compound}]_{T=0}} \times 100. \quad (5)$$

The $t_{1/2}$ and, subsequently, the CLint of the compounds incubated with rCYP were determined as detailed in eqs. 6 and 7:

$$t_{1/2\text{rCYP}}(\text{min}) = \frac{\text{Ln}(2)}{-\text{slope}}. \quad (6)$$

$$\text{CL}_{\text{int,rCYP}}(\mu\text{L}/\text{min}/\text{pmol}) = \frac{\text{Ln}(2) \times V}{t_{1/2}}. \quad (7)$$

In which, V ($\mu\text{L}/\text{mg}$ protein) is the incubation volume divided by the mg of protein in the incubation.

The $\text{CL}_{\text{int,rCYP}}$ was further scaled to account for CYP450 abundance and the intersystem extrapolation factors (ISEFs) utilizing the respective values incorporated in Simcyp (v19) using eq. 8:

$$\text{CL}_{\text{int,CYP}_i}(\mu\text{L}/\text{min}/\text{mg protein}) = \text{CL}_{\text{int,rCYP}_i} \times \text{CYP}_i\text{abundance} \times \text{ISEF}_{\text{CYP}_i}. \quad (8)$$

In which CYP_i is the i th CYP isoform tested out of n CYP isoforms. $\text{CL}_{\text{int,CYP}_i}$ is the scaled CLint for the i th CYP isoform, $\text{CL}_{\text{int,rCYP}_i}$ is the CLint determined for the i th CYP isoform in rCYP ($\mu\text{L}/\text{min}/\text{pmol}$) (eq. 7), CYP_i abundance is the abundance of the i th CYP isoform in the HLM (pmol of cytochrome P450/mg protein), and $\text{ISEF}_{\text{CYP}_i}$ is the ISEF for the i th CYP isoform.

The scaled $\text{CL}_{\text{int,CYP}}$ values were summed to give the total scaled CLint in HLM, and the contribution of each CYP isoform in HLM was determined according to eq. 9.

$$\text{Contribution}_{\text{CYP}_i}(\%) = \frac{\text{CL}_{\text{int,CYP}_i}}{\sum_{i=1}^n \text{CL}_{\text{int,CYP}_i}} \times 100. \quad (9)$$

HLM:HH CLint ratio. Scaled HH and scaled HLM CLint values (ml/min/kg, eq. 10) were compared for each compound to calculate the difference, which was referred to as HLM:HH CLint ratio. Specifically, scaled HLM CLint (ml/min/kg) was divided by scaled HH CLint (ml/min/kg). Assuming incubational binding was consistent between HLM and HH (Chen et al., 2017; Winiwarter et al., 2019), the difference in scaled CLint (ml/min/kg) between the matrices was expected to be ~ 1 (based on the physiologic scaling factors noted in Table 1).

HLM or HH scaled CLint (mL/min/kg)

$$= \left[(\text{microsomal protein (mg/g liver)} \text{ OR } \text{hepatocellularity} (\times 10^6/\text{g liver})) \times \frac{\text{liver weight (g)}}{\text{body weight (kg)}} \right] / 1000. \quad (10)$$

IVIVE. To compare *in vitro* hepatic CLint and *in vivo* CL for the 140 compound set, the WSM (eq. 11) (Rowland et al., 1973; Yang et al., 2007) was applied with a regression offset to correct for the observed systematic underprediction of *in vivo* CL:

$$\text{CL}_{\text{met}}(\text{mL}/\text{min}/\text{kg}) = \frac{(Q_h \times f_u \times \text{CL}_{\text{int,u}})}{(Q_h + f_u \times \text{CL}_{\text{int,u}})}. \quad (11)$$

Where, CLmet is the *in vivo* CL determined in plasma (assuming CL is hepatic metabolic), Q_h is hepatic blood flow (mL/min/kg), f_u is the free fraction determined in plasma, and $\text{CL}_{\text{int,u}}$ is the scaled unbound intrinsic metabolic CL determined from HH or HLM (mL/min/kg).

Regression Offset Approach.

1. HH or HLM CLint values corrected for fuinc were scaled to the whole liver using physiological scaling factors (eq. 10; Table 1) to generate an *in vitro* CLint,u (units: mL/min/kg).
2. *In vivo* CLint (units: mL/min/kg) was back-calculated from human *in vivo* CLtotal values (*in vivo* CLint,u mL/min/kg), assuming hepatic metabolic CL and using the WSM (eq. 11) to deconvolute hepatic blood flow and fu in the blood (Yang et al., 2007).
3. Using a training set of 24 metabolically cleared drugs, the *in vitro* CLint,u and *in vivo* CLint,u values were compared for HH and HLM. A systematic underprediction of *in vivo* CLint,u from *in vitro* CLint,u was observed for both matrices. In our laboratory, the regression offset required to correct the underprediction was 3-fold for HLM and HH (Riley et al., 2005; Sohlenius-Sternbeck et al., 2012).
4. For the 140 compound set, the regression offset, previously defined as 3-fold (see point 3 above), was applied prospectively to the *in vitro* CLint,u from HH and HLM and compared with the *in vivo* CLint,u values. If the CLint,u values (regression offset applied) for a compound were within 3-fold of unity, CLmet was categorized as correctly predicted. Overpredictions and underpredictions of *in vivo* CLint,u were categorized as greater than 3-fold differences.
5. For scaling without the application of a regression offset, the *in vitro* CLint,u was calculated solely using the WSM (eq. 11).

Caco-2 and MDCK-MDR1 Papp (eq. 12) and, subsequently, efflux ratio were determined according to eq. 13:

$$\text{Papp}(\times 10^{-6} \text{ cm/s}) = \frac{V_A}{\text{Area} \times \text{Time}} \times \frac{[\text{Compound}]_{\text{Acceptor}}}{[\text{Compound}]_{\text{Initial donor}}}. \quad (12)$$

In which, V_A is the volume in the acceptor well, Area is the surface area of the membrane, and Time is the total transport time.

$$\text{Efflux ratio} = \frac{\text{Papp}_{(B-A)}}{\text{Papp}_{(A-B)}}. \quad (13)$$

The average fold error (AFE) (eq. 14) and absolute average fold error (AAFE) (eq. 15) were calculated to determine the bias (AFE) and precision (AAFE) of the CL predictions:

$$\text{AFE} = 10^{\frac{1}{n} \sum \log \left(\frac{\text{Observed}}{\text{Predicted}} \right)}. \quad (14)$$

$$\text{AAFE} = 10^{\frac{1}{n} \sum \left| \log \left(\frac{\text{Observed}}{\text{Predicted}} \right) \right|}. \quad (15)$$

HLM:HH CLint ratio data were not normally distributed. Therefore, a Kruskal-Wallis with Dunn's multiple comparison correction was used to determine whether there was a difference in HLM:HH CLint ratio median between compounds for different classes (e.g., ion class, main metabolizing enzymes, etc.). To determine the Kruskal-Wallis statistic (H) and the probability (P), all data were pooled (ignoring the group from which the data belong) and ranked in ascending order. The rank sums were then combined to generate the P value and

a single statistic value termed *H*. A large *H* refers to a large difference between rank sums. If the Kruskal-Wallis test was significant, a Dunn's multiple comparison was used to determine which groups were statistically different from each other by calculating a *P* value (McDonald, 2014a; Dinno, 2015; Weaver et al., 2017).

A paired *T* test was used to compare HLM and HH CLint,u for uridine 5'-diphospho-glucuronosyltransferase (UGT), CYP1A, CYP2C, CYP2D6, and others because the variables were normally distributed, and a Wilcoxon matched-pairs signed rank test (McDonald, 2014b) was used to compare HLM and HH CLint,u for CYP3A because the variable was not normally distributed.

Results

HLM:HH CLint Ratio Dependencies. Assuming similar metabolic rates and routes, scaled HLM and HH CLint (*in vitro* CLint,u ml/min/kg) were expected to be equivalent (HLM:HH CLint ratio = 1). In our data set, 51% of compounds had an HLM:HH CLint ratio ~1, with a maximum observed HLM:HH CLint ratio of 15. The mean HLM:HH CLint ratio was 1.9, and the median was 1.1 for the 140 compound data set. Inherent experimental variability in the HLM and HH *in vitro* assays was assessed (unpublished data), and ~95% of replicate CLint determinations for the same compound were within 2-fold. Thus, we categorized HLM:HH CLint ratio ≥2-fold as a significant biologic difference.

The HLM:HH CLint ratio was significantly different between bases (mean 2.1) and acids (mean 1.0). However, the HLM:HH CLint ratio was not significantly different between neutrals and acids or neutrals and bases (Fig. 1A). HLM:HH CLint ratio was not correlated with MW, LogD, pKa, human Vd, or human CL (Fig. 1, B–F).

The dependence between the main metabolizing enzyme for compounds and HLM:HH CLint ratio was evaluated for the compound data set. The HLM:HH CLint ratio varied between metabolizing enzymes with the highest HLM:HH CLint ratio observed for CYP3A substrates (mean/median HLM:HH CLint ratio = 2.8/2.1) (Fig. 2; Supplemental Table 1). The difference in *in vitro* scaled CLint between HH and HLM was significantly different for CYP3A substrates (Wilcoxon matched pairs: *P* < 0.0001). UGT substrates displayed a mean HLM:HH CLint ratio >1, but the median was 0.8. For CYP2C, CYP2D6, and CYP1A substrates, the HLM:HH CLint ratio was ~1 (Fig. 2). The data set also contained 16 compounds (11%, referred to as “Other” in Fig. 2) that were reported to be cleared via enzymes other than the major CYPs or UGTs, or the route was not defined, and the mean HLM:HH CLint ratio was 1.4. Interestingly, the vast majority (78%) of compounds with an HLM:HH CLint ratio ≥2 were CYP3A substrates. CYP3A HLM:HH CLint ratio was significantly different to the ratio determined for CYP2C, CYP2D6, and “other” substrates.

Overlapping substrate specificities between CYP3A and Pgp were investigated to understand whether efflux in HH provided an explanation for the high HLM:HH CLint ratio (Fig. 3). Caco-2 efflux ratio (ER) and MDCK-MDR1 ER were determined for 41 and 28 compounds, respectively, and represent compounds from all metabolizing enzyme families. For compounds with an ER >2 in Caco-2 and MDCK-MDR1 cells, the mean/median HLM:HH CLint ratio was 3.8/3.1 and 2.7/2.5, respectively. This was in contrast to the compounds with an ER <2, which displayed mean/median HLM:HH CLint ratio of 1.3/1.0 and 1.6/1.1 in Caco-2 and MDCK-MDR1 cells, respectively. For compounds with an HLM:HH CLint ratio ≥2, 50% and 64% of these compounds displayed an ER >2 in Caco-2 and MDCK-MDR1, respectively. For CYP3A substrates that had an HLM:HH CLint ratio ≥2, 55% had an ER >2 in Caco-2 cells, and 67% had an ER >2 in MDCK-MDR1 cells.

In Vitro In Vivo Extrapolation. IVIVE accuracy of *in vivo* CLint,u was evaluated using both the regression offset approach and with no offset applied for HLM and HH as outlined in *Materials and Methods*.

When evaluating the set of 140 compounds as a whole, IVIVE performance using the regression offset showed minimal bias and good precision for both matrices. For HH, AFE was 1.3, AAFE was 2.9, and % compounds overpredicted/correctly predicted/underpredicted was 25/62/13, respectively, whereas for HLM, AFE was 1.6, AAFE 3.6, and % compounds overpredicted/correctly predicted/underpredicted was 34/52/14, respectively.

Prediction accuracy using the regression offset was comparable between HLM and HH when evaluating subcategories of UGT, CYP2D6, CYP2C, CYP1A, and “other” substrates (Figs. 4 and 5). However, using HLM and the regression offset approach for CYP3A substrates demonstrated a clear overprediction bias (AFE 3.1, AAFE 4.8, % compounds overpredicted/correctly predicted/underpredicted 56/33/11, respectively) (Fig. 4E; Table 2). A comparison between CYP3A and non-CYP3A substrates using HLM and the regression offset approach is shown in Fig. 6. Conducting IVIVE using HLM without a regression offset for CYP3A substrates broadly corrected this overprediction bias (AFE 1.0, AAFE 3.2, % compounds overpredicted/correctly predicted/underpredicted 20/61/19, respectively, Fig. 6B; Table 2). However, using this approach (HLM without a regression offset) for the remaining non-CYP3A substrates led to a marked underprediction of *in vivo* CLint,u (AFE 0.3, AAFE 4.4, % compounds overpredicted/correctly predicted/underpredicted 4/34/62, respectively, Fig. 6B; Table 2).

Using HH and the regression offset for CYP3A substrates demonstrated no clear bias (AFE 1.6, AAFE 3.1, % compounds overpredicted/correctly predicted/underpredicted 27/62/11, respectively) (Fig. 5E; Table 2). Likewise, a comparison between CYP3A and non-CYP3A substrates using HH showed no discernible difference in predictive performance (Fig. 6C; Table 2). It followed that conducting IVIVE using HH without a regression offset resulted in a significant underprediction bias for both CYP3A and non-CYP3A compounds (Table 2).

Discussion

To ensure sufficient target engagement, CL is often a key parameter to optimize before progression of oral candidate drugs into clinical development. With the majority of drugs eliminated via hepatic metabolic enzymes (Cerny, 2016), low CLint in HLM and HH is targeted, and the values are used to predict human metabolic CL. A systematic underprediction of *in vivo* CLint from both these hepatic matrices appears to be universally apparent (Riley et al., 2005; Sohlenius-Sternbeck et al., 2012; Bowman and Benet, 2019a), although it can be corrected for via a regression offset approach (Riley et al., 2005; Sohlenius-Sternbeck et al., 2012) and has been used prospectively to allow successful prediction of *in vivo* CL for many candidate drugs in our laboratory (Davies et al., 2020).

Typically, both HLM and HH are used in drug discovery. Once scaled, the HLM:HH CLint ratio should be ~1 for drugs cleared by the same drug metabolism enzyme pathways in both systems. However, significant differences between HLM and HH CLint have been highlighted by several groups (Stringer et al., 2008; Foster et al., 2011; Bowman and Benet, 2019b), and this “HLM:HH disconnect” has also been observed in our laboratory. When encountered in drug discovery, this HLM:HH disconnect phenomenon poses challenges; firstly to understand the reason(s) for differences in CLint for compounds cleared by the same enzymes and secondly to decide how to approach IVIVE for compounds that demonstrate this disconnect.

No correlation was observed between HLM:HH CLint ratio and human *in vivo* CL, human Vd, LogD, MW, or compound pKa for the 140 compound dataset (Fig. 1). The lack of correlation between HLM:HH CLint ratio and pKa is consistent with the hypothesis that the pH

Fig. 1. Relationship between HLM:HH CLint ratio and physicochemical properties or human PK: (A) Ion class, (B) MW, (C) LogD, (D) Predicted pKa, (E) Human Vd and (F) Human CL. Where, in (A) the black box and whisker represent the median and 95% confidence intervals, and the blue solid line is the mean and in (A-F) the solid red horizontal line represents no difference in scaled HLM and HH CLint, and the dashed red horizontal lines represent a 2-fold difference in scaled HLM and HH CLint. HLM:HH CLint ratio was significantly different between bases (mean 2.1) and acids (mean 1.0) (Kruskal-Wallis: $P < 0.0001$, $H = 53.9$, Dunn's: acids-bases $P = 0.036$). The HLM:HH CLint ratio was not significantly different between acids and neutrals or neutrals and bases.

integrity of hepatocytes in suspension is destroyed (Berezhevskiy, 2011), resulting in the same levels of ionized species present in hepatocyte and microsome assays conducted at pH 7.4. However, there was an observation that neutrals and bases (HLM:HH CLint ratio 2.1) demonstrate this HLM:HH disconnect more so than acids (HLM:HH CLint ratio 1.0), but this difference was only significant between acids and bases and would need more data specifically using acids to confirm. Another consideration is the potential for acids to be substrates of the hepatic organic anion transporting polypeptides (OATPs) present on the membrane of HH. Active uptake into HH has the potential to become the rate-limiting step for the CL of these compounds. However, as shown previously (Di et al., 2012) and as further exemplified here, there was no difference in HLM:HH CLint ratio between OATP substrates [including paritaprevir, lesinurad, and repaglinide (Shebley et al., 2017; <https://didb.druginteractionsolutions.org/drug/monograph/11166/#main-transporters>; <https://didb.druginteractionsolutions.org/drug/monograph/1269/#main-transporters>)] and non-OATP substrates, suggesting there is no OATP dependence for the HLM:HH CLint ratio.

Building on the work by Bowman and Benet (2019b), the association of HLM:HH CLint ratio and metabolism via the major hepatic metabolic enzymes was assessed (Fig. 2). For CYP1A, CYP2C, and CYP2D6 substrates, the HLM:HH CLint ratio value was ~ 1 , consistent with the CLint routes and rates being similar between matrices. Although UGT substrates displayed a high mean HLM:HH CLint ratio, this was influenced by two outliers (edaravone and mizolastine). The median HLM:HH CLint ratio was 0.8, < 1 , as would be expected with the additional metabolism routes present in HH versus HLM. In agreement with Bowman and Benet (2019b), CYP3A substrates displayed a significantly higher CLint in HLM versus HH. These data conducted

on such a large number of compounds confirm that this HLM:HH disconnect phenomenon is highly, if not exclusively, associated by cause or effect with CYP3A substrates. This would also explain the emerging trend that acids, which are not typically substrates of CYP3A, tend not to demonstrate this phenomenon. A thorough mechanistic understanding of the basis for the high HLM CLint relative to HH CLint and the explanation for the strong association of this phenomenon toward CYP3A substrates are clearly desirable. Efflux transporter activity in hepatocytes has been hypothesized to restrict compound access to metabolism enzymes in hepatocytes relative to unhindered access to the same enzymes in microsomes, thus leading to a high liver microsome: hepatocyte CLint ratio (Huang et al., 2010). Hence, overlapping substrate specificities between CYP3A and Pgp (Kim et al., 1999) may contribute to the observed HLM:HH CLint disconnect (Bowman and Benet, 2019b). We considered the relationship between ER and HLM:HH CLint ratio and determined that efflux transporter substrates displayed an HLM:HH disconnect (HLM:HH CLint ratio > 2), whereas non-efflux substrates demonstrated an average HLM:HH CLint ratio of ~ 1 , the theoretical expected ratio, irrespective of the cell line used to determine efflux potential (Fig. 3). These data suggest that an HLM:HH disconnect may be apparent for efflux substrates as a result of Pgp activity restricting CLint in HH relative to HLM. However, only $\sim 50\%$ of CYP3A substrates that displayed an HLM:HH CLint ratio ≥ 2 had an ER > 2 , as determined in either MDCK or Caco-2 cells. Therefore, it would appear that the reason CYP3A substrates demonstrate this phenomenon as a class is not exclusively explained by Pgp activity. Additional confirmatory work would be beneficial to explore the dependence on HLM:HH CLint ratio with Pgp and other transporter activity. It should be noted that all efflux substrates in this

	UGT	CYP2C	CYP2D6	CYP1A	CYP3A	Other
No. of compounds	11	15	22	13	63	16
Mean	2.3	0.8	1.1	1	2.8	1.4
Median	0.8	0.7	0.9	1.1	2.1	0.9
Median 95% CI	0.4 - 3.7	0.6 - 1.0	0.7 - 1.6	0.5 - 1.5	1.4 - 2.8	0.3 - 1.6

Fig. 2. Evaluation of HLM:HH CLint ratio and the main metabolizing enzyme. Where, the black box and whisker solid lines represent the median and 95% confidence intervals (CI), and the blue solid line is the mean. The solid red horizontal line represents no difference in scaled HLM and HH CLint, and the dashed red horizontal lines represent a 2-fold difference in scaled HLM and HH CLint. CYP3A HLM:HH CLint ratio was significantly different to CYP2C, CYP2D6, and Other (Kruskal-Wallis $P < 0.0001$, $H = 29.4$, Dunn's: $P = 0.0006$, $P = 0.009$, and $P = 0.03$, respectively).

data set (when measured) were highly permeable in the Caco-2 assay, suggesting passive permeability across a cell membrane was not a rate-limiting factor in hepatocytes. Furthermore, no correlation was observed between HLM:HH CLint ratio and passive permeability (unpublished data). There may be further additional contributory factors other than transporter activity that may lead to high HLM:HH CLint ratio, for example, whether the high HLM:HH CLint for CYP3A substrates is associated with cryopreserved and not freshly prepared HH. Hepatocyte cryopreservation would have to specifically decrease CYP3A isoform activity, possibly because of conformational changes in the enzyme or via affecting cofactor levels. Considering the high proportion of candidate drugs that are CYP3A substrates, more work in our laboratory and others is warranted.

Although the use of a regression offset approach to correct the widely reported systematic underprediction of *in vivo* CLint is a pragmatic solution to predict CL, it is not mechanistically satisfactory given the empirical nature of this approach. Similar to previous reports, from our

laboratory and others (Riley et al., 2005; Sohlenius-Sternbeck et al., 2012), IVIVE predictive capability utilizing the regression offset approach for the whole dataset of compounds was broadly similar between HH and HLM; 62% and 52% of compounds predicted *in vivo* CLint,u within 3-fold from HH (AFE 1.3, AAFE 2.9) and HLM (AFE 1.6, AAFE 3.6).

Approximately 80% of compounds in this data set with an HLM:HH CLint ratio ≥ 2 were CYP3A substrates, and likewise, our in-house experience with propriety AstraZeneca compounds demonstrates this phenomenon being strongly associated with CYP3A substrates. Given this strong association and the preponderance of candidate drugs metabolized primarily by CYP3A, our analysis focused on comparing and contrasting IVIVE performance for CYP3A versus non-CYP3A substrates. It was observed that when using HLM with an IVIVE regression offset approach for CYP3A substrates (Figs. 4E and 6A; Table 2), there was an overprediction (>3 -fold) of *in vivo* CLint,u (AFE 3.1) for 56% compounds, with only 33% correctly predicted. Not using the regression offset corrected this overprediction so that AFE approached 1, and the number of CYP3A substrates overpredicted reduced from 56% to 20%, and those correctly predicted increased from 33% to 61% (Fig. 6B; Table 2). These data are consistent with previous reports for CYP3A substrates, which employed IVIVE without the use of a regression offset and demonstrated good IVIVE accuracy from HLM (Bowman and Benet, 2019b). However, using HLM without a regression offset for non-CYP3A substrates led to a marked underprediction of *in vivo* CLint,u (AFE 0.3) for 62% compounds, with 34% correctly predicted (Fig. 6B; Table 2). These data highlight that when using HLM for IVIVE, a different regression offset factor is optimal for scaling CYP3A substrates versus non-CYP3A substrates. A full mechanistic understanding is not immediately apparent, but the observation of both CYP isoform and matrix dependence on IVIVE methodology to predict CLmet may help to delineate the underlying reasons in the future. Based on these analyses, it is important to carefully consider IVIVE approaches so that CL is appropriately predicted from HLM data for each substrate class (Riley et al., 2005; Chiba et al., 2009; Bowman and Benet, 2016).

In contrast, for HH, no prediction bias using the IVIVE regression offset approach existed for all categories of compound, including CYP3A substrates, with 62% of such compounds correctly predicted (AFE 1.6, AAFE 3.1) (Fig. 6C; Table 2). Overall, using HH, IVIVE performance using the regression offset approach for the whole data set showed minimal bias and good precision (AFE 1.3, AAFE 2.8) and correctly predicted CLint,u within 3-fold for 62% of compounds, a comparable performance to CYP3A substrates.

Fig. 3. HLM:HH CLint ratio compared with ER determined in (A) Caco-2 cells and (B) MDCK-MDR1 cells. Where, the solid red horizontal line represents no difference in scaled HLM and HH CLint, the dashed red horizontal lines represent a 2-fold difference in scaled HLM and HH CLint, and the solid red vertical line represents the efflux transporter substrate categorization; ≥ 2 = efflux substrate, < 2 = not an efflux substrate. Symbols represent the main metabolizing enzyme: ●, UGT; ○, CYP3A; ■, CYP2D6; ▲, CYP2C; ◆, CYP1A; and *, Other. In Caco-2, the number of compounds from each metabolizing enzyme group was: UGT $n = 3$, CYP3A $n = 22$, CYP2D6 $n = 7$, CYP2C $n = 4$, CYP1A $n = 0$, and Other $n = 5$. In MDCK-MDR1, UGT $n = 1$, CYP3A $n = 16$, CYP2D6 $n = 5$, CYP2C $n = 2$, CYP1A $n = 2$, and Other $n = 2$.

Fig. 4. IVIVE with a regression offset using HLM for substrates of drug metabolism enzyme families. (A) UGT, (B) CYP2C, (C) CYP2D6, (D) CYP1A, (E) CYP3A, and (F) Other. Where, the red solid line is the line of unity, and the red dotted lines represent a 3-fold difference.

Furthermore, based on the data obtained herein, it is reassuring that our current approach of employing an IVIVE regression offset from data generated using HH remains a useful and applicable strategy for all compounds, irrespective of metabolism enzyme. However, in the

authors' opinion, uncertainty in CL_{met} prediction would increase for compounds that were relatively unstable in HLM compared with a more favorable HH CL_{int} value, which highlights the need to use both HLM and HH CL_{int} data to optimize against in drug discovery.

Fig. 5. IVIVE with a regression offset using HH for substrates of drug metabolism enzyme families. (A) UGT, (B) CYP2C, (C) CYP2D6, (D) CYP1A, (E) CYP3A, and (F) Other. Where, the red solid line is the line of unity, and the red dotted lines represent a 3-fold difference.

TABLE 2
IVIVE prediction accuracy with and without a regression offset for CYP3A and non-CYP3A substrates

	Substrates	IVIVE Approach	AFE	AAFE	IVIVE Prediction (%)		
					Over	Correct	Under
HLM	CYP3A	Offset	3.1	4.8	56	33	11
		No Offset	1.1	3.2	20	61	19
	Non-CYP3A	Offset	0.9	2.8	16	68	16
		No Offset	0.3	4.4	4	34	62
HH	CYP3A	Offset	1.6	3.1	27	62	11
		No Offset	0.5	3.2	13	54	33
	Non-CYP3A	Offset	1.1	2.8	22	64	14
		No Offset	0.4	3.8	7	48	45

Underprediction of CL could have detrimental effects on the candidate drug progression and may even result in the termination of the clinical program because of insufficient exposure to test the clinical hypothesis. Hence, in drug discovery, it remains an appropriate if risk-averse strategy to focus on lowering HLM in addition to HH CLint to maximize intrinsic metabolic stability. Thus the authors’ recommendation remains to progress candidate drugs into the clinic with a suitable HH derived CLmet prediction and a correspondingly low HLM CLint. In addition, because a different IVIVE regression offset is now emerging for CYP3A substrates using HLM, the relationship may begin to elucidate a more comprehensive mechanistic understanding of IVIVE rather than the current empirical approach, which, although it is broadly successful, can be improved upon.

In summary, this work highlights the correlation between CYP3A substrates and the HLM:HH disconnect and the subsequent deleterious effect on the accuracy of metabolic CL predictions using HLM with our current IVIVE approach for this group of compounds. We demonstrated the HLM:HH CLint ratio is not correlated with MW, LogD, pKa, human CL, or human Vd. However, more work is required to understand the association, if any, of efflux transporter activity with the HLM:HH disconnect. This work suggests a consistent IVIVE approach can be successfully applied to all compounds using HH irrespective of the main contributing metabolic enzyme and provides enhanced scaling methodologies using HLM. However, without a full mechanistic understanding of this HLM:HH disconnect phenomena, it remains our strategy to minimize both HLM as well as HH CLint.

Fig. 6. IVIVE from HLM and HH. Prediction of *in vivo* CLint,u (A) using HLM with a regression offset, (B) using HLM without a regression offset, and (C) using HH with a regression offset. Where, the blue circles represent CYP3A substrates, large blue circles represent CYP3A substrates with an HLM:HH CLint ratio >2, and the red circles represent non-CYP3A substrates. The black solid line is the line of unity, and the black dotted lines represent a 3-fold difference.

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Authorship Contributions

Participated in research design: Williamson, Harlfinger, McGinnity.

Conducted experiments: Williamson.

Performed data analysis: Williamson.

Wrote or contributed to the writing of the manuscript: Williamson, Harlfinger, McGinnity.

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