An Investigation into the Prediction of In Vivo Clearance for a Range of Flavin-containing Monooxygenase Substrates[§]

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Received July 3, 2017; accepted August 4, 2017

ABSTRACT

Flavin-containing monooxygenases (FMO) are metabolic enzymes mediating the oxygenation of nucleophilic atoms such as nitrogen, sulfur, phosphorus, and selenium. These enzymes share similar properties to the cytochrome P450 system but can be differentiated through heat inactivation and selective substrate inhibition by methimazole. This study investigated 10 compounds with varying degrees of FMO involvement to determine the nature of the correlation between human in vitro and in vivo unbound intrinsic clearance. To confirm and quantify the extent of FMO involvement six of the compounds were investigated in human liver microsomal (HLM) in vitro assays using heat inactivation and methimazole substrate inhibition. Under these conditions FMO contribution varied from 21% (imipramine) to 96% (itopride). Human hepatocyte and

HLM intrinsic clearance (CL_{int}) data were scaled using standard methods to determine the predicted unbound intrinsic clearance (predicted $CL_{int,u}$) for each compound. This was compared with observed unbound intrinsic clearance (observed $CL_{int,u}$) values back calculated from human pharmacokinetic studies. A good correlation was observed between the predicted and observed $CL_{int,u}$ using hepatocytes ($R^2 = 0.69$), with 8 of the 10 compounds investigated within or close to a factor of 2. For HLM the in vitro-in vivo correlation was maintained ($R^2 = 0.84$) but the accuracy was reduced with only 3 out of 10 compounds falling within, or close to, twofold. This study demonstrates that human hepatocytes and HLM can be used with standard scaling approaches to predict the human in vivo clearance for FMO substrates.

Introduction

Flavin-containing monooxygenases (FMO) are a major enzyme system mediating oxygenation of nucleophilic atoms typically nitrogen, sulfur, phosphorus, and selenium. FMOs have an important role in the metabolism of exogenous compounds including many drugs but also endogenous compounds such as trimethylamine (Phillips and Shephard, 2008). These enzymes are similar to cytochrome P450s (CYPs) in cellular location and cofactor requirement (oxygen and NADPH). However, FMOs can be differentiated from CYPs by heat inactivation (maximum of 5 minutes at 40–50°C in the absence of NADPH) and inhibition by specific competitive substrate inhibitors such as methimazole (Cashman and Zhang, 2006; Taniguchi-Takizawa et al., 2015).

There are five FMOs expressed in humans with the major hepatic enzyme being FMO3, which is regarded as having the greatest role in drug metabolism. FMOs are also expressed in extrahepatic tissue, including lung and kidney with FMO1 the major form expressed in kidney and FMO2 mainly expressed in the lung (Cashman and Zhang, 2006). However, in studies that use hepatocytes and human liver microsomes (HLM) only, clearance by FMO may be underestimated due to the lack of expression of FMO1 and 2 in human liver.

Along with differential tissue distribution, the FMOs also have differing substrate specificity. For instance, the FMO1 active site is reported to

accommodate larger tertiary amines and sulfur-containing substrates such as imipramine and chlorpromazine (Kim and Ziegler, 2000). This is supported by studies on tamoxifen (Parte and Kupfer, 2005), which show a higher rate of metabolism in FMO1 versus FMO3. In contrast ranitidine shows a higher rate for both the N and S-oxygenations in FMO3 versus FMO1 (Chung et al., 2000). FMO3 has been shown to metabolize many primary, secondary, and tertiary amines along with various sulfur-containing substrates and is viewed as the principal drug metabolizing FMO in human liver (Cashman and Zhang, 2006). FMO2 metabolizes a variety of sulfur-containing compounds such as thioureas along with various primary, secondary, and tertiary amines including trifluoroperazine (Geier et al., 2015). Little is known about the substrate specific for FMO4 because the protein is unstable. Finally, FMO5, which is expressed at similar levels to FMO3 in the liver (Cashman and Zhang, 2006), shows little reported activity toward drug like molecules (Krueger et al., 2006), although it has been shown that the purified enzyme can carry out Baeyer-Villiger reactions (Fiorentini et al., 2016).

For drugs undergoing oxygenation by FMOs, it is important to be able to quantify their contribution to metabolism and clearance to be able to assess the impact on drug-drug interaction (DDI) profile. When FMO is contributing to metabolism, this may serve to mitigate DDIs from other enzymes such as the CYPs, impacting the overall DDI profile of the drug.

The ability to predict the in vivo clearance (CL) of compounds is a key part of drug metabolism activities. The methods by which clearance is predicted differ depending on the route of elimination, e.g., renal, hepatic,

<https://doi.org/10.1124/dmd.117.077396>.

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

ABBREVIATIONS: ACN, acetonitrile; AO, aldehyde oxidase; CL, clearance; CL_b , blood clearance; CL_{int} , intrinsic clearance; $CL_{int,u}$, unbound intrinsic clearance; CYP, cytochrome P450; DDI, drug-drug interaction; FA, formic acid; FMO, flavin-containing monooxygenases; fu, unbound fraction in plasma; fu_{inc} , unbound fraction in the incubation; HLM, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; $R_{b/p}$, blood/plasma ratio.

etc. (Di et al., 2013; Grime et al., 2013). The method most routinely used for hepatic metabolic clearance is scaling the unbound intrinsic clearance ($CL_{int,u}$) from in vitro systems. Hepatocytes or HLM $CL_{int,u}$ values are scaled to the in vivo situation using factors for the number of cells or milligram protein per gram liver and the ratio of liver weight to body weight and then applying the well-stirred model (Riley et al., 2005; Di et al., 2013). The observation that these in vitro systems can produce systematic under prediction of in vivo CL can be addressed by use of a regression offset method as described previously (Grime and Riley, 2006). However, although this approach generally works well for CYP it has also been shown not to work well for other enzymes substrates such as aldehyde oxidase (AO) where the in vitro data produces a significant under prediction of the in vivo CL (Zientek et al., 2010).

FMO activity in hepatocytes has been demonstrated using benzydamine as marker substrate (Fisher et al., 2002). In this study, rat and human hepatocytes were used to predict in vivo blood clearances (CL_b) using the scaling methodology described above. In this case, the in vivo CL in rat was predicted well from the in vitro CL_{int} (within 30% of the measured value), whereas there was an over prediction of the human CL [>10.5 vs. 2.4 (ml/min)/kg]. However, this is for only one compound and to date there has not been a comprehensive assessment of FMO scaling using a series of substrates with differing FMO contributions.

The aim of this study was to use human in vitro data to predict the CL for a set of compounds for which all or part of the in vivo CL has been determined to be mediated by FMO.

Materials and Methods

Pooled (150 donors; equal sex mix) HLM were obtained from BD Biosciences (Oxford, UK). Human hepatocytes (mixed sex, 10 donors) were obtained from BioreclamationIVT (Baltimore, MD). Frozen human plasma (mixed sex, 78 individuals) generated using K2-EDTA anticoagulant was purchased from BioreclamationIVT. Coculture of the five-donor human hepatocyte pool (lot 1410235; XenoTech (Kansas City, KS)) and nonparenchymal stromal cells (stromal cell type and ratio of hepatocyte per stromal cell are proprietary information) in type I collagen-coated 96-well plates and H μ REL PlatinumHeps medium were purchased from H μ REL (North Brunswick, NJ). FMO substrates benzydamine, tozasertib, itopride, imipramine, clozapine, moclobemide, cimetidine, tamoxifen, ranitidine, and olanzapine used in this study were all synthesized internally at AstraZeneca (Cambridge, UK). β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), methimazole, verapamil, formic acid (FA), ammonium formate, and dimethylsulfoxide were purchased from Sigma-Aldrich (Poole, UK). L-15 medium, methanol, water, and acetonitrile (ACN) were HPLC grade from Thermo Fisher Scientific (Hemel Hempstead, UK). All the other solvents were HPLC grade and, unless otherwise specified, all of the other reagents were purchased from Sigma-Aldrich.

Unless otherwise indicated all experiments were conducted in triplicate.

HLM CL_{int} Incubations

Compounds ($1\ \mu\text{M}$ final concentration) were incubated with HLM ($1\ \text{mg/ml}$) in $100\ \text{mM}$ phosphate buffer (pH 7.4) at 37°C . After a 5-minute preincubation in the presence of NADPH ($1\ \text{mM}$), the reaction was initiated by the addition of the compound in ACN ($<1\%$ final concentration). Samples ($20\ \mu\text{l}$) were removed from the incubation at 0.5, 5, 10, 15, 20, and 30 minutes and added to $100\ \mu\text{l}$ of cold ACN containing internal standard. The samples were then centrifuged at $4000\ \text{rpm}$ and 4°C for 20 minutes to sediment the precipitated proteins before quantitation using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The kinetic data were analyzed using a linear fit of the natural logarithm of the ratio of the compound peak area to the internal standard peak area against time. CL_{int} values were then calculated from the negative slope of the linear fit divided by the microsomal concentration.

Hepatocytes CL_{int} Incubations

The hepatocytes were thawed and resuspended in prewarmed L-15 medium to give a concentration of 1.0×10^6 viable cells/ml ($>80\%$ viability). The incubations were performed in duplicate in a 96-well plate and the reaction

initiated by addition of the compound in ACN ($<1\%$ final concentration) to give a final concentration of $1\ \mu\text{M}$. The plate was incubated at 37°C , and $20\ \mu\text{l}$ samples were removed at 0.5, 5, 15, 30, 45, 60, 80, 100, and 120 minutes and added to $100\ \mu\text{l}$ of cold ACN (containing internal standard). The samples were then centrifuged at $4000\ \text{rpm}$ at 4°C for 20 minutes before quantitation using LC-MS/MS. The kinetic data were analyzed using a linear fit of the natural logarithm of the ratio of the compound peak area to the internal standard peak area against time. CL_{int} values were then calculated from the negative slope of the linear fit divided by the number of hepatocytes.

H μ REL Low CL_{int} Incubations

If the hepatocyte CL_{int} was $<1\ (\mu\text{l/min})/10^6$ cells, then a low intrinsic clearance assay using the H μ REL primary human hepatocyte coculture system was used. This methodology has been described in detail previously (Bonn et al., 2016). Briefly, coculture plates at a seeding density of 30,000 hepatocytes per well were shipped in H μ REL PlatinumHeps shipping medium at 37°C from H μ REL. On arrival, the shipping medium was replaced with PlatinumHeps maintenance medium and the cells left to acclimatize overnight in incubator at 37°C in humidified atmosphere containing 95% air and 5% CO_2 . Before the incubation the cells in each well were washed with $100\ \mu\text{l}$ H μ REL PlatinumHeps dosing medium, which was aspirated and replaced with $50\ \mu\text{l}$ of fresh media before the addition of the test compound, which was added in $50\ \mu\text{l}$ H μ REL PlatinumHeps dosing medium (final concentration $1\ \mu\text{M}$ with $<1\%$ ACN). Samples ($45\ \mu\text{l}$) were taken at six time points (1, 3, 5, 24, 48 and 70 hours) and added to $180\ \mu\text{l}$ ice-cold acetonitrile. Samples were centrifuged (20 minutes 4°C , $4000\ \text{g}$), and $50\ \mu\text{l}$ of the supernatant was diluted within $100\ \mu\text{l}$ water before LC-MS/MS analysis. The kinetic data were analyzed using a linear fit of the natural logarithm of the compound peak area against time. CL_{int} values were then calculated from the negative slope of the linear fit divided by the hepatocyte concentration.

HLM Incubations for Determination of FMO Contribution

For heat inactivation of FMO, HLM were heated using a thermal cycler that was set to start at 4°C and programmed to heat to 50°C for 1 minute and revert to 4°C immediately after. Compounds ($1\ \mu\text{M}$ final concentration) were incubated with control and heat inactivated HLM ($1\ \text{mg/ml}$) in $100\ \text{mM}$ phosphate buffer (pH 7.4) at 37°C . After a 5-minute preincubation in the presence of NADPH ($1\ \text{mM}$) the reaction was initiated by the addition of the compound in ACN ($<1\%$ final concentration). In parallel, compounds ($1\ \mu\text{M}$ final concentration) were also incubated with methimazole ($500\ \mu\text{M}$) to determine the fraction metabolized by FMO. Incubations were performed for 0, 5, 10, 20, 30, and 50 minutes in a shaking incubator at 37°C . Reactions were terminated by the addition of four volumes of ice-cold ACN (containing internal standard), vortexed, and centrifuged at $3500\ \text{rpm}$ for 10 minutes and samples analyzed by LC-MS/MS. The data were analyzed using a linear fit of the natural logarithm of the ratio of the compound peak area to the internal standard peak area against time. CL_{int} values were then calculated from the negative slope of the linear fit divided by the microsomal concentration. The fraction metabolized by FMO was determined by comparing control incubations with incubations where methimazole or heat inactivation was used.

Determination of Unbound Fraction in Human Plasma (fu)

The extent of binding of compounds to plasma proteins was determined by equilibrium dialysis at a compound concentration of $5\ \mu\text{M}$ using the Rapid Equilibrium Device from ThermoScientific Pierce (Hemel Hempstead, UK). Phosphate buffer ($100\ \text{mM}$, pH 7.4) was added to the buffer chamber, and $300\ \mu\text{l}$ of plasma with compound to the sample chamber. The unit was covered with a gas permeable lid and incubated for 18 hours at 37°C at $300\ \text{rpm}$ with 5% CO_2 . At the end of incubation, samples ($50\ \mu\text{l}$) from both buffer and plasma chambers were removed for analysis. Samples and standards were matrix matched and analyzed using LC-MS/MS. The unbound fraction in plasma (fu) was calculated as follows:

$$fu = \text{Conc. buffer chamber} / \text{Conc. plasma chamber}$$

Determination of Unbound Fraction in HLM (fu_{inc})

The extent of binding of compounds to HLM was determined by equilibrium dialysis using the HTDialysis LLC device (Gales Ferry, CT) with HLM at a

concentration of 1 mg/ml and a final compound concentration of 1 μ M. Phosphate-buffered saline (150 μ l) was added to the buffer well and 150 μ l HLM containing the compound to the sample well and incubated at 37°C for 4 hours. After the incubation, 50- μ l aliquots from both donor and receiver wells were removed for analysis. Samples and standards were matrix matched and analyzed by LC-MS/MS. The unbound fraction in the incubation ($f_{u,inc}$) was calculated as follows:

$$f_{u,inc} = \text{Conc. buffer well} / \text{Conc. microsomal suspension well}$$

Determination of Unbound Fraction in Rat Hepatocytes ($f_{u,inc}$)

The extent of binding of compounds to rat hepatocytes was also determined by equilibrium dialysis using the HTDialysis LLC device. Rat hepatocytes were resuspended with prewarmed L-15 medium at 1.0×10^6 viable cells/ml. The test compounds, at a final concentration of 1 μ M, were added to the hepatocyte suspension. One hundred and fifty microliters of the hepatocytes suspension containing the compound was added to each of the sample wells of the 96-well dialysis device and phosphate-buffered saline (150 μ l) to the receiver well and incubated at 37°C for 4 hours. After the incubation the samples and standards were matrix matched and analyzed by LC-MS/MS analysis. The unbound fraction ($f_{u,inc}$) was calculated as follows:

$$f_{u,inc} = \text{Conc. buffer well} / \text{Conc. hepatocyte suspension well}$$

Determination of Blood/Plasma Ratio ($R_{b/p}$)

A volume of plasma sufficient for the assay was obtained from whole human blood by centrifugation (3220 g for 10 minutes at 4°C). The test compound (10 μ M) was added to 398 μ l of the prewarmed human plasma and blood separately and incubated for 30 minutes. After incubation, the blood samples were centrifuged for 10 minutes at 4000 rpm (37°C) and the plasma samples stored at 37°C. Aliquots (400 μ l) of ice-cold acetonitrile containing internal standard were added to 100- μ l samples of plasma separated from centrifuged whole blood and to reference plasma samples. This was then centrifuged, diluted with distilled water, and analyzed by LC-MS/MS to determine the compound concentration. $R_{b/p}$ was calculated as follows:

$$R_{b/p} = \text{Conc reference plasma} / \text{Conc plasma from blood}$$

LC-MS/MS Analysis

The concentration of all compounds in the incubations was determined by LC-MS/MS. An Acquity ultraperformance liquid chromatography system (Waters, Elstree, UK) coupled to a triple-quadrupole mass spectrometer (Xevo TQ-S; Waters, Milford, MA) was used to carry out the sample analysis. The analytes were separated by reverse-phase liquid chromatography using a Waters Atlantis T3, 3 μ m, 2.1 $\times$ 50 mm column (Waters). Mobile phases A and B

consisted of water (containing 0.1% FA v/v) and ACN (containing 0.1% FA v/v), respectively. The flow rate was held constant at 0.73 ml/min throughout the gradient run. The initial mobile phase composition of 95% A and 5% B was held for 0.3 minutes. Mobile phase B was then increased linearly to 70% until 1.5 minutes, followed by further increase to 99% B until 1.59 minutes. At 1.6 minutes, the composition of A and B was reversed to the initial 95% A and 5% B and was held until 2 minutes. Analyte quantitation was achieved by MS-MS detection in positive electrospray ionization mode. The MS operating conditions were as follows: the capillary voltage was 1.14 kV and source offset was 50 V. The desolvation temperature was set to 600°C. Nitrogen was used as the desolvation gas (800 l/hour) and cone gas (150 l/hour). Argon was used as the collision gas at a flow rate of 0.15 ml/min. Detection of the ions was performed in the multiple reaction monitoring mode using the transitions described in Table 1. Peak integration and calibrations were performed using TargetLynx software (Version 4.1; Waters).

Unbound Intrinsic Clearance Calculations.

In vitro. The unbound in vitro CL_{int} ($CL_{int,u}$) using hepatocytes or microsomes were compared with the in vivo $CL_{int,u}$ values using the regression scaling method (Grime and Riley, 2006). Briefly, the unbound intrinsic clearance was determined using the following equation:

$$\text{Predicted } CL_{int,u} = ((CL_{int}/f_{u,inc}) \times SF1 \times SF2) \times 3/1000 \quad (1)$$

where SF1 is the scaling factor for the number of cells per gram of liver (120×10^6 cells/g for humans) or the milligrams of microsomal protein per gram of liver (40 mg/g for humans), SF2 is the scaling factor for the liver-to-body weight ratio (24 g/kg for humans) and $f_{u,inc}$ is the unbound fraction in the incubation. Note the unbound CL_{int} is multiplied by three by way of applying the regression offset correction. Blood clearance (CL_b) was calculated using the well-stirred model in the following equation:

$$CL_b = \frac{Q \times CL_{int,u} \times f_{u,R_{b/p}}}{Q + CL_{int,u} \times f_{u,R_{b/p}}} \quad (2)$$

In vivo. The in vivo, observed, unbound intrinsic clearance was calculated from the in vivo plasma clearance values by back calculating through the well-stirred liver model using the following equation:

$$CL_h = CL - CL_{renal} \quad (3)$$

$$\text{Observed } CL_{int,u} = \frac{(CL_h \times Q)}{f_u \times (Q - (CL_h/R_{b/p}))} \quad (4)$$

where CL is plasma clearance from intravenous or oral studies, CL_h is the hepatic clearance, CL_{renal} is the renal clearance, Q is hepatic blood flow (23 (ml/min)/kg in humans), f_u is the plasma protein binding, and $R_{b/p}$ is the blood/plasma ratio. When using the oral clearance values it is assumed that the compound is completely absorbed, and that the liver is the major organ of clearance.

For this study, 10 compounds were selected from the literature where the metabolism has been shown to involve FMO: benzydamine (Taniguchi-Takizawa

TABLE 1
Mass spectrometer parameters for FMO substrates

Analyte	MRM (Parent→Daughter) m/z	Dwell	Cone Voltage	Collision Energy
		s	V	
Tamoxifen	372.166→129.175	0.080	10	25
Moclobemide	269.053→182.073	0.080	10	15
Olanzapine	313.127→84.118	0.080	10	25
Imipramine	281.178→86.025	0.080	60	15
Tozasertib	465.195→190.215	0.080	20	40
Ranitidine	315.095→176.243	0.040	10	15
Benzydamine	310.173→86.065	0.080	60	30
Cimetidine	253.06→94.994	0.080	20	20
Clozapine	327.14→270.16	0.080	40	20
Itopride	359.22→71.94	0.80	10	40
Verapamil (IS)	455.198→165.207	0.020	15	25

et al., 2015), imipramine (Adali et al., 1999), olanzapine (Korprasertthaworn et al., 2015), ranitidine (Chung et al., 2000), cimetidine (Cashman et al., 1993), moclobemide (Hoskins et al., 2001), itopride (Mushiroda et al., 2000), clozapine (Tugnait et al., 1997), tamoxifen (Parte and Kupfer, 2005), and tozasertib (Ballard et al., 2007) (Fig. 1).

Results

Determination of FMO Involvement. The contribution of FMO to the intrinsic clearance in HLM was determined using a combination of methimazole competitive substrate inhibition and heat inactivation (Taniguchi-Takizawa et al., 2015). The results of these studies are illustrated in Fig. 2.

The overall FMO contribution was defined as the mean of the inhibition by the competing substrate methimazole (500 μM) and the extent of heat inactivation. Both mechanisms give a broadly similar extent of reduction of FMO activity ($>80\%$ reduction) as determined by benzydamine *N*-oxide formation. Under the same conditions there was minimal impact ($<30\%$ reduction in activity) on CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A. The impact on CYP1A2 was more significant with 37% reduction in activity in the presence of methimazole but only 12% reduction by heat inactivation (for full methods and results, see Supplemental Material).

It was not possible to determine the FMO contribution for ranitidine, cimetidine, olanzapine, and tamoxifen due to the low intrinsic clearance [<3 ($\mu\text{l/min}$)/mg] observed in this study (Fig. 2). However, among the remaining six compounds, the greatest FMO contribution was observed with itopride ($>90\%$ FMO) and the lowest was imipramine ($\sim 20\%$ FMO). Benzydamine had an FMO contribution of 53%, moclobemide and tozasertib both had a contribution of 38%, and clozapine had a contribution of 23%.

Human in Vivo Studies. The 10 FMO substrates with human pharmacokinetic data (Fig. 1) were identified from the literature. The plasma

clearance data for these compounds was combined with the measured plasma protein binding and blood/plasma ratio ($R_{b/p}$) to derive the observed unbound CL_{int} as described in *Materials and Methods* (Table 2).

The only compounds in the set with reported significant renal clearance were ranitidine and cimetidine with renal clearances of 7.4 and 6.7 (ml/min)/kg, respectively (Walkenstein et al., 1978; van Hecken et al., 1982). This was incorporated into the calculations, and the derived hepatic clearance was used to calculate the unbound CL_{int} as detailed in *Materials and Methods*. The resulting metabolic component of clearance was calculated to be 1.8 (ml/min)/kg for ranitidine and 2 (ml/min)/kg for cimetidine (Table 2).

For olanzapine, itopride, clozapine, and tamoxifen no human intravenous data were available. For these compounds, it was assumed that absorption was complete and that the liver is the major organ of clearance such that the observed oral clearance approximates to the intravenous clearance. This resulted in clearance values of 13.9, 7.6, and 19 (ml/min)/kg, respectively.

Human in Vitro Studies. The in vitro CL_{int} was determined for the 10 FMO substrates in human hepatocytes and HLM at a substrate concentration of 1 μM . The hepatocyte and HLM CL_{int} values ranged from 0.1 ($\mu\text{l/min}$)/million cells and <0.3 ($\mu\text{l/min}$)/mg, respectively, for cimetidine to 29.2 ($\mu\text{l/min}$)/million cells and 64.5 ($\mu\text{l/min}$)/mg, respectively, for tozasertib.

The predicted unbound CL_{int} was derived by combining the CL_{int} with the measured fraction unbound in the incubation and applying the regression correction (eq. 1; Table 3).

For olanzapine, ranitidine, and cimetidine the HLM CL_{int} was beyond the limit of the assay [<3 ($\mu\text{l/min}$)/mg protein] so a conservative value of 3 ($\mu\text{l/min}$)/mg protein was used in the subsequent calculations.

The HLM CL_{int} values were on average 1.7-fold high than the hepatocyte values, although this was influenced by inclusion of olanzapine, ranitidine, and cimetidine, which had HLM CL_{int} values

Fig. 1. Structures of the 10 FMO substrates used in the current study.

Fig. 2. FMO contribution to microsomal metabolism for the FMO substrates.

set to 3 ($\mu\text{L}/\text{min}/\text{mg}$). If these three compounds are omitted from the analysis then this drops to 0.8-fold so the unbound CL_{int} values from both systems compare very favorably.

Comparison of Predicted and Observed Unbound Intrinsic Clearance. The CL_{int} was calculated for each compound from the hepatocyte or HLM in vitro CL_{int} and compared with the corresponding value calculated from CL values from intravenous or, if no intravenous study was available, from an oral study, assuming a value for the bioavailability. The resulting CL_{int} values were then compared for each of the systems studied (Fig. 3, A and B; Fig. 4, A and B).

Human hepatocytes produced a good concordance ($R^2 = 0.69$) between the predicted and observed unbound intrinsic clearance values, with 8 of 10 compounds falling within or very close to twofold of the line of unity (Fig. 3A). There was also a strong correlation between predicted and observed CL_b with an R^2 of 0.75 (Fig. 3B). Benzydamine showed approximately sixfold overprediction in terms of CL_{int} [predicted $\text{CL}_{\text{int}} = 104$ ($\text{mL}/\text{min}/\text{kg}$) versus observed $\text{CL}_{\text{int}} = 18$ ($\text{mL}/\text{min}/\text{kg}$)], which in turn equates to approximately a fourfold over prediction in blood clearance [predicted $\text{CL}_b = 11$ ($\text{mL}/\text{min}/\text{kg}$) versus observed $\text{CL}_b = 3$ ($\text{mL}/\text{min}/\text{kg}$)]. This is a similar degree of over prediction observed previously (Fisher et al., 2002). In contrast, tozasertib showed an under prediction of around threefold in CL_{int} [predicted $\text{CL}_{\text{int}} = 1177$ ($\text{mL}/\text{min}/\text{kg}$) versus observed $\text{CL}_{\text{int}} = 3370$ ($\text{mL}/\text{min}/\text{kg}$)], which in turn translates to

less than twofold in blood clearance [predicted $\text{CL}_b = 19$ ($\text{mL}/\text{min}/\text{kg}$) versus observed $\text{CL}_b = 22$ ($\text{mL}/\text{min}/\text{kg}$)].

For HLM, the correlation between the in vitro and in vivo unbound intrinsic clearance values was maintained and also showed a good concordance ($R^2 = 0.84$) with only three compounds falling within or close to twofold of the line of unity (Fig. 4A). However, the correlation between predicted and observed CL_b (Fig. 4B) was weaker ($R^2 = 0.32$) compared with hepatocytes. Benzydamine produced a greater degree of overprediction in CL_{int} than was seen with hepatocytes (8-fold) [predicted $\text{CL}_{\text{int}} = 148$ ($\text{mL}/\text{min}/\text{kg}$) versus observed $\text{CL}_{\text{int}} = 18$ ($\text{mL}/\text{min}/\text{kg}$)]. In addition, ranitidine and cimetidine showed significant overpredictions of 4.5-fold [predicted $\text{CL}_{\text{int}} = 9$ and 9 ($\text{mL}/\text{min}/\text{kg}$), respectively, versus observed $\text{CL}_{\text{int}} = 2$ and 2 ($\text{mL}/\text{min}/\text{kg}$), respectively]. This is likely due to the overestimation of the in vitro HLM CL_{int} , which were set at 3 ($\mu\text{L}/\text{min}/\text{mg}$), because the true values were below the limit of quantification of the assay. There was a more significant degree of underprediction in HLM with four compounds (tamoxifen, itopride, tozasertib, and moclobemide) all underpredicted by around sevenfold.

Discussion

The prediction of human pharmacokinetics is an important part of drug discovery. The use of human in vitro systems such as hepatocytes or HLM combined with the appropriate scaling factors have been routinely used to predict human in vivo CL (Riley et al., 2005; Di et al., 2013).

It has been shown that not all enzyme systems are fully recovered when the in vitro preparation is produced from the original tissue. Specifically, AO activity has been shown to be lower in isolated in vitro systems compared with the original tissue (Hutzler et al., 2014). This reduced activity significantly impaired the ability of these in vitro systems to predict adequately the clearance of a set of AO substrates (Zientek et al., 2010). The current study determined the in vitro CL_{int} values in hepatocytes and HLM for a set compounds that had differential degrees of FMO involvement in their metabolism. The aim was to determine whether FMOs were subject to similar recovery issues, because AO and the potential downstream consequences for the prediction on in vivo CL.

The 10 compounds used here have all been shown to have metabolic routes that are mediated by FMO. Generally, FMO investigations have been related to specific metabolic pathways such as the *N*-oxygenation of moclobemide (Hoskins et al., 2001) without necessarily looking at the contribution the pathway makes to the overall hepatic in vitro CL_{int} . When this was determined for 6 of the 10 FMO substrates in this study,

TABLE 2
Human in vivo plasma clearance, plasma protein, and blood plasma ratio data for 10 FMO substrates

	CL i.v. or oral	fu	$R_{\text{b/p}}$	Observed CL_{int}	Reference
	($\text{mL}/\text{min}/\text{kg}$)			($\text{mL}/\text{min}/\text{kg}$)	
Benzydamine	2.3 ^a	0.148	0.76	18	Baldock et al. (1991)
Imipramine	12.8	0.221	0.93	192	Abernethyl et al. (1984)
Olanzapine	6.2 ^b	0.294	0.73	30	Callaghan et al. (1999)
Ranitidine	1.8 ^{a,c}	0.874	0.90	2	van Hecken et al. (1982)
Cimetidine	2 ^{a,c}	0.899	1.02	2	Walkenstein et al. (1978)
Moclobemide	13.2 ^a	0.622	0.84	61	Raaflaub et al. (1984)
Itopride	13.9 ^b	0.246	0.72	296	Cho et al. (2010)
Clozapine	7.6 ^b	0.106	0.83	131	Tassaneeyakul et al. (2005)
Tamoxifen	1.9 ^b	0.0003	0.89	4293	Fuchs et al. (1996)
Tozasertib	20.7 ^a	0.076	0.94	3370	Traynor et al. (2011)

^aFrom intravenous study.

^bFrom oral study.

^cCorrected for renal clearance.

TABLE 3
Human in vitro data for 10 FMO substrates

All data $n = 3$ except for data marked with *, which is $n = 2$.

	Human Hepatocytes				Human Liver Microsomes			
	CL _{int}	fu _{inc} ^a	Predicted CL _{int u}	Predicted CL _b	CL _{int}	fu _{inc}	Predicted CL _{int u}	Predicted CL _b
	($\mu\text{L}/\text{min}/10^6$ cells)			($\text{mL}/\text{min}/\text{kg}$)			($\text{mL}/\text{min}/\text{kg}$)	
Benzylamine	9	0.74	104	11	18	0.35	148	13
Imipramine	9	0.65	122	13	14	0.31	129	13
Olanzapine	2	0.86	18	5	<3 ^b	0.73	12	4
Ranitidine	0.6*	0.98	5	4	<3 ^b	0.97	9	6
Cimetidine	0.1*	1.01	1	1	<3 ^b	1.01	9	6
Moclobemide	3	0.87	30	11	3	0.98	9	5
Itopride	11	0.88	107	14	18	1.05	49	10
Clozapine	5	0.33	131	10	18	0.30	173	11
Tamoxifen	4	0.00	9706	3	4	0.02	662	0.2
Tozasertib	29	0.21	1177	19	61	0.18	971	18

^aData from rat hepatocytes.

^bValue of 3 ($\mu\text{L}/\text{min}/\text{mg}$) used in calculations.

the FMO contribution was seen to vary from itopride (>90%) to imipramine and clozapine, where the FMO contribution is just over 20%. This reflects the situation seen in many drug discovery programs where the contribution to hepatic metabolism for any given enzyme will vary across compounds.

This study demonstrated consistency when scaling FMO substrates from either hepatocytes or HLM. It has been argued that hepatocytes are the most suitable system to use for scaling clearance because of the presences of the full complement of drug metabolizing enzymes (Riley et al., 2005). This aspect is important for compounds such as olanzapine, which have been shown to form phase 2 metabolites, such as *N*-glucuronides, which would not be seen and so accounted for in the HLM (Korprasertthaworn et al., 2015). Interestingly, in this study olanzapine predicted clearance equally well from both hepatocytes and HLM, suggesting that the *N*-glucuronide is not playing a major role in the rate of metabolism in these systems.

The only compound that was significantly overpredicted in both hepatocyte and HLM was benzylamine. This was also the case in HLM. This was observed previously by Fisher et al. (2002) who speculated that the reported intravenous human pharmacokinetics may be in error possibly

due to enterohepatic recycling of the *N*-oxide metabolite, an analytical error or an unknown enhancement of FMO activity in the in vitro systems. The potential for the *N*- or *S*-oxide to be reduced back to the original drug molecule in vivo would confound this type of in vitro-in vivo assessment and lead to an underprediction of in vivo CL_{int}. It has been shown that both imipramine and desipramine are circulating in the plasma of healthy volunteers after intravenous, intramuscular, and oral dosing of imipramine *N*-oxide (Nagy and Hansen, 1978). Whether one of these reasons or a different reason is behind the underprediction of benzylamine is unclear.

FMOs are differentially distributed in the body with FMO3 and FMO5 expressed in the liver and FMO1 and FMO2 mainly expressed in the kidney and lung, respectively (Cashman and Zhang, 2006). Hence, studies that use human hepatocytes and HLM will reflect the involvement of FMO3, and to a lesser extent FMO5, but not FMO1 and FMO2. As such these studies may underestimate the contribution of FMO to the overall clearance of a compound if the latter two enzymes play a significant role in its metabolism. However, based on the *S*-oxygenation of a disulfiram metabolite, it has been estimated that the human kidney has 14-fold lower FMO metabolic capacity compared

Fig. 3. Unbound intrinsic clearance in vitro/in vivo correlation (A) and blood in vitro/in vivo clearance (B) correlations for human hepatocytes.

Fig. 4. Unbound intrinsic clearance in vitro/in vivo correlation (A) and blood in vitro/in vivo clearance (B) correlations for human liver microsomes.

with the liver so the impact of FMO1 may not be highly significant (Cashman and Zhang, 2006).

In general, HLM did not perform as well as hepatocytes in predicting CL_b in this study, supporting the view that the intact hepatocyte is a better system for predicting human clearance. There were a number of compounds that were both over- and underpredicted. The reason for this is not clear but may relate to other clearance mechanisms not reflected in HLM or low recovery of the microsomal clearance enzymes after cryopreservation.

The approach adopted in this study uses point estimates for the scaling factors—the number of cells per gram of liver or the milligrams of microsomal protein per gram of liver and the liver-to-body weight ratio. This does not take into account the population variability in these scaling factors, which in turn can lead to variability in the subsequent clearance estimates (Kenyon et al., 2016). However, in the context of drug discovery where the challenge is to optimize and select a small number of drug molecules from hundreds of compounds, it can be argued that using such point estimates can facilitate this selection, because it reduces the number of parameters contributing to the overall variability to those related to the molecule and not to the system. While this allows rank ordering of compounds, it does not give a full understanding of the variability in clearance that might occur in vivo. Once compounds are selected, then it is appropriate to use more sophisticated approaches such as physiologically based pharmacokinetic modeling to account for variability in these scaling factors along with the variability in other system-related parameters such as the variability in the enzyme expression. This is also important for FMO3, in particular given the polymorphic nature of its expression (Cashman and Zhang, 2006). At this stage it is also important to consider the variability that might be seen in pediatric populations, because it is reported that FMO1 is expressed in the liver up to 72 hours postnatally when it is suppressed, whereas FMO3 expression increases in a variable manner until full expression is reached around 10 years of age (Hines and McCarver, 2002). Thus the physiologically based pharmacokinetic approach will give a much greater understanding of the anticipated overall variability in clearance that could be seen in vivo.

This study clearly demonstrates, with a range of substrates, that the routine methodologies employed to scale in vitro metabolic data to in vivo clearance adequately capture and scale the activity of FMO.

Authorship Contributions

Participated in research design: Jones, Srivastava, Reddy, Colclough.

Conducted experiments: Srivastava, Amberntsson, Li.

Performed data analysis: Jones, Srivastava, Colclough, Wilson, Reddy, Amberntsson, Li.

Wrote or contributed to the writing of the manuscript: Jones, Srivastava, Colclough, Wilson, Reddy, Amberntsson, Li.

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