

# Development and Validation of a Higher-Throughput Cytochrome P450 Inhibition Assay with the Novel Cofactor-Supplemented Permeabilized Cryopreserved Human Hepatocytes (MetMax Human Hepatocytes)

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Received June 6, 2019; accepted July 22, 2019

## ABSTRACT

Here, we report the application of a novel hepatocyte system, the cofactor-supplemented permeabilized cryopreserved human hepatocytes [MetMax human hepatocytes (MMHs)] in a higher-throughput 384-well plate assay for the evaluation of cytochrome P450 (P450) inhibition. The assay was created to develop physiologically relevant P450 inhibition information, taking advantage of the complete organelle composition and their associated drug-metabolizing enzymes of the MMH but with the ease of use of human liver microsomes, including storage at  $-80^{\circ}\text{C}$  instead of in liquid nitrogen, and thaw and use without centrifugation and microscopic evaluation as required for intact hepatocytes. Nine key P450 isoforms for drug metabolism (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) were evaluated using multiple isoform-selective inhibitors. Results with MMH were found to be comparable to those obtained with intact cryopreserved human hepatocytes (CHHs). Isoform-selective drug-metabolizing enzyme pathways evaluated were phenacetin *O*-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), bupropion hydroxylation (CYP2B6), amodiaquine *N*-deethylation (CYP2C8), diclofenac 4-hydroxylation (CYP2C9), *s*-mephenytoin 4'-hydroxylation (CYP2C19), dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and midazolam 1'-hydroxylation and testosterone

6 $\beta$ -hydroxylation (CYP3A4). The  $K_m$  values obtained with MMHs were comparable with those reported in the literature for CHHs. Using substrate concentrations at or near  $K_m$  values, the  $\text{IC}_{50}$  values for the standard inhibitors against the P450 activities were found to be comparable between MMHs and CHHs, with 73% and 84% of values falling within 2-fold and 3-fold, respectively, from the line of unity. The results indicate that MMHs can be an efficient experimental system for the evaluation of P450 inhibition in hepatocytes.

## SIGNIFICANCE STATEMENT

MetMax human hepatocytes (MMHs) are cofactor-supplemented cryopreserved human hepatocytes with the complete drug-metabolizing enzyme pathways of the conventional hepatocytes but with the convenience of human liver microsomes, including storage at  $-80^{\circ}\text{C}$  instead of in liquid nitrogen, and direct thaw and use without a need for centrifugation and microscopic examination. Here, we report the application of MMH in a high-throughput assay in a 384-well plate format for the evaluation of cytochrome P450 (P450) inhibition. Our results show that data obtained with MMH are similar to those with conventional hepatocytes, suggesting that the MMH 384-well P450 inhibition assay can be used routinely for the evaluation of drug-drug interaction potential of new chemical entities in drug development.

## Introduction

During drug discovery and development in the pharmaceutical industry, new chemical entities are routinely screened for their drug-drug interaction (DD) potential. Human liver microsomes (HLMs) are widely used for high-throughput screening of cytochrome P450 (P450) inhibitory potential (Obach et al., 2005, 2006), with throughput enhanced via the use of automated liquid-handling systems coupled with liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis (Lim et al., 2013; Kozakai et al., 2014; Li et al., 2015), yielding in vitro reversible ( $\text{IC}_{50}$ ,  $K_i$ ) and irreversible inhibition constants

[maximal inactivation rate constant ( $k_{\text{inact}}$ ); inactivation rate constant ( $K_i$ )]. The inhibition constants, along with the unbound concentrations of the inhibitors in plasma ( $f_{u,\text{plasma}}$ ) and microsomes ( $f_{u,\text{mic}}$ ), serve as an input for the mechanistic static or dynamic models that predict the magnitude of an in vivo DDI for a given victim-perpetrator pair (Fahmi et al., 2009). Although widely applied, there are concerns that the accuracy of the prediction of clinical DDI potential based on HLM results may be hampered by the lack of uptake transporters, cytosolic proteins, and incompleteness of the drug-metabolizing enzyme pathways, leading to overestimation of the inhibitory potential (Brown et al., 2007b, 2010; Xu et al., 2009; Mao et al., 2013). To overcome this major deficiency of HLMs, cryopreserved human hepatocytes (CHHs) have been applied toward the evaluation of reversible (Oleson et al., 2004;

<https://doi.org/10.1124/dmd.119.088237>

**ABBREVIATIONS:** CHH, cryopreserved human hepatocyte; DDI, drug-drug interaction; HLM, human liver microsome; HQM, Hepatocyte Incubation Medium; IVAL, In Vitro ADMET Laboratories; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MMH, MetMax human hepatocyte; P450, cytochrome P450.

Li, 2009; Brown *et al.*, 2010; Doshi and Li, 2011; Mao *et al.*, 2012) and irreversible P450 inhibition parameters (Zhao *et al.*, 2005; McGinness *et al.*, 2006; Li and Doshi, 2011) to improve the accuracy of the prediction of drug interaction magnitude *in vivo*. The use of CHHs in higher-throughput P450 inhibition assays is hampered by the fragility of the cells and the complicated handling procedures, including centrifugation, microscopic examination, and storage in liquid nitrogen freezers, which may not be available in laboratories equipped mainly for analytical chemistry.

Here, we report the development of a higher-throughput hepatocyte-based P450 inhibition assay using a novel human hepatocyte system, the cofactor-supplemented permeabilized human hepatocytes [MetMax human hepatocytes (MMHHs)] (Li *et al.*, 2018). MMHHs have the desirable properties of both intact hepatocytes and HLMs—the completeness of the drug metabolizing enzymes in hepatocytes and the robustness and ease of handling of HLMs, including storage in a  $-80^{\circ}\text{C}$  freezer instead of liquid nitrogen, and use directly after thawing without a need for centrifugation and microscopic examination as required for CHHs. Our results show that MMHHs can be used in a 384-well-plate format for the evaluation of P450 inhibition, yielding results similar to those obtained with intact human hepatocytes. The MMHH 384-well-plate P450 inhibition assay may represent an effective approach for the early screening of DDI potential of new chemical entities.

### Materials and Methods

MMHHs (mixed gender, pool of 10, cell density of  $5 \times 10^6$  cells·ml $^{-1}$ , catalog number 82130, and lot number PHHX-8012), cofactor N10 for MetMax Hepatocytes (Catalog number 82187), CHHs (pool of 10, lot number PHH 8008), Universal Cryopreservation Recovery Medium, and Hepatocyte Incubation Medium (HQM, serum free, catalog number 81040) were provided by In Vitro ADMET Laboratories Inc. (IVAL, Columbia, MD) as part of a collaboration to develop and validate high-throughput P450 inhibition assays. The P450 substrates phenacetin, coumarin, amodiaquine, bupropion, diclofenac, mephenytoin, dextromethorphan, and chlorzoxazone were obtained from Sigma-Aldrich (St. Louis, MO). Midazolam was obtained from Lake Chemicals Private Limited (Bangalore, Karnataka), and testosterone was obtained from Acros Organics. The metabolites acetaminophen, hydroxy coumarin, hydroxy midazolam, and 6-hydroxy testosterone were obtained from Sigma-Aldrich. Hydroxy bupropion, hydroxy mephenytoin, hydroxy chlorzoxazone, and dextropran were obtained from Corning (Woburn, MA). Desethyl amodiaquine was obtained from Cypex (Dundee, UK). All chemical inhibitors used in this study were obtained from Sigma-Aldrich except for paroxetine and mibefradil, which were obtained from Tocris (Ellisville, MO), and troleandomycin, which was obtained from Enzo Life Sciences (Farmingdale, NY). The 384-deep-well MASTERBLOCK polypropylene plates (170- $\mu\text{l}$  capacity) were obtained from Greiner Bio-One North America Inc. (Monroe, NC). The Axygen AxyMat silicone sealing mats (part number AM-384-DW-SQ) for 384-deep-well plates and microplates were obtained from Corning Inc. (Salt Lake City, UT). All other reagents were obtained from standard suppliers.

**Thawing Procedures for MMHHs and CHHs.** The MMHH vial was thawed and used directly without the need for centrifugation or cell viability determination (Li *et al.*, 2018). In brief, MMHH vials were removed from the freezer ( $-80^{\circ}\text{C}$ ), thawed, and the contents were transferred to cofactor N10 vials (IVAL) with gentle mixing. The cell suspension was diluted to  $2\times$  the final cell density with HQM. The final cell densities of MMHHs were 0.15, 0.3, and  $0.6 \times 10^6$  cells·ml $^{-1}$  for time or cell density optimization study and  $0.2 \times 10^6$  cells·ml $^{-1}$  for enzyme kinetic ( $V_{\text{max}}$  and  $K_m$ ) and inhibition ( $\text{IC}_{50}$ ) studies. CHHs were thawed according to the instructions provided by the supplier (IVAL). In brief, the hepatocytes were thawed in a  $37^{\circ}\text{C}$  water bath, and the contents were transferred to a 50-ml conical tube containing 50 ml of Universal Cryopreservation Recovery Medium and centrifuged at 100g for 10 minutes. The cell pellet was resuspended in 1 ml of HQM for the determination of viability by trypan blue exclusion method and for cell concentration determination. The cell suspension was then adjusted with HQM to  $2\times$  the final cell density. The final cell density of CHHs was 0.2 million cells·ml $^{-1}$  for the  $\text{IC}_{50}$  experiments.

**Enzyme Kinetic Studies.** Phenacetin *O*-deethylation, coumarin 7-hydroxylation, bupropion hydroxylation, amodiaquine *N*-deethylation, diclofenac 4-hydroxylation, *s*-mephenytoin 4'-hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone 6-hydroxylation, midazolam 1'-hydroxylation, and testosterone 6 $\beta$ -hydroxylation were used as isoform-specific pathways for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A4, respectively, to determine the kinetics of metabolite formation. The stock solutions of P450 substrates were prepared in dimethylsulfoxide (DMSO) except for phenacetin, bupropion, mephenytoin, and testosterone, which were prepared in mixtures of H<sub>2</sub>O:CH<sub>3</sub>CN (50:50, v/v), H<sub>2</sub>O:DMSO (75:25, v/v), H<sub>2</sub>O:acetonitrile (50:50, v/v), and ACN:DMSO (40:60, v/v), respectively. For the optimization of time and cell density, the working dilutions of all substrates were prepared at a single concentration in HQM at  $2\times$  the final concentration in the assay. The final concentrations of the substrates phenacetin, coumarin, bupropion, amodiaquine, diclofenac, mephenytoin, dextromethorphan, chlorzoxazone, midazolam, and testosterone in the incubation were 10, 10, 50, 10, 10, 50, 10, 50, 5, and 10  $\mu\text{M}$ , respectively. The working dilutions of the substrates were added at a volume of 10  $\mu\text{l}$  to 384-well plates in triplicate at each cell density. The plates were prewarmed to  $37^{\circ}\text{C}$  for 10 minutes in a water bath (Julabo SW23; Julabo Labor Technik GmbH, Seelbach, Germany). The reactions were initiated by the addition of 10  $\mu\text{l}$  of prewarmed MMHH suspension to the 384-well plates containing the substrates, and the plates were transferred to an incubator (Thermo Fisher Scientific, Marietta, OH) maintained at 95% humidity and 5% CO<sub>2</sub>. A separate 384-well plate was prepared for each time point (2, 4, 6, 8, 12, 16, 20, 24, and 28 minutes). For the determination of  $V_{\text{max}}$  and  $K_m$ , a range of concentrations (eight concentrations, including zero) of the marker probe substrate was used. The stock solutions of the substrates were serially diluted in the identical solvent, and working dilutions were prepared in HQM at  $2\times$  the final concentration in the assay. The working dilutions of the substrates were added at a volume of 10  $\mu\text{l}$  to 384-well plates in triplicate for each substrate concentration. A separate 384-well plate was prepared for each time point (4, 8, 12, 16, and 20 minutes). The reactions were initiated by the addition of 10  $\mu\text{l}$  of MMHH cell suspension to the 384-well plates. The incubations were terminated by the addition of 100  $\mu\text{l}$  of stop solution [100% CH<sub>3</sub>CN containing 4-OH butyranilide (100 ng/ml) and diclofenac (150 ng/ml)]. The samples were mixed well, and the plates were centrifuged at 6000 rpm (4-16KS; Sigma-Aldrich) for 20 minutes. The supernatants were transferred to a separate 384-well plate and were analyzed by LC-MS/MS. Two independent studies were performed for all of the enzymes except for CYP2A6 and CYP2C9, where the first study resulted in saturation of the enzymes due to high concentrations of the substrate used.

**P450 Inhibition Assay Using MMHHs and CHHs.** The P450 inhibition assay was performed with MMHHs and CHHs using a set of standard inhibitors for each enzyme. Inhibitor stock solutions were prepared in DMSO at  $1000\times$  the final concentration in the assay. Serial dilutions were performed in 384-well plates. In brief, 125  $\mu\text{l}$  of acetonitrile was added to each well of the last row (H or P considering the 384-well plate as two 96-well plates with respect to rows, two columns similar to the 96-well plate) in 384-deep-well plates. An aliquot (50  $\mu\text{l}$ ) of a mixture of CH<sub>3</sub>CN and DMSO (90:10, v/v) was added to all of the wells through rows A–G or I–O. An aliquot (12.5  $\mu\text{l}$ ) of the inhibitor stock at  $1000\times$  was added to the last well of each column and mixed well. Twenty-five microliters of the solutions was serially diluted through rows H–B or P–J. All of the wells in row A or I contained solvent only without inhibitor. Working dilutions of the inhibitors were prepared at  $4\times$  by adding 5  $\mu\text{l}$  of the serial dilutions prepared earlier to the 120  $\mu\text{l}$  of HQM/HIM. The working dilutions were prepared in HQM/HIM at  $4\times$  the final concentration in the assay. The working dilutions of the substrates and inhibitors were added at a volume of 5  $\mu\text{l}$  each to 384-well plates in sextets ( $n = 6$  per compound). The concentrations of the substrates, including phenacetin, coumarin, bupropion, amodiaquine, diclofenac, mephenytoin, dextromethorphan, chlorzoxazone, midazolam, and testosterone, in the final incubation were 10, 1, 50, 1, 2, 40, 2, 100, 2, and 50  $\mu\text{M}$ , respectively, with all values at or below the  $K_m$  values of the substrates. The plates were prewarmed to  $37^{\circ}\text{C}$  for 10 minutes in a water bath. The reactions were initiated by the addition of 10  $\mu\text{l}$  of prewarmed MMHH or CHH cell suspension to the 384-well plates and were transferred to an incubator maintained at 95% humidity and 5% CO<sub>2</sub>. A separate 384-well plate was prepared for each enzyme. The percentages of DMSO and CH<sub>3</sub>CN in the final incubation were 0.1% and 0.9%, respectively. The reactions were terminated, after 20 minutes of incubation, by the

addition of 100  $\mu$ l of stop solution. The samples were treated in a similar manner as described in the earlier sections.

**Sample Analysis.** Metabolite formation was measured by LC-MS/MS using an API4000 mass spectrometer (MDS SCIEX) operating in positive or negative ion mode connected to Nexera  $\times$ 2 series ultrahigh-performance liquid chromatography (Shimadzu Corporation). An Acquity UPLC BEH C18 (2.1  $\times$  50 mm, 1.7  $\mu$ m) column was used for the chromatographic separation of hydroxy bupropion, dextropran, hydroxy diclofenac, hydroxy midazolam, hydroxy coumarin, hydroxy chlorzoxazone, and desethyl amodiaquine with a mobile phase composition of 10 mM ammonium formate (0.2% formic acid) (A) and 100% acetonitrile (B) at a flow rate of 0.5 ml·min<sup>-1</sup>. The gradient program for the separation was as follows: 0–0.2 minutes 90% A and 10% B, a linear increase of B from 10% to 80% over 0.60 minutes, maintained at 80% until 1.2 minutes, and then decreased to 10% at 1.21 minutes, with a total run time of 2.0 minutes except for hydroxy coumarin and hydroxy chlorzoxazone, where the B concentration was increased from 10% to 90% over 0.50 minutes, maintained at 90% for 1.2 minutes, and decreased to 10% at 1.21 minutes, with a total run time of 2.0 minutes. For the separation of acetaminophen, hydroxy testosterone, and hydroxy mephenytoin, an Atlantis T3 (2.1  $\times$  50 mm, 3.0  $\mu$ m) column was used with a mobile phase composition of 0.1% formic acid (A) and 100% acetonitrile (B) at a flow rate of 0.4 ml·min<sup>-1</sup>. The gradient program for the separation was as follows: 0–0.6 minutes 98% A and 2% B, a linear increase of B from 2% to 50% over 0.80 minutes, maintained at 50% up to 1.8 minutes, and decreased to 2% at 1.81 minutes, with a total run time of 2.5 minutes. Data acquisition and analysis were performed with the Analyst software version 1.6.3 (AB Sciex, Ontario, Canada). The marker metabolites quantified, mass transitions, and linearity range (nanomolar) used are summarized in Table 1.

**Data Analysis.** Kinetic parameters for P450 marker reactions were estimated by analyzing the data with Michaelis-Menten (eq. 1), Hill (eq. 2), substrate inhibition (eq. 3), or two enzyme Michaelis-Menten (eq. 4) equations using GraphPad Prism v.4.0 (GraphPad Software, San Diego, CA). The best-fit equation was selected based on Akaike Information Criteria.

The equations used were as follows:

$$v = V_{\max} * [S] / (K_m + [S]) \quad (1)$$

$$v = V_{\max} * [S]^n / (S_{50}^n + [S]^n) \quad (2)$$

$$v = V_{\max} / (1 + K_m / [S] + [S] / K_{si}) \quad (3)$$

$$v = (V_{\max, LA} * [S]) / (K_{m, LA} + [S]) + (V_{\max, HA} * [S]) / (K_{m, HA} + [S]) \quad (4)$$

where  $v$  is the initial rate of metabolite formation;  $V_{\max}$  is maximal velocity;  $K_m$  is Michaelis-Menten constant;  $[S]$  is the substrate concentration;  $S_{50}$  is analogous to  $K_m$ ;  $n$  is the Hill coefficient indicative of the degree of curve sigmoidicity and/or cooperativity;  $K_{si}$  is the constant describing the substrate inhibition interaction;  $V_{\max, LA}$ ,  $K_{m, LA}$ ,  $V_{\max, HA}$ , and  $K_{m, HA}$  are the maximal velocity and Michaelis-Menten constants for low-affinity and high-affinity enzymes, respectively.

For the determination of  $IC_{50}$ , the metabolite formation (nanomolar) was converted to activity (picomoles per minute per million cells). The percentage of remaining activity in the presence of inhibitor at each

concentration compared with that in the absence of inhibitor was calculated as follows:

$$\text{activity remaining (\%)} = (\text{activity in the presence of inhibitor} / \text{activity in the absence of inhibitor}) * 100. \quad (5)$$

The percentage of activity remaining was plotted as a function of the logarithm of the inhibitor concentration, and  $IC_{50}$  values were calculated by nonlinear regression using the sigmoidal dose-response variable slope equation:

$$Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{((\text{Log}IC_{50} - X) * \text{Hill slope}))} \quad (6)$$

where  $Y$  is the percentage of activity remaining, and  $X$  is the corresponding concentration.

## Results

**Enzyme Kinetics.** MMHs were incubated with various P450 selective substrates at 0.15, 0.3, and 0.6  $\times$  10<sup>6</sup> cells·ml<sup>-1</sup> for 2, 4, 6, 8, 12, 16, 20, 24, and 28 minutes. The final concentrations of the substrates phenacetin, coumarin, bupropion, amodiaquine, diclofenac, mephenytoin, dextromethorphan, chlorzoxazone, midazolam, and testosterone in the incubation were 10, 10, 50, 10, 10, 50, 10, 50, 5, and 10  $\mu$ M, respectively. Metabolite formation was found to increase with cell concentration for all substrates. The metabolite formation was linear for all enzymes and was  $\leq$ 10% of the nominal incubated substrate concentration at 0.15  $\times$  10<sup>6</sup> cells·ml<sup>-1</sup> for all enzymes except for CYP2C8-mediated amodiaquine *O*-deethylation, for which 16% of metabolite formation was observed above 20 minutes. The metabolite formation was linear with respect to time up to 28 minutes except for CYP3A4-mediated midazolam and testosterone hydroxylation, where the product formation at 0.6  $\times$  10<sup>6</sup> cells·ml<sup>-1</sup> was linear up to 8 and 24 minutes, respectively. Based on these results, the cell density and time were fixed at 0.2  $\times$  10<sup>6</sup> cells·ml<sup>-1</sup> (rounded off from 0.15) and 20 minutes, respectively, for subsequent  $V_{\max}$  and  $K_m$  determinations. The  $V_{\max}$  and  $K_m$  values from the independent studies are summarized in Table 2, and a comparison with the values for hepatocytes reported in the literature is summarized in Table 3. The CYP2B6-mediated bupropion hydroxylation, CYP2C8-mediated amodiaquine *N*-deethylation, CYP2C9-mediated diclofenac 4-hydroxylation, CYP2C19-mediated *s*-mephenytoin 4'-hydroxylation, and CYP2E1-mediated chlorzoxazone 6-hydroxylation followed Michaelis-Menten kinetics. The CYP1A2-mediated phenacetin *O*-deethylation and CYP2D6-mediated dextromethorphan *O*-demethylation followed two-enzyme-mediated kinetics with low-affinity enzymes not saturated at the concentrations used in the kinetic experiments. The CYP2A6-mediated coumarin hydroxylation and CYP3A4-mediated midazolam hydroxylation followed substrate inhibition kinetics. The CYP3A4-mediated

TABLE 1

Ion mode application, mass transitions, and linearity range used for the quantification of marker metabolites of the isoform-specific pathways used for kinetic experiments in MMHs and inhibition experiments in both MMHs and CHHs

P450 Isozyme	Marker Substrate	Marker Metabolite	Ion Mode	Mass Transition	Linearity Range
				<i>m/z</i>	<i>nM</i>
CYP1A2	Phenacetin	Acetaminophen	Positive	152.2→110	6.6–6622.5
CYP2A6	Coumarin	Hydroxy coumarin	Negative	160.8→132.7	6.2–6211.2
CYP2B6	Bupropion	Hydroxy bupropion	Positive	256→139	3.9–3921.6
CYP2C8	Amodiaquine	Desethyl amodiaquine	Positive	328.3→283.3	3.1–3058.1
CYP2C9	Diclofenac	Hydroxy diclofenac	Positive	311.927→266	3.2–3215.4
CYP2C19	Mephenytoin	Hydroxy mephenytoin	Positive	235.1→150.2	4.3–4273.5
CYP2D6	Dextromethorphan	Dextropran	Positive	258.3→157.2	3.9–3891.1
CYP2E1	Chlorzoxazone	Hydroxy chlorzoxazone	Negative	184→120.1	5.4–5434.8
CYP3A4	Midazolam	Hydroxy midazolam	Positive	342→203	2.9–2932.6
CYP3A4	Testosterone	Hydroxy testosterone	Positive	305.1→269.1	3.9–3289.5

TABLE 2

Substrate concentrations,  $V_{max}$ , and  $K_m$  values determined for the marker reactions in MMHs from two independent studies $V_{max}$  units are picomoles per minute per million cells;  $K_m$  units are micromolars.

P450	Marker Reaction	Study	Substrate Concentrations	$V_{max}$	Mean $V_{max}$	$K_m$	Mean $K_m$	Kinetics
			$\mu M$					
CYP1A2	Phenacetin deethylation <sup>a</sup>	1	0, 15.625, 31.25, 62.5, 125, 250, 500, 1000	51	80	11.4	11	TS (atypical)
		2	0, 7.81, 15.625, 31.25, 62.5, 125, 250, 500	109		10.7		TS (atypical)
CYP2A6	Coumarin hydroxylation	1	0, 0.937, 1.875, 3.75, 7.5, 15, 30, 60	—	112	—	1.5	ND
		2	0, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10	112		1.5		SI (atypical)
CYP2B6	Bupropion hydroxylation	1	0, 15.625, 31.25, 62.5, 125, 250, 500, 1000	131	136	69	67	MM (typical)
		2	0, 15.625, 31.25, 62.5, 125, 250, 500, 1000	140		66		MM (typical)
CYP2C8	Amodiaquine deethylation	1	0, 0.937, 1.875, 3.75, 7.5, 15, 30, 60	758	517	1.4	1.4	MM (typical)
		2	0, 0.937, 1.875, 3.75, 7.5, 15, 30, 60	277		1.4		MM (typical)
CYP2C9	Diclofenac hydroxylation	1	0, 3.125, 6.25, 12.5, 25, 50, 100, 200	—	3273	—	2.4	ND
		2	0, 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50	3273		2.4		MM (typical)
CYP2C19	Mephenytoin hydroxylation	1	0, 14.06, 28.12, 56.25, 112.5, 225, 450, 900	49	50	55	45	MM (typical)
		2	0, 14.06, 28.12, 56.25, 112.5, 225, 450, 900	52		35		MM (typical)
CYP2D6	Dextromethorphan <i>O</i> -demethylation <sup>a</sup>	1	0, 3.125, 6.25, 12.5, 25, 50, 100, 200	28	37	2.6	2.0	TS (atypical)
		2	0, 0.47, 0.937, 1.875, 3.75, 7.5, 15, 30	47		1.5		TS (atypical)
CYP2E1	Chlorzoxazone hydroxylation	1	0, 31.25, 62.5, 125, 250, 500, 1000, 2000	693	462	469	465	MM (typical)
		2	0, 15.62, 31.25, 62.5, 125, 250, 500, 1000	231		462		MM (typical)
CYP3A4	Midazolam hydroxylation	1	0, 0.937, 1.875, 3.75, 7.5, 15, 30, 60	187	215	3.7	3.5	SI (atypical)
		2	0, 0.312, 0.625, 1.25, 2.5, 5, 10, 20	243		3.3		MM (typical)
CYP3A4	Testosterone hydroxylation	1	0, 15.62, 31.25, 62.5, 125, 250, 500, 1000	765	592	73 <sup>b</sup>	70	HK (atypical)
		2	0, 15.62, 31.25, 62.5, 125, 250, 500, 1000	418		67 <sup>b</sup>		HK (atypical)

HK, Hill kinetics; MM, Michaelis-Menten; ND, not determined; SI, substrate inhibition; TS, two-site.

<sup>a</sup>Only high-affinity enzyme values were reported for pathways following two-site kinetics.<sup>b</sup>Values are  $S_{50}$  values equivalent to  $K_m$ ; Hill equation  $n_1 = 1.432$  and  $n_2 = 1.778$ .

testosterone hydroxylation followed Hill kinetics. The substrate saturation curves for each enzyme are depicted in Fig. 1.

**P450 Inhibition Assay Using MMHs and CHHs.** The inhibitors were incubated with MMHs and CHHs for 20 minutes in the presence of marker probe substrate, and the  $IC_{50}$  values were calculated using eq. 6. The  $IC_{50}$  values for each inhibitor against the respective enzymes are summarized in Table 4. A comparison of  $IC_{50}$  values generated from MMHs and CHHs is depicted in Fig. 2. The inhibition curves for a set of representative controls against each enzyme are depicted in Fig. 3.

## Discussion

To develop and validate P450 inhibition assays in MMHs, a complete characterization of kinetics for major P450 isoform-specific

pathways has been performed. The enzyme kinetic parameters,  $V_{max}$  and  $K_m$ , for nine P450 selective pathways were determined using marker probe substrate reactions that are well established for HLMs and CHHs. Both midazolam and testosterone hydroxylation kinetics were evaluated for the CYP3A4 enzyme. All kinetic and inhibition experiments were performed in 384-well plates to develop a high-throughput P450 inhibition assay using MMHs.

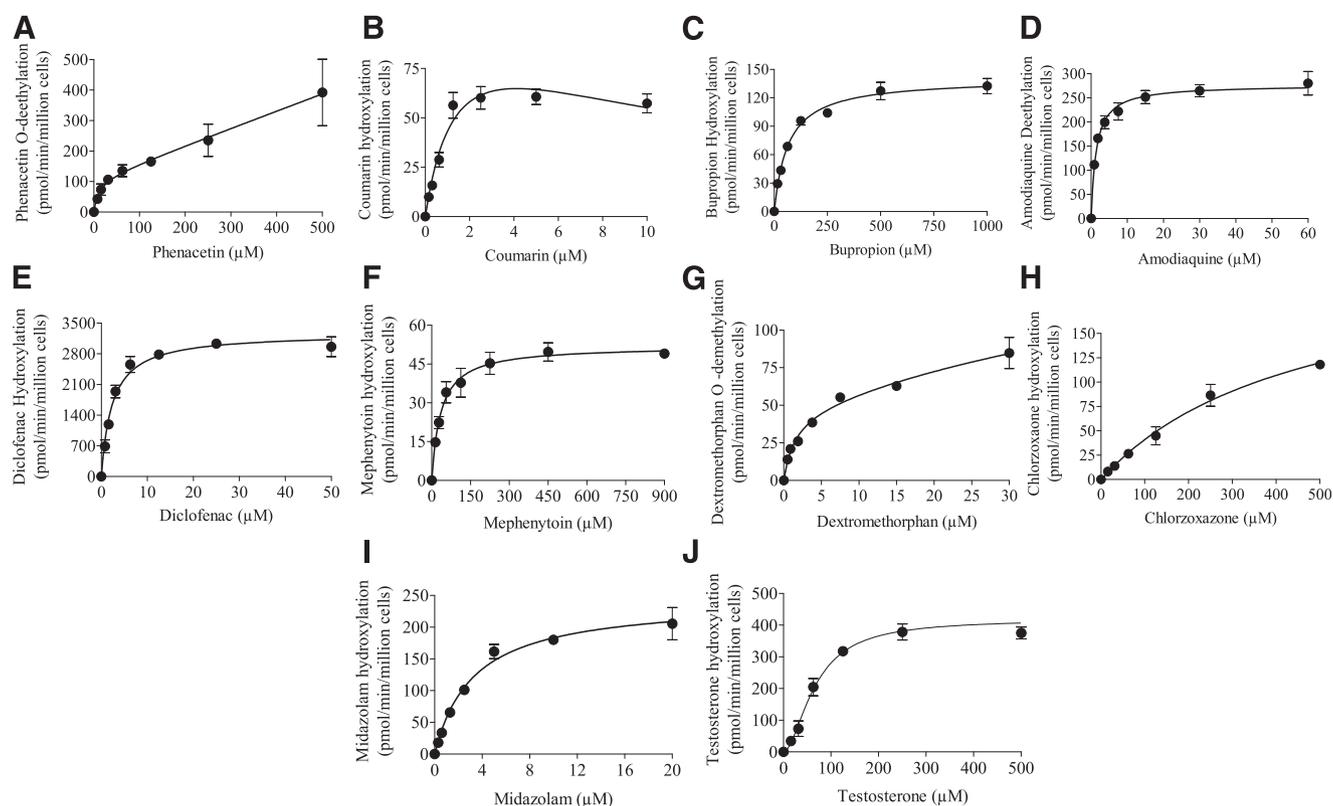
The linear formation of an isoform-specific metabolite with time and cell density is established in the preliminary experiments, and a cell density of 0.2 million cells·ml<sup>-1</sup> and an incubation time of 20 minutes were used for the kinetic experiments. The  $K_m$  values of isoform-specific pathways determined in this study using MMHs are compared with the values reported in the literature that are determined in hepatocytes and human liver microsomes using similar isoform-specific

TABLE 3

A comparison of  $V_{max}$  and  $K_m$  values determined using MMHs from this study with those determined in the cryopreserved human hepatocytes and HLMsThe  $V_{max}$  and  $K_m$  values and the calculated ratio of the values for MMH to that for hepatocytes and human liver microsomes are shown.  $V_{max}$  values are in pmol/min per million cells for MMH and hepatocytes;  $K_m$  values are in  $\mu M$ .

P450 Isoforms	Marker Reaction	MMH		Hepatocytes		HLM $K_m$	Reference for Hepatocytes	Reference for Human Liver Microsomes	MMH/Hepatocyte Ratio		MMH/HLM Ratio
		$V_{max}$	$K_m$	$V_{max}$	$K_m$				$V_{max}$	$K_m$	
CYP1A2	Phenacetin deethylation	80	11	NA	NA	112.7	NA	Li et al. (2015)	NA	NA	0.10
CYP2A6	Coumarin hydroxylation	112	2	NA	NA	1.9	NA	Hosono et al. (2017)	NA	NA	1.05
CYP2B6	Bupropion hydroxylation	136	67	21	41	130	Li and Schlicht (2014)	Faucette et al. (2000)	6.5	1.6	0.52
CYP2C8	Amodiaquine deethylation	517	1	334	10	1	Li and Schlicht (2014)	Li et al. (2015)	1.5	0.1	1.00
CYP2C9	Diclofenac hydroxylation	3273	2	290	7	22.4	Brown et al. (2007)	Siu and Lai (2017)	11.3	0.3	0.09
CYP2C19	Mephenytoin hydroxylation	50	45	16	13	56.8	Brown et al. (2007)	Siu and Lai (2017)	3.1	3.5	0.79
CYP2D6	Dextromethorphan demethylation	37	2	50	1	2.9	Brown et al. (2007)	Li et al. (2015)	0.7	2.0	0.69
CYP2E1	Chlorzoxazone hydroxylation	462	465	NA	NA	149.8	NA	Li et al. (2015)	NA	NA	3.10
CYP3A4	Midazolam hydroxylation	215	4	20	4	8.4	Li and Schlicht (2014)	Siu and Lai (2017)	10.8	1.0	0.48
CYP3A4	Testosterone hydroxylation	592	70	1800	24	10.2	Brown et al. (2007)	Siu and Lai (2017)	0.3	2.9	6.86

NA, not Available.



**Fig. 1.** Substrate saturation curves for phenacetin *O*-deethylation (A), coumarin hydroxylation (B), bupropion hydroxylation (C), amodiaquine *N*-deethylation (D), diclofenac hydroxylation (E), mephenytoin hydroxylation (F), dextromethorphan *O*-demethylation (G), chlorzoxazone 6-hydroxylation (H), midazolam 1-hydroxylation (I), and testosterone 6-hydroxylation (J) in MMHs. Each data point is the mean  $\pm$  S.E.M. of triplicates from a single experiment.

pathways (Table 3). The  $K_m$  values determined in MMHs for phenacetin *O*-deethylation, coumarin 7-hydroxylation, bupropion hydroxylation, *s*-mephenytoin 4'-hydroxylation, dextromethorphan *O*-demethylation, chlorzoxazone hydroxylation, and midazolam 1'-hydroxylation are within 2- to 4-fold of those reported for hepatocytes and/or human liver microsomes. The MMHH  $K_m$  of 1  $\mu$ M for amodiaquine *O*-deethylation is the same as that reported for human liver microsomes but lower than the 10  $\mu$ M reported for human hepatocytes. The MMHH  $K_m$  of 70  $\mu$ M for testosterone 6 $\beta$ -hydroxylation is within 4-fold of that reported for hepatocytes but is approximately 7-fold of that for human liver microsomes.

The MMHH  $V_{max}$  values for the various P450 pathways are compared with that available in the published literature for hepatocytes (Table 3). Over 5-fold higher  $V_{max}$  values were observed in MMHs than in hepatocytes for bupropion hydroxylation (6.5-fold), diclofenac hydroxylation (11.3-fold), and testosterone (10.8-fold). The  $V_{max}$  values for MMHH for mephenytoin hydroxylation, dextromethorphan demethylation, and testosterone hydroxylation are within 5-fold of that reported for hepatocytes.

It is interesting to note that the  $K_m$  for diclofenac hydroxylation (4.2  $\mu$ M) is within 2-fold compared with that determined in hepatocytes suspended in plasma (Mao et al., 2012) after correction for plasma protein binding. The  $V_{max}$  value (3293 pmol $\cdot$ min $^{-1}$  $\cdot$ million cells $^{-1}$ ) is 11-fold higher in MMHs compared with that determined in hepatocytes [450 pmol/min/million cells (Mao et al., 2012); 290 pmol/min/million cells (Brown et al., 2007b)]. The  $V_{max}$  for mephenytoin hydroxylation is 3.5-fold higher, whereas the  $V_{max}$  is 3.1-fold higher than that determined in hepatocytes (Brown et al., 2007). Dextromethorphan *O*-demethylation followed two-enzyme-mediated kinetics and is consistent with the kinetics reported in the literature (Brown et al., 2007). The  $K_m$  and  $V_{max}$  for

dextromethorphan *O*-demethylation in MMHs are within 2-fold compared to that determined in hepatocytes (Brown et al., 2007). The  $K_m$  for midazolam hydroxylation (3.5  $\mu$ M) is within 2-fold compared to that determined in hepatocytes (4  $\mu$ M), whereas the  $V_{max}$  is 11-fold higher in MMHs (215 pmol/min/million cells) compared to that in hepatocytes (Li and Schlicht, 2014). The  $K_m$  for midazolam hydroxylation (3.5  $\mu$ M) is 2.3-fold lower compared to that in hepatocytes (8.1  $\mu$ M) and 2.3-fold higher compared to that determined in hepatocytes suspended (1.5  $\mu$ M) in human plasma in a different study (Mao et al., 2012).

Overall, the results indicate that the  $K_m$  values are comparable with those reported in the literature determined using hepatocytes and human liver microsomes. It is of interest that higher  $V_{max}$  values were observed in MMHs compared with those determined in the results reported by others for hepatocytes. The difference may be a result of the use of livers from different donors used in the preparation of the hepatocytes, as large interindividual differences in P450 activities is a well established phenomenon. However, ease of substrate entrance into the MMHs due to the permeabilized plasma membranes may also be a possible mechanism for the higher activities. A direct comparison of  $V_{max}$  values between MMHs and CHHs using hepatocytes from the same donors will be determined in the laboratory of one of the coauthors of this report (A. P. Li, personal communication).

The inhibition experiments were performed in MMHs and CHHs using the isoform-specific pathways using the substrate concentrations that are  $\leq K_m$  in MMHs. Phenacetin *O*-deethylation was used as the isoform-specific pathway to evaluate the P450 inhibition of standard inhibitors against the CYP1A2 enzyme. The  $IC_{50}$  values of the standard inhibitors furafylline,  $\alpha$ -naphthoflavone, propranolol, and methoxsalen are comparable ( $\leq 2$ -fold difference) between MMHs and CHHs with

TABLE 4

Absolute IC<sub>50</sub> values of standard inhibitors for nine major P450 enzymes determined in MMHs and CHHs and their corresponding fold difference values

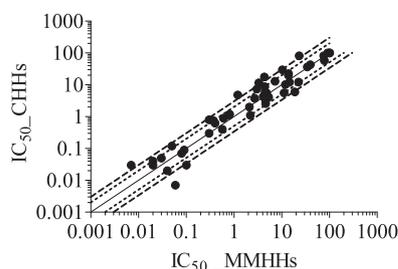
P450 Isoform	Marker Reaction	Inhibitors	Absolute IC <sub>50</sub>		Fold Difference MMHH/CHH
			MMHH	CHH	
			$\mu\text{M}$	$\mu\text{M}$	
1A2	Phenacetin <i>O</i> -deethylation	Fluvoxamine	0.007	0.025	0.28 <sup>a</sup>
		Furafylline	0.81	1.2	0.67
		Methoxsalen	0.015	0.025	0.60
		$\alpha$ -Naphthoflavone	0.029	0.047	0.62
2A6	Coumarin hydroxylation	Propranolol	0.57	0.39	1.46
		Ketoconazole	91.4	100	0.91
		Methoxsalen	0.10	0.03	3.33 <sup>a</sup>
		Phenelzine	2.1	2.0	1.07
2B6	Bupropion hydroxylation	Tranlycypromine	0.059	0.007	8.43 <sup>a</sup>
		Ketoconazole	2.7	3.8	0.72
		Methoxsalen	4.5	5.9	0.76
		Phenelzine	11.2	5.6	2.01
2C8	Amodiaquine <i>N</i> -deethylation	PPP	4.8	5.5	0.87
		Thiotepa	4.3	17.7	0.24 <sup>a</sup>
		Ticlopidine	22.4	12.3	1.82
		Ketoconazole	11.9	10.2	1.17
2C9	Diclofenac hydroxylation	Methoxsalen	>100	>100	NC
		Montelukast	4.1	4.3	0.95
		Tranlycypromine	77.8	58	1.35
		Fluconazole	23.2	82.0	0.28 <sup>a</sup>
2C19	Mephenytoin hydroxylation	Ketoconazole	7.2	13.0	0.55
		Methoxsalen	34.2	36.7	0.93
		Sulfaphenazole	0.4	0.63	0.59
		Fluconazole	4.6	3.2	1.46
2D6	Dextromethorphan <i>O</i> -demethylation	Fluoxetine	1.2	4.8	0.24 <sup>a</sup>
		Fluvoxamine	0.02	0.04	0.64
		Methoxsalen	14.5	12.1	1.19
		Omeprazole	4.6	2.6	1.80
3A4	Testosterone hydroxylation	Ticlopidine	13.8	20.1	0.69
		Cinacalcet	3.3	11.5	0.29 <sup>a</sup>
		Fluoxetine	0.63	0.92	0.68
		Methoxsalen	39.6	43.1	0.92
3A4	Midazolam hydroxylation	Paroxetine	0.37	0.75	0.49
		Propafenone	0.08	0.07	1.14
		Quinidine	0.04	0.02	2.33
		Methoxsalen	75.7	81.5	0.93
3A4	Erythromycin hydroxylation	Methylpyrazole	1.4	1.1	1.24
		Erythromycin	5.3	4.1	1.31
		Ketoconazole	0.09	0.09	1.11
		Methoxsalen	>100	>100	NC
3A4	Testosterone hydroxylation	Mibefradil	0.32	0.32	1.00
		Troleandomycin	2.2	1.1	1.98
		Verapamil	18.8	5.97	3.15 <sup>a</sup>
		Erythromycin	13.9	22.4	0.62
3A4	Testosterone hydroxylation	Ketoconazole	0.045	0.12	0.38
		Methoxsalen	10.3	29.8	0.35
		Mibefradil	0.25	0.75	0.33
		Troleandomycin	3.0	7.4	0.40
3A4	Testosterone hydroxylation	Verapamil	4.4	8.8	0.50

NC, fold difference not calculated; PPP, 2-phenyl-2-(1-piperidinyl)propane.

<sup>a</sup>Fold difference of  $\geq 3$  or  $\leq 0.3$  between MMHs and CHHs.

the exception of fluvoxamine, which showed a 3.5-fold potent value in MMHs. The IC<sub>50</sub> values against CYP2B6 for the standard inhibitors were determined using bupropion hydroxylation as a marker reaction in MMHs and CHHs. The IC<sub>50</sub> values showed no major differences and are comparable between MMHs and CHHs ( $\leq 2$ -fold) except for thiotepa, which has a 4-fold higher inhibitory potency in MMHs, with an IC<sub>50</sub> value of 4.3  $\mu\text{M}$  in MMHs compared with 17.8  $\mu\text{M}$  in CHHs. Thiotepa has been reported to be a P-glycoprotein substrate (Liang et al., 2015), which could be the possible reason for a weaker IC<sub>50</sub> in CHHs, possibly due to an efflux mechanism. CYP2C8 inhibition was evaluated using amodiaquine *N*-deethylation as a marker reaction, and no differences ( $\leq 2$ -fold) are observed in IC<sub>50</sub> values of the standard inhibitors. The potency of sulfaphenazole, ketoconazole, and methoxsalen against CYP2C9-mediated diclofenac hydroxylation is comparable between

MMHs and CHHs with the exception of fluconazole having a 3.5-fold higher potency in MMHs. The IC<sub>50</sub> values for standard inhibitors against CYP2C19 were determined using mephenytoin hydroxylation as a marker reaction in MMHs and CHHs. The IC<sub>50</sub> values are comparable and showed no major differences ( $\leq 2$ -fold) except for fluoxetine, which has a 4-fold potent IC<sub>50</sub> in MMHs (1.2  $\mu\text{M}$ ) compared with CHHs (4.8  $\mu\text{M}$ ). The IC<sub>50</sub> of omeprazole against CYP2C19 from CHHs in this study (2.6  $\mu\text{M}$ ) is comparable with that reported in the literature (4.7  $\mu\text{M}$ ) using cryopreserved human hepatocytes (Ogilvie et al., 2011). No other information is available in the literature on the determination of IC<sub>50</sub> against CYP2C19 using human hepatocytes for a direct comparison. Dextromethorphan *O*-demethylation is the marker probe reaction used for the IC<sub>50</sub> determination against CYP2D6 enzyme. The IC<sub>50</sub> values of the



**Fig. 2.** Comparison of  $IC_{50}$  values generated using isoform-specific marker probe reactions from CHHs and MMHs. The solid line represents the line of unity, the dotted line represents a 2-fold deviation from the line of unity, and the dashed line represents a 3-fold deviation from the line of unity.

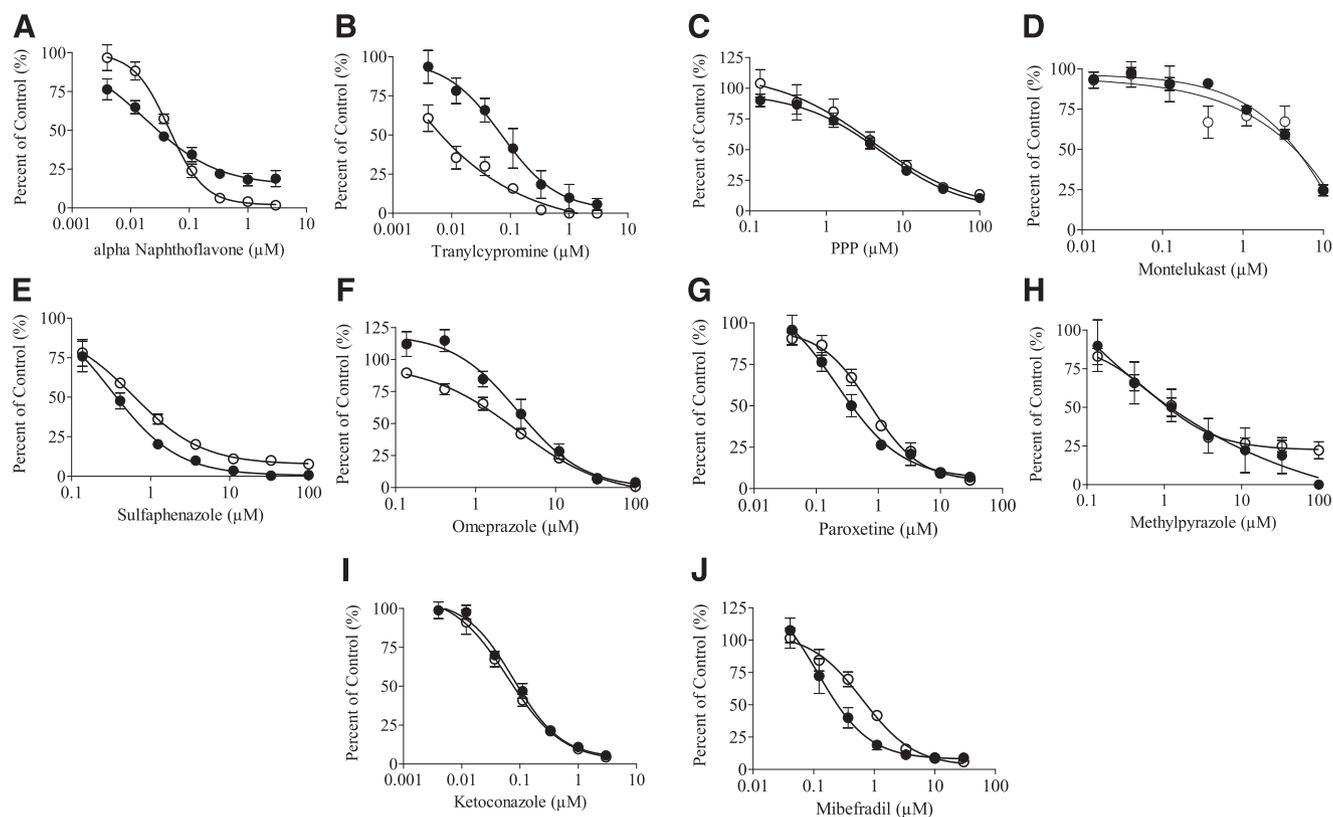
CYP2D6 inhibitors determined from MMHs and CHHs are comparable and showed no major differences, with a less than 2-fold difference for fluoxetine, methoxsalen, paroxetine, and propafenone, with the exception of quinidine (2.3-fold potent in CHHs) and cinacalcet (3-fold potent in MMHs). The  $IC_{50}$  values of 0.035 and 0.015  $\mu\text{M}$  for quinidine derived from MMHs and CHHs, respectively, are consistent with the reported  $IC_{50}$  value of 0.03  $\mu\text{M}$  using bufuralol hydroxylation as a marker probe reaction with a 20-minute preincubation step before the addition of the substrate (Mao et al., 2012), a result consistent with the non-time dependent inhibition property of quinidine. The  $IC_{50}$  values of fluoxetine and paroxetine in MMHs are 0.63 and 0.37  $\mu\text{M}$ , respectively, versus the reported  $IC_{50}$  values of 0.04  $\mu\text{M}$  for fluoxetine and 0.03  $\mu\text{M}$  for paroxetine determined with a 20-minute preincubation step (Mao et al., 2012), a result that can be attributed to the fact that paroxetine and fluoxetine are time dependent inhibitions of

CYP2D6 enzyme, and a 20-minute preincubation step will result in shift of the  $IC_{50}$  toward higher potency. No other literature is available on the  $IC_{50}$  values determined in human hepatocytes for a direct comparison with  $IC_{50}$  determined in MMHs or CHHs. The potency of methylpyrazole, a positive control inhibitor against CYP2E1, is comparable between MMHs and CHHs.

CYP3A4 inhibition by standard inhibitors was evaluated using two isoform-specific pathways, namely, midazolam hydroxylation and testosterone hydroxylation. The  $IC_{50}$  values of the standard inhibitors are comparable, with ketoconazole, erythromycin, mibefradil, and troleandomycin having less than 2-fold difference and verapamil having 3.2-fold differences between MMHs and CHHs, with potent  $IC_{50}$  values against CYP3A4 in CHHs using midazolam hydroxylation. The  $IC_{50}$  values of ketoconazole, troleandomycin, and verapamil are comparable between MMHs and CHHs (within 2- to 3-fold), with consistently higher inhibitory potencies in MMHs using testosterone hydroxylation as a marker probe reaction against CYP3A4 enzyme.

Overall, the differences in  $IC_{50}$  values for the majority of the inhibitors against several enzymes between MMHs and CHHs are within 2-fold (73%) and 3-fold (84%), with the exception of fluvoxamine (CYP1A2), methoxsalen and tranlycypromine (CYP2A6), thiotepa (CYP2B6), fluconazole (CYP2C9), fluoxetine (CYP2C19), cinacalcet (CYP2D6), and verapamil (CYP3A4) having more than 3-fold differences.

Here, we report data comparing the MMHs to traditional cryopreserved hepatocytes in evaluating a series of P450 probe substrates and inhibitors. Although, in general, the results are similar between the two systems, we will continue to compare the two experimental systems to explore potential differences which may further our understanding of key determinants of drug metabolism. The following are areas that are



**Fig. 3.** The inhibition curves in MMHs (●) and CHHs (○) for a set of representative inhibitors, including  $\alpha$ -naphthoflavone (A), tranlycypromine (B), 2-phenyl-2-(1-piperidinyl)propane (PPP) (C), montelukast (D), sulfaphenazole (E), omeprazole (F), paroxetine (G), methylpyrazole (H), ketoconazole (I), and mibefradil (J), against CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 (midazolam hydroxylation), and CYP3A4 (testosterone hydroxylation) enzyme-mediated activities, respectively. Each data point is the mean  $\pm$  S.E.M. of six replicates in a single experiment.

being investigated in the laboratory of one of the coauthors (A. P. Li, personal communication):

1. MMHs are fully permeabilized. For instance, the commonly used trypan blue exclusion assay would yield 100% blue cells (A. P. Li, personal communication). A comparison of MMHs and intact human hepatocytes may allow the evaluation of the role of membrane permeability in metabolic clearance. Furthermore, the commonly used pooled human hepatocytes, due to the thawing and re-cryopreservation processes in their preparation, may have compromised uptake transporter activity (A. P. Li, personal communication); MMHs may be used for the evaluation of uptake transporter substrates which may have a low rate of metabolic clearance due to this artifact of the pooled donor human hepatocytes.
2. Using phase-contrast microscopy, MMHs are observed to possess a visually visible plasma membrane. It is not yet fully determined if the cytosolic proteins still remained at physiologic concentrations within the cell membrane, as the commonly used procedure of centrifugation may artifactually lead to pressure on the cells, leading to exudation of the cytosolic proteins through the permeabilized plasma membrane. The extent of cytosolic protein leakage in MMHs is now being investigated.

To our knowledge, this is the first study evaluating the P450 inhibition potential of several inhibitors against nine major P450 enzymes using either CHHs or MMHs. The results indicate that MMHs can be a valuable tool in place of the CHHs for the evaluation of P450 inhibition without the need for time-consuming procedures. The complete drug-metabolizing enzyme pathways in MMHs may allow the results to be complementary to those obtained with HLMs, allowing a more accurate assessment of clinical P450 inhibitory potential.

#### Authorship Contributions

*Participated in research design:* Palacharla, Chunduru, Ajjala, Bhyrapuneni, Nirogi, Li.

*Conducted experiments:* Palacharla, Chunduru.

*Performed data analysis:* Palacharla, Chunduru, Ajjala, Li.

*Wrote or contributed to the writing of the manuscript:* Palacharla, Chunduru, Ajjala, Bhyrapuneni, Li.

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