MULTIPLEXED METHOD TO SCREEN FOR CHEMICAL-INDUCED HEPATOTOXICITY USING A NOVEL MICROPATTERNED HUMAN HEPATOCYTE CO-CULTURE PLATFORM

Edward LeCluyse[1], Kristina K. Wolf[1], O. Joseph Trask[1], Kelly Rose[1], Okechukwu Ukairo[2], Amanda Moore[2], Jeannemarie Gaffney[2], and Melvin E. Andersen[1]

[1]The Hamner Institutes for Health Sciences, Research Triangle Park, NC; [2]Hepregen Corporation, Medford, MA

INTRODUCTION

- Current toxicity testing paradigms have shifted from focusing initially on traditional *in vivo* models to more high-throughput *in vitro* systems.
- Profiling strategies utilized by Tox21, ToxCast, and the EU Joint Research Centre (JRC) focus on identifying modes-of-action (MOAs) – discrete toxicological endpoints such as specific cellular targets or pathway perturbations – with the goal of prioritizing compounds for more in-depth testing.[1,2,3]
- A recent analysis of results from ToxCast's Phase I studies indicated that high-throughput screening (HTS) assays have limited ability to predict *in vivo* toxicity.[4]
- HTS primarily utilizes cell lines in monoculture, which may not model heterotypic cell-cell interactions or maintain requisite *in vivo* biochemical functionality.
- Primary rat and human hepatocytes in micropatterned co-cultures (MPCC; HepatoPac™) surrounded by mouse embryonic fibroblasts (3T3-J2) maintain morphologic characteristics and more physiologically relevant biochemical functions long-term.[5,6,7]
- We have previously shown human MPCC to be amenable to high content screening (HCS) imaging applications.[8]

The objective of this study was to determine the fidelity of multiplexed HCS endpoint assays in human MPCC for MOA-based screening.

METHODS

Culture Models

- Cryopreserved primary human hepatocytes from a single donor (QHu0030; ***Table 1***) were provided by QPS Hepatic Biosciences (Research Triangle Park, NC). Cryopreserved fractions enriched for primary human Kupffer cells (KCs), not donor matched (HU8150; ***Table 1***), were a generous gift from Life Technologies (Carlsbad, CA).
- MPCC were created by first seeding human hepatocytes in serum-free HepatoPac culture medium (HPCM) on collagen "islands" in Grenier Bio-One 96-well black-wall, clear bottom plates (Monroe, NC); 3T3-J2 cells were added in serum (10% v/v)-supplemented HPCM 12-18 h later. Cultures stabilized for ~1 week prior to shipment. Upon receipt, MPCC acclimated for ~3-4 d before treatment.
- Human KCs were seeded in HepatoMune culture medium (HMCM) at a ratio of 1 KC:2.5 hepatocytes 1 d prior to treatment.

Table 1. *Characteristics of hepatocyte (QHu0030) and Kupffer cell (HU8150) donors.*

Donor	Characteristics								
	Gender	Race	Age	BMI	Tobacco History	Alcohol History	Drug History	Medication	Cause of Death
QHu0030	Male	Caucasian	30	20.9	Yes	2/day	No	None	Stroke
HU8150	Female	Caucasian	21	25	1-1½ ppd	Socially	Marijuana	Tylenol	Head Trauma

Treatment

- A subset of compounds with varying MOAs were selected from the JRC and ToxCast hepatotoxicant lists, in addition to the prototypic compounds valinomycin (apoptosis) and menadione (oxidative stress) as positive controls.
- Cultures were exposed to test compounds in 5% (v/v) serum-supplemented HPCM or HMCM for 4, 24, or 72 h without subsequent medium changes. Eight 10-fold dilutions, from 10 pM to 100 µM, were utilized.

High-Content Imaging (HCI) and Toxicity Analysis

- At the end of each treatment period, medium was collected; urea was measured.
- HCI probes for measuring oxidative stress (CellROX® Green) and membrane permeability (TOTO-3®) were added to the cultures for 30 min. Cells were then fixed with 4% (v/v) paraformaldehyde. Following cell permeabilization with Triton X-100, HCI probes for measuring apoptosis and identifying hepatocytes (cytochrome c) and nuclei (Hoechst 33342) were introduced.
- HCI images, up to 25 fields, were captured within 48 h on a Cellomics ArrayScan® VTI reader (Thermo Scientific, Pittsburgh, PA) equipped with a Zeiss 10x/0.45 NA Plan-ApoChromat objective lens.
- Image analysis was performed using the Compartmental Analysis V4 bioapplication algorithm (Thermo Scientific; ***Figure 1)*** with defined thresholds to determine percent response.

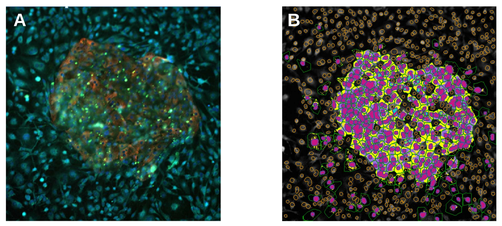

Figure 1. *HCI image analysis.* ***(A)*** *Composite image from 72 h DMSO-treated well. Image labeled with Hoechst 33342 (blue), CellROX® green (green), cytochrome c (red), and TOTO-3® (aqua).* ***(B)*** *Algorithm mask overlay.*

RESULTS

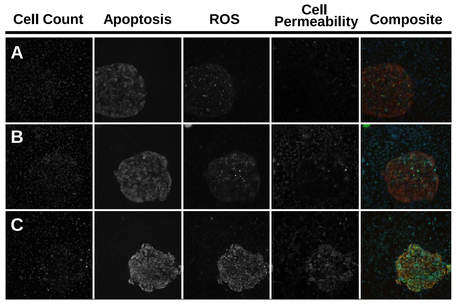

Figure 2. *Representative single channel and composite images of MPCC treated with vehicle control (0.1% [v/v] DMSO;* ***A****), caffeine (100 µM,* ***B****), or rotenone (10 µM,* ***C****) for 72 h without subsequent medium changes. Images labeled with Hoechst 33342 (to detect nuclei, blue), cytochrome c (to detect apoptosis and identify hepatocytes, red), CellROX (to detect ROS, green), and TOTO-3 (to detect cell permeability, aqua).*

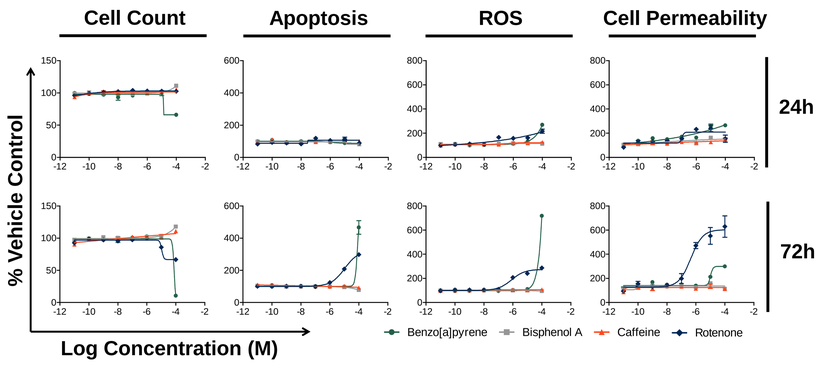

Figure 3. *Cell count, apoptosis, ROS generation, and cell permeability in MPCC 24 and 72 h after treatment with hepatotoxicants. Symbols and error bars denote means and ranges, respectively, of 2 replicate wells.*

Table 2. *Cell count, cytochrome c response, ROS response, and cell permeability following hepatotoxicant treatment.*

Compound	HCI Feature							
	Cell Count (Valid Object Count)		Cytochrome c Response (MEAN_RingSpotTotalIntenCh2)		ROS Response (MEAN_CircSpotAvgIntenCh3)		Cell Permeability (MEAN_CircAvgIntenCh4)	
	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Acetaminophen	NC	NC	NC	NC	0.001 µM	NC	0.001 µM	0.0001 µM
Amiodarone	NC	100 µM	NC	NC	10 µM	10 µM	0.0001 µM	0.00001 µM
Benzo[a]pyrene	100 µM	100 µM	NC	100 µM	10 µM	100 µM	0.0001 µM	0.0001 µM
Bisphenol A	NC	NC	NC	NC	NC	NC	0.001 µM	0.0001 µM
Caffeine	NC	NC	NC	NC	100 µM	NC	0.001 µM	0.0001 µM
Cypermethrin	NC	NC	NC	0.1 µM	NC	NC	0.00001 µM	0.0001 µM
Dibutyl Phthalate	NC	NC	NC	NC	NC	NC	0.00001 µM	0.00001 µM
Dichlorvos	NC	NC	NC	NC	NC	NC	0.0001 µM	0.0001 µM
Haloperidol	NC	NC	NC	NC	100 µM	NC	0.001 µM	0.0001 µM
Hexaconazol	NC	NC	NC	NC	100 µM	NC	0.001 µM	0.001 µM
2-Naphthylamine	NC	NC	NC	NC	NC	NC	0.0001 µM	0.00001 µM
Nilutamide	NC	NC	NC	NC	NC	NC	0.0001 µM	0.00001 µM
PFOA	NC	NC	NC	NC	NC	NC	0.00001 µM	0.0001 µM
Permethrin	NC	NC	NC	NC	NC	NC	0.0001 µM	0.0001 µM
Rotenone	NC	100 µM	NC	1 µM	0.1 µM	1 µM	0.0001 µM	0.0001 µM
Tamoxifen	NC	100 µM	NC	0.1 µM	NC	100 µM	0.001 µM	0.01 µM
Triclosan	NC	NC	0.1 µM	NC	NC	NC	0.0001 µM	0.01 µM
Troglitazone	NC	NC	1 µM	NC	NC	NC	0.0001 µM	0.0001 µM
Valproic Acid	NC	NC	NC	NC	NC	NC	0.0001 µM	0.0001 µM
Menadione	NC	100 µM	NC	100 µM	NC	100 µM	0.0001 µM	0.0001 µM

*Compound concentrations indicate a ≥20% decrease (cell count) or increase (neutral lipid and phospholipid accumulation) relative to vehicle control (DMSO).
NC, not changed.

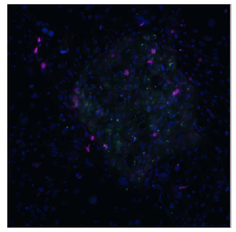

Figure 4. *Representative composite image of MPCC with Kupffer cells (KCs) following treatment with trovafloxacin and lipopolysaccharide (LPS) for 72 h without subsequent medium changes. Image labeled with Hoechst 33342 (to detect nuclei, blue), CellROX (to detect ROS, green), and CD68 (to detect KCs, pink).*

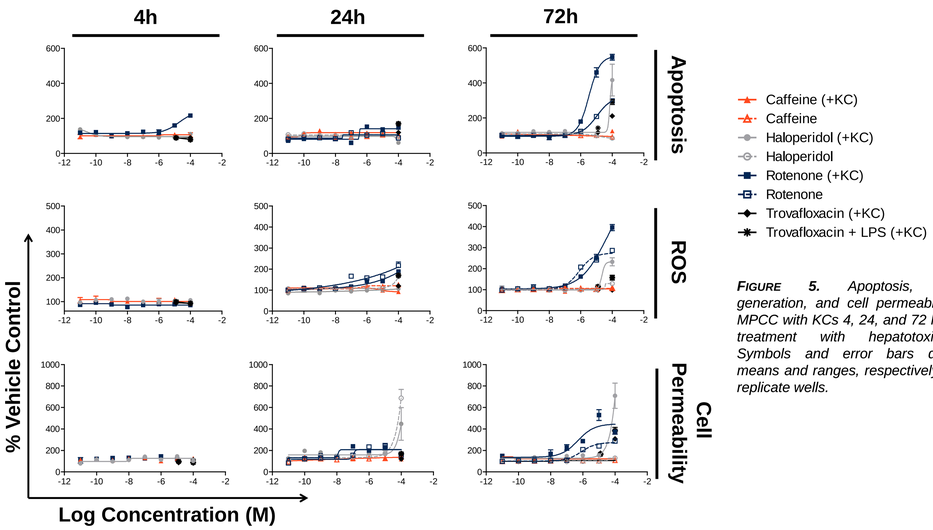

Figure 5. *Apoptosis, ROS generation, and cell permeability in MPCC with KCs 4, 24, and 72 h after treatment with hepatotoxicants. Symbols and error bars denote means and ranges, respectively, of 2 replicate wells.*

Table 3. *Cell count, cytochrome c response, ROS response, and cell permeability following hepatotoxicant treatment in the presence of Kupffer cells.*

Compound	HCI Feature											
	Cell Count (Valid Object Count)			Cytochrome c Response (MEAN_RingSpotTotalIntenCh2)			ROS Response (MEAN_CircSpotAvgIntenCh3)			Cell Permeability (MEAN_CircAvgIntenCh4)		
	4h	24 h	72 h	4h	24 h	72 h	4h	24 h	72 h	4h	24 h	72 h
Acetaminophen	NC	NC	NC	NC	NC	NC	NC	0.0001 µM	NC	0.00001 µM	0.0001 µM	NC
+LPS	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	0.00001 µM	NC
Bisphenol A	NC	NC	NC	NC	NC	0.1 µM	NC	NC	NC	0.00001 µM	0.00001 µM	0.0001 µM
Caffeine	NC	NC	NC	NC	NC	0.0001 µM	NC	NC	NC	0.0001 µM	0.00001 µM	0.00001 µM
+LPS	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	0.00001 µM	0.00001 µM
Dibutyl Phthalate	NC	NC	NC	0.00001 µM	NC	0.1 µM	0.1 µM	NC	NC	0.001 µM	0.00001 µM	0.00001 µM
Dichlorvos	NC	NC	NC	0.0001 µM	NC	0.01 µM	10 µM	NC	NC	0.001 µM	0.00001 µM	0.00001 µM
Haloperidol	NC	100 µM	100 µM	0.00001 µM	NC	0.001 µM	NC	NC	100 µM	0.1 µM	0.00001 µM	0.001 µM
2-Naphthylamine	NC	NC	NC	0.00001 µM	NC	0.01 µM	NC	NC	NC	0.001 µM	0.00001 µM	0.0001 µM
PFOA	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	0.001 µM	0.0001 µM
Permethrin	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	0.0001 µM	0.00001 µM
Rotenone	NC	NC	10 µM	0.0001 µM	1 µM	1 µM	NC	1 µM	1 µM	0.0001 µM	0.0001 µM	0.00001 µM
Troglitazone	NC	NC	NC	0.00001 µM	NC	NC	NC	0.00001 µM	NC	0.001 µM	0.00001 µM	0.00001 µM
Trovafloxacin	NC	NC	50 µM	NC	25 µM	25 µM	NC	NC	NC	NC	50 µM	12.5 µM
+LPS	NC	25 µM	25 µM	NC	25 µM	12.5 µM	NC	50 µM	50 µM	NC	12.5 µM	12.5 µM
Valproic Acid	NC	NC	NC	1 µM	NC	NC	NC	NC	NC	0.01 µM	0.00001 µM	0.00001 µM

*Compound concentrations indicate a ≥20% decrease (cell count) or increase (neutral lipid and phospholipid accumulation) relative to vehicle control (DMSO).
NC, not changed.

CONCLUSIONS

- **The HepatoPac MPCC model has successfully been utilized to measure biological endpoints by HCI with the purpose of profiling chemicals at 24 and 72 h.**
- **The addition of KCs to the MPCC model increased the sensitivity of assay endpoints.**
- **Further testing with additional donors is required to validate these findings.**
- **Future directions could include examining biological responses to treatment when the KC-to-hepatocyte ratio is modulated.**

REFERENCES

1. Attene-Ramos MS *et al.* (2013) The Tox21 robotic platform for the assessment of environmental chemicals – from vision to reality. *Drug Discov Today* **18:** 716-723.
2. Mennecozzi M *et al.* (2012) Hepatotoxicity screening taking a mode-of-action approach using HepaRG cells and HCA. *ALTEX Proceedings of WC8* **1/12:** 193-204.
3. Judson RS *et al.* (2010) In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. *Environ Health Perspect* **118:** 485-492.
4. Thomas RS *et al.* (2012) A comprehensive statistical analysis of predicting in vivo hazard using high-throughput in vitro screening. *Toxicol Sci* **128:** 398-417.
5. Khetani SR and Bhatia SN (2008) Microscale culture of human liver cells for drug development. *Nat Biotechnol* **26:** 120-126.
6. Ukairo O *et al.* (2013) Long-term stability of primary rat hepatocytes in micropatterned cocultures. *J Biochem Mol Toxicol* **27:** 204-212.
7. Ukairo O *et al.* (2013) Bioactivation and toxicity of acetaminophen in a rat hepatocyte micropatterned coculture system. *J Biochem Mol Toxicol* **27:** 471-478.
8. Trask Jr OJ *et al.* (2014) A micropatterned hepatocyte coculture model for assessment of liver toxicity using high-content imaging analysis. *Assay Drug Dev Technol* **12:** 16-27.

ACKNOWLEDGEMENTS

This work was supported by the American Chemistry Council's Long-Range Research Initiative (ACC-LRI). We thank Natalie Holman for assistance with the poster.