Cytotoxic Reactive Metabolite Assay with Permeabilized Cryopreserved Human Hepatocytes for the Identification of Drug Candidates With Potential to Cause Idiosyncratic Drug-induced Liver Injuries

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INTRODUCTION

- Drug induced liver injuries (DILI) resulting in deaths or a need for liver transplantation of marketed drugs is a major challenge in drug development.
- A large majority of DILI drugs are known to be metabolized to highly reactive metabolites which, because of covalent binding to key biological molecules, may lead to a cascade of events ultimately resulting in severe liver toxicity.
- Current experimental approaches in drug development for the identification drug candidates with potential to form reactive metabolite involve the incubation of the drug candidates 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 conjugates as well as quantification of covalent binding to human liver microsomal proteins.
- While it is generally believed that early identification of chemical structures prone to reactive metabolite formation for the selection of chemical structures and direction of structural modification may minimize toxic liability in drug discovery, there are concerns with the possibility of inadvertent removal of drug candidates without toxicological consequences with this approach.
- We present here a novel experimental assay, the Metabolism-Dependent Cytotoxicity Assay (MDCA) to identify reactive metabolites with toxicological consequences, therefore allowing a more accurate identification of structures with toxic liability.

MATERIALS & METHODS

MDCA assay. The principle of the assay is to evaluate cytotoxicity of the various drugs in HEK293 in the presence and absence of MMHH as an exogenous metabolic system, and in the presence and absence of cofactors. 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 500000 cells per mL. A volume of 10 uL (containing approximately 5000 cellls) was delivered into each of the wells in the 384-well white plates (treatment plates) employed for the assay. After a 4-hour culturing duration to allow cell attachment, 10 uL of MMHH supplemented with the designated cofactors were added into each of the treatment wells, followed by 10 uL of the drugs to be evaluated (at 3X of the desired final concentration). The treatment plates were returned to the cell culture incubator for a treatment duration of 24 hours followed by viability determination via quantification of cellular ATP contents.

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

Relative viability (%) = (Luminescence (treatment))/Luminescence (solvent control)) x 100

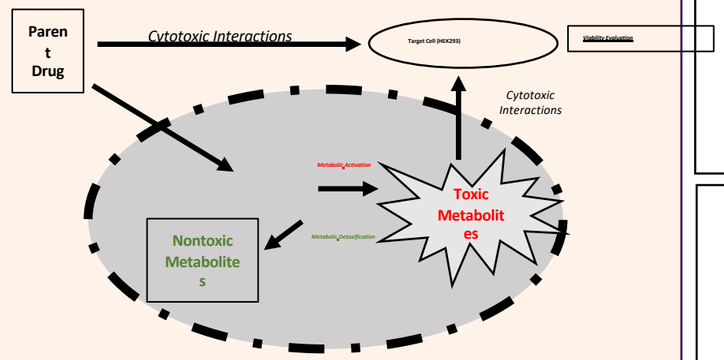
Data Analysis.

1. Statistical analysis: Statistical analysis was performed using student's t-test with the Microsoft Excel 6. software, with the probability of $p<0.05$ to be considered statistically significant.
2. IC_{50} determination: Graphpad Prism 9.0.2 software was used for the determination of IC_{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_{50} values in the absence and presence of a cofactor for a specific pathway of drug metabolism, with values >1 representing activation, and <1 representing detoxification. MDCI was calculated using the following equation:

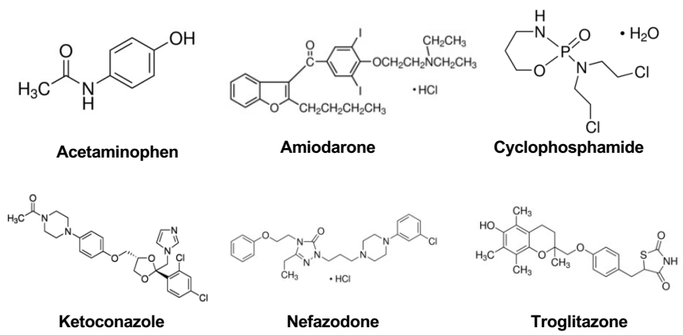
MDCI = IC_{50} (without cofactor)/IC_{50} (with cofactor).

Metabolism-Dependent Cytotoxicity Assay (MDCA)

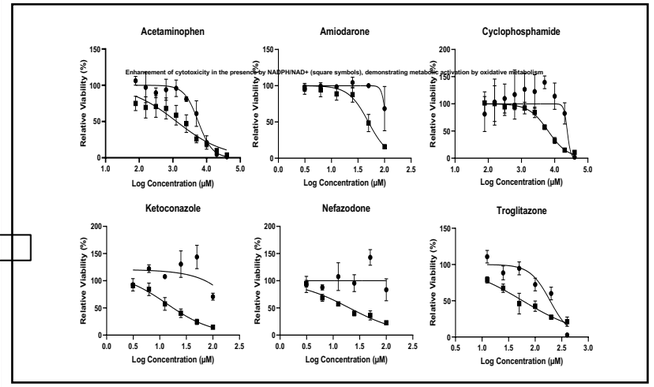
In this assay, drug toxicity towards the metabolically incompetent HEK293 cells is evaluated in the presence of permeabilized cryopreserved human hepatocytes (MetMax™ Human Hepatocytes (MMHH)) as an exogenous metabolic activating system in the presence and absence of cofactors required for oxidative metabolism and cofactors for detoxification



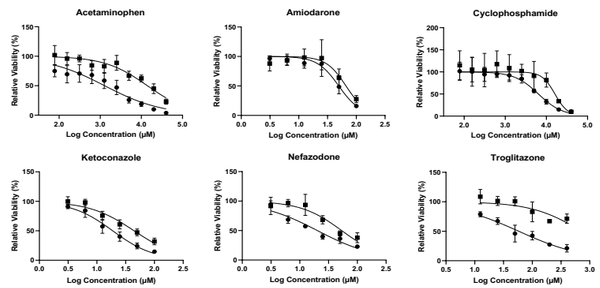
Model DILI Drugs



RESULTS: Cytotoxicity in the Presence (square symbols) and Absence (circle symbols) of Cofactors



Attenuation of cytotoxicity by GSH (square symbols), demonstrating cytotoxic reactive metabolite formation – a hall mark of DILI drugs



Metabolic dependent cytotoxicity index (MDCI) values for oxidative metabolism, UGT, SULT and GST based on the IC_{50} values of the drugs in the presence and absence of NADPH/NAD+ (oxidative metabolism), UDPGA (UGT-dependent glucuronidation), PAPS (SULT-dependent sulfate conjugation) and GSH (GST-dependent GSH conjugation). MDCI is calculated as the ratio of IC_{50} values in the absence and presence of the cofactors. MDCI values greater than 1 indicates metabolic activation, less than 1 indicates detoxification.

Drug	Metabolism Dependent Cytotoxicity Index (MDCI)			
	Oxidative Metabolism	UGT	SULT	GST
Acetaminophen	4.41	0.32	0.19	0.09
Amiodarone	2.16	1.08	0.95	0.75
Cyclophosphamide	3.76	1.02	1.06	0.40
Ketoconazole	5.48	0.66	0.74	0.42
Nefazodone	>9.90	0.67	1.05	0.38
Troglitazone	3.28	0.48	0.65	<0.15

SUMMARY AND CONCLUSIONS

- 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 HEK-293 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 various cofactors for drug metabolism: nicotinamide adenine dinucleotide phosphate (NADPH) for phase 1 oxidation, uridine 5′-diphosphoglucuronic acid (UDPGA) for uridine 5'-diphospho-glucuronosyltransferase (UGT) mediated glucuronidation, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) for sulfotransferase (SULT) mediated sulfation, and L-glutathione (GSH) for glutathione S-transferase (GST) mediated GSH conjugation.
- Six drugs with clinically significant hepatoxicity, 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, suggesting metabolic activation via phase 1 oxidation. Attenuation of cytotoxicity by UDPGA was observed for acetaminophen and troglitazone, by PAPS for acetaminophen 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.***

REFERENCES

- Li AP, Ho MD, Amaral K, and 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:**1608-1616.
- Wei H and Li AP (2022) Permeabilized Cryopreserved Human Hepatocytes as an Exogenous Metabolic System in a Novel Metabolism-Dependent Cytotoxicity Assay 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. *Drug Metab Dispos* **50:**140-149.

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