Permeabilized cryopreserved human hepatocytes as an exogenous metabolic system in a novel metabolism-dependent cytotoxicity assay (MDCA) for the evaluation of metabolic activation and detoxification of drugs associated with drug induced liver injuries: Results with acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone, and troglitazone

Author names

Hong Wei and Albert P. Li

Author affiliations

In Vitro ADMET Laboratories Inc., Columbia, MD
Running title: Metabolic activation and detoxification of DILI drugs

Corresponding author: Albert P. Li, Ph. D., In Vitro ADMET Laboratories Inc., 9221 Rumsey Road Suite 8, Columbia, MD 21045, USA. Telephone: (410)869-9037. Fax: (410)869-9034. Email: lialbert@invitroadmet.com.

Number of text pages: 31

Number of tables: 6

Number of figures: 7

Number of references: 84

Number of words (Abstract): 243

Number of words (Introduction): 749

Number of words (Discussion): 1438

List of Abbreviations: DILI (drug-induced liver injuries), DME (drug metabolizing enzymes), MDCA (metabolism-dependent cytotoxicity assay), GSH (L-glutathione), GST (glutathione S-transferase), MMHH (MetMax cryopreserved human hepatocytes), HIM (Hepatocyte Induction Medium), NAD+ (Nicotinamide adenine dinucleotide), NADPH (nicotinamide adenine dinucleotide phosphate), PAPS (3'-phosphoadenosine-5'-phosphosulfate), SULT (cytosolic sulfotransferase), UCPM (Universal Cryopreservation Plating Medium), UDPGA (uridine 5'-diphosphoglucuronic acid, UGT (uridine 5'-diphospho-glucuronosyltransferase),
Abstract:
We report here a novel in vitro experimental system, the metabolism-dependent cytotoxicity assay (MDCA), for the definition of the roles of hepatic drug metabolism in toxicity. MDCA employs permeabilized cofactor-supplemented cryopreserved human hepatocytes (MetMax™ human hepatocytes, MMHH), as an exogenous metabolic activating system, and HEK293 cells, a cell line devoid of drug metabolizing enzyme activity, as target cells for the quantification of drug toxicity. The assay was performed in the presence and absence of cofactors for key drug metabolism pathways known to play key roles in drug toxicity: NADPH/NAD+ for phase 1 oxidation, UDPGA for UGT mediated glucuronidation, PAPS for SULT mediated sulfation, and GSH for GST mediated GSH conjugation. Six drugs with clinically significant hepatotoxicity, resulting in liver failure or a need for liver transplantation: acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone and troglitazone were evaluated. All six drugs exhibited cytotoxicity enhancement by NADPH/NAD+, suggesting metabolic activation via phase 1 oxidation. Attenuation of cytotoxicity by UDPGA was observed for acetaminophen, ketoconazole and troglitazone, by PAPS for acetaminophen, ketoconazole and troglitazone, and by GSH for all six drugs. Our results suggest that MDCA can be applied towards the elucidation of metabolic activation and detoxification pathways, providing information that can be applied in drug development to guide structure optimization to reduce toxicity and to aid the assessment of metabolism-based risk factors for drug toxicity. GSH detoxification represents an endpoint for the identification of drugs forming cytotoxic reactive metabolites, a key property of drugs with idiosyncratic hepatotoxicity.
**Significance Statement:** Application of the metabolism-dependent cytotoxicity assay (MDCA) for the elucidation of the roles of metabolic activation and detoxification pathways in drug toxicity may provide information to guide structure optimization in drug development to reduce hepatotoxic potential, and to aid the assessment of metabolism-based risk factors. GSH detoxification represents an endpoint for the identification of drugs forming cytotoxic reactive metabolites that may be applied towards the evaluation of idiosyncratic hepatotoxicity.
Introduction

Metabolic activation – metabolism of a relatively nontoxic parent molecule to toxic metabolites, and detoxification – biotransformation of the toxic parent and metabolite molecules to nontoxic metabolites, are key determinants of drug toxicity (Hinson et al., 1994). Definition of metabolic activation and detoxification pathways of drug candidates is an important aspect of drug development, providing information that can be applied towards structural design and drug candidate selection to minimize toxicological liability, identification of animal species for preclinical evaluation to improve the accuracy of assessment of human toxicity, identification of at risk patient populations based on metabolic activation and detoxification capacity, and identification of environmental factors that may exacerbate drug toxicity via their induction of metabolic activating pathways and inhibition of detoxification pathways (Spielberg, 1984; Nebert et al., 1996; Li, 2002; Tuschi et al., 2008; Baillie and Rettie, 2011).

We report here a novel in vitro experimental system for the evaluation of the roles of drug metabolizing enzymes in drug toxicity, the Metabolism Dependent Cytotoxicity assay (MDCA). MDCA utilizes a novel in vitro model for the evaluation of hepatic drug metabolism developed in our laboratory, the cofactor-supplemented permeabilized human hepatocytes (MetMax™ Human Hepatocytes, MMHH) (Li et al., 2018), as an exogenous hepatic metabolic system, with human embryonic kidney 293 (HEK293) cells, a cell line deficient in drug-metabolizing enzyme activities (Westlind et al., 2001; Koga et al., 2011), as target cells for the quantification of cytotoxicity (Figure 1). MMHH was originally developed to enhance the ease of use of human hepatocytes for drug metabolism studies, with features that can overcome several limitations of intact human hepatocytes: 1. The drug metabolizing enzyme (DME) activities of MMHH are not affected by drug toxicity, a major limitation of intact hepatocytes in the evaluation of toxic drugs. 2. Due to permeabilization, plasma membranes no longer serve as a barrier for drug entry and metabolite exit, thereby allowing the evaluation of the metabolic fate of drugs with low permeability (Keefer et al., 2020). 3. As permeabilization drastically reduced endogenous cofactor
concentrations due to leakage, drug metabolism by MMHH requires cofactor supplementation and thereby can be directed via cofactor specification, for instance, reduced nicotinamide adenine dinucleotide phosphate (NADPH) for phase 1 oxidation, uridine 5'-diphosphoglucuronic acid (UDPGA) for uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT) mediated glucuronidation, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) for cytosolic sulfotransferase (SULT)-mediated sulfate conjugation, and reduced glutathione (GSH) for glutathione S-transferase (GST)-mediated GSH conjugation). These features suggest that MMHH may also be applicable as an exogenous activating system for protoxicant activation for a co-cultured target cells, akin to the application of induced rat liver S9 in the evaluation of promutagens in genotoxicity assays (McCann et al., 1975; Li, 1984; Mitchell et al., 1997). Furthermore, the contribution of specific drug metabolism pathways towards drug toxicity may be defined via supplementation of MMHH with selected cofactors.

We report here MDCA results with six drugs associated with drug-induced liver injuries (DILI): acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone, and troglitazone (Figure 2). Acetaminophen is a widely used over-the-counter analgesic and antipyretic drug which is known to cause severe liver injury, mainly due to overdose (Clark and Taubman, 2016; Bouvet et al., 2020). Amiodarone is an antiarrhythmic that was withdrawn from the market due to adverse toxicity in multiple tissues, including fatalities due to hepatotoxicity (Agozzino et al., 2002; Kang et al., 2007; Babatin et al., 2008; Wu et al., 2021). Cyclophosphamide is a widely used alkylating anticancer agent with toxic consequences including hepatotoxicity (Subramaniam et al., 2013), cardiotoxicity (Liu et al., 2018), hemorrhagic cystitis (Kolb et al., 1994), hepatic venoocclusive disease (Ortega et al., 1997), immunosuppression (Hauser et al., 1983), and genotoxicity/carcinogenicity (Hansel et al., 1997). Ketoconazole is an orally administered azole antifungal known to cause severe hepatotoxicity, resulting in liver failure (Findor et al., 1998; Zollner et al., 2001; Li et al., 2021). Nefazodone, an antagonist for the 5-hydroxytryptamine receptor for the treatment of depression, was withdrawn from the market due to
its association with severe hepatotoxicity, resulting in liver failure (Stewart, 2002). Troglitazone, the first oral peroxisome proliferator-associated receptor gamma agonist for the treatment of type 2 diabetes, was marketed in March 1997 and was removed from the U.S. market 36 months later after 90 cases of liver failure. Troglitazone is often regarded as a prototypical drug for research to further the mechanistic understanding of idiosyncratic drug toxicity as well as the development of predictive approaches for the identification of structures with idiosyncratic hepatotoxic potential in drug development (Goda et al., 2016; Takemura et al., 2016; Kim et al., 2017a; Mak et al., 2018).

Materials and Methods

**Chemicals.** The hepatotoxic drugs acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone, troglitazone and the cofactors NAD+, NADPH, UDPGA, PAPS and GSH were obtained from Sigma Aldrich (St. Louis, MO).

**Cell Culture Reagents and Plates.** Hepatocyte Induction Medium (HIM), Hepatocyte Incubation Medium (HQM), and Universal Cryopreservation Plating Medium (UCPM) were obtained from In Vitro ADMET Laboratories Inc. (Columbia, MD). 384-well white plates (Falcon® 384-Well Tissue Culture Treated Microplates, us.vwr.com) and 75 cm² tissue culture flasks (Falcon® Tissue Culture Flasks) were obtained from VWR Inc. (www.usvwr.com). Trypsin (0.25% trypsin solution from bovine pancreas) was obtained from Sigma Aldrich (St. Louis, MO).

**MMHH.** MMHH (lot number: PHHX8012) was prepared from cryopreserved human hepatocytes pooled from 10 donors as previously described (Li et al., 2018). The demographics of the 10 donors are shown in Table 1. For the MDCA assay, MMHH was supplemented with and without the following pathway-specific cofactors for the evaluation of the roles of specific DME pathways on drug toxicity: NADPH/NAD+ (2 mM) for phase 1 oxidative metabolism, UDPGA (1 mM) for UDP-
glucuronosyltransferase (UGT)-dependent glucuronidation, PAPS (0.1 mM) for sulfotransferases (SULT)-
dependent sulfate conjugation, and GSH (1 mM) for GST-dependent GSH conjugation.

**HEK293 cells.** HEK293 cells (human embryo kidney cells) were a gift from United States Environmental
Protection Agency. The cells were routinely maintained at approximately 50% confluency in UCPM in 75
cm² tissue culture flasks in a cell culture incubator at 37 deg. C in a highly humidified atmosphere of 5%
CO₂ and 95% air.

**Evaluation of cofactor-directed acetaminophen metabolism by MMHH.** Incubation of MMHH with
acetaminophen for the quantification of metabolite formation was performed as previously reported (Li
et al., 2018). Briefly, acetaminophen was dissolved in HQM at 2 mM (2X of the final concentration of 1
mM), added at a volume of 50 µL per well in triplicate (metabolism wells) of a 96-well cell culture plate
(metabolism plate) and prewarmed to 37° C for approximately 15 minutes in a cell culture incubator.
Cofactor-supplemented MMHH (Li et al., 2018) was thawed from cryopreservation and placed in a cell
culture incubator to prewarm the reagent to approximately 37° C. Metabolism of acetaminophen was
initiated by adding 50 µL of MMHH at a cell density of 2 x 10⁶ cells/mL (2X of the final cell density of 1 x
10⁶ cells/mL) into the metabolism wells (containing 50 µL of 2 mM acetaminophen) and returned to the
cell culture incubator without shaking for 30 minutes. At the end of the incubation, 200 µL of
acetonitrile was added into each well for the termination of the metabolism. The 96-well plate was
stored in a -80° C freezer for later LC/MS-MS quantification of metabolite formation.

**LC/MS/MS Quantification of Metabolite Formation.** Upon thawing of the contents of the reaction plate,
100 µL aliquots of acetonitrile solution containing 250 nM of tolbutamide (internal standard) were
added to each of the metabolism wells followed by centrifugation at 3,500 rpm for 5 minutes to remove
the cellular debris. An aliquot of 100 µL of supernatant from each well was transferred to a 96 well plate
and was diluted with 200 µL of deionized water for the quantification of metabolites using an API 4000
QTRAP mass spectrometer with an electrospray ionization source (AB SCIEX, Framingham, MA) connected to Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA) using LC/MS/MS MRM mode, monitoring the mass transitions (parent to daughter ion) (Table 2). An Agilent Zorbax Eclipse Plus C18 column (4.6 x 75 mm i.d., 3.5 μm; Agilent Technologies, Santa Clara, CA) at a flow rate of 1 mL/min was used for the chromatography separation. The mobile phase consisted of 0.1 % formic acid in acetonitrile (A) and 0.1 % formic acid in water (B). The gradient for the positive ion mode operation was programed as: 0 to 2.5 min, increase B from 5 to 95%; 2.5 to 3.5 min, 95% B; 3.5 to 3.6 min, decrease B to 5%; run-time 5 min. The gradient program for the negative ion mode was: 0 to 3 min, increase B from 5 to 95%; 3 to 4 min, 95% B; 4 to 4.2 min, decrease B to 5%; run-time 6 min. Data acquisition and data processing were performed with the software Analyst 1.6.2 (AB SCIEX, Framingham, MA).

**MDCA assay.** The principle of the assay is to evaluate cytotoxicity of the various drugs in HEK293 in the presence MMHH as an exogenous metabolic system, and in the presence and absence of cofactors for specific metabolic pathways: NADPH/NAD+ for oxidative metabolism, UDPGA for UGT-mediated glucuronidation, PAPS for SULT-mediated sulfation, and GSH for GST-mediated GSH conjugation. For the assay, HEK293 cells were trypsinized from the stock cultures and suspended in UCPM. Cell density was quantified using a hemocytometer and adjusted with UCPM to approximately 520000 cells per mL. A volume of 10 μL (containing approximately 5200 cells) was delivered into each of the wells in the 384-well white plates (treatment plates) and placed in the cell culture incubator for approximately 4 hours to allow cell attachment. The drugs to be evaluated were prepared in culture medium at 3 times of the final desired concentrations (3X treatment media). Acetaminophen and cyclophosphamide were dissolved directly in HIM. Amiodarone, ketoconazole, nefazodone, and troglitazone were firstly dissolved in DMSO as 3000X stock solutions followed by 1:1000 (v/v) dilution in HIM to constitute the 3X treatment media. Treatment was initiated by the addition of 10 μL of MMHH supplemented with the designated cofactors into each of the treatment wells containing HEK293 cells in 10 μL of culture.
medium, followed by 10 μL of the 3X treatment media. HIM was used as the negative control for acetaminophen and cyclophosphamide. HIM with 0.1% DMSO was used as the negative control (solvent control) for amiodarone, ketoconazole, nefazodone and troglitazone. The treatment plates were then returned to the cell culture incubator for a treatment duration of 24 hours followed by viability determination.

**Quantification of cell viability.** Viability of the HEK293 cells after treatment was determined via quantification of cellular ATP contents using a luminescence-based reagent (Perkin Elmer ATPLite Luminescence Assay System, www.perkinelmer.com). Luminescence was quantified using a Victor3V Multiwell plate reader (Perkin Elmer, Waltham, MA, USA). Results are expressed as relative viability using the following equation:

\[
\text{Relative viability (\%)} = \frac{\text{Luminescence (treatment)}}{\text{Luminescence (solvent control)}} \times 100
\]

**Data Analysis.**

1. **Statistical analysis:** Statistical analysis was performed using student’s t-test with the Microsoft Excel 6.0 software, with the probability of p<0.05 to be considered statistically significant.

2. **IC\textsubscript{50} determination:** Graphpad Prism 9.0.2 software was used for the determination of IC\textsubscript{50} values from nonlinear regression analysis of plots of relative viability versus log drug concentrations.

3. **Metabolic dependent cytotoxicity index (MDCI) calculation:** MDCI values were calculated as a ratio of the IC\textsubscript{50} values in the absence and presence of a cofactor for a specific pathway of drug metabolism using the following equation,

\[
\text{MDCI} = \frac{\text{IC}_{50} \text{ (without cofactor)}}{\text{IC}_{50} \text{ (with cofactor)}}
\]
Metabolic activation is indicated by MDCI values > 1 (lower IC\textsubscript{50} values in the presence of cofactor than that without cofactor), and metabolic detoxification is indicated by MDCI values <1 (higher IC\textsubscript{50} values in the presence of cofactor than that without cofactor).

Results

Cofactor-specification of acetaminophen metabolism. Acetaminophen metabolism was evaluated in MMHH supplemented with NADPH/NAD+ in the presence and absence of each of the phase 2 conjugating cofactors. Significantly higher rates of formation of glucuronide, sulfate, and GSH conjugate were observed in the presence of UDPGA, PAPS, and GSH, respectively, than that in the absence the cofactors (Figure 3). The relative rates of metabolite formation expressed as percentages of that in the absence of each of the cofactors were 5721% ± 437% for UDPGA-mediated glucuronidation, 1411% ± 83% for PAPS-mediated sulfation, and 2907% ± 466% for GST-mediated GSH conjugation. The results demonstrate the specification of drug metabolism pathways by cofactor selection.

Cofactor-specification of drug toxicity in MDCA. The cytotoxicity of acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone, and troglitazone in the presence and absence of NADPH/NAD+, and in the presence of NADPH/NAD+ with either UDPGA, PAPS, or GSH is as follows:

\textbf{NADPH/NAD+}: Statistically significant enhancement of cytotoxicity by NADPH/NAD+ was observed for all six drugs (Figure 4) as demonstrated by decreases in IC\textsubscript{50} values (Table 3). The MDCI values ranged from 2.16 (amiodarone) to 5.48 (ketoconazole) (Table 4). The MDCI for nefazodone was determined to be >4.95 due to the lack of cytotoxicity in the absence of NADPH, thereby not allowing an accurate calculation of MDCI value for oxidative metabolism.

\textbf{UDPGA}: Statistically significant attenuation of cytotoxicity by UDPGA was observed for acetaminophen, ketoconazole, nefazodone, and troglitazone at one or more of the drug concentrations evaluated (Figure 5), resulting in increases in IC\textsubscript{50} values (Table 3). No attenuation was observed for amiodarone and
cyclophosphamide. The MDCI values ranged from 0.32 (acetaminophen) to >1 (amiodarone and cyclophosphamide) (Table 4).

**PAPS:** Statistically significant attenuation of cytotoxicity by PAPS was only observed for acetaminophen at the concentrations evaluated (Figure 6) resulting in an increase in IC$_{50}$ value (Table 3). While none of the other treatments yielded statistically significant attenuation by PAPS, increases in IC50 values were also observed for ketoconazole and troglitazone (Table 3). The MDCI values ranged from 0.19 (acetaminophen) to 1.06 (cyclophosphamide) (Table 4).

**GSH:** Statistically significant attenuation of cytotoxicity by GSH was observed at multiple concentrations for all drugs except amiodarone where attenuation was observed at only one concentration (Figure 7), resulting in increases in IC$_{50}$ values (Table 3). The MDCI values ranged from <0.15 (troglitazone) to 0.75 (amiodarone) (Table 4).

**MDCI ranking.** Ranking of the MDCI values of the six drugs for each of the cofactor-dependent metabolic pathways is shown in Table 5, demonstrating the drug-dependent effects of each cofactor on cytotoxicity. Ranking of the MDCI values for each detoxification pathway for each of the drugs is shown in Table 6, demonstrating that GSH was the most effective cofactor for all six drugs in the attenuation of drug toxicity.
Discussion

The major objective of our study was to investigate the utility of MDCA, a novel assay with HEK293 as target cells and MMHH supplemented by specific cofactors as an exogenous metabolic system, as an experimental tool to define key metabolic activating and detoxifying pathways of drug toxicity. The effectiveness of pathway specification by cofactor selection was clearly demonstrated by results with acetaminophen metabolism where each of the cofactors NADPH/NAD+, UDPGA, PAPS, and GSH was found to enable glucuronidation, sulfation, and GSH conjugation, respectively, at levels substantially higher than that observed in the absence of the factors.

The higher cytotoxicity of acetaminophen in the presence of NADPH/NAD+ in MDCA is consistent with the known metabolic activation of acetaminophen to the “ultimate” toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by P450 isoforms CYP1A2 (Laine et al., 2009), CYP2D6 (Dong et al., 2000), CYP2E1 (Laine et al., 2009), and CYP3A4 (Thummel et al., 1993; Laine et al., 2009). UDPGA and PAPS attenuation of acetaminophen cytotoxicity observed in MDCA is consistent with the established protective roles of UGT and SULT conjugation via removal of the parent molecule from metabolic activation (Price et al., 1986; Ziemniak et al., 1987; Kane et al., 1990; Dalhoff and Poulsen, 1993; Court and Greenblatt, 1997; Mutlib et al., 2006; McGill and Jaeschke, 2013). Attenuation of acetaminophen cytotoxicity by GSH is consistent with the known detoxification of NAPQI via GSH conjugation (Mitchell et al., 1973; Hoffmann and Baillie, 1988; Henderson et al., 2000; Zhao et al., 2002). An interesting observation is that GSH supplementation resulted in an IC$_{50}$ value of 13.98 mM which is 2.47X that in the absence of NADPH. A plausible explanation of the observation is that by detoxifying NAPQI via GSH conjugation, P450 metabolism serves as a detoxifying rather than activating pathway by reducing the concentration and the accompanying cytotoxic effects of the parent chemical.
NADPH/NAD+ enhancement of amiodarone cytotoxicity is consistent with previous findings that CYP3A4 induction by rifampin resulted in its higher cytotoxicity in cultured human hepatocytes and that cytotoxicity in HepG2 cells was enhanced via supplementation with exogenous CYP3A4 supersomes (Zahno et al., 2011). GSH attenuation of amiodarone cytotoxicity is consistent with the detection of its reactive metabolites in rat bile (Parmar et al., 2016) and upon incubation with human and rat liver homogenates (Ramesh Varkhede et al., 2014). The lack of effects of UDPGA and PAPS on amiodarone in MDCA suggest that the cytotoxic metabolites of amiodarone are not significantly detoxified by glucuronidation and sulfation. As of this writing, there are no reports on the detoxification of amiodarone by glucuronidation and sulfation.

Our observation of metabolic activation of cyclophosphamide by oxidative metabolism and detoxification by GSH-conjugation is consistent with previous findings of its metabolism by various P450 isoforms to the toxic metabolites 4-hydroxycyclophosphamide, phosphoramide mustard, and acroleins (Rodriguez-Antona and Ingelman-Sundberg, 2006; Doloff et al., 2010; Bachanova et al., 2015; Nishikawa et al., 2015), and detoxification by GSH-conjugation and antioxidants (Dirven et al., 1994; DeLeve, 1996; Terakura et al., 2020). Cyclophosphamide cytotoxicity was not attenuated by UDPGA and PAPS in MDCA, suggesting that glucuronidation and sulfation are not detoxifying pathways.

Ketoconazole is now known to undergo complex metabolic schemes, resulting in myriads of metabolites. CYP1A1 (Korashy et al., 2007), CYP3A4 (Fitch et al., 2009) and flavin-containing monooxygenase (Rodriguez and Acosta, 1997; Rodriguez and Buckholz, 2003) have been reported to be involved in ketoconazole metabolism. Our results with MDCA suggest that ketoconazole was metabolically activated by oxidative metabolism and detoxified by glucuronidation, sulfation, and GSH conjugation. Our results are consistent with a recent report showing that ketoconazole is metabolized to 28 metabolites, including 3 reactive metabolites based on cyanide trapping (Kim et al., 2017b). It is to be
noted that ketoconazole-GSH conjugates have not yet been identified. Further research will be performed in our laboratory to evaluate if ketoconazole-GSH conjugates can be detected in MDCA.

Nefazodone cytotoxicity was enhanced by NADPH/NAD+ and attenuated by UDPGA and GSH, suggesting that it is metabolically activated by oxidative metabolism, and detoxified by glucuronidation and GSH-conjugation. The results are consistent with previous reports that it is metabolized by CYP3A4 to quinone-imine, a reactive metabolite that is detoxified by GSH conjugation (Kalgutkar et al., 2005; Bauman et al., 2008), but is different from that reported by Kostrubsky et al (Kostrubsky et al., 2006) that in cultured human hepatocytes, inhibition of P450 metabolism by 1-aminobenzotriazole increased its cytotoxicity. The difference between our results with MDCA and that reported for human hepatocytes will be further evaluated in our laboratory.

Troglitazone cytotoxicity was enhanced by NADPH and attenuated by UDPGA, PAPS, and GSH, with GSH being the most protective. NADPH/NAD+ enhancement of cytotoxicity and detoxification by GSH provides clear evidence with that troglitazone can be metabolized by oxidative metabolism to cytotoxic reactive metabolites, consistent with previous findings of its metabolic activation to reactive metabolites involving the sulfur moiety of thiazolidinedione nucleus firstly reported by Kassahun et al. (Kassahun et al., 2001) and subsequently confirmed by others (Prabhu et al., 2002; Saha et al., 2010; Okada et al., 2011). That troglitazone metabolism to reactive metabolites and detoxification by GSH conjugation are critical to the manifestation of liver injuries is also suggested by clinical data demonstrating increased hepatotoxicity in glutathione-S-transferase (GST) deficient patients (Watanabe et al., 2003), and in an in vitro study in which liver microsomes generated from GST-deficient donors had increased covalent binding levels compared to that from normal livers (Usui et al., 2011). While inhibition of the bile salt export pump by troglitazone sulfate resulting in accumulation of bile salt to hepatotoxic levels has been postulated to be a mechanism of troglitazone hepatotoxicity (Yang and
Brouwer, 2014), our results with MDCA suggest that cytotoxic reactive metabolites may also be involved in the clinically manifested liver injuries upon troglitazone administration.

A large majority of DILI drugs are known to be metabolized to highly reactive metabolites which, as a result of covalent binding to key biological molecules, may lead to a cascade of events ultimately resulting in severe liver toxicity (Gibaldi, 1992; Boelsterli, 2002; Li, 2002; Cho and Uetrecht, 2017). Evaluation of reactive metabolite formation to minimize the risks of developing drugs with drug induced liver injuries (DILI potential) was firstly applied in Merck Research Laboratories (Evans et al., 2004) and now widely adopted in drug development. Commonly used approaches include Incubation of the chemicals in question with human liver microsomes in the presence of a trapping agent such as glutathione, followed by LC-MS/MS identification of the reactive metabolite-GSH conjugate (Ma and Chowdhury, 2012; Huang et al., 2015; Hosaka et al., 2018; Paludetto et al., 2019) as well as quantification of covalent binding to human liver microsomal proteins (Mitrea et al., 2010; Kakutani et al., 2021). However, there are concerns with the inadvertent removal of drug candidates without toxicological consequences with this approach (Obach et al., 2008; Kalgutkar and Didiuk, 2009). Our results suggest that GSH attenuation of cytotoxicity in MDCA represents an experimental approach to identify reactive metabolites with toxicological consequences, therefore allowing a more accurate identification of structures with toxic liability.

We also introduce in this report a novel parameter, MDCI, calculated as a ratio of the IC$_{50}$ values in the absence to that in the presence of each of the cofactors, as a quantitative measure of the effects of each drug metabolism pathway on toxicity. MDCI values of less than 1 indicates metabolic detoxification (higher IC$_{50}$ values in the presence of the cofactor), and that higher than 1 representing metabolic activation (lower IC$_{50}$ values in the presence of the cofactor). MDCI may be useful as a quantitative parameter for the comparison of multiple drugs in the effects of a specific drug metabolism pathway on
toxicity (Table 5) as well as a comparison of the effects of various pathways on the toxicity of a specific drug (Table 6).

Definition of the role of specific drug metabolizing enzyme pathways in drug toxicity, as described in this report with MDCA, is an important aspect of drug development. The information may aid structural optimization approaches to minimize toxicity (e.g., identification and removal of chemical moieties required for cytotoxic reactive metabolite formation), identification of potential environmental factors that may exacerbate drug toxicity (e.g., CYP3A4 induction resulting in enhanced metabolic activation) and compromising metabolic detoxification (e.g. GSH depletion). In our laboratory, we will further apply MDCA to evaluate additional biological molecules such as antioxidants as well as inflammatory cytokines to further our understanding of the key determinants of drug toxicity. Lastly, our results suggest that MMHH may be applicable as an exogenous hepatic metabolic system for in vitro toxicity assays involving nonhepatic organ target cells to allow a more accurate assessment of in vivo drug toxicity.

**Authorship Contributions.**

Participate in research design: Li, Wei
Conducted experiments: Wei
Contributed new reagents or analytical tools: Li, Wei
Performed data analysis: Wei, Li
Wrote or contribute to the writing of the manuscript: Wei, Li
References


Footnotes

1Funding: No external funding was received for the reported research.

2Conflict of interest: The authors, Hong Wei and Albert P. Li, are employees of In Vitro ADMET Laboratories (IVAL). IVAL is a commercial provider of MMHH.
Table 1. Donor demographics of the human hepatocytes used in the preparation of the permeabilized human hepatocytes (Lot PHHX8012) used in the study. PHHX8012 was prepared by combining hepatocytes from ten individual donors followed by permeabilization and cryopreservation.

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>Ethnicity</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Body Mass Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH1004</td>
<td>Caucasian</td>
<td>Male</td>
<td>30</td>
<td>26.9</td>
</tr>
<tr>
<td>HH1006</td>
<td>Hispanic</td>
<td>Male</td>
<td>65</td>
<td>37.0</td>
</tr>
<tr>
<td>HH1009</td>
<td>Caucasian</td>
<td>Female</td>
<td>47</td>
<td>27.3</td>
</tr>
<tr>
<td>HH1012</td>
<td>Caucasian</td>
<td>Male</td>
<td>22</td>
<td>25.2</td>
</tr>
<tr>
<td>HH1014</td>
<td>Caucasian</td>
<td>Male</td>
<td>15</td>
<td>20.0</td>
</tr>
<tr>
<td>HH1016</td>
<td>Hispanic</td>
<td>Female</td>
<td>64</td>
<td>24.7</td>
</tr>
<tr>
<td>HH1019</td>
<td>Caucasian</td>
<td>Male</td>
<td>17</td>
<td>23.0</td>
</tr>
<tr>
<td>HH1034</td>
<td>Caucasian</td>
<td>Male</td>
<td>49</td>
<td>29.9</td>
</tr>
<tr>
<td>HH1035</td>
<td>Caucasian</td>
<td>Female</td>
<td>46</td>
<td>22.0</td>
</tr>
<tr>
<td>HH1039</td>
<td>Caucasian</td>
<td>Male</td>
<td>67</td>
<td>21.7</td>
</tr>
<tr>
<td>Marker Metabolite Analyzed</td>
<td>Ion Mode Application</td>
<td>Mass Transitions Monitoring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>----------------------</td>
<td>-----------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen Glucuronide</td>
<td>Negative</td>
<td>m/z 326.0 to 150.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen Sulfate</td>
<td>Negative</td>
<td>m/z 229.8 to 150.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen Glutathione</td>
<td>Negative</td>
<td>m/z 455.0 to 271.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. IC₅₀, 95% CI, and R-square values of the various model toxicants in the presence of the various cofactors in the MMHH/HEK293 assay. The values were derived by nonlinear regression analysis of the dose-response curves using Graphpad Prism 9.0.2 software. NA: Not applicable due to the lack of a defined dose-response relationship.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Acetaminophen</th>
<th>Amiodarone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chem</td>
<td>IC₅₀</td>
<td>95% CI</td>
</tr>
<tr>
<td>None</td>
<td>5.65 mM</td>
<td>4.701 to 6.759 mM</td>
</tr>
<tr>
<td>NADPH</td>
<td>1.28 mM</td>
<td>0.8725 to 1.835 mM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>4.01 mM</td>
<td>3.300 to 4.85 mM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>6.60 mM</td>
<td>5.298 to 8.200 mM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>13.98 mM</td>
<td>10.59 to 19.10 mM</td>
</tr>
<tr>
<td>None</td>
<td>107.3 µM</td>
<td>42.94 to 57.34 µM</td>
</tr>
<tr>
<td>NADPH</td>
<td>49.6 µM</td>
<td>34.58 to 61.63 µM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>52.44 µM</td>
<td>61.63 to 67.61 µM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>66.21 µM</td>
<td>51.54 to 66.21 µM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>107.3 µM</td>
<td>107.3 µM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Cyclophosphamide</th>
<th>Ketoconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chem</td>
<td>IC₅₀</td>
<td>95% CI</td>
</tr>
<tr>
<td>None</td>
<td>24.00 mM</td>
<td>5.001 to 8.191 mM</td>
</tr>
<tr>
<td>NADPH</td>
<td>6.39 mM</td>
<td>5.066 to 7.773 mM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>6.26 mM</td>
<td>4.831 to 7.578 mM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>6.04 mM</td>
<td>11.35 to 23.06 mM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>16.13 mM</td>
<td>15.81 to 22.48 µM</td>
</tr>
<tr>
<td>None</td>
<td>103.20 µM</td>
<td>15.31 to 22.48 µM</td>
</tr>
<tr>
<td>NADPH</td>
<td>18.83 µM</td>
<td>21.29 to 30.30 µM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>28.45 µM</td>
<td>21.29 to 30.30 µM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>25.32 µM</td>
<td>21.29 to 30.30 µM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>44.36 µM</td>
<td>36.37 to 55.35 µM</td>
</tr>
<tr>
<td>None</td>
<td>191.00 µM</td>
<td>45.77 to 73.75 µM</td>
</tr>
<tr>
<td>NADPH</td>
<td>28.45 µM</td>
<td>80.81 to 201.5 µM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>52.44 µM</td>
<td>70.57 to 113.8 µM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>66.21 µM</td>
<td>70.57 to 113.8 µM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>107.3 µM</td>
<td>70.57 to 113.8 µM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Nefazodone</th>
<th>Troglitazone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chem</td>
<td>IC₅₀</td>
<td>95% CI</td>
</tr>
<tr>
<td>None</td>
<td>&gt;100 µM</td>
<td>16.14 to 25.52 µM</td>
</tr>
<tr>
<td>NADPH</td>
<td>20.21 µM</td>
<td>21.09 to 47.22 µM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>30.28 µM</td>
<td>15.45 to 24.29 µM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>19.33 µM</td>
<td>40.62 to 75.42 µM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>53.47 µM</td>
<td>151.1 to 237.1 µM</td>
</tr>
<tr>
<td>None</td>
<td>191.00 µM</td>
<td>45.77 to 73.75 µM</td>
</tr>
<tr>
<td>NADPH</td>
<td>58.24 µM</td>
<td>80.81 to 201.5 µM</td>
</tr>
<tr>
<td>NADPH/UDPGA</td>
<td>121.40 µM</td>
<td>70.57 to 113.8 µM</td>
</tr>
<tr>
<td>NADPH/PAPS</td>
<td>89.28 µM</td>
<td>70.57 to 113.8 µM</td>
</tr>
<tr>
<td>NADPH/GSH</td>
<td>&gt;400 µM</td>
<td>&gt;400 µM</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.00511</td>
<td>0.9033</td>
</tr>
</tbody>
</table>
Table 4. Metabolic dependent cytotoxicity index (MDCI) values for NADPH/NAD+-dependent oxidative metabolism (oxidative metabolism), UGT-dependent glucuronidation (UGT), PAPS-dependent sulfation (SULT) and GST-dependent GSH conjugation (GST) for acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone, and troglitazone. MDCI is calculated as the ratio of IC_{50} values in the absence and presence of the cofactors (in the absence and presence of NADPH for oxidative metabolism; with NADPH and absence and presence of each of the phase 2 conjugating cofactors for UGT, SULT and GST). MDCI values greater than 1 indicates metabolic activation, less than 1 indicates detoxification. (The IC50 value of nefazodone in the absence of NADPH was >100 uM, thereby yielding MDCE of oxidative metabolism (cytotoxicity in the presence of NADPH) of >4.95. The IC50 of troglitazone in the presence of GSH was found to be >400 uM, thereby yielding MDCI of <0.15).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Metabolism Dependent Cytotoxicity Index (MDCI)</th>
<th>Oxidative Metabolism</th>
<th>UGT</th>
<th>SULT</th>
<th>GST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td></td>
<td>4.41</td>
<td>0.32</td>
<td>0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>Amiodarone</td>
<td></td>
<td>2.16</td>
<td>1.08</td>
<td>0.95</td>
<td>0.75</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td></td>
<td>3.76</td>
<td>1.02</td>
<td>1.06</td>
<td>0.40</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td></td>
<td>5.48</td>
<td>0.66</td>
<td>0.74</td>
<td>0.42</td>
</tr>
<tr>
<td>Nefazodone</td>
<td></td>
<td>&gt;4.95</td>
<td>0.67</td>
<td>1.05</td>
<td>0.38</td>
</tr>
<tr>
<td>Troglitazone</td>
<td></td>
<td>3.28</td>
<td>0.48</td>
<td>0.65</td>
<td>&lt;0.15</td>
</tr>
</tbody>
</table>
Table 5. Rank order of the MCDI values of acetaminophen (APAP), amiodarone (AMD), cyclophosphamide (CPA), ketoconazole (KTZ), nefazodone (NFZ) and troglitazone (TGZ) for the various metabolic activation and detoxification pathways.

<table>
<thead>
<tr>
<th>Metabolic Activation/Detoxification Pathways</th>
<th>Rank Order</th>
<th>MCDI Ranking Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic activation by oxidative metabolism</td>
<td>NFZ &gt; KTZ &gt; APAP &gt; CPA &gt; TGZ &gt; AMD</td>
<td>Highest to lowest MCDI, representing highest to lowest levels of metabolic activation</td>
</tr>
<tr>
<td>UGT detoxification</td>
<td>APAP &gt; TGZ &gt; KTZ = NFZ &gt; CPA, &gt; AMD</td>
<td>Lowest to highest MCDI, representing highest to lowest levels of detoxification</td>
</tr>
<tr>
<td>SULT detoxification</td>
<td>APAP &gt; TGZ &gt; KTZ (No apparent SULT detoxification was observed for AMD, NFZ, CPA, NFZ)</td>
<td>Lowest to highest MCDI, representing highest to lowest levels of detoxification</td>
</tr>
<tr>
<td>GST detoxification</td>
<td>TGZ &gt; APAP &gt; CPA = KTZ = NFZ &gt; AMD</td>
<td>Lowest to highest MCDI, representing highest to lowest levels of detoxification</td>
</tr>
</tbody>
</table>
Table 6. Rank order of the MCDI values of the phase 2 detoxification pathways (UGT, SULT and GST) for acetaminophen (APAP), amiodarone (AMD), cyclophosphamide (CPA), ketoconazole (KTZ), nefazodone (NFZ) and troglitazone (TGZ).

<table>
<thead>
<tr>
<th>DILI Drugs</th>
<th>Rank Order</th>
<th>MDCI Ranking Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>GST&gt;SULT&gt;UGT</td>
<td>Lowest to highest MDCI, representing highest to lowest levels of detoxification</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>GST&gt;SULT&gt;UGT</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>GST&gt;UGT&gt;SULT</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>GST&gt;UGT&gt;SULT</td>
<td></td>
</tr>
<tr>
<td>Nefazodone</td>
<td>GST&gt;UGT&gt;SULT</td>
<td></td>
</tr>
<tr>
<td>Troglitazone</td>
<td>GST&gt;UGT&gt;SULT</td>
<td></td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1: A schematic representation of the metabolism-dependent cytotoxicity assay (MDCA). MDCA employs permeabilized human hepatocytes (MetMax Human Hepatocytes, MMHH) as an exogenous metabolic system, and HEK293 cells as the target of cytotoxicity. Upon drug treatment, the final cytotoxicity will be a result of metabolic clearance of the parent drug, metabolic activation of the parent drug to cytotoxic metabolites, and the detoxification of the parent drug and its cytotoxic metabolites.

Figure 2. Chemical structures of the six DILI drugs evaluated in MDCA. Acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone and troglitazone are drugs known to be associated with severe clinically significant liver toxicity resulting in deaths or a need for liver transplantation.

Figure 3. Rates of formation of acetaminophen metabolites in the presence of NADPH/NAD+ and each of the phase 2 cofactors: UDPGA (top left); PAPS (top right) and GSH (bottom). Data represent mean ± S.D. (n = 3). (*: statistically significant (p<0.05) difference in metabolite formation rates between incubation with NADPH/NAD+ only and that with UDPGA, PAPS, and GSH)

Figure 4. Drug cytotoxicity in the presence (squares) and absence (circles) of the cofactor mixture for oxidative metabolism, NADPH/NAD+. Data represent mean ± S.D. (n = 3). (*: statistically significant (p<0.05) difference in cytotoxicity between treatment with and without NADPH/NAD+)

Figure 5. Drug toxicity in the presence of NADPH/NAD+ with (squares) and without (circles) UDPGA, the cofactor for UGT-mediated glucuronidation. Data represent mean ± S.D. (n = 3). (*: statistically significant (p<0.05) difference in cytotoxicity between treatment with and without UDPGA).

Figure 6. Drug toxicity in the presence of NADPH/NAD+ with (squares) and without (circles) PAPS, the cofactor for SULT-mediated sulfation. Data represent mean ± S.D. (n = 3). (*: statistically significant (p<0.05) difference in cytotoxicity between treatment with and without PAPS).
Figure 7. Drug toxicity in the presence of NADPH/NAD+ with (squares) and without (circles) GSH, the cofactor for GSH-conjugation. Data represent mean ± S.D. (n = 3). (*: statistically significant (p<0.05) difference in cytotoxicity between treatment with and without GSH).
Figure 1.

Cytotoxic Interactions

Parent Drug

Target Cell (HEK293)

Viability Evaluation

Drug Metabolizing Enzymes/cofactors

Metabolic Activation

Toxic Metabolites

Metabolic Detoxification

Nontoxic Metabolites

MMHH (Exogenous Metabolic System)
Figure 2

Acetaminophen

Amiodarone

Cyclophosphamide

Ketoconazole

Nefazodone

Troglitazone
Figure 6

Acetaminophen

Amiodarone

Cyclophosphamide

Ketoconazole

Nefazodone

Troglitazone
Figure 7

Acetaminophen

Amiodarone

Cyclophosphamide

Ketoconazole

Nefazodone

Troglitazone

This article has not been copyedited and formatted. The final version may differ from this version.

DMD Fast Forward. Published on November 8, 2021 as DOI: 10.1124/dmd.121.000645

Downloaded from dmd.aspetjournals.org at ASJF Journals on November 14, 2021