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Title Page

An investigation into the prediction of *in vivo* clearance for a range of Flavin-containing monooxygenase substrates

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Running Title Page

Running title: In vitro-in vivo correlation for FMO substrates in human

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Abbreviations: ACN, acetonitrile; AO, aldehyde oxidase; CL, clearance; CLb, blood clearance; CLh, hepatic clearance; Clint, intrinsic clearance; CLint u, unbound intrinsic clearance; CLrenal, renal clearance; CYP, cytochrome P450; DDI, drug-drug interaction; FA, formic acid; FMO, Flavin-containing monooxygenases; fu inc, unbound fraction in the incubation; fu, unbound fraction in plasma; HLM, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; NADPH, β-nicotinamide adenine dinucleotide 2'-

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phosphate reduced tetrasodium salt; PBS, phosphate-buffered saline; Q, hepatic blood flow; Rb/p, blood/plasma ratio; RED, rapid equilibrium dialysis; UPLC, ultra performance liquid chromatography

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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 (CYP) 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. In order to confirm and quantify the extent of FMO involvement 6 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 (CLint) data was scaled using standard methods to determine the predicted unbound intrinsic clearance (predicted CLint u) for each compound. This was compared with observed unbound intrinsic clearance (observed CLint u) values back calculated from human pharmacokinetic studies. A good correlation was observed between the predicted and observed CLint u using hepatocytes (R^2 = 0.69) with 8 out 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, 2-fold. 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.

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Introduction

Flavin-containing monooxygenase (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 to 50^{o}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 5 FMOs expressed in human 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 which 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 vs FMO3. In contrast ranitidine shows a higher rate for both the N and S-oxygenations in FMO3 vs FMO1 (Chung et al., 2000). FMO3 has been shown to metabolise many primary, secondary and tertiary amines along with various sulfur-containing substrates and is viewed as the principal

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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 trifluroperazine (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 in order 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 etc. (Di et al., 2013; Grime et al., 2013). The method most routinely used for hepatic metabolic clearance is scaling the unbound intrinsic clearance (CLint u) from in vitro systems. Hepatocytes or HLM Clint U values are scaled to the in vivo situation using factors for the number of cells or milligram protein per g 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 predication 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 (CLb) using the scaling methodology described above. In this case the in vivo CL in rat was predicted well from the in vitro CLint (within 30% of the measured value) whereas there was an over prediction of the human CL (>10.5 vs 2.4ml/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 gender mix) HLM were obtained from BD Biosciences (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 (Baltimore, MD). Co-culture of the five-donor human hepatocyte pool (lot 1410235, XenoTech) 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 HμREL PlatinumHeps medium were purchased from HμREL (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 (DMSO) were purchased from Sigma-Aldrich (Poole, UK). L-15 medium, methanol, water and acetonitrile (ACN) were HPLC grade from Thermo Fisher Scientific (UK). All the other solvents were HPLC grade and, unless otherwise specified, all of the other reagents were purchased from Sigma-Aldrich (Poole, UK).

Unless otherwise indicated all experiments were conducted in triplicate.

HLM CLint Incubations: Compounds (1μM final concentration) were incubated with HLM (1mg/ml) in 100mM phosphate buffer (pH 7.4) at 37°C. After an 5 minute pre-incubation in the presence of NADPH (1mM) the reaction was initiated by the addition of the compound in ACN (less than 1% final concentration). Samples (20μl) were removed from the incubation at 0.5, 5, 10, 15, 20 and 30 minutes and added to 100 μL of cold ACN containing internal standard. The samples were then centrifuged at 4000rpm 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. CLint values were then calculated from the negative slope of the linear fit divided by the microsomal concentration.

Hepatocytes Clint incubations: The hepatocytes were thawed and re-suspended in pre-warmed 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 (less than 1% final concentration) to give a final concentration of 1µM. The plate was incubated at 37 °C and 20µl samples removed at 0.5, 5, 15, 30, 45, 60, 80, 100 and 120 minutes and added to 100 µL of cold ACN (containing internal standard). The samples were then centrifuged at 4000 rpm and 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. CLint values were then calculated from the negative slope of the linear fit divided by the number of hepatocytes.

HµREL low CLint incubations: If the hepatocyte CLint was less than 1µl/min/million cells then a low intrinsic clearance assay using the HµREL primary human hepatocyte co-culture system was used. This methodology has been described in detail previously (Bonn et al., 2016). Briefly, co-culture plates at a seeding density of 30,000 hepatocytes per well were shipped in HµREL PlatinumHeps shipping medium, at 37^{o}C, from HµREL (North Brunswick, NJ, USA). On arrival the shipping medium was replaced with PlatinumHeps maintenance medium and the cells left to acclimatize overnight in incubator at 37^{o}C in humidified atmosphere containing 95% air and 5% CO_2. Prior to the incubation the cells in each well were washed with 100µl HµREL PlatinumHeps dosing medium which was aspirated and replaced with 50µl of fresh media prior to the addition of the test compound which was added in 50µl HµREL PlatinumHeps dosing medium (final concentration 1µM with less than 1% ACN). Samples (45µl) were taken at 6 time points (1, 3, 5, 24, 48 and 70 hours) and added to 180µl ice cold acetonitrile. Samples were centrifuged (20 minutes 4°C, 4000g) and 50µl of the supernatant was diluted within 100µl water prior to LC-MS/MS analysis. The kinetic data were analyzed using a

linear fit of the natural logarithm of the compound peak area against time. CLint 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 which was set to start at 4^{o}C and programmed to heat to 50^{o}C for 1 minute and revert to 4^{o}C immediately after. Compounds (1µM final concentration) were incubated with control and heat inactivated HLM (1mg/ml) in 100mM phosphate buffer (pH 7.4) at 37°C. After an 5 minute pre-incubation in the presence of NADPH (1mM) the reaction was initiated by the addition of the compound in ACN (less than 1% final concentration).In parallel, compounds (1µM final concentration) were also incubated with methimazole (500µM) to determine the fraction metabolised 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 rpm for 10 minutes and samples analysed 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. CLint values were then calculated from the negative slope of the linear fit divided by the microsomal concentration. The fraction metabolised 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 5µM using the Rapid Equilibrium Device (RED Thermoscientific Pierce). Phosphate buffer (100mM pH 7.4) was added to the buffer chamber, and 300µl 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 rpm with 5% CO2. At the end of incubation, samples (50µl) from both buffer and plasma chambers were removed for analysis. Samples and standards were matrix matched and analysed using LC-MS/MS. The unbound fraction in plasma (fu) was calculated as follows:

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

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 1mg/ml and a final compound concentration of 1µM. PBS (150µl) was added to the buffer well and 150µl HLM containing the compound to the sample well and incubated at 37°C for 4 hours. After the incubation, 50µl aliquots from both donor and receiver wells were removed for analysis. Samples and standards were matrix matched and analysed by LC-MS/MS. The unbound fraction in the incubation (fu inc) was calculated as follows:

$$fu\ inc = Conc._{\text{buffer well}} / Conc._{\text{microsomal suspension well}}$$

Determination of unbound fraction in rat hepatocytes (fu inc). The extent of binding of compounds to rat hepatocytes was also determined by equilibrium dialysis using the HTDialysis LLC device (Gales Ferry, CT). Rat Hepatocytes were re-suspended with pre-warmed L-15 medium at 1.0×10^6 viable cells/ml. The test compounds, at a final concentration of 1µM, were added to the hepatocyte suspension. 150 µl of the hepatocytes suspension containing the compound was added to each of the sample wells of the 96-well dialysis device and PBS (150 µl) to the receiver well and incubated at 37°C for 4 hours. After the incubation the samples and standards were matrixed matched and analysed by LC-MS/MS analysis. The unbound fraction (fu inc) was calculated as follows:

$$fu\ inc = Conc._{\text{buffer well}} / Conc._{\text{hepatocyte suspension well}}$$

Determination of blood/plasma ratio ($R_{b/p}$): A volume of plasma sufficient for the assay was obtained from whole human blood by centrifugation (3,220 g for 10 minutes at 4°C). The test compound (10µM) was added to 398µl of the pre-warmed human plasma and blood separately and

incubated for 30 minutes. After incubation, the blood samples were centrifuged for 10 minutes at 4,000 rpm (37°C) and the plasma samples stored at 37°C. Aliquots (400µl) of ice cold acetonitrile containing internal standard were added to 100µl 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:

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

LC-MS/MS analysis: The concentration of all compounds in the incubations was determined by LC-MS/MS. An Acquity ultra performance liquid chromatography (UPLC) system, (Waters, 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, 3µm, 2.1X50mm column (Waters, UK). 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/Hr) and cone gas (150 L/Hr). Argon was used as the collision gas at a flow rate of 0.15 ml/min. Detection of the ions was performed in the MRM mode using the transitions described in Table 1. Peak integration and calibrations were performed using TargetLynx software (Version 4.1, Waters, Milford, MA).

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Unbound intrinsic clearance calculations

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

$$\text{Predicted CLint u} = ((\text{CLint/fu inc}) \times \text{SF1} \times \text{SF2}) \text{ X } 3 /1000 \quad (1)$$

where SF1 is the scaling factor for the number of cells per gram of liver ($120x10^6$ cells/g for human) or the milligrams of microsomal protein per gram of liver (40mg/g for human), SF2 is the scaling factor for the liver to body weight ratio (24g/kg for human) and fu inc is the unbound fraction in the incubation. Note the unbound CLint is multiplied by 3 by way of applying the regression offset correction. Blood clearance (CLb) was calculated using the well stirred model in the following equation:

$$\text{CLb} = \frac{Q \text{ X CLint u X } {}^{Fu}/_{R_{b/p}}}{Q + \text{CLint u X } {}^{Fu}/_{R_{b/p}}} \quad (2)$$

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:

$$\text{CLh} = \text{CL} - \text{CLrenal} \quad (3)$$

$$\text{Observed CLint u} = \frac{(CL_h \text{ X } Q)}{fu \text{ X } (Q-(CL_h/R_{b/p}))} \quad (4)$$

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Where CL is plasma clearance from intravenous or oral studies, CLh is the hepatic clearance, CLrenal is the renal clearance, Q is hepatic blood flow (23ml/min/kg in human), fu 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 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).

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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 (500µM) and the extent of heat inactivation. Both mechanisms give a broadly similar extent of reduction of FMO activity (greater than 80% reduction) as determined by benzydamine N-oxide formation. Under the same conditions there was minimal impact (less than 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 data).

It was not possible to determine the FMO contribution for ranitidine, cimetidine, olanzapine and tamoxifen due to the low intrinsic clearance (< 3µl/min/mg) observed in this study (fig. 2). However, amongst the remaining 6 compounds the greatest FMO contribution was observed with itropride (>90% FMO) and the lowest was imipramine (~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 CLint as described in the 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 6.7ml/min/kg respectively (Walkenstein et al., 1978; van Hecken et al., 1982). This was incorporated into the calculations and the derived hepatic clearance used to calculate the unbound CLint as detailed in the methods section. The resulting metabolic component of clearance was calculated to be 1.8ml/min/kg for ranitidine and 2ml/min/kg for cimetidine (table 2).

For olanzapine, itopride, clozapine and tamoxifen no human intravenous data was 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 19ml/min/kg respectively.

Human in vitro studies

The in vitro CLint was determined for the 10 FMO substrates in human hepatocytes and HLM at a substrate concentration of 1µM. The hepatocyte and HLM CLint values ranged from 0.1µl/min/million cells and <0.3µl/min/mg respectively for cimetidine to 29.2µl/min/million cells and 64.5µl/min/mg respectively for tozasertib.

The predicted unbound CLint was derived by combining the CLint with the measured fraction unbound in the incubation and applying the regression correction (table 3 & equation 1).

For olanzapine, ranitidine and cimetidine the HLM CLint was beyond the limit of the assay (<3µl/min/mg protein) so a conservative value of 3µl/min/mg protein was used in the subsequent calculations.

The HLM CLint u 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 Clint values set to 3 µl/min/mg. If these three compounds are omitted from the analysis the then this drops to 0.8-fold so the unbound CLints from both systems compare very favourably.

Comparison of predicted and observed unbound intrinsic clearance

The Clint u was calculated for each compound from the hepatocyte or HLM in vitro CLint 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 Clint u values were then compared for each of the systems studied (Fig. 3 A and B and 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 out of 10 compounds falling within or very close to 2-fold of the line of unity (fig. 3A). There was also a strong correlation between predicted and observed CLb with an R^2 of 0.75 (fig. 3B). Benzydamine showed approximately 6-fold over prediction in terms of CLint u (predicted CLint u = 104ml/min/kg vs. observed CLint u = 18ml/min/kg) which in turn equates to approximately a 4-fold over prediction in blood clearance (predicated CLb 11ml/min/kg vs observed CLb 3ml/min/kg). This is a similar degree of over prediction observed previously (Fisher et al., 2002). In contrast, tozasertib showed an under prediction of around 3 fold in CLint u (predicted

CLint u = 1177ml/min/kg vs observed CLint u = 3370ml/min/kg) which in turn translates to less than 2-fold in blood clearance (predicted CLb = 19ml/min/kg vs. observed CLb = 22ml/min/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 3 compounds falling within or close to 2-fold of the line of unity (fig. 4A). However, the correlation between predicted and observed CLb (fig. 4B) was weaker (R^2 = 0.32) compared to hepatocytes. Benzydamine produced a greater degree of over prediction in CLint u than was seen with hepatocytes (8-fold) (predicted CLint u = 148ml/min/kg vs observed CLint u = 18ml/min/kg). In addition ranitidine and cimetidine showed significant over predictions of 4.5-fold (predicted CLint u = 9 and 9ml/min/kg respectively vs. observed CLint u = 2 and 2ml/min/kg respectively). This is likely due to the over estimation of the in vitro HLM CLint which were set at 3µl/min/mg as the true values were below the limit of quantification of the assay. There was a more significant degree of under-prediction in HLM with four compounds (tamoxifen, itopride, tozasertib and moclobemide) all under predicted by around 7-fold

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 to the original tissue (Hutzler et al., 2014). This reduced activity significantly impaired the ability of these in vitro systems to adequately predict the clearance of a set of AO substrates (Zientek et al., 2010). The current study determined the in vitro CLints in hepatocytes and HLM for a set compounds which had differential degrees of FMO involvement in their metabolism. The aim was to determine whether FMOs were subject to similar recovery issues as 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 CLint. When this was determined for 6 of the 10 FMO substrates in this study 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 metabolising 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 over predicted in both hepatocyte and HLM was benzydamine. 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 re-cycling 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 which would confound this type of in vitro-in vivo assessment and lead to an under prediction of in vivo CLint. It has been shown that both imipramine and desipramine are circulating in the plasma of healthy volunteers following intravenous, intra muscular and oral dosing of imipramine N-oxide (Nagy and Hansen, 1978). Whether one of these reasons or a different reason is behind the under-prediction of benzydamine 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 to 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 CLb 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 under predicted. 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 optimise and select a small number of drug molecules from 100s 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 system. Whilst 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 (PBPK) modelling 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 important to consider the variability that might be seen in pediatric populations as it is reported that FMO1 is expressed in the liver up to 72 hours postnatally when it is suppressed whilst FMO3 expression increases in a variable manner until full expression is reached around 10 years of age (Hines and McCarver, 2002). Thus the PBPK

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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 and Colclough

Conducted experiments: Srivastava, Amberntsson and Li

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

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

References

Abernethyl DR, Divoll M, Greenblatt DJ, Harmatz JS, and Shader RI (1984) Absolute bioavailability of imipramine: influence of food. *Psychopharmacology* **83:**104-106.

Adali O, Carver GC, and Philpot RM (1999) The effect of arginine-428 mutation on modulation of activity of human liver flavin monooxygenase 3 (FMO3) by imipramine and chlorpromazine. *Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie* **51:**271-276.

Baldock GA, Brodie RR, Chasseaud LF, Taylor T, Walmsley LM, and Catanese B (1991) Pharmacokinetics of benzydamine after intravenous, oral, and topical doses to human subjects. *Biopharmaceutics & drug disposition* **12:**481-492.

Ballard JE, Prueksaritanont T, and Tang C (2007) Hepatic metabolism of MK-0457, a potent aurora kinase inhibitor: interspecies comparison and role of human cytochrome P450 and flavin-containing monooxygenase. *Drug metabolism and disposition: the biological fate of chemicals* **35:**1447-1451.

Bonn B, Svanberg P, Janefeldt A, Hultman I, and Grime K (2016) Determination of Human Hepatocyte Intrinsic Clearance for Slowly Metabolized Compounds: Comparison of a Primary Hepatocyte/Stromal Cell Co-culture with Plated Primary Hepatocytes and HepaRG. *Drug metabolism and disposition: the biological fate of chemicals* **44:**527-533.

Callaghan JT, Bergstrom RF, Ptak LR, and Beasley CM (1999) Olanzapine. Pharmacokinetic and pharmacodynamic profile. *Clinical pharmacokinetics* **37:**177-193.

Cashman JR, Park SB, Yang ZC, Washington CB, Gomez DY, Giacomini KM, and Brett CM (1993) Chemical, enzymatic, and human enantioselective S-oxygenation of cimetidine. *Drug metabolism and disposition: the biological fate of chemicals* **21:**587-597.

Cashman JR and Zhang J (2006) Human flavin-containing monooxygenases. *Annual review of pharmacology and toxicology* **46:**65-100.

Cho KJ, Cho W, Cha KH, Park J, Kim MS, Kim JS, and Hwang SJ (2010) Pharmacokinetic and bioequivalence study of itopride HCl in healthy volunteers. *Arzneimittel-Forschung* **60:**137-140.

Chung WG, Park CS, Roh HK, Lee WK, and Cha YN (2000) Oxidation of ranitidine by isozymes of flavin-containing monooxygenase and cytochrome P450. *Japanese journal of pharmacology* **84:**213-220.

Di L, Feng B, Goosen TC, Lai Y, Steyn SJ, Varma MV, and Obach RS (2013) A perspective on the prediction of drug pharmacokinetics and disposition in drug research and development. *Drug metabolism and disposition: the biological fate of chemicals* **41:**1975-1993.

Fiorentini F, Geier M, Binda C, Winkler M, Faber K, Hall M, and Mattevi A (2016) Biocatalytic Characterization of Human FMO5: Unearthing Baeyer-Villiger Reactions in Humans. *ACS chemical biology* **11:**1039-1048.

Fisher MB, Yoon K, Vaughn ML, Strelevitz TJ, and Foti RS (2002) Flavin-containing monooxygenase activity in hepatocytes and microsomes: in vitro characterization and in vivo scaling of benzydamine clearance. *Drug metabolism and disposition: the biological fate of chemicals* **30:**1087-1093.

Fuchs WS, Leary WP, van der Meer MJ, Gay S, Witschital K, and von Nieciecki A (1996) Pharmacokinetics and bioavailability of tamoxifen in postmenopausal healthy women. *Arzneimittel-Forschung* **46:**418-422.

Geier M, Bachler T, Hanlon SP, Eggimann FK, Kittelmann M, Weber H, Lutz S, Wirz B, and Winkler M (2015) Human FMO2-based microbial whole-cell catalysts for drug metabolite synthesis. *Microbial cell factories* **14:**82.

Grime K and Riley RJ (2006) The impact of in vitro binding on in vitro-in vivo extrapolations, projections of metabolic clearance and clinical drug-drug interactions. *Current drug metabolism* **7:**251-264.

Grime KH, Barton P, and McGinnity DF (2013) Application of in silico, in vitro and preclinical pharmacokinetic data for the effective and efficient prediction of human pharmacokinetics. *Molecular pharmaceutics* **10:**1191-1206.

Hines RN and McCarver DG (2002) The ontogeny of human drug-metabolizing enzymes: phase I oxidative enzymes. *The Journal of pharmacology and experimental therapeutics* **300:**355-360.

Hoskins J, Shenfield G, Murray M, and Gross A (2001) Characterization of moclobemide N-oxidation in human liver microsomes. *Xenobiotica; the fate of foreign compounds in biological systems* **31:**387-397.

Hutzler JM, Yang YS, Brown C, Heyward S, and Moeller T (2014) Aldehyde oxidase activity in donor-matched fresh and cryopreserved human hepatocytes and assessment of variability in 75 donors. *Drug metabolism and disposition: the biological fate of chemicals* **42:**1090-1097.

Kenyon EM, Eklund C, Lipscomb JC, and Pegram RA (2016) The impact of variation in scaling factors on the estimation of internal dose metrics: a case study using bromodichloromethane (BDCM). *Toxicology mechanisms and methods* **26:**620-626.

Kim YM and Ziegler DM (2000) Size limits of thiocarbamides accepted as substrates by human flavin-containing monooxygenase 1. *Drug metabolism and disposition: the biological fate of chemicals* **28:**1003-1006.

Korprasertthaworn P, Polasek TM, Sorich MJ, McLachlan AJ, Miners JO, Tucker GT, and Rowland A (2015) In Vitro Characterization of the Human Liver Microsomal Kinetics and Reaction Phenotyping of Olanzapine Metabolism. *Drug metabolism and disposition: the biological fate of chemicals* **43:**1806-1814.

Krueger SK, Vandyke JE, Williams DE, and Hines RN (2006) The role of flavin-containing monooxygenase (FMO) in the metabolism of tamoxifen and other tertiary amines. *Drug metabolism reviews* **38:**139-147.

Mushiroda T, Douya R, Takahara E, and Nagata O (2000) The involvement of flavin-containing monooxygenase but not CYP3A4 in metabolism of itopride hydrochloride, a gastroprokinetic agent: comparison with cisapride and mosapride citrate. *Drug metabolism and disposition: the biological fate of chemicals* **28:**1231-1237.

Parte P and Kupfer D (2005) Oxidation of tamoxifen by human flavin-containing monooxygenase (FMO) 1 and FMO3 to tamoxifen-N-oxide and its novel reduction back to tamoxifen by human cytochromes P450 and hemoglobin. *Drug metabolism and disposition: the biological fate of chemicals* **33:**1446-1452.

Phillips IR and Shephard EA (2008) Flavin-containing monooxygenases: mutations, disease and drug response. *Trends in pharmacological sciences* **29:**294-301.

Raaflaub J, Haefelfinger P, and Trautmann KH (1984) Single-dose pharmacokinetics of the MAO-inhibitor moclobemide in man. *Arzneimittel-Forschung* **34:**80-82.

Riley RJ, McGinnity DF, and Austin RP (2005) A unified model for predicting human hepatic, metabolic clearance from in vitro intrinsic clearance data in hepatocytes and microsomes. *Drug metabolism and disposition: the biological fate of chemicals* **33:**1304-1311.

Taniguchi-Takizawa T, Shimizu M, Kume T, and Yamazaki H (2015) Benzydamine N-oxygenation as an index for flavin-containing monooxygenase activity and benzydamine N-demethylation by cytochrome P450 enzymes in liver microsomes from rats, dogs, monkeys, and humans. *Drug metabolism and pharmacokinetics* **30:**64-69.

Tassaneeyakul W, Kittiwattanagul K, Vannaprasaht S, Kampan J, Tawalee A, Puapairoj P, Tiamkao S, Juthagridsada S, Kukongviriyapan V, and Tassaneeyakul W (2005) Steady-state bioequivalence study of clozapine tablet in schizophrenic patients. *Journal of pharmacy & pharmaceutical sciences : a publication of the Canadian Society for Pharmaceutical Sciences, Societe canadienne des sciences pharmaceutiques* **8:**47-53.

Traynor AM, Hewitt M, Liu G, Flaherty KT, Clark J, Freedman SJ, Scott BB, Leighton AM, Watson PA, Zhao B, O'Dwyer PJ, and Wilding G (2011) Phase I dose escalation study of MK-0457, a novel

Aurora kinase inhibitor, in adult patients with advanced solid tumors. *Cancer chemotherapy and pharmacology* **67:**305-314.

Tugnait M, Hawes EM, McKay G, Rettie AE, Haining RL, and Midha KK (1997) N-oxygenation of clozapine by flavin-containing monooxygenase. *Drug metabolism and disposition: the biological fate of chemicals* **25:**524-527.

van Hecken AM, Tjandramaga TB, Mullie A, Verbesselt R, and de Schepper PJ (1982) Ranitidine: single dose pharmacokinetics and absolute bioavailability in man. *British journal of clinical pharmacology* **14:**195-200.

Walkenstein SS, Dubb JW, Randolph WC, Westlake WJ, Stote RM, and Intoccia AP (1978) Bioavailability of cimetidine in man. *Gastroenterology* **74:**360-365.

Zientek M, Jiang Y, Youdim K, and Obach RS (2010) In vitro-in vivo correlation for intrinsic clearance for drugs metabolized by human aldehyde oxidase. *Drug metabolism and disposition: the biological fate of chemicals* **38:**1322-1327.

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Legends for Figures

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

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

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

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Table 1 Mass Spectrometer parameters for FMO substrates

Analyte	**MRM (Parent→Daughter) m/z**	**Dwell (s)**	**Cone voltage (V)**	**Collision energy (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

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

	CL iv or po (ml/min/kg)	fu	$R_{b/p}$	Observed CLint u (ml/min/kg)	Reference
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[ac]	0.874	0.90	2	(van Hecken et al., 1982)
Cimetidine	2[ac]	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)

[a] from intravenous study
[b] from oral study
[c] corrected for renal clearance

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Table 3 Human in vitro data for 10 FMO substrates

	Human hepatocytes				Human liver microsomes			
	CLint (µl/min/10^6 cells)	fu inc[a]	Predicted CLint u (ml/min/kg)	Predicted CLb (ml/min/kg)	CLint (µl/min/mg)	fu inc	Predicted CLint u (ml/min/kg)	Predicted CLb (ml/min/kg
Benzydamine	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

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

[a]Data from rat hepatocytes

[b]Value of 3µl/min/mg used in calculations

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Figure 1

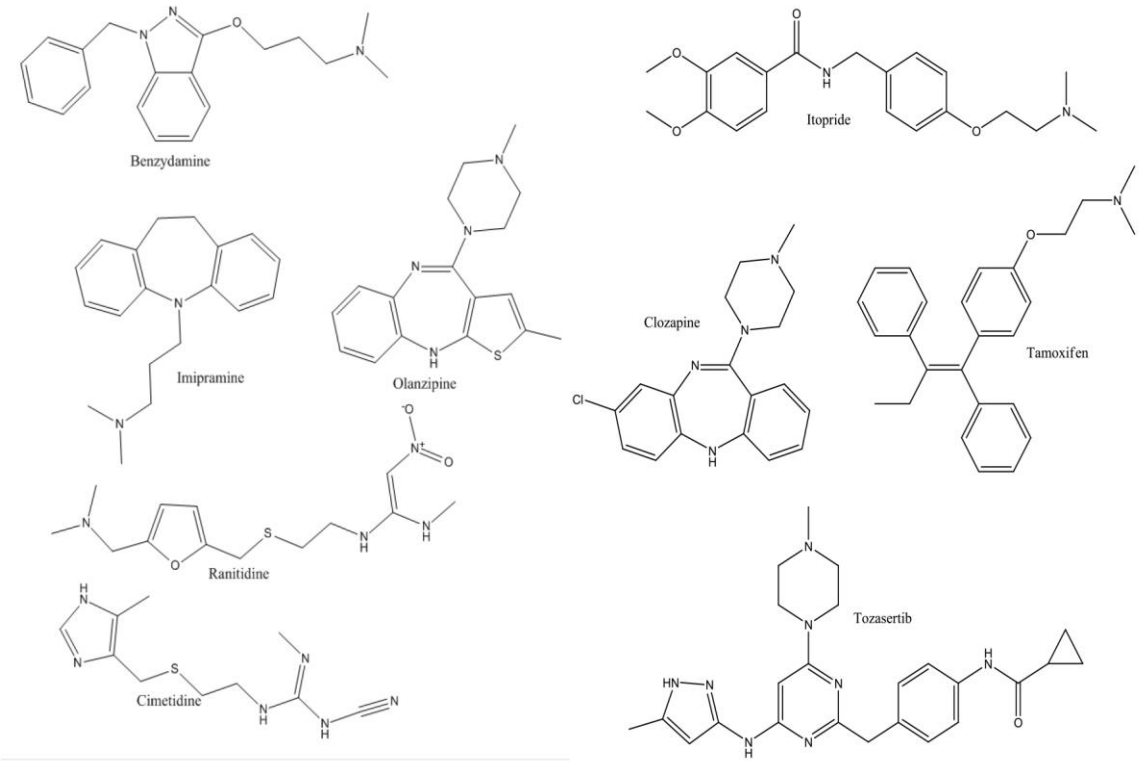

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Figure 2

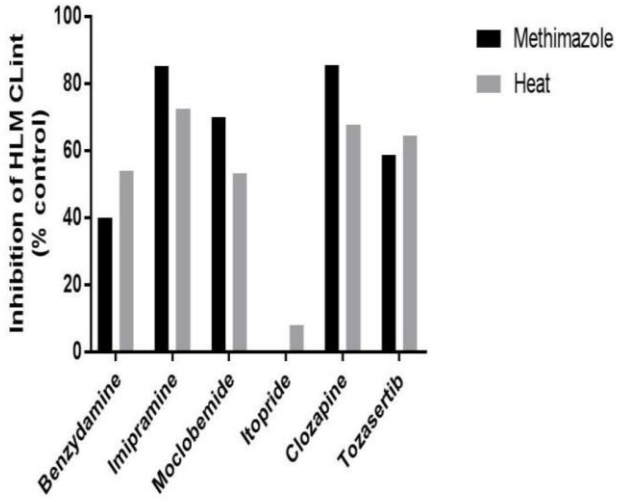

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Figure 3

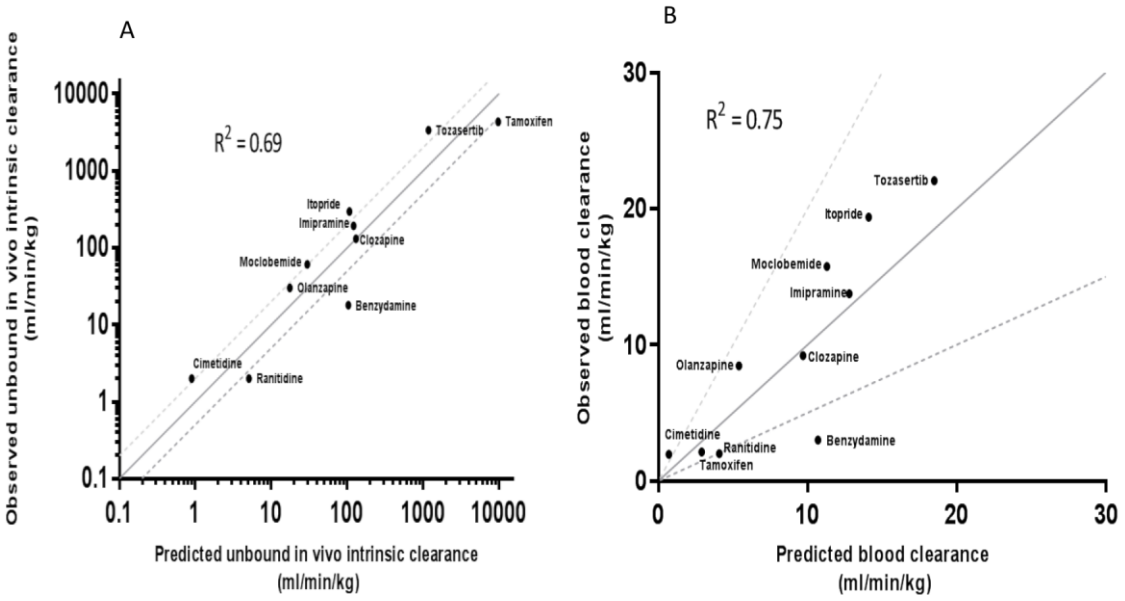

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Figure 4