Short Communication

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In Vitro – In Vivo Inaccuracy: The CYP3A4 Anomaly

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Running Title Page

IVIVE Inaccuracy: The CYP3A4 Anomaly

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Text pages: 8

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Words in Abstract: 133

Words in Introduction: 326

Words in Discussion: 873

Abbreviations: AFE average full error; AO aldehyde oxidase; CL_{int} intrinsic clearance; CL_H total hepatic clearance; ECCS Extended Clearance Classification System; IVIVE in vitro to in vivo extrapolation; P-gp P-glycoprotein

Significance Statement

For CYP3A4 substrates microsomes give markedly higher predictive IVIVE than for other metabolic enzymes, which is not found for hepatocytes. We hypothesize that this could be a result of CYP3A4—P-gp interplay or coordinated regulation in hepatocytes that would not be observed in microsomes.

Abstract

When predicting hepatic clearance using *in vitro* to *in vivo* extrapolation (IVIVE), microsomes or hepatocytes are commonly utilized. Here we examine intrinsic clearance values and IVIVE results in human hepatocytes and microsomes for compounds metabolized by a variety of enzymes. The great majority of CYP3A4 substrates examined had higher intrinsic clearance values in microsomes compared to hepatocytes, while the values were more similar between the two incubations for substrates of other enzymes. We hypothesize that this may be due to interplay between CYP3A4 and the efflux transporter P-glycoprotein, as they have been shown to exhibit coordinated regulation. When examining the prediction accuracy for substrates of other enzymes between microsomes and hepatocytes, average fold errors as well as overall error were similar, demonstrating once again that IVIVE methods are not adequately defined and understood.

Introduction

Despite hepatic clearance playing an important role in the pharmacokinetics and pharmacodynamics of molecules, accurately predicting the parameter during drug discovery is still challenging. Many have found inaccuracies when implementing *in vitro* to *in vivo* extrapolation (IVIVE), where intrinsic clearance (CL_{int}) is measured using microsomes or hepatocytes and scaled to a predicted *in vivo* hepatic clearance (CL_H) using scaling factors and a model of hepatic disposition (Bowman and Benet, 2016; Wood et al., 2017).

It has become apparent that the mechanisms behind the current IVIVE disconnect must be discovered and considered during the IVIVE process. Many ideas surrounding the systematic underprediction have been presented including donor variability (Floby et al., 2009), liver sample preparation and viability (Fisher et al., 2001), protein-binding discrepancies (Obach 1999; Kochansky et al., 2008), and ignoring extra-hepatic metabolism (Houston and Carlile, 1997; Chiba et al., 2009).

While microsomes are routinely used for IVIVE, as these subcellular fractions are easy to prepare and store, it would be expected that hepatocytes would yield more accurate clearance predictions given that hepatic transporters are present in hepatocytes and not in microsomes (Lam and Benet, 2004). One review found that human hepatocytes underpredict clearance by 3-6 fold, while microsomes underpredict by 9 fold (Chiba et al., 2009). However, more recent studies have found the overall error between the systems to be more similar (Bowman and Benet, 2016; Wood et al., 2017). Furthermore, it has recently been noted that there is CL_{int}-dependent underprediction (Hallifax et al., 2010) as well as CL_H-dependent underprediction (Bowman and Benet, 2019) with increasing clearance, a finding that is more marked for data generated in hepatocytes.

Recently Wood et al. (2017) compiled predicted clearance values from human microsomes and hepatocytes and El-Kattan et al. (2016) compiled primary enzyme information for compounds classified in the Extended Clearance Classification System (ECCS). Using both datasets, here we examine the role different metabolic enzymes may play in the values generated in the two systems and in IVIVE accuracy.

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Methods

Scaled *in vitro* CL_{int} values generated in human hepatocytes and microsomes (and corrected for incubational binding) and *in vivo* CL_{int} values were taken from Wood et al. (2017). In this source, *in vivo* CL_{int} was back-calculated using the well-stirred model accounting for protein binding. CL_H was determined by subtracting renal clearance from total CL in relevant cases where data were available.

Primary enzyme information was taken from El-Kattan et al. (2016). Of the 101 compounds with human hepatocyte values listed in Wood et al. (2017), 48 had primary metabolizing enzyme information in El-Kattan et al. (2016) and of the 83 compounds with human microsome values, 45 had primary metabolizing information reported. It should be noted that the enzyme assignments are qualitative and more than one enzyme could be involved.

The bias in predictions was determined by calculating the average fold error (AFE):

$$AFE=10^{\frac{1}{N}\sum\log(\frac{observed}{predicted})}$$

The accuracy of predictions was determined based on whether the predicted CL_{int} values fell within two fold of the observed CL_{int} values (Houston and Carlile, 1997):

$$0.5 \leq \frac{observed\ CL_{int}}{predicted\ CL_{int}} \leq 2$$

The 54 drugs investigated, organized by main metabolizing enzyme, are listed in Supplemental Table S1, with human hepatocyte and human microsome CL_{int} values and the AFE for both hepatocyte and microsome predictions.

Results

When comparing CL_{int} values generated in hepatocytes vs. microsomes, it became apparent that the values generated in microsomes for CYP3A4 substrates are often higher than those generated in hepatocytes (Figure 1A). Of the 14 CYP3A4 substrates that had values generated in both systems, 13 had higher CL_{int} values in microsomes, where diltiazem was the only example where the value was higher in hepatocytes (35 vs. 27 mL/min/kg in hepatocytes vs. microsomes). In comparison, the values between the two systems were more similar for CYP2C (n=7) and CYP2D6 (n=7) substrates and fell on both sides of the line of unity (Figure 1B). For UGT substrates (n=8) (Figure 1C), lower clearance compounds fell on both sides of the line of unity, while hepatocytes yielded higher values as CL_{int} increased.

These CL_{int} values generated for the same drugs in both hepatocytes and microsomes were then compared to *in vivo* CL_{int} to see the effect on IVIVE accuracy (Figure 2, Table 1). There were 39 overlapping compounds and CYP3A4 substrates had the highest AFE in hepatocytes of 5.88 (compared to 2.03 in microsomes). Although only three drugs were substrates of CYP1A2, it is interesting to note that the AFE was the lowest in both systems.

Given the potential of CL_{int}-dependent underprediction with hepatocytes in particular, the highest CL_{int} compounds were examined across enzymes. With the drugs examined here, substrates of CYP3A4 and CYP2D6 had similar observed highest CL_{int} values (Table 2). Despite having similar observed values, the difference in predicted values between microsomes and hepatocytes was not as marked for CYP2D6 substrates as for CYP3A4. For instance, with midazolam (CYP3A4) with an observed CL_{int} of 390 ml/min/kg, the CL_{int} measured in microsomes was 7.50 fold higher than the value measured in hepatocytes. With carvedilol (CYP2D6) with a similar observed CL_{int} value of 427 ml/min/kg, the CL_{int} measured in

microsomes was only 1.30 fold higher than the value measured in hepatocytes. In Figure 3, the difference between the values generated in the two systems appeared to more notably increase with observed CL_{int} only with CYP3A4 substrates.

Finally, all compounds with primary enzyme information were examined (n=48, hepatocytes; n=45, microsomes). When examining the number of compounds with accurate, under-, and overpredictions (Table 3), almost all of the errors were due to underprediction, agreeing with the systematic underprediction noted throughout the field. Substrates of CYP3A4 had the most accurate predictions in microsomes. When examining the human hepatocyte AFEs (Table 4), AO was an obvious outlier with only one compound as an example (zaleplon) that had a 22.1 fold error. Excluding AO and with the additional compounds added, CYP3A4 still had the highest AFE of 7.87. Upon further inspection, there was an outlier in this category as well (nitrendipine has a 668 fold error); however, after removing this drug, the AFE remained the highest at 5.96. When examining the AFE for human microsomes, CYP3A4 had the second lowest AFE of 2.08. The highest AFE was for UGT with 7.54, however here again there was an outlier (fenoprofen had a 159 fold error), and after removing it, the AFE dropped to 4.88, a value more comparable to that of the other enzymes.

Discussion

While predicting hepatic clearance with IVIVE using hepatocytes and microsomes is commonly done, there is still systematic underprediction. When comparing CL_{int} values measured in microsomes and hepatocytes, it became apparent that CYP3A4 substrates frequently had higher CL_{int} values in microsomes. Stringer et al. (2008) also saw similar results for 5 CYP3A4 substrates and found that the CL_{int} values for the same drugs were 10-50 fold higher in microsomes than hepatocytes. Foster et al. (2011) measured the clearance of compounds in hepatocytes and microsomes from the same donor livers and found that the CL_{int} values for the highest clearance substrates, in their case substrates of CYP3A4, were higher when measured in microsomes vs. hepatocytes, but the values were comparable between the systems for a low clearance CYP3A4 substrate. The authors hypothesized there could be cofactor rate limitation or permeation limitation for high clearance compounds in hepatocytes. For the compounds examined here, substrates of CYP3A4 and CYP2D6 had similar observed high CL_{int} values, but the difference in predicted values between the systems was not as marked for CYP2D6 substrates as for CYP3A4, making the cofactor limitation hypothesis less likely.

Although CYP3A4 substrates yielded higher CL_{int} values in microsomes, inherently microsomes are not mechanistically better predictors. The overall percentage of inaccurate predictions (77% for hepatocytes and 69% for microsomes) and the AFE (5.19 for hepatocytes and 3.47 for microsomes) were still high, emphasizing that present IVIVE methods are not adequately understood.

When examining the AFE for overlapping compounds as well as all compounds, CYP3A4 had the highest AFE in hepatocytes and CYP1A2 had the lowest AFE in both systems. If extrahepatic metabolism is ignored and hepatic clearance is assumed to be total clearance (or if

only renal clearance is subtracted from total clearance), then only using measurements from liver microsomes or hepatocytes could lead to IVIVE underpredictions. De Kanter et al. (2004) found that multi-organ (liver, lung, kidney, small intestine, colon) precision-cut slices from rats could predict drug clearance better than when only considering the contribution of the liver. Given that CYP1A2 is the only enzyme of those examined with no evidence of intestinal metabolism while CYP3A4 contributes 80% to the total CYP abundance in the intestine (Paine et al., 2006), this could potentially explain the trend noted with hepatocytes. However, a similar trend would have been expected in microsomes, and *in vivo* CL_H values are typically taken from intravenous studies when available, making a potential contribution of intestinal metabolism smaller. While genetic polymorphisms could also be an explanation for the errors seen for several of the examined enzymes, both over- and underpredictions would be expected (Chiba et al., 2009), which was not found here.

When considering the difference in CYP3A4 CL_{int} values between hepatocytes and microsomes (and the corresponding different AFEs between the systems), a possible explanation could be due to transporter-enzyme interplay that is present in hepatocytes, but not in microsomes. Several publications have noted the common substrate specificity, tissue localization, and coinducibility of CYP3A4 and the efflux transporter P-glycoprotein (P-gp) and proposed that the enzyme and transporter could play complementary roles in the absorption, distribution, metabolism, and elimination of compounds (Wacher et al., 1995; Benet et al., 1996; Hall et al., 1999; Zhang and Benet, 2001). In the intestine where drugs will contact P-gp prior to CYP3A4, they can be effluxed back into the lumen before diffusing into enterocytes to be metabolized, forming more metabolites than without P-gp (Benet, 2009). In the liver where drugs will contact CYP3A4 prior to P-gp, drugs will be pumped out by P-gp, forming less

metabolites than without P-gp (Benet, 2009). Therefore, CYP3A4 substrates evaluated in hepatocytes may have lower CL_{int} values because they can be effluxed by P-gp, whereas when they are evaluated in microsomes with no P-gp present, they are subject to more metabolism by CYP3A4. Lam and Benet (2004) found that elacridar, a P-gp inhibitor, had no effect on digoxin metabolism in rat microsomes, while in rat hepatocytes the P-gp inhibitor, at low concentrations (1 μM) that did not change digoxin uptake, caused increased metabolism. Cummins et al. (2002) also demonstrated that in CYP3A4-transfected Caco-2 cells, inhibiting P-gp reduced CYP3A metabolism in the apical to basolateral direction (similar to the intestine), but increased metabolism in the basolateral to apical direction (similar to the liver). Bow et al. (2007) found that P-gp is internalized after hepatocyte isolation and suggested that "drug efflux from suspended hepatocytes are not an appropriate system to study apical efflux/canalicular excretion of drugs." However, they did not negate the finding of Lam and Benet (2004) and others that there can be a transporter effect potentially from the internalized proteins in hepatocytes that is different than in microsomes. We hypothesize and will test whether this transporter internalization could have a negative effect on metabolic activity of CYP3A4, potentially even for drugs that are not strong substrates of P-gp as a result of coordinated regulation.

In conclusion, when examining CL_{int} values generated in microsomes and hepatocytes, values were almost always larger for CYP3A4 substrates in microsomes, perhaps due to CYP3A4-Pgp interplay present in hepatocytes but not microsomes. While IVIVE predictions were better for these substrates in microsomes, overall % inaccuracies were similar between the two systems, highlighting that IVIVE methods are not adequately understood.

Authorship Contributions

Participated in research design: Bowman, Benet

Conducted experiments: Bowman

Performed data analysis: Bowman, Benet

Wrote or contributed to the writing of the manuscript: Bowman, Benet

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Footnotes

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Legends for Figures:

Figure 1: CL_{int} values generated in hepatocytes vs. microsomes for CYP3A4 substrates (A), CYP2C and CYP2D6 substrates (B), and UGT substrates (C).

Figure 2: The error in CL_{int} predictions for overlapping compounds grouped by main enzyme in hepatocytes (A) and microsomes (B).

Figure 3: Observed CL_{int} vs. the difference in predicted CL_{int} from microsomes vs. hepatocytes with CYP3A4 substrates depicted as red squares.

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Table 1: AFE across enzymes for compounds with both hepatocyte and microsome CL_{int} predictions.

Enzyme	CYP1A2	CYP2C	CYP2D6	CYP3A4	UGT	Total
AFE Hepatocytes	0.97	5.35	5.03	5.88	5.13	4.76
AFE Microsomes	1.94	4.21	6.18	2.03	7.54	3.68
n	3	7	7	14	8	39

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Table 2: The CYP3A4 and CYP2D6 substrates with the highest observed CL_{int} values and the difference in their predicted values between microsomes and hepatocytes.

Drug	Enzyme	CL_{int} Observed	Predicted CL_{int} Microsomes / Hepatocytes
Sildenafil	CYP3A4	298	4.29
Midazolam	CYP3A4	390	7.50
Verapamil	CYP3A4	750	2.23
Buprenorphine	CYP3A4	1354	8.47
Propranolol	CYP2D6	333	0.36
Diphenhydramine	CYP2D6	360	0.73
Carvedilol	CYP2D6	427	1.30
Propafenone	CYP2D6	4672	3.43

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Table 3: The number of compounds with accurate predictions, under-, and overpredictions grouped by main metabolizing enzyme in human hepatocytes and microsomes.

	Enzyme	AO	CYP1A2	CYP2C	CYP2D6	CYP3A4	UGT	Total
Human Hepatocytes	**#Accurate**	0	1	2	3	1	4	11
	#Underpredicted	1	1	6	5	16	7	36
	#Overpredicted	0	1	0	0	0	0	1
Human Microsomes	**#Accurate**	0	0	2	2	8	2	14
	#Underpredicted	0	2	6	5	9	6	28
	#Overpredicted	0	1	0	0	2	0	3

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Table 4: Average fold error and percentage of inaccurate predictions (those falling outside two fold of observed values) grouped by main metabolizing enzyme for human hepatocytes and microsomes.

	Enzyme	AO	CYP1A2	CYP2C	CYP2D6	CYP3A4	UGT	Total
Human Hepatocytes	**AFE**	22.1	0.97	4.84	4.75	7.87	4.25	5.19
	% inaccurate	100%	66.7%	75.0%	62.5%	94.1%	63.6%	77.1%
	n	1	3	8	8	17	11	48
Human Microsomes	**AFE**	NA	1.94	4.04	6.18	2.08	7.54	3.47
	% inaccurate	NA	100%	75.0%	71.4%	57.9%	75.0%	68.9%
	n	NA	3	8	7	19	8	45

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Figure 1

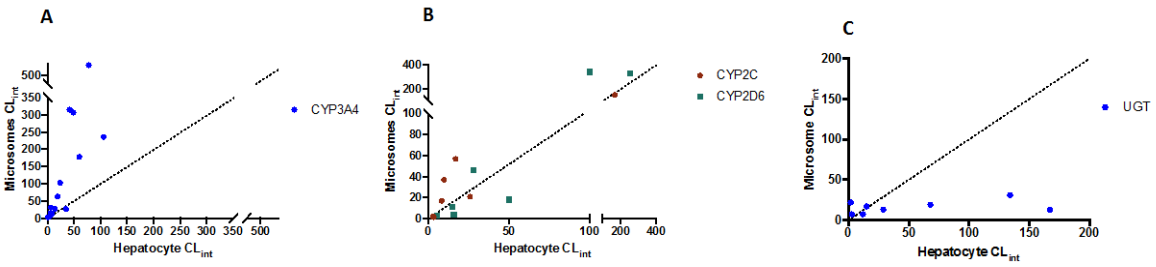

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Figure 2

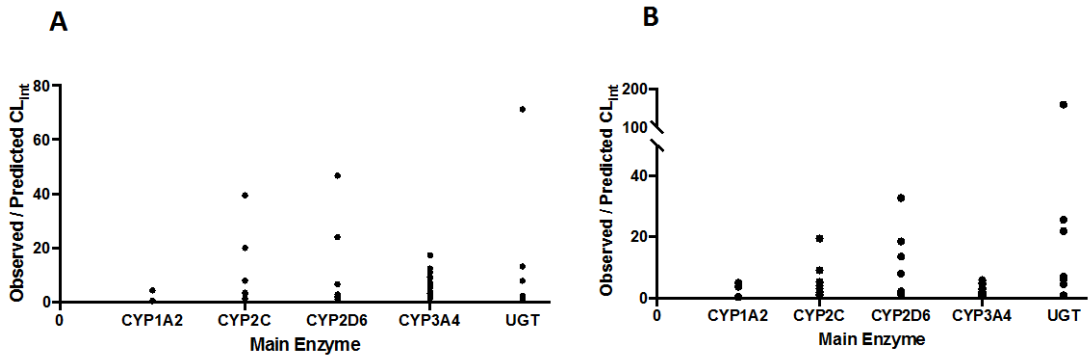

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Figure 3