A MICROPATTERNED CULTURE WITH PRIMARY HEPATOCYTES AND KUPFFER MACROPHAGES FOR STUDYING INFLAMMATION-DRUG INTERACTIONS

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ABSTRACT

The occurrence of inflammation has been shown to alter the activity of enzymes involved in drug metabolism. This altered activity demands special consideration upon co-administration of drugs due to potential pharmacological and toxicological consequences. An *in vitro* model that mimics liver inflammation may provide better predictive data in preclinical testing. We have developed a micropatterned co-culture of primary hepatocytes and embryonic fibroblasts (HepatoPac™) that retains high levels of phenotypic functions such as drug metabolism enzymes for 4 weeks *in vitro*. Here, we supplement the HepatoPac platform with primary Kupffer macrophages in order to mimic one component of inflammation. Species-matched Kupffer cells were added to human and rat HepatoPac at multiple ratios (to mimic both the normal and inflamed state of the liver) after stabilization to generate a tri-culture with primary hepatocytes and embryonic fibroblasts (HepatoPac- Kupffer cell co-culture). Recent evidence suggests that interaction between inflammatory stress and certain drugs may precipitate toxic responses. Here, we assess whether stimulation of HepatoPac-Kupffer cell co-cultures with LPS sensitizes the cultures to trovafloxacin (TVX) toxicity. Rat or human HepatoPac- Kupffer cell co-cultures were treated with increasing concentrations of TVX (+/- LPS) and assessed for changes in hepatic ATP content. TVX caused a concentration-dependent toxicity in the HepatoPac-Kupffer cell co-cultures which was potentiated by addition of 50ng/mL LPS to the cultures (TC50= 87.29 vs 27.77 Cmax for the rat platform and 92.03 vs 23.15 Cmax for the human platform). This effect was not observed with the non-toxic analog, levofloxacin. Treatment with pentoxifylline (an inhibitor of TNFα transcription) significantly decreased TVX/LPS- induced rat HepatoPac toxicity suggesting a synergistic effect between TNFα and trovafloxacin (TC50= 19.73 vs. 76.36 Cmax). In conclusion, rat or human HepatoPac-Kupffer cell co-cultures may be used to predict drug induced liver injury mediated by inflammatory stress.

INTRODUCTION

Isolated primary human hepatocytes in adherent culture are widely considered to be the most suitable for *in vitro* testing [1].They are relatively simple to use and maintain an intact cellular architecture with complete, undisrupted enzymes and cofactors. We have utilized microtechnology tools to both optimize and miniaturize, in a multi-well format (up to 96-well), *in vitro* models of human and animal (i.e. rat, dog, monkey) livers called HepatoPac [2]. Specifically, primary hepatocytes are organized into colonies of prescribed, empirically-optimized dimensions and subsequently surrounded by supportive stromal cells. Hepatocytes in HepatoPac retain their *in vivo*-like morphology, express a complete complement of liver-specific genes, metabolize compounds using active Phase I/II drug metabolism enzymes [3], secrete diverse liver-specific products, and display functional bile canaliculi for 4-6 weeks *in vitro*. In the current study, we supplemented the rat and human HepatoPac model with primary rat or human Kupffer cells for utility in evaluating inflammation- drug interactions. Previously, we have shown that addition of Kupffer cells do not compromise hepatic functionality in our model (figure 1 below) and remained functional (as assessed via phagocytosis, CD68 staining [6], Cyp450 inhibition, and cytokine release) for up to 10 days post addition to HepatoPac.

It has been proposed that inflammatory stress may precipitate an idiosyncratic adverse drug reaction (IADR) in the liver such as that observed during the administration of the fluoroquinolone antibiotic trovafloxacin (TVX) [4]. Previous work by Shaw *et al*. in an *in vivo* mouse model demonstrated that LPS-induced inflammatory stress rendered TVX, but not its non-toxic analog levofloxacin (LVX), toxic. TNFα was implicated as the pro-inflammatory mediator of this toxic response, an observation supported by toxicity abrogation in the presence of pentoxifylline and etanercept [5]. **Using TVX as a prototype compound, we evaluated the ability of our HepatoPac- Kupffer cell co-culture model to detect inflammation- mediated toxicities.**

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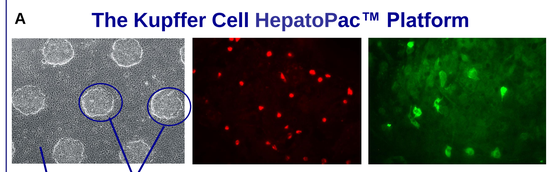

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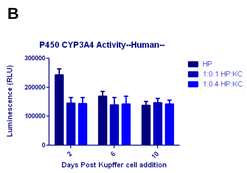

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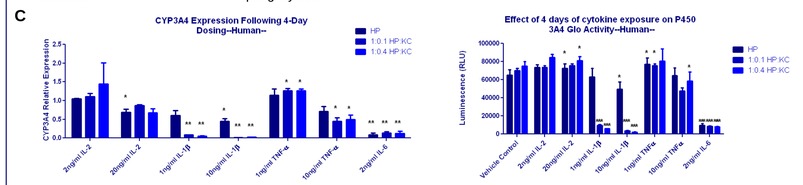


Figure 1. **The HepatoPac-Kupffer cell co-culture.** HepatoPac-Kupffer cell co-culture was created using patented microfabrication tools and consists of primary hepatocytes arranged in optimized domains and surrounded by stromal fibroblasts. Cryopreserved primary Kupffer cells were added on day 7 of HepatoPac culture. **A.** pHrodo and CD68 staining illustrates the presence of functional Kupffer cells after 10 days of culture (17 days of HepatoPac culture). **B.** Addition of primary human Kupffer cells to HepatoPac does not affect functionality of human hepatocytes as determined by CYP3A4 activity. **C**. Addition of IL-1β to HepatoPac produces a Kupffer cell-dependent decrease in CYP3A4 expression as well as Cyp3A4 activity. *HP = HepatoPac alone, HP:KC = HepatoPac/Kupffer co-cultures at various ratios.*

MATERIALS & METHODS

- Cryopreserved human hepatocytes were obtained from Celsis In Vitro Technologies. Cryopreserved rat hepatocytes, rat kupffer cells, and human kupffer cells were obtained from Life Technologies. Cytokine ELISA kits were purchased from BD Biosciences while pHrodo™ S.*aureus* Bioparticles were obtained from Life Technologies. Trovafloxacin, Levofloxacin, Pentoxifylline, and Lipopolysaccharide were obtained from Sigma-Aldrich. Cell-titer Glo (ATP) and CYP3A4-Glo Kits were obtained from Promega.
- Rat and human HepatoPac cultures were created using cryopreserved hepatocytes and 3T3-J2 murine embryonic fibroblasts following Hepregen's proprietary protocols and quality controls. HepatoPac cultures were stabilized in serum-supplemented media for 7 days prior to addition of Kupffer cells at hepatocyte:Kupffer cell ratios of 1:0.2 and 1:0.5 to mimic physiological and inflamed liver states, respectively in rat. Human hepatocyte:Kupffer cell ratios of 1:0.1 and 1:0.4 were used to mimic the *in vivo* physiological and inflammatory state, respectively.
- HepatoPac-Kupffer cell co-cultures were treated with 50 ng/mL Lipopolysaccharide (LPS) for 20 hours and cell supernatants were analyzed for cytokine secretion. Cultures were dosed with Trovafloxacin (TVX) and Levofloxacin (LVX) in serum-free media at multiples of their Cmax, 4.08uM and 5.7uM, respectively, for a total of 72 hours to investigate compound toxicity. Cultures were stimulated with 50ng/ml of LPS 24 hours after the initiation of compound dosing.

RESULTS

RAT HEPATOPAC-KUPFFER CELL CO-CULTURES

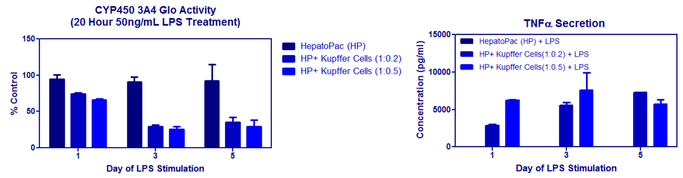
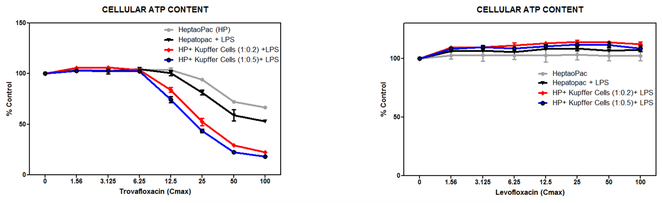
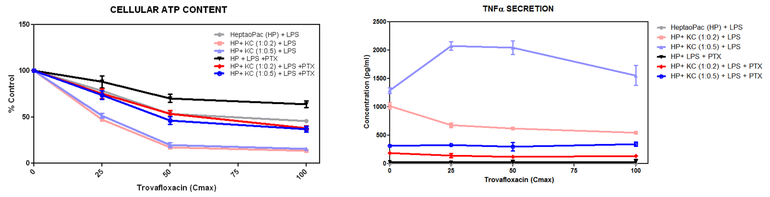


Figure 2. **Characterization of Rat HepatoPac-Kupffer cell co-cultures**. Stimulation of rat Kupffer-HepatoPac at different time-points with 50ng/mL LPS for 20 hours caused robust TNFα secretion and down-regulation of CYP3A4 activity .



Trovafloxacin TC50 (Cmax)		
Rat	**- LPS**	**+ LPS**
HepatoPac (HP)	>100	>100
HP + Kupffer cell (1:0.2)	90.95	36.33
HP + Kupffer cell (1:0.5)	87.29	27.77

Figure 3. **Trovafloxacin (TVX) toxicity is potentiated in LPS- treated rat HepatoPac-Kupffer cell co-cultures.** TVX showed characteristic dose-dependent cytotoxicity when added to rat HepatoPac-Kupffer cell co-cultures. Stimulation of the cultures with LPS exacerbated TVX-induced toxicity as seen above where there's a leftward shift (lower TC50 values) in the dose- response curves for ATP content. Levofloxacin was not toxic to rat HepatoPac-Kupffer cell cultures even when stimulated with LPS.



Trovafloxacin TC50 (Cmax)		
Rat	**+ LPS**	**+ LPS + PTX**
HepatoPac	74.33	>100
HP + Kupffer cell (1:0.2)	16.96	78.73
HP + Kupffer cell (1:0.5)	19.73	76.36

Figure 4. **Treatment with pentoxifylline (an inhibitor of TNFα transcription) significantly decreased TVX/LPS-induced HepatoPac toxicity and TNFα secretion**. Cultures were treated with Trovafloxacin and 5mM Pentoxifylline for 72 hours. After 24 hours of dosing, Kupffer cells were activated with 50ng/ml of LPS.

HUMAN HEPATOPAC-KUPFFER CELL CO-CULTURES

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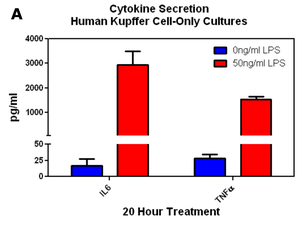

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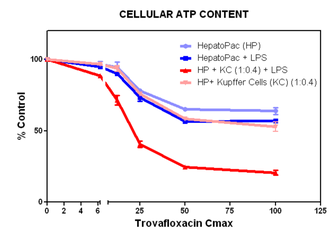

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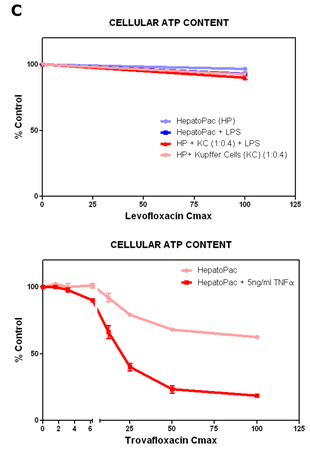

Figure 5. **Trovafloxacin (TVX) toxicity is potentiated in LPS- treated human HepatoPac-Kupffer cell co-cultures.**. **(A)** LPS stimulation of human Kupffer cell-only cultures causes robust secretion of TNFα and IL6. **(B)** Stimulation of HepatoPac-Kupffer cell co-cultures with LPS exacerbated TVX-induced toxicity as seen above where there's a leftward shift (lower TC50 values) in the dose-response curves for ATP content. **(C)** Levofloxacin remained non-toxic in all of the tested conditions (as measured by total cellular ATP content).

Figure 6. **Co-administration of TNFα and TVX potentiates TVX toxicity in Human HepatoPac cultures.** Simultaneous treatment of human HepatoPac cultures with TNFα and TVX exacerbates TVX toxicity in a manner similar to that observed in LPS-TVX treatment of human HepatoPac-Kupffer cell co-cultures.

CONCLUSIONS & FUTURE DIRECTIONS

- HepatoPac™ is a multi-well (i.e. 96-well plates) platform that consists of primary hepatocytes organized in empirically optimized clusters and subsequently surrounded by 3T3-J2 murine embryonic fibroblasts. Here, both human and rat HepatoPac were supplemented with primary human or rat Kupffer cells for utility in assessment of inflammation-drug interactions**.**
- Kupffer cells seeded at physiologically-relevant or inflamed ratios in HepatoPac did *not* affect hepatocyte functionality as assessed via Cyp3A4 activity and urea synthesis in either species (data for rat not shown).
- Kupffer cells in human HepatoPac-Kupffer co-cultures remained viable and functional for up to 10 days post addition to the wells as assessed via positive phagocytosis of pH-sensitive S.*aureus* bioparticles and CD68 staining.
- Trovafloxacin toxicity was potentiated in LPS-treated HepatoPac-Kupffer cell cultures in both rat and human.
- LPS-induced potentiation of trovafloxacin toxicity in the rat HepatoPac-Kupffer cell cultures was reversed by treatment of the cultures with pentoxifylline (an inhibitor of TNFα transcription), while TNFα treatment in humans potentiated Trovafloxacin toxicity.
- Future studies on the HepatoPac-Kupffer cell co-culture will seek to evaluate the toxicity profiles of compounds known to cause immune-mediated liver toxicities.
- The HepatoPac-Kupffer cell co-culture platform could find utility in assessment of clinically relevant interactions between therapeutic biologics and small molecule drugs as well as evaluation of inflammation-mediated toxicities.

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