DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

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

Veera Raghava Choudary Palacharla, Prathyusha Chunduru, Devender Reddy Ajjala, Gopinadh Bhyrapuneni, Ramakrishna Nirogi, and Albert P. Li

Drug Metabolism and Pharmacokinetics, Suven Life Sciences Ltd, Jeedimetla, Hyderabad, India (Nirogi, Bhyrapuneni, and Palacharla), Bio-analysis, Suven Life Sciences Ltd, Pashamylaram, Medak, India (Chunduru and Ajjala), and In Vitro ADMET Laboratories Inc., Columbia, Maryland (Li)

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

High throughput P450 inhibition assay with MetMax Hepatocytes

Corresponding author # 1

Dr. Ramakrishna Nirogi

Discovery Research, Suven Life Sciences Ltd,

Serene Chambers, Road # 7, Banjara Hills,

Hyderabad 500034, India

E-mail: nvsrk@suven.com;

Corresponding author # 2

Dr. Albert P. Li,

In Vitro ADMET Laboratories Inc

9221 Rumsey Road Suite 8,

Columbia, MD 21045.

E-mail: lialbert@invitroadmet.com

Number of Text pages: 28
Number of tables: 4
Number of figures: 3
Number of references: 25
Number of words in the abstract: 252
Number of words in the introduction: 458
Number of words in the discussion: 1309

Abbreviations:
MMHHs- MetMax Human Hepatocytes, CHHs-Cryopreserved Human Hepatocytes, V_{max} – Maximal Velocity, K_m – Substrate concentration at half maximal velocity, IC50- Inhibitor concentration required to inhibit 50% of the metabolism of probe substrate, PPP- 2-phenyl-2-(1-piperidinyl)propane, K_{inact} – Inactivation rate constant, K_I – Inactivation constant, K_i – reversible inhibition constant, $f_{u,\ plasma}$- fraction unbound in plasma, $f_{u,\ mic}$-fraction unbound in microsomes, IVAL- In Vitro ADMET Laboratories.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

We report here 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 P450 inhibition. The assay was developed 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 deg C instead of liquid nitrogen, and thaw and use without centrifugation and microscopic evaluation as required for intact hepatocytes. Nine key cytochrome P450 (CYP) 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 that 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, for CYP3A4, midazolam 1'-hydroxylation and testosterone 6β-hydroxylation. The K_m values obtained with MMHHs were comparable with those reported in the literature for CHHs. Using substrate concentrations at or near Km 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 two-fold and three-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.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

Significance Statement

MMHH are co-factor supplemented cryopreserved human hepatocytes with the complete drug metabolizing enzyme pathways of the conventional hepatocytes, but with the convenience of HLM including storage at -80 deg. C instead of in liquid nitrogen, and direct thaw and use without a need for centrifugation and microscopic examination. We report here the application of MMHH in a high throughput assay in a 384-well plate format for the evaluation of P450 inhibition. Our results show that data obtained with MMHH are similar to that 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.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

During drug discovery and development in the pharmaceutical industry, new chemical entities (NCEs) are routinely screened for their drug-drug interaction potential (DDI). Human liver microsomes (HLMs) are widely used for high throughput screening of P450 inhibitory potential (Obach et al., 2006; Obach et al., 2005), with throughput enhanced via the use of automated liquid handling systems coupled with LC/MS-MS analysis (Kozakai et al., 2014; Li G et al., 2015; Lim et al., 2013), 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., 2008). While 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 over-estimation of the inhibitory potential (Brown et al., 2007a, ; Brown et al., 2007b; Brown et al., 2010; Mao et al., 2013; Xu et al., 2009) To overcome this major deficiency of HLMs, CHHs have been applied towards the evaluation of reversible (Brown et al., 2007a; Brown et al., 2010; Doshi and Li, 2011; Li AP, 2009; Mao et al., 2012; Oleson et al., 2004) and irreversible P450 inhibition parameters (Li & Doshi, 2011; McGinnity et al., 2006; Zhao et al., 2005) 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.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

We report here 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; MMHH) (Li et al., 2018). MMHHs have the desirable properties of both intact hepatocytes and HLMs - the completeness of the DME's in hepatocytes and the robustness and ease of handling of HLMs including storage at -80 deg. 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 MMHH can be used in a 384-well plate format for the evaluation of P450 inhibition, yielding results similar to that 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 NCEs.

Materials and methods

MetMax Human Hepatocytes (MMHHs, *mixed gender, pool of 10, cell density of 5 x 10^6 cells.mL^{-1}, Catalog # 82130, and Lot #PHHX-8012*), Cofactor N10 for MetMax Hepatocytes (*Catalog # 82187)*, Cryopreserved Human Hepatocytes (CHHs, Pool of 10, Lot # PHH 8008), Universal Cryopreservation Recovery Medium (UCRM) and Hepatocyte incubation medium (*HQM, serum free, Catalog #81040*) were provided by In Vitro ADMET Laboratories Inc (IVAL, Columbia, MD) as a part of 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 (NJ, USA). The metabolites, acetaminophen, hydroxy coumarin, hydroxy midazolam and 6-hydroxy testosterone were obtained from Sigma-

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

Aldrich (St. Louis, MO). Hydroxy bupropion, hydroxy mephenytoin, hydroxy chlorzoxazone, and dextrorphan were obtained from Corning (Woburn, MA). Desethyl amodiaquine was obtained from Cypex (Dundee, UK). All the chemical inhibitors used in this study were obtained from Sigma-Aldrich (St. Louis, MO) 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 master block polypropylene plates (170 µL capacity) were obtained from Greiner Bio-one North America Inc (Monroe, NC). The Axygen® Axymat silicone sealing mats (Part #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

MMHH vial was thawed and used directly without the need for centrifugation or cell viability determination (A. P. Li et al., 2018). Briefly, MMHH vials were removed from the freezer (-80°C), thawed and the contents were transferred to Cofactor N10 vials (IVAL, Columbia, MD) with gentle mixing. The cell suspension was diluted to 2x the final cell density with HQM. The final cell density of MMHHs was 0.15, 0.3 and 0.6 x 10^6 cells.mL^{-1} for time or cell density optimization study and 0.2 x 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, Columbia. MD). Briefly, 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 UCRM and centrifuged at 100 x 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

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

then adjusted with HQM to 2x 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, and 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 dimethyl sulfoxide (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: ACN (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 the substrates were prepared at a single concentration in HQM at 2x 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 triplicates at each cell density. The plates were prewarmed to 37°C for 10 min 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, Mariette, OH) maintained at 95% humidity and 5% CO_2. A

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

separate 384 well plate was prepared for each time point (2, 4, 6, 8, 12, 16, 20, 24 and 28 min). 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 2x 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 triplicates for each substrate concentration. A separate 384 well plate was prepared for each time point (4, 8, 12, 16, and 20 min). The reactions were initiated by the addition of 10 µL of MMHH cell suspension tot the 384 well plates. The incubations were terminated by the addition of 100 µL of stop solution (100% CH_3CN containing 4-OH butyranilide (100ng/mL) and diclofenac (150ng/mL)). The samples were mixed well and the plates were centrifuged at 6000 rpm (Sigma 4-16KS, Germany) for 20 min. The supernatants were transferred to a separate 384-well plate and were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Two independent studies were performed for all 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 1000x the final concentration in the assay. Serial dilutions were performed in 384 well plates. Briefly, 125 µL of acetonitrile was added to each well of the last row (H or P considering 384 well plate as two 96

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

well plates with respect to rows) two columns similar to 96 well plate) in 384 deep well plates. An aliquot (50 µL) of mixture of CH_3CN and DMSO (90:10 v/v) was added to all the wells through rows A - G or I - O. An aliquot (12.5 µL) of the inhibitor stock at 1000x was added to last well of each column and mixed well. 25 µL of the solutions were serially diluted through the rows H to B or P to J. All the wells in row A or I contain solvent only without inhibitor. Working dilutions of the inhibitors were prepared at 4x by adding 5 µL of the serial dilutions prepared above to the 120 µL of HQM/HIM. The working dilutions were prepared in HQM/HIM at 4x 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 the values at or below the Km values of the substrates. The plates were prewarmed to 37°C for 10 min 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 percentage of DMSO and CH_3CN in the final incubation was 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, Canada) operating in positive or negative ion mode connected to Nexera X2 series Ultra

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

high performance liquid chromatography (UHPLC, Shimadzu corporation). An Acquity UPLC BEH C18 (2.1 x 50mm, 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 min 90% A and 10% B, a linear increase of B from 10% to 80% over 0.60 min, maintained at 80% until 1.2 min and then decreased to 10% at 1.21 minutes with a total run time of 2.0 mins except for hydroxy coumarin and hydroxy chlorzoxazone where the B concentration is increased from 10% to 90% over 0.50 min, maintained at 90% for 1.2 min 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 x 50 mm, 3.0 µm) column was used with the 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 min 98% A and 2% B, a linear increase of B from 2% to 50% over 0.80 min, maintained at 50% upto 1.8 min, and decreased to 2% at 1.81 min with a total run time of 2.5 minutes. Data acquisition and analysis was performed with the Analyst software version 1.6.3 (ABSciex, Ontario, Canada). The marker metabolites quantified, mass transitions, and linearity range (nM) used are summarized in Table 1.

Data analysis

Kinetic parameters for P450 marker reactions were estimated by analyzing the data with Michaelis-Menten (Equation 1) or Hill (Equation 2) or substrate inhibition (Equation 3), or two

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

enzyme Michaelis-Menten (Equation 4) equations using GraphPad Prism v.4.0 (San Diego, CA). The best fit equation was selected based on Akaike Information Criteria (AIC).

The equations used were as follows:

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

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

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

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

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, Ksi is the constant describing the substrate inhibition interaction, $V_{max, LA}$, $K_{m, LA}$, and $V_{max, HA}$, $K_{m, HA}$ 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 (nM) was converted to activity (pmol/min/million cells). The percent remaining activity in the presence of inhibitor at each concentration compared to that in the absence of inhibitor was calculated as follows.

Activity Remaining (%) = (Activity in the presence of inhibitor/Activity in the absence of inhibitor) * 100 Equation 5

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

$$Y=Bottom + (Top-Bottom)/ (1+10^{\wedge} ((LogIC_{50}-X)*Hill\ Slope)) \quad \text{Equation 6}$$

Where Y is the percent activity remaining and X is the corresponding concentration.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Enzyme kinetics

MMHH's were incubated with various P450 selective substrates at 0.15, 0.3, and 0.6 x 10^6 cells.mL^{-1} for 2, 4, 6, 8, 12, 16, 20, 24 and 28 min. 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 the substrates. The metabolites formation was linear for all the enzymes and was ≤ 10% of the nominal incubated substrate concentration at 0.15 x 10^6 cells.mL^{-1} for all the enzymes except for CYP2C8 mediated amodiaquine O-deethylation for which 16% of metabolite formation was observed above 20 min. The metabolite formation was linear with respect to time up to 28 min except for CYP3A4 mediated midazolam and testosterone hydroxylation where the product formation at 0.6 x 10^6 cells.mL^{-1} was linear up to 8 and 24 min respectively. Based on these results, the cell density and time was fixed at 0.2 x 10^6 cells.mL^{-1} (rounded off from 0.15) and 20 min 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 reported in literature from hepatocytes 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

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

midazolam hydroxylation followed substrate inhibition kinetics. The CYP3A4 mediated testosterone hydroxylation followed Hill kinetics. The substrate saturation curves for each enzyme are depicted in Figure 1.

P450 inhibition assay using MMHH and CHH

The inhibitors were incubated with MMHHs and CHHs for 20 min in the presence of marker probe substrate and the IC_{50} values were calculated using equation 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 Figure 2. The inhibition curves for a set of representative controls against each enzyme are depicted in Figure 3.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

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 HLM and CHHs. Both midazolam and testosterone hydroxylation kinetics were evaluated for CYP3A4 enzyme. All the kinetic and inhibition experiments were performed in 384 well plates to develop a high throughput P450 inhibition assay using MMHHs.

The linear formation of 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 min was used for the kinetic experiments. The K_m 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 pathways (Table 3). The K_m determined in MMHH for phenacetin O-deethylation, coumarin 7-hydroxylation, bupropion hydroxylation, *s*-mephenytoin 4'-hydroxylation, dextromethorphan O-demethylation, chlorzoxazone hydroxylation and midazolam 1'-hydroxylation are within 2-4 fold of that reported for hepatocytes and/or human liver microsomes. The MMHH K_m for amiodiaquine O-deethylation of 1 uM is the same as that reported for human liver microsomes but lower than the 10 uM reported for human hepatocytes. MMHH K_m for testosterone 6β-hydroxylation of 70 uM is within 4 fold of that reported for hepatocytes but is approximately 7 fold of that for human liver microsomes.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

The MMHH V_{max} for the various P450 pathways are compared to that available in published literature for hepatocytes (Table 3). Over 5 fold higher V_{max} values were observed in MMHH than that 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 two fold 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 to that determined in hepatocytes (450 pmol/min/million cells, (Mao et al., 2012); 290 pmol/min/million cells (Brown et al., 2007). The Vmax 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 a 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 two fold to that determined in hepatocytes (Brown et al., 2007). The K_m for midazolam hydroxylation (3.5 µM) is within two fold to that determined in hepatocytes (4 µM) whereas the V_{max} is 11 fold higher in MMHHs (215 pmol/min/million cells) to that in hepatocytes (Li and Schlicht, 2014). The K_m for midazolam hydroxylation (3.5 µM) is 2.3 fold lower to that in hepatocytes (8.1 µM) and 2.3 fold higher to that determined in hepatocytes suspended (1.5 µM) in human plasma in a different study (Mao et al., 2012).

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

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 to 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 inter-individual 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 CHH using hepatocytes from the same donors will be determined in the laboratory of one of the co-authors of this report (Li, A. P., 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 CYP1A2 enzyme. The IC_{50} values of the standard inhibitors, furafylline, alpha-Naphthoflavone, Propranolol, and methoxsalen, are comparable ($\leq$ 2 fold differences) between MMHHs and CHHs with 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 ($\leq$ 2 fold) except for thiotepa which has four fold higher inhibitory potency in MMHHs, with an IC_{50} value of 4.3 µM in MMHHs compared to 17.8 µM in CHHs. Thiotepa has been reported to be a P-glycoprotein (P-gp) substrate (Liang et al, 2015) which could be the possible reason for a weaker IC_{50} in CHHs

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

possibly due to 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 are comparable between MMHHs and CHHs with the exception of fluconazole having 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} are comparable and showed no major differences (≤ 2 fold) except for fluoxetine which has four fold potent IC_{50} in MMHHs (1.2 µM) compared to CHHs (4.8 µM). The IC_{50} of omeprazole against CYP2C19 in this study from CHHs (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 CYP2D6 inhibitors determined from MMHHs and CHHs are comparable and showed no major differences with a less than two 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 quinidine derived from MMHHs and CHHs of 0.035 and 0.015 µM, respectively, are consistent with reported IC_{50} value of 0.03 µM using bufuralol hydroxylation as marker probe reaction with a 20 min pre-incubation step before the addition of the substrate (Mao et al, 2012), a result consistent with the non-TDI 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 min pre-incubation step (Mao et al., 2012), a result that can be

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

attributed to that paroxetine and fluoxetine are TDI's of CYP2D6 enzyme and a 20 min pre-incubation step will result in shift of IC_{50} towards 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- 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 two fold (73%) and three 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 three-fold differences.

We report here data comparing the MMHHs to traditional cryopreserved hepatocytes in evaluating a series of P450 probe substrates and inhibitors. While in general the results are similar between

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

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

1. MMHH are fully permeabilized. For instance, the commonly used trypan blue exclusion assay would yield 100% blue cells (Li, A. P., personal communication). A comparison of MMHH and intact human hepatocytes may allow the evaluation of the role of membrane permeability on 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 (Li, A. P. personal communication), MMHH may be used for the evaluation of uptake transporter substrates which may have low rate of metabolic clearance due to this artifact of the pooled donor human hepatocytes.
2. Using phase-contrast microscopy, MMHH are observed to possess a visually visible plasma membrane. It is not yet fully determined if the cytosolic proteins still retained at physiological concentrations within the cell membrane as the commonly used procedure of centrifugation may artefactually 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 MMHH 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

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

pathways in MMHHs may allow the results to be complementary to that obtained with HLMs, allowing a more accurate assessment of clinical P450 inhibitory potential.

References

Brown HS, Chadwick A, Houston JB. (2007a) Use of isolated hepatocyte preparations for cytochrome P450 inhibition studies: comparison with microsomes for Ki determination. Drug Metab Dispos. 35(11):2119-26.

Brown HS, Griffin M, Houston JB. (2007b) Evaluation of cryopreserved human hepatocytes as an alternative in vitro system to microsomes for the prediction of metabolic clearance. *Drug Metab Dispos* 35(2):293-301.

Brown HS, Wilby AJ, Alder J, Houston JB. (2010) Comparative use of isolated hepatocytes and hepatic microsomes for cytochrome P450 inhibition studies: transporter-enzyme interplay. Drug Metab Dispos. 38(12):2139-46.

Doshi U, Li AP. (2011) Luciferin IPA-based higher throughput human hepatocyte screening assays for CYP3A4 inhibition and induction. *J Biomol Screen* 16(8):903-9.

Fahmi OA, Hurst S, Plowchalk D, Cook J, Guo F, Youdim K, Dickins M, Phipps A, Darekar A, Hyland R, Obach RS. (2009) Comparison of different algorithms for predicting clinical drug-drug interactions, based on the use of CYP3A4 in vitro data: predictions of compounds as precipitants of interaction. *Drug Metab Dispos* 37(8):1658-66.

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM, Lindley CM. (2000) Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos*. 28(10):1222-30.

Hosono H, Kumondai M, Maekawa M, Yamaguchi H, Mano N, Oda A, Hirasawa N, Hiratsuka M. (2017). Functional characterization of 34 CYP2A6 allelic variants by assessment of nicotine C-oxidation and coumarin 7-hydroxylation activities. *Drug Metab Dispos*. 45(3):279-85.

Kozakai K, Yamada Y, Oshikata M, Kawase T, Suzuki E, Haramaki Y, Taniguchi H (2014) Cocktail-substrate approach-based high-throughput assay for evaluation of direct and time-dependent inhibition of multiple cytochrome P450 isoforms. Drug Metab Pharmacokinet. 29(2):198-207.

Li AP (2009) Evaluation of luciferin-isopropyl acetal as a CYP3A4 substrate for human hepatocytes: effects of organic solvents, cytochrome P450 (P450) inhibitors, and P450 inducers. Drug Metab Dispos. 37(8):1598-603.

Li AP, Doshi U (2011) Higher throughput human hepatocyte assays for the evaluation of time-dependent inhibition of CYP3A4. *Drug Metab Lett* 5(3):183-91.

Li AP, Schlicht KE (2014) Application of a higher throughput approach to derive apparent Michaelis-Menten constants of isoform-selective p450-mediated biotransformation reactions in human hepatocytes. *Drug Metab Lett* 8(1):2-11.

Li AP, Ho MD, Amaral K, Loretz C (2018) A Novel In Vitro Experimental System for the Evaluation of Drug Metabolism: Cofactor-Supplemented Permeabilized Cryopreserved Human Hepatocytes (MetMax Cryopreserved Human Hepatocytes). Drug Metab Dispos 46(11):1608-1616.

Liang Y, Li S, Chen L (2015) The physiological role of drug transporters. *Protein Cell* 6(5):334-50.

Li G, Huang K, Nikolic D, van Breemen RB (2015) High-Throughput Cytochrome P450 Cocktail Inhibition Assay for Assessing Drug-Drug and Drug-Botanical Interactions. Drug Metab Dispos. 43(11):1670-8.

Lim KB, Ozbal CC, Kassel DB (2013) High-throughput mass spectrometric cytochrome P450 inhibition screening. Methods Mol Biol. 987:25-50.

Mao J, Mohutsky MA, Harrelson JP, Wrighton SA, Hall SD (2011) Prediction of CYP3A-mediated drug-drug interactions using human hepatocytes suspended in human plasma. *Drug Metab Dispos* 39(4):591-602.

Mao J, Mohutsky MA, Harrelson JP, Wrighton SA, Hall SD (2012) Predictions of cytochrome P450-mediated drug-drug interactions using cryopreserved human hepatocytes: comparison of plasma and protein-free media incubation conditions. *Drug Metab Dispos* 40(4):706-16.

Mao J, Johnson TR, Shen Z, Yamazaki S.(2013) Prediction of crizotinib-midazolam interaction using the Simcyp population-based simulator: comparison of CYP3A time-dependent inhibition between human liver microsomes versus hepatocytes. Drug Metab Dispos. 41(2):343-52.

McGinnity DF, Tucker J, Trigg S, Riley RJ (2005) Prediction of CYP2C9-mediated drug-drug interactions: a comparison using data from recombinant enzymes and human hepatocytes. *Drug Metab Dispos* 33(11):1700-7.

McGinnity DF, Berry AJ, Kenny JR, Grime K, Riley RJ. (2006) Evaluation of time-dependent cytochrome P450 inhibition using cultured human hepatocytes. Drug Metab Dispos. 34(8):1291-300.

Moeller T, Worzella T, Sobol M, Ma D, Cali JJ, Larson B (2013). Comparison of inhibition of CYP1A2, 2C9 and 3A4 using human liver microsomes and hepatocytes. *ISSX*, Toronto

Obach RS, Walsky RL, Venkatakrishnan K, Gaman EA, Houston JB, Tremaine LM. (2006). The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. J Pharmacol Exp Ther. 316(1):336-48.

Obach RS, Walsky RL, Venkatakrishnan K, Houston JB, Tremaine LM (2005). In vitro

cytochrome P450 inhibition data and the prediction of drug-drug interactions: qualitative relationships, quantitative predictions, and the rank-order approach. Clin Pharmacol Ther. 78(6):582-92.

Oleson FB, Berman CL, Li AP (2004) An evaluation of the P450 inhibition and induction potential of daptomycin in primary human hepatocytes. Chem Biol Interact. 150(2):137-47.

Ogilvie BW, Yerino P, Kazmi F, Buckley DB, Rostami-Hodjegan A, Paris BL, Toren P, Parkinson A (2011) The proton pump inhibitor, omeprazole, but not lansoprazole or pantoprazole, is a metabolism-dependent inhibitor of CYP2C19: implications for coadministration with clopidogrel. *Drug Metab Dispos* 39(11):2020-33.

Siu, AY and Lai, G (2017) Impact of probe substrate selection on cytochrome P450 reaction phenotyping using the relative activity factor. *Drug Metab Dispos* 45(2):183-189.

Xu L, Chen Y, Pan Y, Skiles GL, Shou M. (2009) Prediction of human drug-drug interactions from time-dependent inactivation of CYP3A4 in primary hepatocytes using a population-based simulator. *Drug Metab Dispos* 37(12):2330-9.

Zhao P, Kunze KL, Lee CA (2005) Evaluation of time-dependent inactivation of CYP3A in Cryopreserved human hepatocytes. *Drug Metab Dispos* 33(6):853-61.

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

Legends to Figures

Figure 1

Substrate saturation curves for a) Phenacetin O-deethylation, b) Coumarin hydroxylation, c) Bupropion hydroxylation, d) Amodiaquine N-deethylation, e) Diclofenac hydroxylation, f) Mephenytoin hydroxylation, g) Dextromethorphan O-demethylation, h) Chlorzoxazone 6-hydroxylation, i) Midazolam 1-hydroxylation and j) Testosterone 6-hydroxylation in MMHHs. Each data point is the Mean ± SEM of triplicates from a single experiment.

Figure 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 short dashed black line represent a 2-fold deviation from the line of unity and the long dashed line represents a 3-fold deviation from the line of unity.

Figure 3

The Inhibition curves in MMHHs (●) and CHHs (○) for a set of representative inhibitors including alpha Naphthoflavone (a), Tranylcypromine (b), PPP (c), Montelukast (d), Sulfaphenazole (e), Omeprazole (f), Paroxetine (g), Methyl pyrazole (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 ± SEM of six replicates in a single experiment.

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 (*m/z*)	Linearity Range (nM)
CYP1A2	Phenacetin	Acetaminophen	Positive	152.2 → 110	6.6 to 6622.5
CYP2A6	Coumarin	Hydroxy Coumarin	Negative	160.8→132.7	6.2 to 6211.2
CYP2B6	Bupropion	Hydroxy Bupropion	Positive	256→139	3.9 to 3921.6
CYP2C8	Amodiaquine	Desethyl Amodiaquine	Positive	328.3→283.3	3.1 to 3058.1
CYP2C9	Diclofenac	Hydroxy Diclofenac	Positive	311.927→266	3.2 to 3215.4
CYP2C19	Mephenytoin	Hydroxy Mephenytoin	Positive	235.1→150.2	4.3 to 4273.5
CYP2D6	Dextromethorphan	Dextrorphan	Positive	258.3→157.2	3.9 to 3891.1
CYP2E1	Chlorzoxazone	Hydroxy Chlorzoxazone	Negative	184→120.1	5.4 to 5434.8
CYP3A4	Midazolam	Hydroxy Midazolam	Positive	342→203	2.9 to 2932.6
CYP3A4	Testosterone	Hydroxy Testosterone	Positive	305.1→269.1	3.9 to 3289.5

Table 2 Substrate concentrations, Vmax and Km values determined for the marker reactions in MMHHs from two independent studies

CYP	Marker Reaction	Study	Substrate concentrations (µM)	V_{max}	Mean V_{max}	K_m	Mean K_m	Kinetics
1A2	Phenacetin deethylation**	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)
2A6	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)
2B6	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)
2C8	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)
2C9	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)
2C19	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)
2D6	Dextromethorphan O-Demethylation**	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)
2 E1	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)
3A4	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)
3A4	Testosterone hydroxylation	1	0, 15.62, 31.25, 62.5, 125, 250, 500, 1000	765	592	73*	70	HK (Atypical)
		2	0, 15.62, 31.25, 62.5, 125, 250, 500, 1000	418		67*		HK (Atypical)

ND-Not determined; TS-Two-Site; SI-Substrate Inhibition; MM-Michaelis-Menten; HK-Hill Kinetics

V_{max} units are pmol/min/million cells; Km_{units} are µM

**only high affinity enzyme values were reported for pathways following two-site kinetics

*Values are S_{50} values equivalent to Km; Hill equation_n1= 1.432 and n2 =1.778;

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 human liver microsomes (HLM). The V_{max} and K_m values (top) and the calculated ratio of the values for MMHH to that for hepatocytes and human liver microsomes (bottom) are shown.

CYP Isoforms	Marker Reaction	MMHH		Hepatocytes		HLM	Reference for Hepatocytes	Reference for Human Liver Microsomes
		V_{max}	K_m	V_{max}	K_m	Km		
1A2	Phenacetin deethylation	80	11	NA	NA	112.7	NA	Li et al., 2015
2A6	Coumarin hydroxylation	112	2	NA	NA	1.9	NA	Honsono et al., 2017
2B6	Bupropion hydroxylation	136	67	21	41	130	Li et al., 2014	Faucette et al., 2000
2C8	Amodiaquine deethylation	517	1	334	10	1	Li et al., 2014	Li et al., 2015
2C9	Diclofenac hydroxylation	3273	2	290	7	22.4	Brown et al., 2007	Siu and Lai, 2017
2C19	Mephenytoin hydroxylation	50	45	16	13	56.8	Brown et al., 2007	Siu and Lai, 2017
2D6	Dextromethorphan Demethylation	37	2	50	1	2.9	Brown et al., 2007	Li et al., 2015
2 E1	Chlorzoxazone hydroxylation	462	465	NA	NA	149.8	NA	Li et al., 2015
3A4	Midazolam hydroxylation	215	4	20	4	8.4	Li et al., 2014	Siu and Lai, 2017
3A4	Testosterone hydroxylation	592	70	1800	24	10.2	Brown et al., 2007	Siu and Lai, 2017

V_{max} in pmol/min/million cells for MMHH and hepatocytes; K_m in µM; NA-Not Available

CYP Isoforms	Marker Reaction	MMHH/Hepatocyte Ratio		MMHH/HLM Ratio
		V_{max}	K_m	V_{max}
1A2	Phenacetin deethylation	NA	NA	0.10
2A6	Coumarin hydroxylation	NA	NA	1.05
2B6	Bupropion hydroxylation	6.5	1.6	0.52
2C8	Amodiaquine deethylation	1.5	0.1	1.00
2C9	Diclofenac hydroxylation	11.3	0.3	0.09
2C19	Mephenytoin hydroxylation	3.1	3.5	0.79
2D6	Dextromethorphan Demethylation	0.7	2.0	0.69
2 E1	Chlorzoxazone hydroxylation	NA	NA	3.10

3A4	Midazolam hydroxylation	10.8	1.0	0.48
3A4	Testosterone hydroxylation	0.3	2.9	6.86

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

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} (μM)		Fold difference MMHH/ CHH
			MMHH	CHH	
1A2	Phenacetin O-deethylation	Fluvoxamine	0.007	0.025	**0.28**
		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**
		Phenelzine	2.1	2.0	1.07
		Tranylcypromine	0.059	0.007	**8.43**
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**
		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**
		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**
		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**
		Fluoxetine	0.63	0.92	0.68
		Methoxsalen	39.6	43.1	0.92
		Paroxetine	0.37	0.75	0.49

DMD Fast Forward. Published on August 2, 2019 as DOI: 10.1124/dmd.119.088237
This article has not been copyedited and formatted. The final version may differ from this version.

		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**
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 ; Fold difference of ≥ 3 or ≤ 0.3 between MMHHs and CHHs is indicated in bold

Figure 1

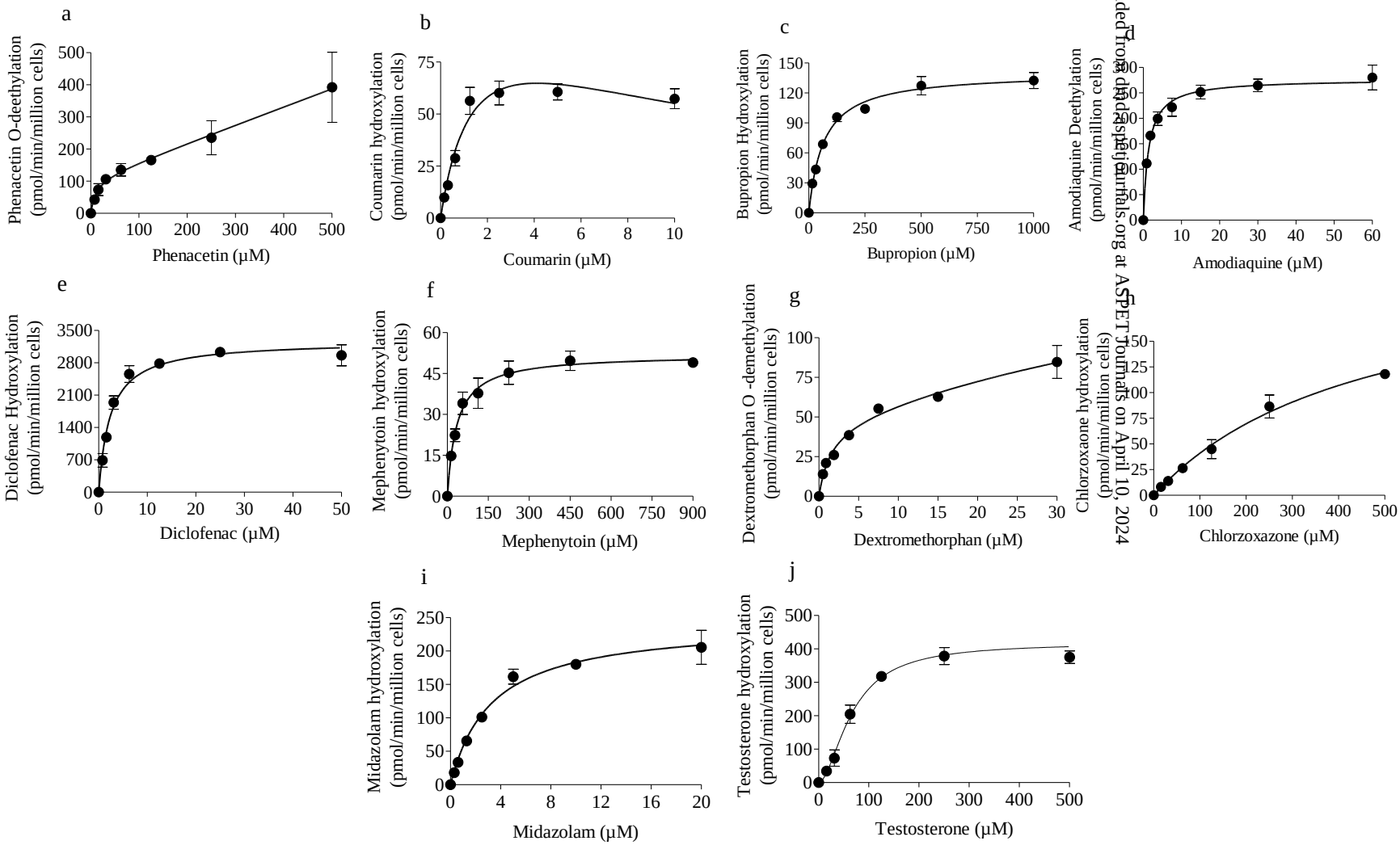

Fig. 2

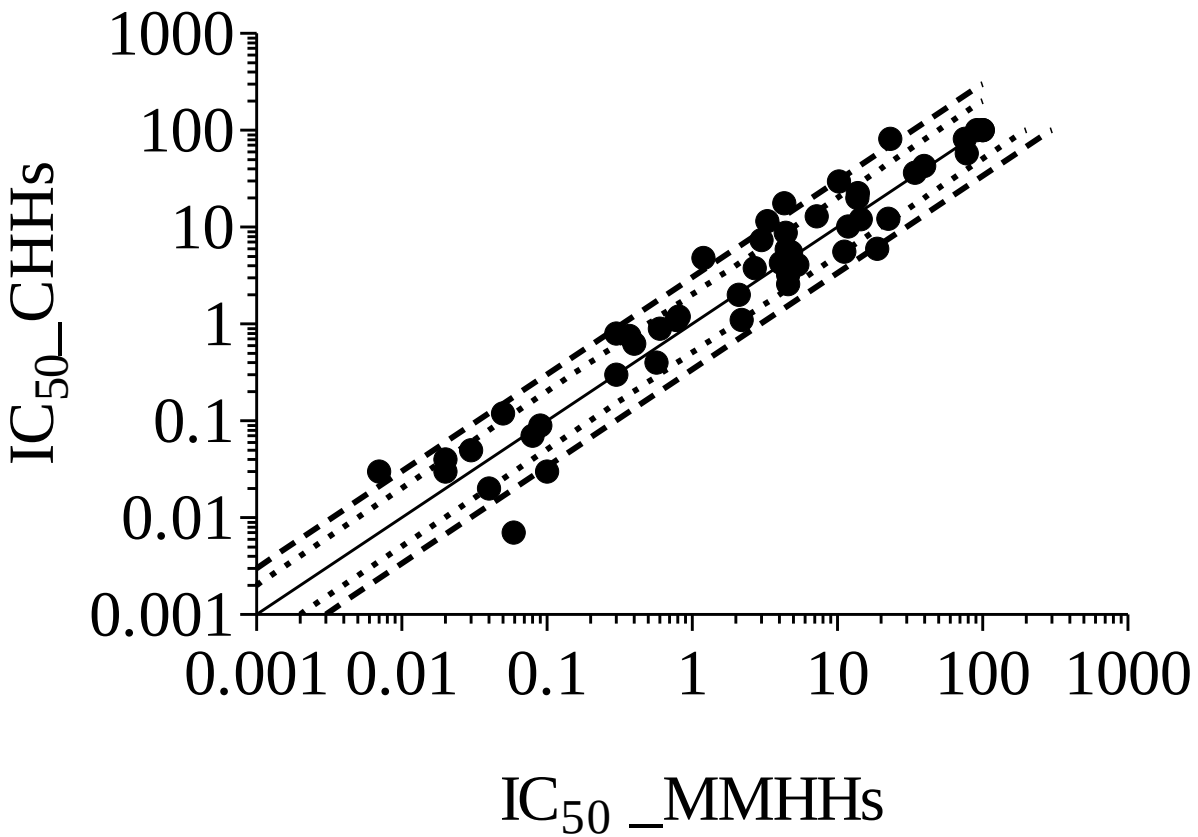

Fig. 3