MetMax Human Hepatocyte/HEK293 Cytotoxic Reactive Metabolite Assay as a Potential In Vitro Experimental System for the Identification of DILI Drugs

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Introduction

- **Hepatic xenobiotic metabolism may lead to higher toxicity via the formation of metabolites with cytotoxicity significantly higher than that of the parent chemical (metabolic activation), or conversion of a cytotoxic parent chemical to noncytotoxic metabolites (metabolic detoxification).**
- ***We report here on a novel in vitro cytotoxicity assay, the MMHH/HEK293 assay for the evaluation of metabolism-dependent drug toxicity:***
  - ***MetMax™ Human Hepatocytes (MMHH) is used as an exogenous metabolic system***
  - ***HEK293, a human embryonic kidney cell line devoid of drug metabolizing enzyme activities, is used as the target cells for drug toxicity***
- ***The following key observations were made:***
  - ***Drug metabolizing enzyme (DME) pathways could be specified via the selection of cofactors***
  - ***GSH-mediated detoxification can be used as an endpoint for the identification of drugs that are metabolized to cytotoxic reactive metabolites, a hall mark of drugs with idiosyncratic hepatotoxicity***

Experimental Procedures

MetMax™ Human Hepatocytes (MMHH): MMHH were prepared from a pool of 10 (5 male and 5 female) human donors using proprietary procedures (patent pending). The use of a multiple donor pool eliminates results unique to an individual, allowing results to be representative of the human population.

Evaluation of metabolic capacity: MMHH were incubated with various drug metabolizing enzyme-selective substrates for 30 minutes followed by LC/MS-MS quantification of metabolites.

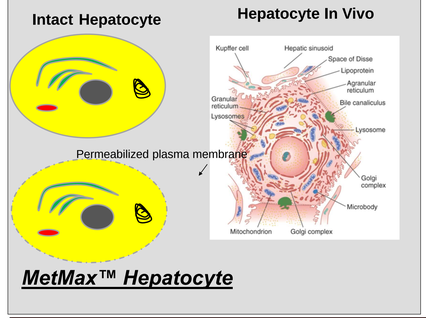
HEK 293 cytotoxicity assay: HEK-293 cells, a commonly used cell line for in vitro cytotoxicity assays, were supplied to us by US EPA. The cells were maintained in T-75 flasks as log growing cells in DMEM containing 10% fetal bovine serum. On the day of experimentation, the cells were trypsinized and resuspended in the maintenance medium. Cell concentration was quantified via hemocytometer counting. Cell concentration was adjusted to 500,000 cells per mL. The cell suspension was plated into 384-well white cell culture plates with transparent bottom at a cell density of 5000 cells/well (10 uL of 500,000 cells/ml) and incubated for 4 hrs in a cell culture incubator with a humidified atmosphere of 5% carbon dioxide and 95% air followed by treatment. An addition-only high throughput procedure was used for the cytotoxicity assay. There were three treatment conditions: 1. HEK 293 cells without metabolic activation; 2. HEK 293 cells with metabolic activation; and 3. HEK 293 cells with inactivated metabolic activation. To group 1, 10 uL of hepatocyte medium but without MetMax™ human hepatocytes was added. To group 2, 10 uL of MetMax™ human hepatocytes was added. To group 3, 10 uL of inactive MetMax™ human hepatocytes (inactivated by heating for 15 minutes in boiling water) was added. Treatment was initiated by an addition of 10 uL of test articles at 3X of the final desired concentration. After the final additional of test articles, the plates were returned to the cell culture incubator where they were incubated for treatment duration of 24 hrs. Cell viability was determined at the end of the treatment duration using cellular ATP as endpoint quantified by luminescence using the PerkinElmer ATPLite kit. as per manufacturer's instructions, and the PerkinElmer Victor3 Multimode Plate Reader.

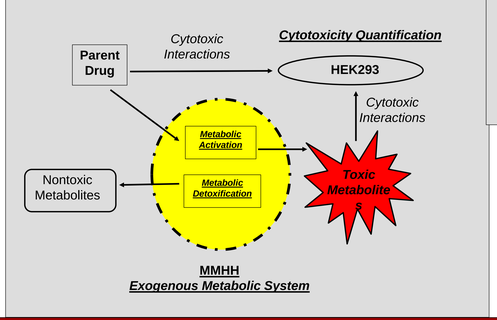
Results are presented as *Relative Viability* calculated using the following equation:

Relative Viability (%) =[Luminescence (treatment)/Luminescence (solvent control)] x 100

MetMax™ Human Hepatocytes/HEK293 Cyotoxicity Assay

Permeabilized Hepatocytes Supplemented with Metabolic Enzyme Cofactors





Demonstration of cofactor-dependent specification of phase 2 conjugative drug metabolizing enzyme (DME) activities (pmol/min/million hepatocytes) in MetMax™ Hepatocytes (MMHH)

MMHH Classification		MMHH-Oxidation		MMHH-Sulfation		MMHH-Glucuronidation		MMHH-GSH Conjugation	
Cofactors		*NADPH*		*NADPH/PAPS*		*NADPH/UDPGA*		*NADPH/GSH*	
Phase 2 DME	Substrate/ Metabolite	mean	sd	mean	sd	mean	sd	mean	sd
UGT	7-Hydroxycoumarin/ 7HC-Glucuronide	0.15	0.02	0.42	0.02	60.11	3.40	0.18	0.02
SULT	7-HC/ 7HC-Sulfate	0.02	0.00	13.93	0.24	0.01	0.00	0.14	0.17
GST	Acetaminophen/ APAP-GSH	0.55	0.03	0.66	0.30	0.43	0.03	14.77	2.54
UGT	APAP/ APAP-Glucuronide	0.21	0.02	0.23	0.01	11.87	0.91	0.09	0.02
SULT	APAP/ APAP-Sulfate	0.98	0.02	13.82	0.82	1.01	0.06	0.82	0.23

- **MetMax™ Human Hepatocytes Metabolic Activation Assay**
  - **Target cells:** HEK293, devoid of xenobiotic drug metabolism activities
  - **Exogenous metabolic activation system**: MetMax™ human hepatocytes
- MMHH cofactors (DMEs): NADPH (phase 1 oxidation)., UDPGA (UGT), PAPS (SULT), GSH (GST

Model Hepatotoxic Drugs

Acetaminophen, Amiodarone, Cyclophosphamide, Ketoconazole, Nefazodone, Troglitazone

Results

Fig. 1. NADPH enhanced cytotoxicity of all 6 drugs, suggesting formation of toxic metabolites from the less toxic parent molecules via oxidative metabolism

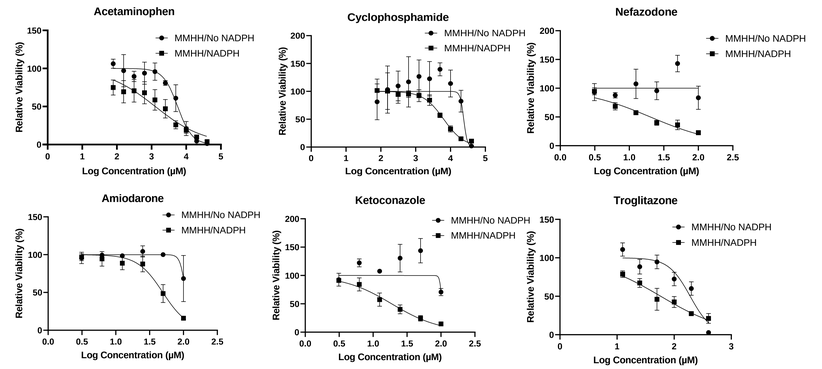
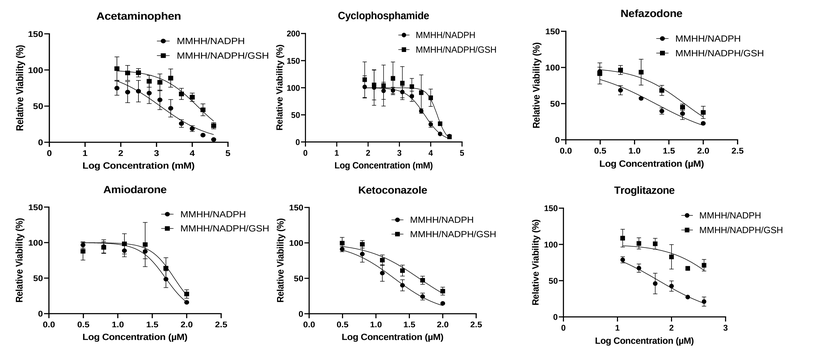


Fig. 2. GSH attenuated cytotoxicity of all 6 drugs, suggesting conjugation of reactive toxic metabolites formed by oxidative metabolism



Summary and Conclusion

- Six hepatotoxic drugs – acetaminophen, amiodarone, cyclophosphamide, ketoconazole, nefazodone, and troglitazone were evaluated for their cytotoxicity in the MMHH/HEK293 in vitro cytotoxicity assay in the presence of specific cofactors
- The cytotoxicity of all six hepatotoxicants was enhanced by NADPH, thereby suggesting formation of toxic metabolites by phase 1 oxidation. This observation is consistent with known metabolic activation of these six drugs.
- Inclusion of GSH in additional to NADPH attenuated cytotoxicity of all drugs except amiodarone. The results suggesting detoxification of acetaminophen, cyclophosphamide, ketoconazole, nefazodone, and troglitazone via GSH conjugation, a property of reactive metabolites. GSH attenuation was most prominent for troglitazone, a known idiosyncratic hepatotoxicant.
- *Results suggest that MetMax™ Human Hepatocytes represent an effective exogenous metabolic activation system for the provision of human-specific drug metabolizing enzyme activities for the evaluation of chemical toxicity in cell based in vitro cytotoxicity assays as represented here by the MMHH/HEK293 assay.*
- *As GSH is known to conjugate reactive metabolites, GSH attenuation of cytotoxicity may be a useful endpoint for the identification of hepatotoxicants that are metabolized to Cytotoxic Reactive Metabolites, a common property of drugs with idiosyncratic hepatotoxicity*