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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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ABSTRACT

Here, we report the application of a novel hepatocyte system, the cofactor-supplemented permeabilized cryopreserved human hepatocytes [MetMax human hepatocytes (MMHHs)] 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 MMHH but with the ease of use of human liver microsomes, including storage at −80°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 MMHH 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 6β-hydroxylation (CYP3A4). The K_m values obtained with MMHHs were comparable with those reported in the literature for CHHs. Using substrate concentrations at or near K_m values, the IC_{50} values for the standard inhibitors against the P450 activities were found to be comparable between MMHHs 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 MMHHs can be an efficient experimental system for the evaluation of P450 inhibition in hepatocytes.

SIGNIFICANCE STATEMENT

MetMax human hepatocytes (MMHHs) 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°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 MMHH 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 MMHH are similar to those with conventional hepatocytes, suggesting that the MMHH 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 (IC_{50}, K_i) and irreversible inhibition constants [maximal inactivation rate constant (k_{inact}); inactivation rate constant (K_I)]. The inhibition constants, along with the unbound concentrations of the inhibitors in plasma ($f_{u,plasma}$) and microsomes ($f_{u,\ mic}$), 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;

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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; MMHH, 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; McGinnity 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 enzymess in hepatocytes and the robustness and ease of handling of HLMs, including storage in a −80°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 5×10^6 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 dextrorphan 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-μl 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°C), thawed, and the contents were transferred to cofactor N10 vials (IVAL) with gentle mixing. The cell suspension was diluted to 2× the final cell density with HQM. The final cell densities of MMHHs were 0.15, 0.3, and 0.6×10^6 cells·ml^{-1} for time or cell density optimization study and 0.2×10^6 cells·ml^{-1} for enzyme kinetic (V_{max} and K_m) and inhibition (IC_{50}) studies. CHHs were thawed according to the instructions provided by the supplier (IVAL). In brief, the hepatocytes were thawed in a 37°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 100*g* 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× the final cell density. The final cell density of CHHs was 0.2 million cells·ml^{-1} for the 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 6β-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_2O:CH_3CN (50:50, *v/v*), H_2O:DMSO (75:25, *v/v*), H_2O:acetylnitrile (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× 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 μM, respectively. The working dilutions of the substrates were added at a volume of 10 μl to 384-well plates in triplicate at each cell density. The plates were prewarmed to 37°C for 10 minutes in a water bath (Julabo SW23; Julabo Labortechnik GmbH, Seelbach, Germany). The reactions were initiated by the addition of 10 μl 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_2. 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_{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× the final concentration in the assay. The working dilutions of the substrates were added at a volume of 10 μl 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 μl of MMHH cell suspension to the 384-well plates. The incubations were terminated by the addition of 100 μl of stop solution [100% CH_3CN 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× the final concentration in the assay. Serial dilutions were performed in 384-well plates. In brief, 125 μl 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 μl) of a mixture of CH_3CN and DMSO (90:10, v/v) was added to all of the wells through rows A–G or I–O. An aliquot (12.5 μl) of the inhibitor stock at 1000× 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× by adding 5 μl of the serial dilutions prepared earlier to the 120 μl of HQM/HIM. The working dilutions were prepared in HQM/HIM at 4× the final concentration in the assay. The working dilutions of the substrates and inhibitors were added at a volume of 5 μl 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 μM, respectively, with all values at or below the K_m values of the substrates. The plates were prewarmed to 37°C for 10 minutes in a water bath. The reactions were initiated by the addition of 10 μl 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_2. A separate 384-well plate was prepared for each enzyme. The percentages of DMSO and CH_3CN 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 μl 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 ×2 series ultrahigh-performance liquid chromatography (Shimadzu Corporation). An Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 μm) column was used for the chromatographic separation of hydroxy bupropion, dextrorphan, 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^{-1}. 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 × 50 mm, 3.0 μm) 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^{-1}. 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. MMHHs were incubated with various P450 selective substrates at 0.15, 0.3, and 0.6 × 10^6 cells·ml^{-1} 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 μM, respectively. Metabolite formation was found to increase with cell concentration for all substrates. The metabolite formation was linear for all enzymes and was ≤10% of the nominal incubated substrate concentration at 0.15 × 10^6 cells·ml^{-1} 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 × 10^6 cells·ml^{-1} was linear up to 8 and 24 minutes, respectively. Based on these results, the cell density and time were fixed at 0.2 × 10^6 cells·ml^{-1} (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 MMHHs and inhibition experiments in both MMHHs and CHHs

P450 Isozyme	Marker Substrate	Marker Metabolite	Ion Mode	Mass Transition	Linearity Range
				m/z	*nM*
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	Dextrorphan	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 MMHHs 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
			μM					
CYP1A2	Phenacetin deethylation[a]	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 *O*-demethylation[a]	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[b]	70	HK (atypical)
		2	0, 15.62, 31.25, 62.5, 125, 250, 500, 1000	418		67[b]		HK (atypical)

HK, Hill kinetics; MM, Michaelis-Menten; ND, not determined; SI, substrate inhibition; TS, two-site.
[a]Only high-affinity enzyme values were reported for pathways following two-site kinetics.
[b]Values are S_{50} values equivalent to K_m; Hill equation_n1 = 1.432 and n2 = 1.778.

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

P450 Inhibition Assay Using MMHHs and CHHs. The inhibitors were incubated with MMHHs 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 MMHHs 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 MMHHs, 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 MMHHs.

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^{-1} 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 MMHHs 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 MMHHs from this study with those determined in the cryopreserved human hepatocytes and HLMs

The V_{max} and K_m values and the calculated ratio of the values for MMHH to that for hepatocytes and human liver microsomes are shown. V_{max} values are in pmol/min per million cells for MMHH and hepatocytes; K_m values are in μM.

P450 Isoforms	Marker Reaction	MMHH		Hepatocytes		HLM	Reference for Hepatocytes	Reference for Human Liver Microsomes	MMHH/ Hepatocyte Ratio		MMHH/HLM Ratio
		V_{max}	K_m	V_{max}	K_m	K_m			V_{max}	K_m	V_{max}
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.

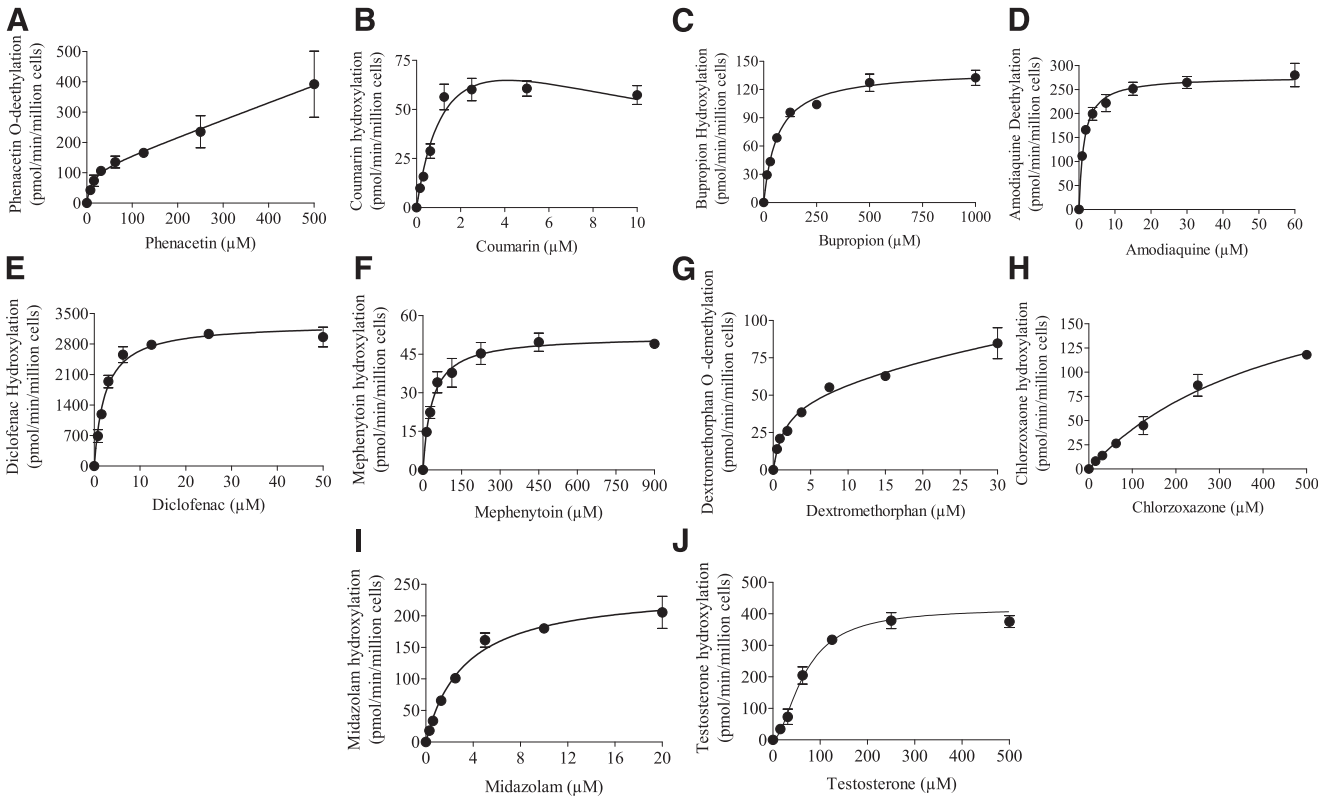

Fig. 1. Substrate saturation curves for phenacetin *O*-deethylation (A), coumarin hydroxylation (B), bupropion hydroxylation (C), amodiaquine *N*-deethylation (D), diclofenac hydroxylation (E), mephenytoin hydroxylation (F), dextromethorphan *O*-demethylation (G), chlorzoxazone 6-hydroxylation (H), midazolam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

pathways (Table 3). The K_m values determined in MMHHs 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 μM for amodiaquine *O*-deethylation is the same as that reported for human liver microsomes but lower than the 10 μM reported for human hepatocytes. The MMHH K_m of 70 μM for testosterone 6β-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 MMHHs 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 μM) 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·min^{-1}·million cells^{-1}) is 11-fold higher in MMHHs 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 MMHHs are within 2-fold compared to that determined in hepatocytes (Brown et al., 2007). The K_m for midazolam hydroxylation (3.5 μM) is within 2-fold compared to that determined in hepatocytes (4 μM), whereas the V_{max} is 11-fold higher in MMHHs (215 pmol/min/million cells) compared to that in hepatocytes (Li and Schlicht, 2014). The K_m for midazolam hydroxylation (3.5 μM) is 2.3-fold lower compared to that in hepatocytes (8.1 μM) and 2.3-fold higher compared to that determined in hepatocytes suspended (1.5 μM) 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 MMHHs 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 MMHHs due to the permeabilized plasma membranes may also be a possible mechanism for the higher activities. A direct comparison of V_{max} values between MMHHs 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 MMHHs and CHHs using the isoform-specific pathways using the substrate concentrations that are $\leq K_m$ in MMHHs. 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, α-naphthoflavone, propranolol, and methoxsalen are comparable ($\leq$2-fold difference) between MMHHs and CHHs with

TABLE 4

Absolute IC_{50} values of standard inhibitors for nine major P450 enzymes determined in MMHHs and CHHs and their corresponding fold difference values

P450 Isoform	Maker Reaction	Inhibitors	Absolute IC_{50}		Fold Difference MMHH/CHH
			MMHH	CHH	
			μM	μM	
1A2	Phenacetin *O*-deethylation	Fluvoxamine	0.007	0.025	0.28[a]
		Furafylline	0.81	1.2	0.67
		Methoxsalen	0.015	0.025	0.60
		α-Naphthoflavone	0.029	0.047	0.62
		Propranolol	0.57	0.39	1.46
2A6	Coumarin hydroxylation	Ketoconazole	91.4	100	0.91
		Methoxsalen	0.10	0.03	3.33[a]
		Phenelzine	2.1	2.0	1.07
		Tranylcypromine	0.059	0.007	8.43[a]
2B6	Bupropion hydroxylation	Ketoconazole	2.7	3.8	0.72
		Methoxsalen	4.5	5.9	0.76
		Phenelzine	11.2	5.6	2.01
		PPP	4.8	5.5	0.87
		Thiotepa	4.3	17.7	0.24[a]
		Ticlopidine	22.4	12.3	1.82
2C8	Amodiaquine *N*-deethylation	Ketoconazole	11.9	10.2	1.17
		Methoxsalen	>100	>100	NC
		Montelukast	4.1	4.3	0.95
		Tranylcypromine	77.8	58	1.35
2C9	Diclofenac hydroxylation	Fluconazole	23.2	82.0	0.28[a]
		Ketoconazole	7.2	13.0	0.55
		Methoxsalen	34.2	36.7	0.93
		Sulfaphenazole	0.4	0.63	0.59
2C19	Mephenytoin hydroxylation	Fluconazole	4.6	3.2	1.46
		Fluoxetine	1.2	4.8	0.24[a]
		Fluvoxamine	0.02	0.04	0.64
		Methoxsalen	14.5	12.1	1.19
		Omeprazole	4.6	2.6	1.80
		Ticlopidine	13.8	20.1	0.69
2D6	Dextromethorphan *O*-demethylation	Cinacalcet	3.3	11.5	0.29[a]
		Fluoxetine	0.63	0.92	0.68
		Methoxsalen	39.6	43.1	0.92
		Paroxetine	0.37	0.75	0.49
		Propafenone	0.08	0.07	1.14
		Quinidine	0.04	0.02	2.33
2 E1	Chlorzoxazone hydroxylation	Methoxsalen	75.7	81.5	0.93
		Methylpyrazole	1.4	1.1	1.24
3A4	Midazolam hydroxylation	Erythromycin	5.3	4.1	1.31
		Ketoconazole	0.09	0.09	1.11
		Methoxsalen	>100	>100	NC
		Mibefradil	0.32	0.32	1.00
		Troleandomycin	2.2	1.1	1.98
		Verapamil	18.8	5.97	3.15[a]
3A4	Testosterone hydroxylation	Erythromycin	13.9	22.4	0.62
		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
		Verapamil	4.4	8.8	0.50

NC, fold difference not calculated; PPP, 2-phenyl-2-(1-piperidinyl)propane.
[a]Fold difference of ≥3 or ≤0.3 between MMHHs and CHHs.

the exception of fluvoxamine, which showed a 3.5-fold potent value in MMHHs. The IC_{50} values against CYP2B6 for the standard inhibitors were determined using bupropion hydroxylation as a marker reaction in MMHHs and CHHs. The IC_{50} values showed no major differences and are comparable between MMHHs and CHHs (≤2-fold) except for thiotepa, which has a 4-fold higher inhibitory potency in MMHHs, with an IC_{50} value of 4.3 μM in MMHHs compared with 17.8 μM 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_{50} in CHHs, possibly due to an efflux mechanism. CYP2C8 inhibition was evaluated using amodiaquine *N*-deethylation as a marker reaction, and no differences (≤2-fold) are observed in IC_{50} values of the standard inhibitors. The potency of sulfaphenazole, ketoconazole, and methoxsalen against CYP2C9-mediated diclofenac hydroxylation is comparable between MMHHs and CHHs with the exception of fluconazole having a 3.5-fold higher potency in MMHHs. The IC_{50} values for standard inhibitors against CYP2C19 were determined using mephenytoin hydroxylation as a marker reaction in MMHHs and CHHs. The IC_{50} values are comparable and showed no major differences (≤2-fold) except for fluoxetine, which has a 4-fold potent IC_{50} in MMHHs (1.2 μM) compared with CHHs (4.8 μM). The IC_{50} of omeprazole against CYP2C19 from CHHs in this study (2.6 μM) is comparable with that reported in the literature (4.7 μM) using cryopreserved human hepatocytes (Ogilvie et al., 2011). No other information is available in the literature on the determination of IC_{50} against CYP2C19 using human hepatocytes for a direct comparison. Dextromethorphan *O*-demethylation is the marker probe reaction used for the IC_{50} determination against CYP2D6 enzyme. The IC_{50} values of the

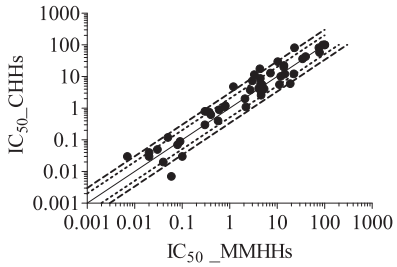

Fig. 2. Comparison of IC_{50} values generated using isoform-specific marker probe reactions from CHHs and MMHHs. The solid line represents the line of unity, the dotted line represents a 2-fold deviation from the line of unity, and the dashed line represents a 3-fold deviation from the line of unity.

CYP2D6 inhibitors determined from MMHHs 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 MMHHs). The IC_{50} values of 0.035 and 0.015 μM for quinidine derived from MMHHs and CHHs, respectively, are consistent with the reported IC_{50} value of 0.03 μM 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 MMHHs are 0.63 and 0.37 μM, respectively, versus the reported IC_{50} values of 0.04 μM for fluoxetine and 0.03 μM 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 MMHHs or CHHs. The potency of methylpyrazole, a positive control inhibitor against CYP2E1, is comparable between MMHHs 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 MMHHs 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 MMHHs and CHHs (within 2- to 3-fold), with consistently higher inhibitory potencies in MMHHs 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 MMHHs and CHHs are within 2-fold (73%) and 3-fold (84%), with the exception of fluvoxamine (CYP1A2), methoxsalen and tranylcypromine (CYP2A6), thiotepa (CYP2B6), fluconazole (CYP2C9), fluoxetine (CYP2C19), cinacalcet (CYP2D6), and verapamil (CYP3A4) having more than 3-fold differences.

Here, we report data comparing the MMHHs 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

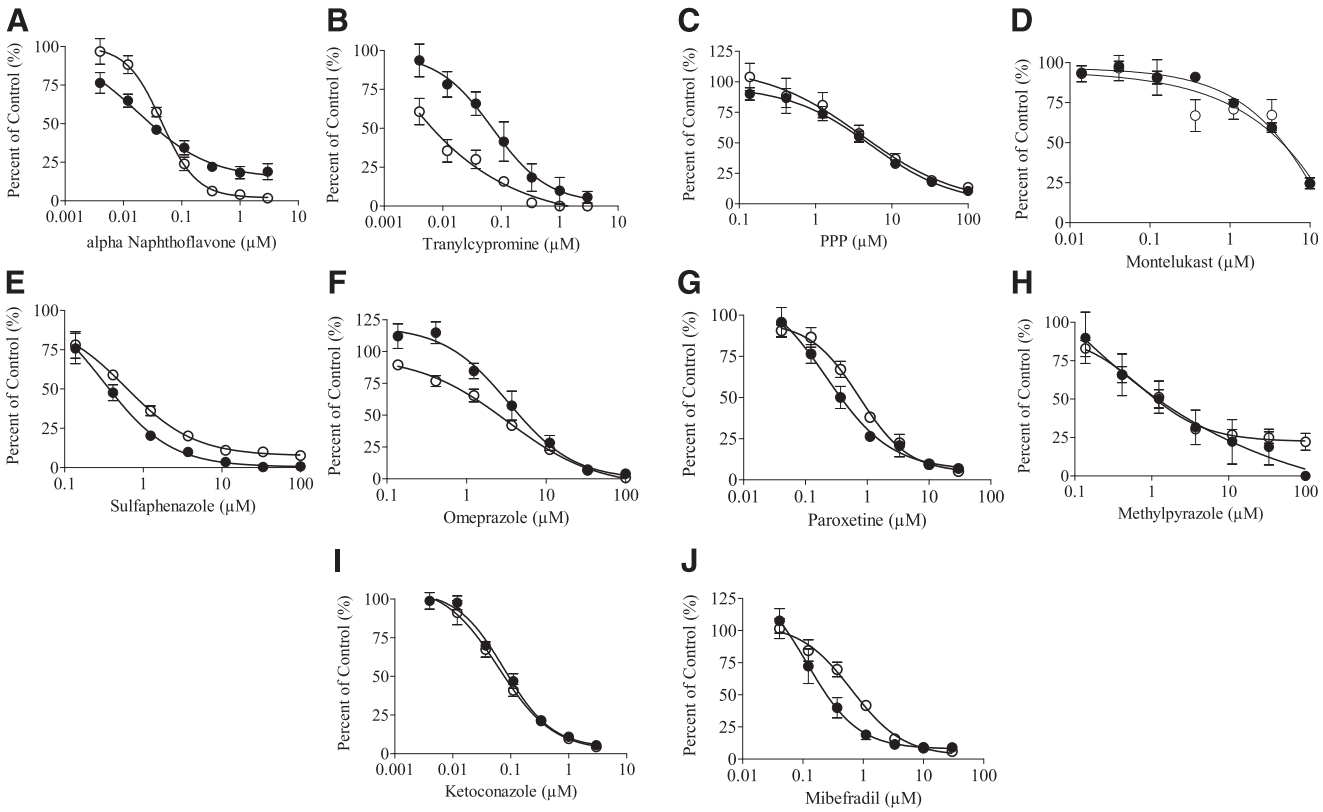

Fig. 3. The inhibition curves in MMHHs (●) and CHHs (○) for a set of representative inhibitors, including α-naphthoflavone (A), tranylcypromine (B), 2-phenyl-2-(1-piperidinyl)propane (PPP) (C), montelukast (D), sulfaphenazole (E), omeprazole (F), paroxetine (G), methylpyrazole (H), ketoconazole (I), 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 ± 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. MMHHs 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 MMHHs 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); MMHHs 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, MMHHs 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 MMHHs 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 MMHHs. The results indicate that MMHHs 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 MMHHs 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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