An Improved *In Vitro* Model for Safety Screening of Drugs in Development: Accurate Evaluation of Drug-Mediated Cytochrome P450 Inhibition Using H-Class *InVitro*CYP Microsomes

Timothy A. Moeller, Ji Young Lee, Dan Dryden, Christopher M. Terrell, and Jennifer Fleischer BioreclamationIVT, 1450 South Rolling Road, Baltimore, MD

ABSTRACT

Human liver microsomes are a well-characterized *in vitro* model for identifying cytochrome P450 (CYP) inhibition during drug development. Use of hepatic microsomes from human donors reduces concerns about species differences and also may reduce the number of animals used during preclinical development. However, the utility of this model is limited by variations in enzyme activity among different microsomal preparations, variations among the isozymes within a single preparation, and also by overall low CYP isoform enzyme activity. In drug safety studies, low microsomal CYP enzyme activity can preclude accurate quantitation of CYP inhibition. Selective pooling of well-characterized microsomes can overcome these limitations. Using a proprietary algorithm, we created H-class microsomes that provide consistently higher activity across the panel of clinically relevant CYP isoforms, including those that are lower in activity. IC_{50} values of known inhibitors of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were determined using isoform-selective substrates. The rate of CYP metabolite formation was measured using validated LC/MS/MS assays. The data indicate that the average dynamic range of CYP enzyme activity increased by 90% in H-class microsomes relative to microsomes with lower activity, avoiding the occurrence of below quantifiable limit (BQL) values and allowing for accurate calculation of IC_{50} values. H-class microsomes are an important emerging tool for accurate CYP inhibition characterization during drug development.

INTRODUCTION

Human liver microsomes (HLM) are the gold standard for ADME applications since the discovery of cytochrome P450 enzymes and their clinical importance by pioneers like the Millers, Axelrod, and Brodie[1]. The identification of isozyme-selective substrates and inhibitors for human CYPs and the development of specific and sensitive analytical methods have positioned microsomes as key reagents for screening new chemical entities (NCEs) in a rapid and cost-effective manner for drug-drug interaction (DDI) potential. HLM are generally preferred over hepatocytes for CYP inhibition studies due to their ease of use, availability, and presence of clinically relevant CYP enzymes that metabolize the majority of marketed drugs[4]. Moreover, determination of CYP inhibition and metabolism kinetics, both of which may be affected by uptake and excretion processes in hepatocytes, is facilitated in HLM.

DDI screening studies are of critical importance for establishing the safety profile of an NCE during the drug discovery and early development processes. Using microsomes to identify CYP inhibition or unfavorable pharmacokinetic properties early in the development process is an effective means of reducing animal use in preclinical studies and also may prevent the need for costly clinical DDI trials. Because of the demonstrated importance of *in vitro* ADME studies, the pharmaceutical industry (PhRMA) and FDA have published scientific reports and guidances concerning recommendations on the execution and regulatory implications of DDI studies[2,3,4]. In determining the CYP inhibition potential of an NCE, the FDA recommends using isoform-selective substrates at concentrations below the K_m in order to correlate inhibitor IC_{50} with K_i. However, insufficient isozyme metabolic rate, as observed in conventional microsome preparations, often necessitates using substrates at higher than recommended concentrations in order to obtain quantifiable substrate metabolite data, potentially resulting in inaccurate estimates of K_i. To overcome this limitation, analytical detection methods must become more sensitive or isozyme activities must be increased.

To directly address this limitation, we have developed a proprietary algorithm that strategically combines characterized microsomes from individual human donors in a manner that consistently and reliably produces microsomes with high CYP isozyme activities as compared to traditional microsome preparations. *InVitro*CYP H-class is a pool of microsomes with high CYP activities across clinically relevant CYP isoforms. However, because high CYP activity may not be appropriate for all ADME applications, *InVitro*CYP M-class was formulated for moderate CYP activity to support studies requiring an average response, like clearance. IC_{50} values for CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 were determined as recommended by the FDA using nine preferred substrates near their respective K_m concentrations with preferred or acceptable inhibitors over a two-log inhibitor concentration range. Cl_{int} values were obtained for piroxicam, warfarin, diltiazem, propranolol, losartan, verapamil, and 7-ethoxycoumarin.

The IC_{50} values determined in the *InVitro*CYP H-class were similar to those determined with M-class microsomes, while the average dynamic range was increased by 90%. *InVitro*CYP H-class microsomes are a new high activity pool of human microsomes that offers researchers a greater dynamic range of activity in determining the inhibition characteristics of an NCE without compromising initial rate conditions or clinical relevance. M-class microsomes may be more appropriate for use in clearance studies as compared to H-class microsomes, although further investigation is needed. With this application-specific, two-class system, researchers have greatly improved tools to evaluate drug ADME profiles.

MATERIALS & METHODS

Reagent preparation.

- Human liver microsomes were prepared by differential centrifugation of liver homogenates[5] and blended selectively to achieve specified ranges of CYP activities, yielding H-class lot JJT and M-class lots XRM and KWO.
- Piroxicam, warfarin, diltiazem, propranolol, losartan, verapamil, and 7-ethoxycoumarin were dissolved in acetonitrile to prepare 100X stock solutions of 0.5 mM.
- Phenacetin, coumarin, bupropion, tolbutamide, *S*-mephenytoin, dextromethorphan, chlorzoxazone, and testosterone were dissolved in acetonitrile to prepare 100X stock solutions of 5, 0.8, 15, 15, 5, 0.5, 5, and 10 mM, respectively; midazolam was dissolved in methanol to prepare a 100X stock solution of 0.8 mM.
- Furafylline, sulfaphenazole, and ketoconazole were dissolved in acetonitrile, tranylcypromine, and diethyldithiocarbamate were dissolved in deionized water, and ticlopidine, nootkatone, and quinidine were dissolved in methanol to prepare 100X stock solutions.
- NADPH regenerating system (NRS) solution consisted of β-NADP+, glucose-6-phosphate, and glucose-6-phophate dehydrogenase in a sodium bicarbonate solution.

Stability and clearance.

Incubation mixtures were prepared in 0.1 M Tris buffer (pH 7.4 ± 0.05) and contained microsomes (0.5 mg protein/mL) and drug or 1% acetonitrile as vehicle control. After a 5-minute pre-incubation at 37ºC, NRS was added to initiate the reactions; 2% bicarbonate solution was added to the no-NADPH control samples, i.e., metabolic negative control (MNC). Incubations were continued for an additional 0, 15, 30, 45, 60, and 90 minutes (0 and 90 minutes only for the MNC samples) and were terminated by adding methanol. All samples were maintained on ice for at least 10 minutes following reaction termination. Samples were stored in cryovials at -70 ± 10°C until analysis by LC/MS/MS. Concentration of drug was determined and loss of parent relative to MNC was calculated. Clearance was determined by the trapezoidal rule using GraphPad Prism®.

Inhibition.

Incubation mixtures were prepared in 0.1 M Tris buffer (pH 7.4 ± 0.05) and contained microsomes (0.25 mg protein/mL for CYP2C9, CYP2D6, CYP2E1, and CYP3A4; 0.5 mg protein/mL for CYP2A6, CYP2B6, and CYP2C19), inhibitor or 1% inhibitor solvent as the vehicle control, and a CYP isoform-selective substrate. After a 5-minute pre-incubation at 37ºC, NRS was added to initiate the reactions; 2% bicarbonate solution was added to the no-NADPH control samples. CYP1A2 incubations contained microsomes (0.5 mg protein/mL), inhibitor or 1% acetonitrile as the vehicle control, and NRS (or 2% bicarbonate solution) incubated for 15 ± 2 minutes prior to adding the substrate. CYP2A6 and CYP3A4 (testosterone) incubations were conducted for an additional 10 minutes. CYP3A4 (midazolam) incubations were conducted for an additional 20 minutes. All other incubations were conducted for an additional 30 minutes. The incubations were terminated by adding methanol. All samples were maintained on ice for at least 10 minutes following reaction termination. The samples were stored in cryovials at -70 ± 10°C until analysis by LC/MS/MS. Substrate metabolite formation was quantitated from which specific activities were calculated (as pmol/mg protein/min). Percent inhibition was calculated by comparing specific activity without and with inhibitor for each isozyme. IC_{50} values were calculated by nonlinear regression using GraphPad Prism®.

REFERENCES

1. Estabrook, R.W. (2003). Drug Metab Disp. 31:1461-1473.
2. Draft Guidance for Industry – Drug Interaction Studies – Study Design, Data Analysis, and Implications for Dosing and Labeling. (September 2006). U.S. Department of Health and Human Services, Food and Drug Administration.
3. Tucker, G.T., *et al.* (2001). Cl Pharm Ther 70:103-114.
4. Bjornsson, T.D., *et al.* (2003). Drug Metab Disp. 31:815-832.
5. Guengerich, F.P. (1989). Analysis and characterization of enzymes. In Principles and Methods of Toxicology. Raven Press, New York, pp.777–813.
6. Tarrus, *et al.* Accumulation of Caffeine in Healthy Volunteers Treated with Furafylline. *Br J Clin Pharmacol.* 49, 49-58 (1987).
7. Zhang, *et al. Drug Metab Disp.* 26, 897-902 (2001).
8. Richter, *et al.* J Pharmacol Exp Ther. 308, 189-97. (2004).
9. Bourrie, *et al. J Pharmacol Exp Ther.* 277, 321-32 (1996).
10. von Moltke, *et al. J Pharm Sci.* 87, 1184-9. (1998).
11. Thummel, *et al. Drug Metab Disp.* 21, 350-357 (1993).
12. McConn 2nd, *et al. Drug Metab Disp.* 32, 1083-91 (2004).
13. Obach S and Reed-Hagen A. *Drug Metab Disp.* 30, 831-837 (2002).

CYP Activity Dynamic Range is Increased in H-Class Microsomes

Lots JJT and XRM (H-class and M-class, respectively) were characterized for CYP isozyme activity and UGT activity (Table 1). The dynamic range of activity as measured from full, uninhibited activity to activity at highest inhibitor concentration or lower limit of quantitation was consistently higher in H-class microsomes. The dynamic range was on average 90% greater in H-Class over M-Class with a minimum increase of approximately 20% (CYP2E1) and a maximum of approximately 275% (CYP2B6) (Figure 1). As expected, calculated IC_{50} values were similar between M-class and H-class microsomes (Table 2).

Table 1. Comparative characterization of *InVitro*CYP H-class lot JJT and M-class lot XRM. (ECOD, 7-ethoxycoumarin-O-deethylase and UGT, uridine diphosphate-glucuronosyltransferase)

Class	Lot	Description	Total P450	1A2	2A6	2B6	2C19	2C9	2D6	2E1	3A4M	3A4T	ECOD	UGT
H-Class	JJT	Mixed Gender Pool (25-donor)	0.328	459	157	383	46	236	65	391	421	2500	378	1734
M-Class	XRM	Mixed Gender Pool (50-donor)	0.250	220	119	55	15	74	33	260	201	768	64	1774
Fold over M-Class Values			1.3	2.1	1.3	7.0	3.1	3.2	2.0	1.5	2.1	3.3	5.9	1.0

Figure 1. Dynamic range of isozyme activity as measured by preferred isoform-selective substrates in *InVitro*CYP H-class JJT and M-class XRM. Isozymes were categorized into low, mid, and high dynamic range by comparing full and minimum specific activities. Full activity was defined as specific activity without inhibitor. Minimum activity was defined as specific activity with highest concentration of inhibitor. For CYP2B6 and CYP3A4M (midazolam), the LLOQ (analytical lower limit of quantitation) was 1.3 and 0.4 pmol/min/mg protein, respectively, and was too small to be observed using the associated scale.

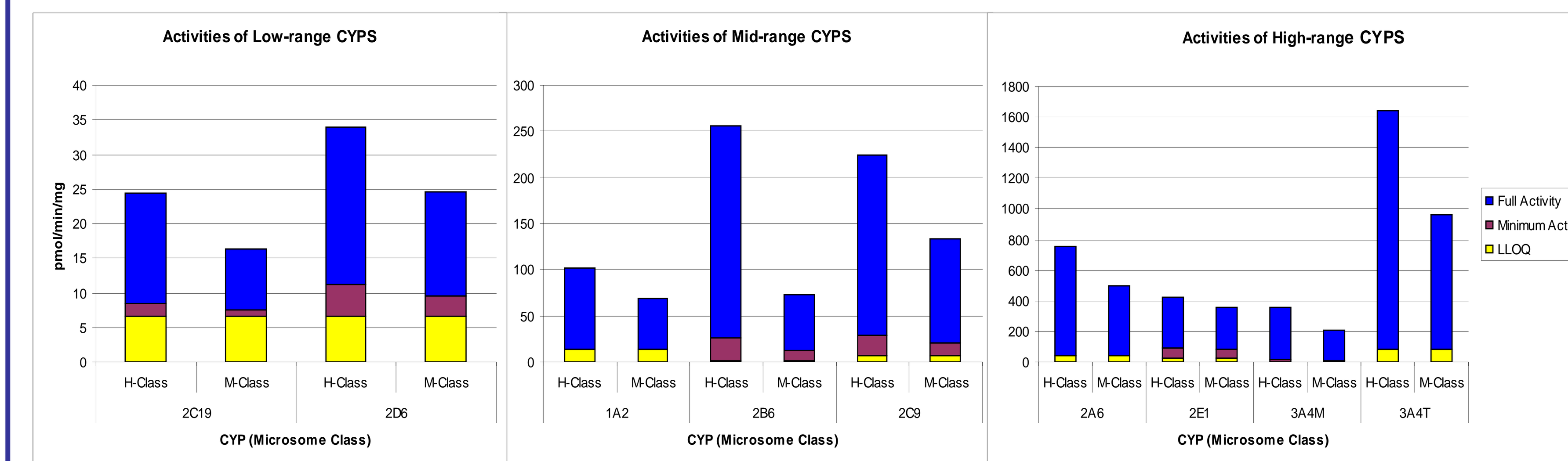


Table 2. CYP isozymes and their respective FDA-preferred substrates and inhibitors with comparative IC_{50} values using *InVitro*CYP H-class lot JJT and M-class lot XRM. (*FDA-acceptable substrate.)

CYP	Substrate	Inhibitor	Type	IC50 H-Class	IC50 M-class
1A2	Phenacetin	Furafylline	Mechanistic[6]	0.504	0.826
2A6	Coumarin	Tranylcypromine	Competitive[7]	1.573	1.508
2B6	Bupropion	*Ticlopidine	Mechanistic[8]	0.701	0.637
2C9	Tolbutamide	Sulfaphenazole	Competitive[9]	0.685	0.883
2C19	s-Mephenytoin	*Nootkatone	Unreported	21.90	9.562
2D6	Dextromethorphan	Quinidine	Competitive[10]	0.127	0.134
2E1	Chlorzoxazone	*Diethyldithiocarbamate	Mechanistic[11]	13.630	5.324
3A4M	Midazolam	Ketoconazole	Competitive[12]	0.088	0.092
3A4T	Testosterone	Ketoconazole	Competitive[12]	0.055	0.056

The Use of InVitro CYPs in Metabolic Stability/Clearance Studies

Lots JJT and KWM (H-class and M-class, respectively) were characterized (Table 3) and used to determine intrinsic clearance by substrate depletion[13] of 7 known compounds representing high (verapamil, 7-ethoxycoumarin), moderate (losartan, propranolol, diltiazem), and low (piroxicam, warfarin) clearance levels. Although H-class microsomes consistently and expectedly had higher clearance values relative to M-class microsomes (Table 4), the segregation into high, moderate and low metabolic stability ranking is maintained (Figure 2). However, a discernible shift is observed within the moderately-cleared group from the high-moderate boundary to the moderate-low boundary.

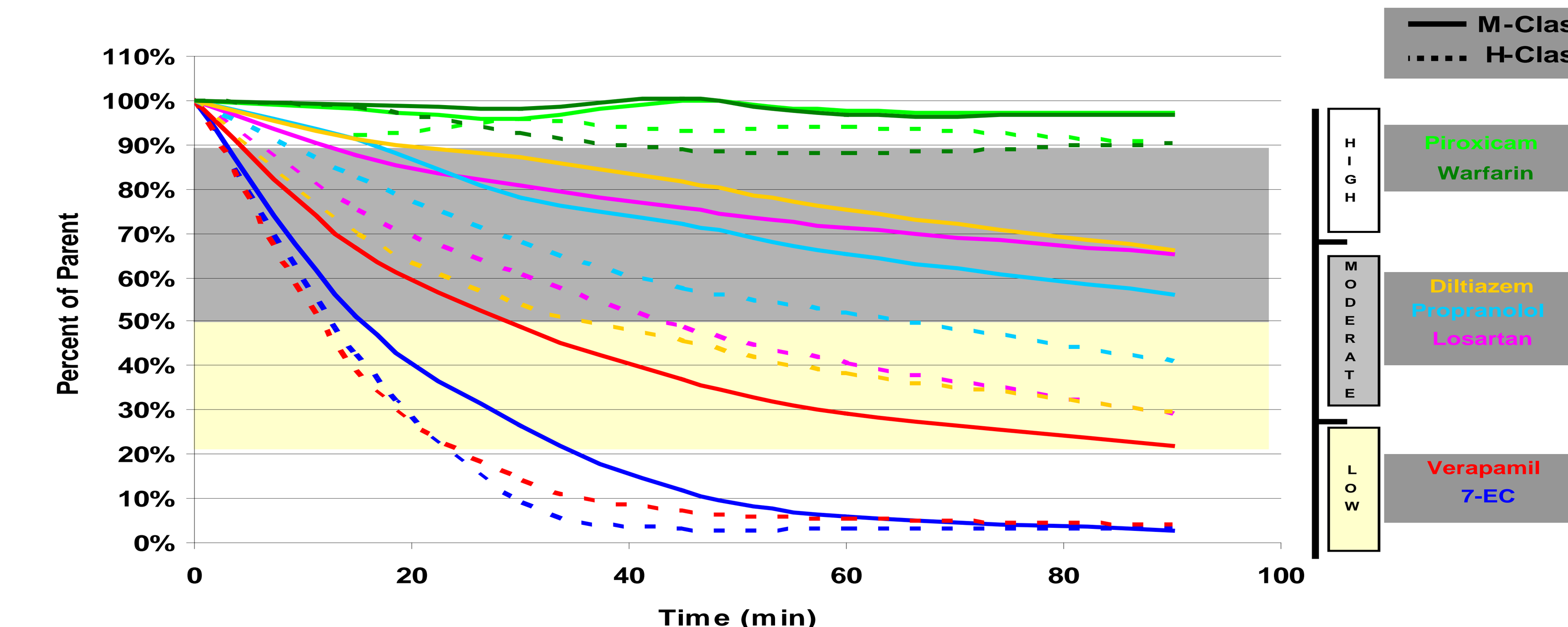
Table 3. Comparative characterization of *InVitro*CYP H-class lot JJT and M-class lot KWO. (ECOD, 7-ethoxycoumarin-O-deethylase and UGT, uridine diphosphate-glucuronosyltransferase)

Class	Lot	Description	Total P450	1A2	2A6	2B6	2C19	2C9	2D6	2E1	3A4M	3A4T	ECOD	UGT
H-Class	JJT	Mixed Gender Pool (25-donor)	0.328	459	157	383	46	236	65	391	421	2500	378	1734
M-Class	KWO	Mixed Gender Pool (50-donor)	0.223	182	112	42	14	70	34	272	208	679	59	1638
Fold over M-Class Values			1.3	2.5	1.4	9.1	3.3	3.4	1.9	1.4	2.0	3.7	6.4	1.1

Table 4. Comparative intrinsic clearance values in *InVitro*CYP H-class lot JJT and M-class lot KWO.

Compound	Clearance (µL/mg/minute) M-class	Clearance (µL/mg/minute) H-class
Piroxicam	0.664	2.28
Warfarin	0.680	2.23
Diltiazem	9.13	29.5
Propanolol	13.0	20.3
Losartan	9.68	28.6
Verapamil	37.7	116
7-ethoxycoumarin	88.5	127

Figure 2. Metabolic stability of known compounds as measured by substrate depletion over time using *InVitro*CYP H-class JJT and M-class KWO microsomes.



CONCLUSIONS

1. *InVitro*CYP H-class human liver microsomes are strategically blended from pre-characterized donors to provide higher activities for clinically relevant CYPs, which results in a greater dynamic range for inhibition studies. IC_{50} values were similar to those obtained from microsomes with more traditional CYP activity levels (M-class), demonstrating the relevance of H-class microsomes in evaluating the inhibition potential of chemicals.
2. Although metabolic stability and intrinsic clearance can be evaluated in H-class microsomes, clearance may be overestimated in some cases. Compounds that border the high-moderate or moderate-low stability designations may be placed in a lower stability class when evaluated in H-class microsomes than when evaluated in M-class microsomes. This illustrates the application-specific nature of the different classes of *InVitro*CYP microsomes.
3. *InVitro*CYP H-class microsomes may show utility for other ADME applications. As a direct consequence of their increased enzymatic activity, metabolite formation may be increased in *InVitro*CYP H-class microsomes, facilitating metabolite profiling and identification and overcoming analytical sensitivity and chemical availability issues. Moreover, high activity could be diluted to more conventional ranges in order to reduce the total protein in HTS or traditional stability assays, thereby lowering non-specific binding of compound to protein and cost per sample. These and other uses warrant further investigation.