A NOVEL IN VITRO MODEL TO ASSESS CHEMICAL-INDUCED STEATOSIS AND PHOSPHOLIPIDOSIS

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INTRODUCTION

- There is a need for modern toxicity testing paradigms that utilize more high-throughput *in vitro* systems to generate accurate, predictive data for assessing liver injury risk and modes-of-action (MOAs) of prolonged chemical exposure.
- Altered lipid homeostasis is one such MOA, which can progress from phospholipidosis (PLD) and/or steatosis to more serious conditions like non-alcoholic fatty liver disease.
- Methods to detect chemically-induced lipid accumulation traditionally include whole-animal systems, cell lines and primary hepatocytes. However, it is difficult to closely study molecular mechanisms *in vivo*, and current *in vitro* models are neither amenable to long-term study nor have the full complement of biochemical functions present *in vivo*.
- Micropatterned co-cultures of human hepatocytes surrounded by mouse fibroblasts (3T3-J2; MPCC; HepatoPac™) represent an advanced multi-cellular model capable of maintaining physiologically relevant morphologic and biochemical hepatocellular characteristics *in vitro for* extended periods of time.
- MPCC enable investigation of chronic exposures and molecular events that may not be recapitulated in more short-term cultures.

The objective of the current study was to utilize MPCC to develop a novel high-content screening (HCS) strategy that can identify a compound's potential for alteration of human lipid dynamics and metabolism.

METHODS

Culture Models

- Cryopreserved primary human hepatocytes from two donors (QHu0030 and QHum13035; ***Table 1***) were provided by QPS Hepatic Biosciences (Research Triangle Park, NC).
- 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.

Table 1. *Characteristics of hepatocyte 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
QHum13035	Male	Caucasian	39	31	No	Yes	No	None	Head Trauma

Identification of High-Content Imaging (HCI) Probes

- MPCC were exposed to the prototypic positive control compounds cyclosporin A (steatosis), propranolol (phospholipidosis) or amiodarone (both) or caffeine in 5% (v/v) serum-supplemented HPCM for 4, 24, or 72 h without subsequent medium changes. For culture subsets to be stained with the LipidTOX™ kit (Life Technologies, Carlsbad, CA), LipidTOX™ Red phospholipid stain was added with the test compounds simultaneously.
- At the end of each treatment period, LysoTracker® Green DND-26 (lysosomal staining) and Hoechst 33342 (nuclear staining) were added to a subset of cultures for 30 min. Immediately after, HCI images of the live cells were captured.
- The remaining cultures were fixed with 4% (v/v) paraformaldehyde. AdipoRed™ (Lonza, Basel, Switzerland) or Bodipy® 493/503 (Life Technologies) were introduced with Hoechst 33342 to other culture subsets. LipidTOX™ Green neutral lipid stain was added to those wells that received LipidTOX™ Red. HCI images were captured 30 min later.

Compound Screening

- MPCC were exposed to a subset of compounds with varying MOAs selected from the JRC and ToxCast hepatotoxicant lists, in addition to prototypic positive control compounds. Test compounds were added in 5% (v/v) serum-supplemented HPCM for 24 or 72 h without subsequent medium changes; LipidTOX™ Red was introduced simultaneously.
- At the end of each treatment period, medium was collected; urea was measured.
- Cultures were fixed with 4% (v/v) paraformaldehyde containing Hoechst 33342. LipidTOX™ Green was added for 30 min prior to capturing HCI images.

Time Course and Serum Study

- MPCC were treated with prototypic positive control compounds or caffeine in 0% or 5% (v/v) serum-supplemented HPCM for 24, 48, 72 or 96 h without subsequent medium changes; LipidTOX™ Red was introduced simultaneously.
- At the end of each treatment period, medium was collected; urea was measured.
- LipidTOX™ Green was added for 30 min prior to capturing HCI images of the live cells.

High-Content Imaging (HCI) and Analysis

- Live cell HCI was performed on a Cellomics ArrayScan® XTI reader (Thermo Scientific, Pittsburgh, PA) equipped with a Zeiss 20x/0.4 NA objective lens and environmental control to maintain temperature, CO_2, and humidity. Up to 36-fields were captured with large camera format. Fixed samples were processed without environmental control.
- Image analysis was performed using the Spot Detector V4 bioapplication algorithm (Thermo Scientific*)* with defined thresholds to determine number of neutral lipid spots and phospholipid intensity in the cytoplasm at the cell level.

REFERENCES

1. Khetani SR and Bhatia SN (2008) Microscale culture of human liver cells for drug development. *Nat Biotechnol* **26:** 120-126.
2. Ukairo O *et al.* (2013) Long-term stability of primary rat hepatocytes in micropatterned cocultures. *J Biochem Mol Toxicol* **27:** 204-212.
3. Ukairo O *et al.* (2013) Bioactivation and toxicity of acetaminophen in a rat hepatocyte micropatterned coculture system. *J Biochem Mol Toxicol* **27:** 471-478.

RESULTS

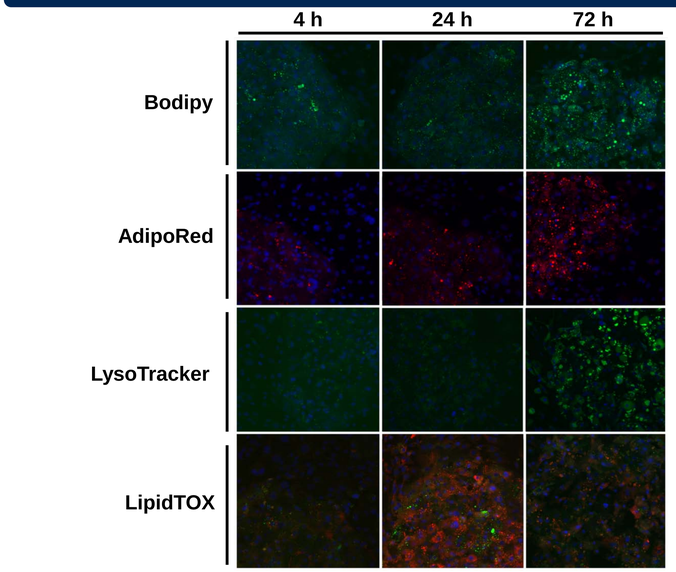

Figure 1. *Representative composite images of MPCC stained with different probes for the detection of steatosis and/or phospholipidosis captured using a 20x objective lens. Cultures were treated with cyclosporin A (30 µM; Bodipy and AdipoRed) or amiodarone (30 µM; LysoTracker and LipidTOX) for 4, 24, or 72 h without subsequent medium changes. Images labeled with Hoechst 33342 (to detect nuclei, blue), Bodipy (to detect neutral lipid, green), AdipoRed (to detect neutral lipid, red), LysoTracker (to detect lysosomes, green), LipidTOX neutral lipid (green) and LipidTOX phospholipid (red).*

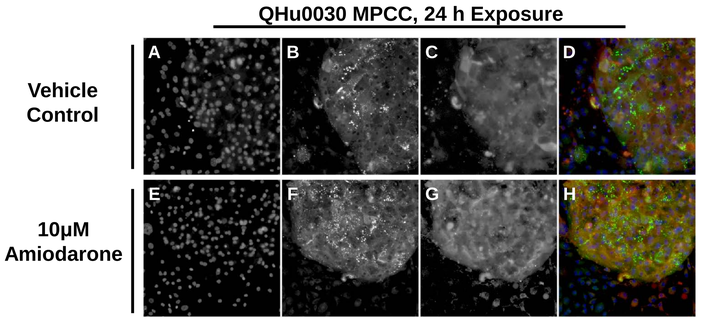

Figure 2. *Representative single channel and composite images of MPCC treated with vehicle control (0.1% [v/v] DMSO; **A-D**) or 10 µM amiodarone (**E-H**) for 24 h. (**A**,**E**) Hoechst 33342. (**B**,**F**) LipidTOX neutral lipid. (**C**,**G**) LipidTOX phospholipid. (**D**,**H**) Composite images labeled with Hoechst 33342 (blue), LipidTOX neutral lipid (green), and LipidTOX phospholipid (red). Magnification, 200x.*

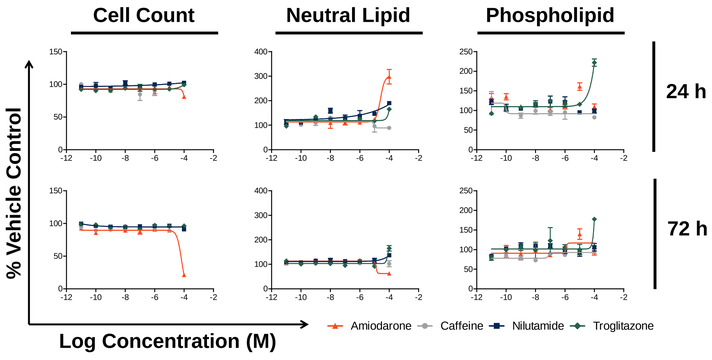

Figure 3. *Cell count, neutral lipid accumulation, and phospholipid accumulation in MPCC 24 and 72 h after treatment with test compounds. Cell count determined from object cell counts of Hoechst 33342-stained nuclei. Neutral lipid accumulation determined from counts of LipidTOX neutral lipid-stained spots. Phospholipid accumulation determined from average intensity of LipidTOX phospholipid-stained spots. Symbols and error bars denote means and ranges, respectively, of 2 replicate wells.*

Table 2. *Cell count and neutral lipid and phospholipid accumulation following hepatotoxicant treatment.*

Compound	HCI Feature					
	Cell Count* (Valid Object Count)		Neutral Lipid Accumulation* (SpotCountCh2)		Phospholipid Accumulation* (SpotAvgIntenCh3)	
	24 h	72 h	24 h	72 h	24 h	72 h
Acetaminophen	NC	NC	0.01 µM	NC	0.00001 µM	NC
Alachlor	NC	NC	0.1 µM	NC	NC	NC
Amiodarone	NC	100 µM	0.001 µM	NC	0.00001 µM	NC
Benzo[a]pyrene	NC	NC	0.001 µM	NC	0.00001 µM	10 µM
Bisphenol A	NC	NC	0.01 µM	NC	NC	NC
Caffeine	NC	NC	0.01 µM	NC	NC	NC
Cypermethrin	NC	NC	1 µM	NC	NC	NC
Dibutyl Phthalate	NC	NC	0.001 µM	NC	NC	1 µM
Dichlorvos	NC	NC	0.001 µM	NC	NC	NC
Haloperidol	NC	100 µM	0.1 µM	NC	NC	0.01 µM
Hexaconazol	NC	NC	100 µM	NC	NC	10 µM
2-Naphthylamine	NC	NC	0.0001 µM	NC	0.01 µM	NC
Nilutamide	NC	NC	0.001 µM	100 µM	0.1 µM	NC
PFOA	NC	NC	0.00001 µM	NC	100 µM	NC
Permethrin	NC	NC	0.0001 µM	NC	0.00001 µM	NC
Rotenone	NC	100 µM	0.01 µM	1 µM	NC	NC
Tamoxifen	NC	100 µM	0.0001 µM	NC	0.001 µM	NC
Triclosan	NC	NC	0.01 µM	NC	0.0001 µM	NC
Troglitazone	NC	NC	0.001 µM	100 µM	0.1 µM	0.1 µM
Valproic Acid	NC	NC	0.1 µM	NC	NC	NC

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

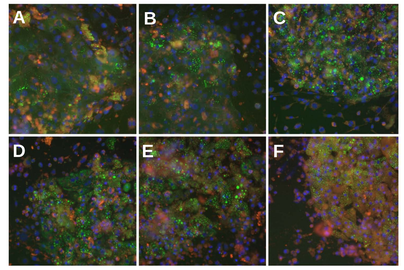

Figure 4. *Representative composite images captured using a 20x objective lens of neutral lipid and phospholipid accumulation over time in a donor with a BMI=20.9% (QHu0030) following treatment with 30 µM amiodarone in 0% (v/v) serum-supplemented HPMC. (**A**) Untreated, 24 h. (**B**) Vehicle control (0.1% [v/v] DMSO), 24 h. Amiodarone (**C**) 24 h, (**D**) 48 h, (**E**) 72 h, (**F**) 96 h.*

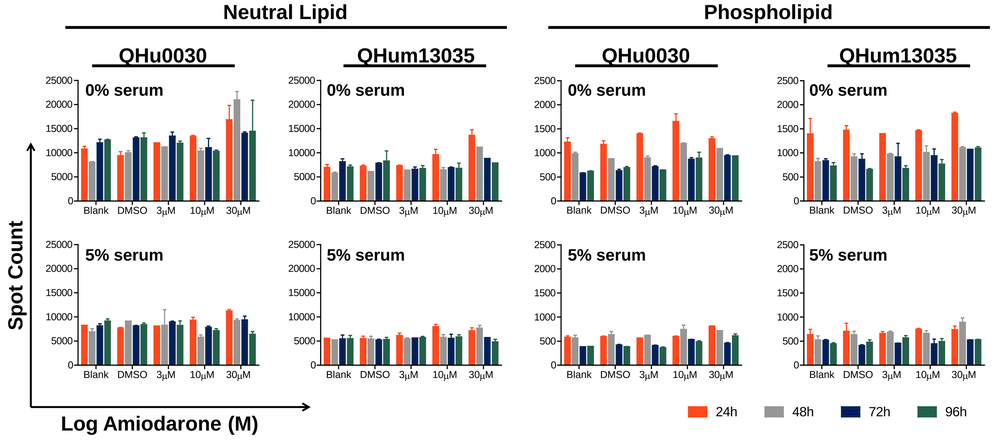

Figure 5. *Neutral lipid and phospholipid accumulation in a donor with a BMI <25 (QHu0030) and a donor with a BMI >25 (QHum13035) over time following amiodarone treatment. Symbols and error bars denote means and ranges, respectively, of 2 replicate wells.*

CONCLUSIONS

- **Live cell HepatoPac MPCC is amenable to testing compound-induced effects on lipid disposition by HCS.**
- **Using the protocol described here, concentration- and time-dependent changes in both neutral lipid and phospholipid accumulation were observed with many of the hepatotoxic compounds tested.**
- **Serum-containing culture medium stunted dose-dependent increases in neutral lipid and phospholipid accumulation.**
- **Future directions include screening the selected subset of hepatotoxicants in additional donors to determine the predictive value of MPCCs for steatosis and/or phospholipidosis potential. Donors with a range of BMIs will be selected to examine the effect of this characteristic on susceptibility to lipid dysregulation *in vitro*.**

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