Enhancement of proliferation in a novel rat hepatocyte co-culture model after mitogenic stimulation.

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ABSTRACT

Primary mouse and rat hepatocyte cultures have long been the gold standard for assessment of cellular changes associated with chemical exposure. While helpful for assessing proliferative and apoptotic responses in vitro, these cultures have limitations due to loss of cells beyond 48hrs incubation. Our motivation was to test whether extending the culture time beyond 3 days using micropatterned primary rat hepatocyte and murine fibroblast co-cultures coupled with downstream quantitative high content imaging (HCI) technology could provide a more accurate assessment of the cell proliferative response following mitogenic stimulation. The HepatoPac™ 96-well co-culture system utilizes primary rat or human hepatocytes seeded on micropatterned islands of collagen and surrounded by non-parenchymal murine embryonic 3T3-J2 fibroblasts (Khetani et al. Nature Biotechnol, 26(1), p120-126, 2007). In this model, hepatocytes have been shown to maintain extended metabolic activity for up to four weeks. Following 8 days of stabilization in serum-supplemented medium, HepatoPac™ cultures were incubated for 3, 5, and 7 days in serum-free medium containing three mitogenic micromolar concentrations of Wyeth 14, 643 (WY) and Phenobarbital (PB) followed by parallel imaging analysis at 2 independent laboratories for Ki67 signal, a marker of proliferation present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but absent from resting cells (G_0). Results showed a maximum enhancement of 140% and 153% over control at 3 d with PB and WY, respectively; 5 d showed a maximum enhancement of 145% and 108% with PB and WY, respectively, over control; 7 d showed the lowest Ki67 response of 81% and 111% over control, respectively. These results collectively resulted in an improvement of proliferative response when compared to previous experiments using conventional mono-layered primary rat cultures that produced a 5-10% hepatocyte proliferative response when held for 48hrs. (This abstract does not reflect EPA policy and mention of trade names is not an endorsement for any product).

BACKGROUND

Scope of the Problem

•The liver is a common target for chemicals and drugs due in part to the role of the liver in xenobiotic metabolism. Analysis of the effects of chemicals in ToxRefDB (Martin et al., 2009), showed 56% and 63% have some effect in the mouse or rat liver, respectively, including liver hypertrophy and effects on cell morphology such as necrosis, apoptosis and cell proliferation.

•In the 2-year bioassay, 30% or 9% of the chemicals were neoplastic in the mouse liver or rat liver, respectively. Importantly, the liver is often the most sensitive target in the 2-year bioassay. NOAELs/LOAELs have been established at least partially based on effects in the liver, i.e., 45% or 37% of all chemicals in mice or rats (Martin et al., 2009).

•Much is known about mechanisms of liver toxicity, but important data gaps remain that preclude accurate risk assessments. These include incomplete knowledge of the key events in individual modes of action (MOA) and the human relevance of many MOA. This proposal outlines a strategy for high-throughput testing and MOA categorization.

Vision

•A biologically-relevant in vitro model of the liver will be used to assess the potential toxicity of hundreds and possibly thousands of chemicals.

•Toxicity will be predicted using a combination of physicochemical information and assays that assess biomarkers of MOA activation.

•Data will be interpreted using virtual liver dose-response models of toxicity networks to predict in vivo dosimetry, mode of action and human relevance.

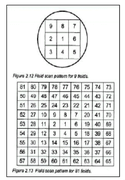
RESEARCH GOALS

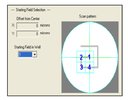
- To characterize proliferative response of rat hepatocytes following mitogenic stimulation with Wyeth 14, 643 (WY) and Phenobarbital using high-content imaging in a 96 well format.
- To contrast primary rat hepatocyte monocultures to the Hepregen HepatoPac™ co-cultures over longer incubation times.
- Assess the utility of using the Cellomics Cell Cycle application to measure the proportion of cells in 2N, 2N-4N, and >4N states.
- Evaluate human HepatoPac™ cultures under mitogenic stimulation with Wyeth14, 643 (WY) and Phenobarbital and contrast with data in rat HepatoPac™ to ascertain species-specific differences.

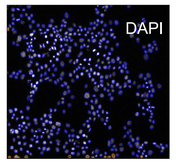
METHODS

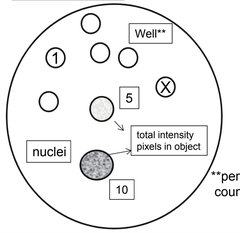
Industry standard 96-well plates were subjected to microfabrication techniques to produce a pattern of extracellular matrix-coated domains at the bottom of each well, followed by seeding of Sprague-Dawley rat hepatocytes (obtained from Celsis) onto the plates. After allowing for complete filling and adherence to the micro-patterned matrix islands, additional hepatocytes were washed away and cultures were allowed to incubate overnight. Murine embryonic fibroblasts (3T3-J2) were added to cultures the following day and allowed to adhere around the hepatocytes, thereby producing micropatterned co-cultures. Cultures were fed serum-supplemented medium up until dosing began on day 8. Cultures were treated with Wyeth 14, 643 (Sigma) at 10, 25, and 50uM, and Phenobarbital (Cayman Chemicals) at 200, 350, and 500uM in serum-free medium. Cultures were dosed every other day for 3, 5, and 7 total days. Cultures were fixed in 3.7% para-formaldehyde for 10 minutes at 37°C. Cultures were treated with Ki67 anti-body (Thermo Scientific) followed by staining with goat Anti-Rabbit Green Alexa Fluorophore Secondary (Invitrogen) and DAPI (Invitrogen). Cultures were assayed using Cellomics VTI imaging.

Object nuclei measured with High-content imaging









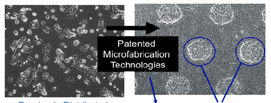
1. Object (cell) data
-Total intensity 100
-Object area (microns) 10
-Mean intensity= 100/10=10

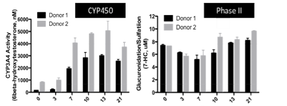
2. Well-based mean-
(independent of # objects)
-Mean of objects 1 thru X
-Average mean intensity

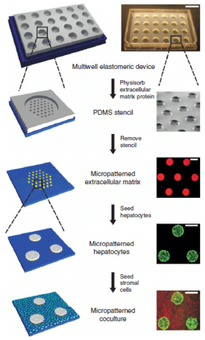
**per 96 well plate over 10 fields counted/each well(>120,000 nuclei total)

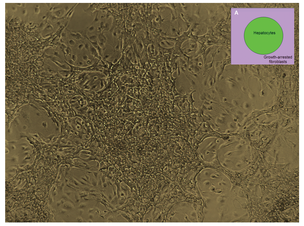
RESULTS

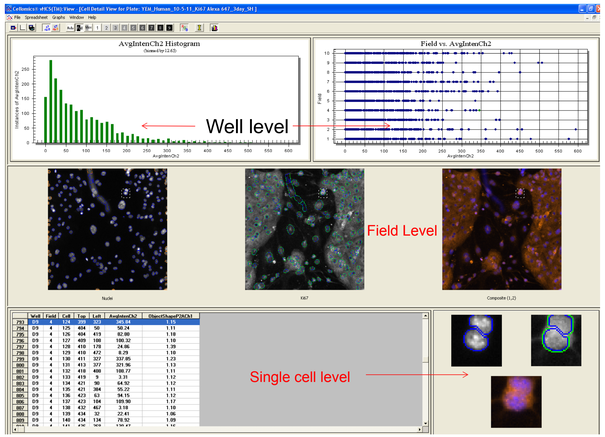
HepatoPac™ is created using Hepregen patented microfabrication tools (soft lithography) and consists of primary hepatocytes arranged in clusters of empirically optimized dimensions and subsequently surrounded by 3T3-J2 murine embryonic fibroblasts. The stencil-based process for creating HepatoPac™ is shown below with photographs taken at each step. HepatoPac™ cultures created using multiple hepatocyte donors retain long- term functionality for several weeks in vitro as shown on the right. Representative enzymes are shown, whereas other major enzymes show similar stability.



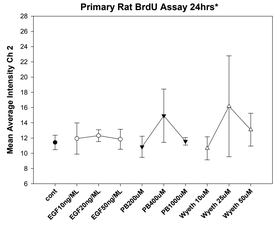




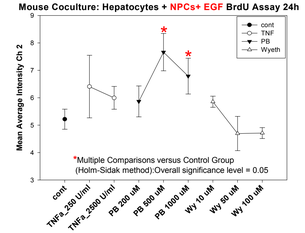




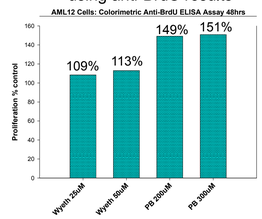
Primary Mouse & Rat Fail to show a proliferative response 24h



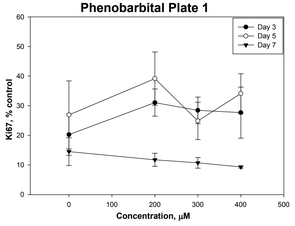
Mouse co-cultures show enhanced proliferation 24h



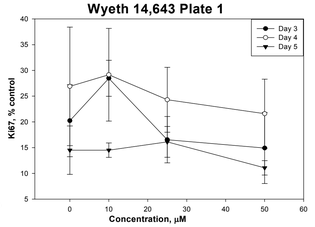
Independent validation of HCI cell counts using anti-BrdU results



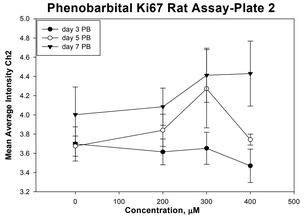
Phenobarbital Plate 1



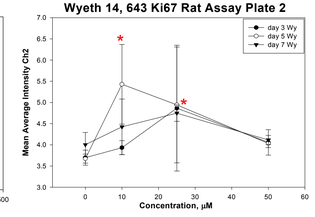
Wyeth 14,643 Plate 1



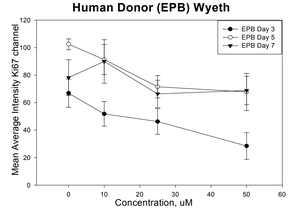
Phenobarbital Ki67 Rat Assay-Plate 2



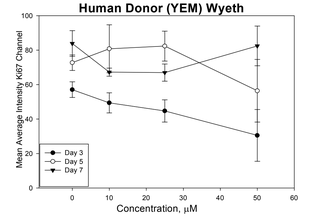
Wyeth 14, 643 Ki67 Rat Assay Plate 2



Human Donor (EPB) Wyeth



Human Donor (YEM) Wyeth



Conclusions

- We examined several hepatocyte models to assess their usefulness to measure cell proliferation following mitogenic stimulation.
- We used reference mitogens; Phenobarbital (PB) and Wyeth 14, 643 (Wy) over various concentrations.
- Primary CD1 mouse and Sprague Dawley rat hepatocyte monocultures failed to show a proliferative response at 24hrs.
- We investigated the Rat HepatoPac™ System for its ability to respond to mitogenic stimulation at 3, 5, and 7 days.
- Repeated plating of rat hepatocytes showed a trend of increased proliferation following mitogenic stimulation at 5 and 7 days.
- Unlike rodent hepatocytes, human hepatocyte cultures are known to be refractory to mitogenic stimulation by the CAR agonist (PB) and the PPARα agonist (Wy) as shown by humanized mouse hepatocyte studies.*
- The expected lack of response in human hepatocytes was confirmed following stimulation with PB and Wy in the Human HepatoPac™ model.

*Ross, J. Human Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) Support the Hypertrophic but not the Hyperplastic Response to the Murine Nongenotoxic Hepatocarcinogens Phenobarbital and Chlordane In Vivo. TOXICOLOGICAL SCIENCES 116(2), 452–466 (2010)