IN VITRO MODELING OF INFLAMMATION-DRUG INTERACTIONS USING MICROPATTERNED CO-CULTURES OF PRIMARY HEPATOCYTES AND KUPFFER MACROPHAGES

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ABSTRACT

Drug induced liver injury (DILI) is a major health problem in the United States and accounts for the majority of clinical holds and post-marketing use restrictions by the FDA. The majority of adverse liver reactions are idiosyncratic and their underlying mechanisms are still not well understood. Better predictive models for iDILI would enable the preclinical elimination of drug candidates with hepatotoxic liabilities. We have previously developed a model in which primary hepatocytes (rat, human, dog, or monkey) are seeded onto ECM-coated domains of optimized dimensions and subsequently co-cultivated with murine embryonic fibroblasts (HepatoPac®). 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, secrete diverse liver-specific products, and display functional bile canaliculi for several weeks *in vitro.* Here, we supplement the HepatoPac co-cultures with primary Kupffer macrophages for use in evaluating inflammation- drug interactions. Kupffer cells were added to human HepatoPac cultures at a precise hepatocyte: Kupffer cell ratio of 10:4 to generate HepatoMune™ co-cultures. We then assessed whether stimulation of HepatoMune co-cultures with 50ng/mL bacteria lipopolysaccharide (LPS) sensitizes the cultures to trovafloxacin (TVX), clozapine (CLP) or chlorpromazine (CPMZ) toxicities. Human HepatoMune co-cultures were treated with increasing concentrations of drugs (+/- LPS) and assessed for changes in hepatic ATP content. TVX, CLP and CPMZ caused concentration dependent depletion of cellular ATP in HepatoMune cultures which was exacerbated by addition of LPS to the cultures (TC50= 375 vs. 94µM for TVX, 68 vs. 39µM for CLP, and 29 vs. 17µM for CPMZ respectively). The potentiation of CLP and CPMZ toxicities were more pronounced after repeated co-administration of drug/ LPS over 6 days. In conclusion, human HepatoMune co-cultures may be used to predict drug induced liver injury mediated by inflammatory stress.

METHODS

Human micropatterned co-cultures (HepatoPac) were created using patented microfabrication tools and consists of primary hepatocytes arranged in optimized domains and surrounded by 3T3-J2 murine embryonic fibroblasts. In this configuration, human hepatocytes retain long-term functionality for several weeks *in vitro* . Human HepatoPac co-cultures were allowed to stabilize for 7 days in serum-supplemented medium prior to the addition of human Kupffer cells (from unmatched donors) to create Human HepatoMune™ cultures. Kupffer cells were added on day 8 of culture at a precise hepatocyte:Kupffer cell ratio of 10: 4 to mimic an inflamed liver state.

Initial investigations were performed to assess the functionality of the HepatoMune platform by evaluating i) the phagocytosis of Staphylococcus aureus labeled bioparticles by the Kupffer macrophages, ii) the basal CYP450 activity of the hepatocytes in the presence and absence of the Kupffer cells, iii) secretion of cytokines by LPS-stimulated Kupffer cells and iv) suppression of CYP450 activity and gene expression bin cytokine-stimulated HepatoMune cultures.

HepatoMune cultures were treated with 50 ng/mL LPS for 20 hours and cell supernatants were analyzed for cytokine secretion. Cultures were dosed with Trovafloxacin (TVX) and Levofloxacin (LVX) in the presence or absence of LPS in serum-free medium at multiples of their Cmax, 4.08µM and 15.77µM, respectively, for a total of 72 hours to investigate compound toxicity. In a follow-up study, the cultures were dosed with increasing concentrations of antipsychotics, clozapine and chlorpromazine in the presence or absence of 50ng/mL LPS in serum-free medium at multiples of their Cmax, up to a 100*Cmax. The Cmax values for clozapine and chlorpromazine are 1.2µM and 0.5µM respectively. Both Chlorpromazine[4] and Clozapine[5] have been reported to exhibit immune-mediated hepatotoxicity

RESULTS

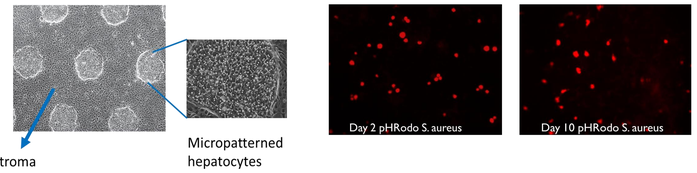

Figure 1 HepatoMune™ co-culture platform. A) HepatoPac co-cultures were created using patented microfabrication tools and consists of primary hepatocytes arranged in optimized domains and surrounded by stromal fibroblasts. Cryopreserved primary Kupffer cells were added on day 7 of HepatoPac culture. **B.** pHrodo dye staining illustrates the presence of functional Kupffer cells after 10 days of culture (17 days of HepatoPac culture). pHrodo dye is a pH sensitive dye . Acidification of the phagosome after phagocytosis by the macrophages is marked by red fluorescence.

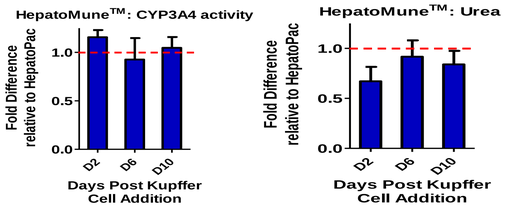

Figure 2: Functionality of hepatocytes in HepatoMune cocultures. Addition of Kupffer macrophages did not affect the function of hepatocytes as measured by **A)** CYP3A4 activity and **B**) urea production

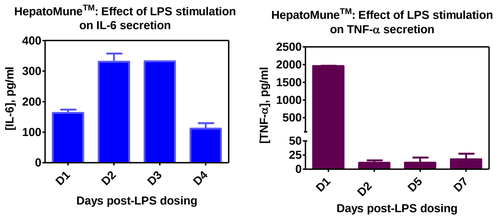

Figure 3: Functionality of Kupffer macrophages: Effect of LPS stimulation on Cytokine secretion by HepatoMune co-cultures over time. HepatoMune co-cultures were treated with a single dose of 50ng/mL LPS and supernatants were assayed for the presence of cytokines, IL-6 and TNF-α days after dosing cultures with LPS

RESULTS

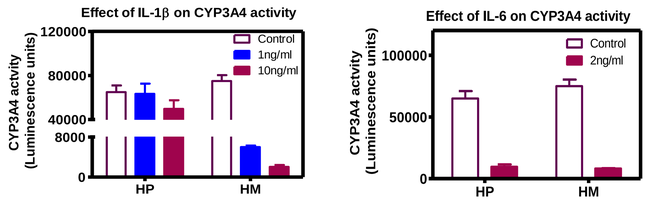

Figure 4: Down-regulation of CYP3A4 protein activity levels in cytokine-treated human HepatoMune co-cultures. A) IL-1β stimulation of cultures inhibited CYP3A4 activity in both HepatoPac (HP) and HepatoMune (HM) co-cultures in a concentration-dependent manner. Significantly greater down-regulation of CYP3A4 activity was observed in the HepatoMune cultures. **B)** IL-6 stimulation of cultures down-regulated CYP3A4 activity similarly in both HepatoPac and HepatoMune co-cultures in a concentration-dependent manner.

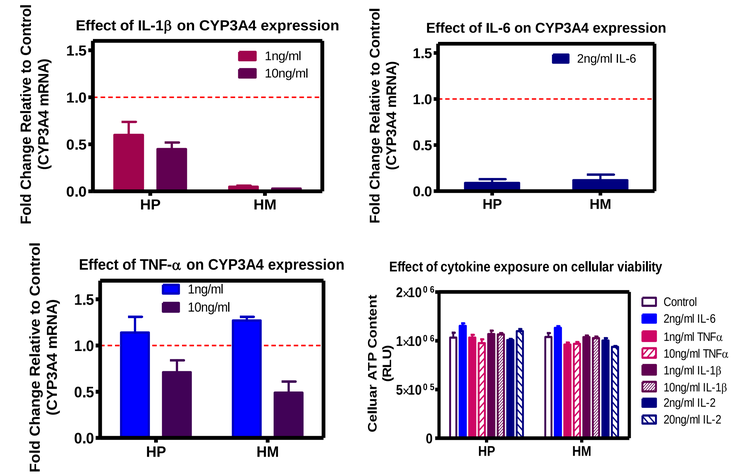

Figure 5: Down-regulation of CYP3A4 mRNA levels in cytokine-treated human HepatoMune co-cultures is exacerbated in the presence of Kupffer macrophages and effect of cytokine exposure on cellular viability. mRNA levels were normalized to the housekeeping gene GAPDH and compared to the respective untreated controls. **A)** IL-1β stimulation of cultures down-regulated CYP3A4 gene expression in both HepatoPac and HepatoMune cocultures, with a significantly greater down-regulation see in HepatoMune cocultures. **B**) IL-6 stimulation of cultures down-regulated CYP3A4 mRNA levels equally in both HepatoPac and HepatoMune cultures. **C)** TNF-α stimulation of cultures down-regulated CYP3A4 gene expression in both HepatoPac and HepatoMune cocultures, with a significantly greater down-regulation see in HepatoMune co-cultures. **D)** Treatment of the co-cultures with cytokines at the test concentrations did not affect cellular viability.

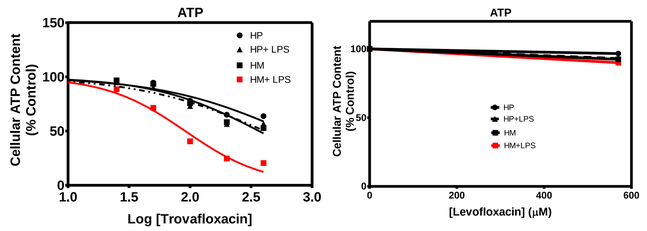

Figure 6: Trovafloxacin (TVX) toxicity is potentiated in LPS- treated human HepatoMune co-cultures. (A) Stimulation of HepatoMune (HM) co-cultures with LPS exacerbated TVX-induced toxicity as seen above where there's a leftward shift (lower TC50 values) in the dose-response curves for ATP content away from the curves for the HepatoPac (HP) cultures. **(B)** Levofloxacin, the non-toxic analog of TVX remained non-toxic (as measured by total cellular ATP content)

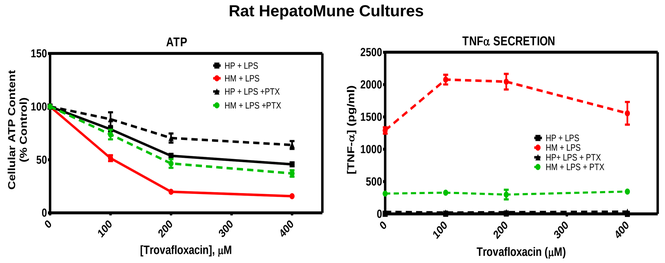

Figure 7: Treatment with pentoxifylline (an inhibitor of TNFα transcription) significantly decreased TVX/LPS-induced HepatoMune toxicity and TNFα secretion. Cultures were treated with Trovafloxacin and 5mM Pentoxifylline for 72 hours in the presence and absence of 50ng/ml LPS.
TNFα has been implicated as the pro-inflammatory mediator of Trovafloxacin toxicity, an observation supported by toxicity abrogation in the presence of pentoxifylline and etanercept[7]

RESULTS

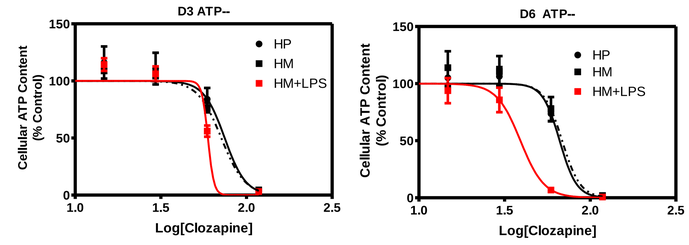

Figure 8. Clozapine toxicity is potentiated in LPS- treated human HepatoMune co-cultures. (A) Stimulation of HepatoMune (HM) co-cultures with LPS for 72 hours resulted in a mild exacerbation of Clozapine-induced toxicity as seen above, **B**) Treatment of HepatoMune cc-cultures with LPS for 144 hours resulted in greater exacerbation of toxicity as shown by the larger leftward shift of the dose-response curve, IC50 values decreased from 68µM (day 3 post dosing) to 39µM (day 6 post dosing)

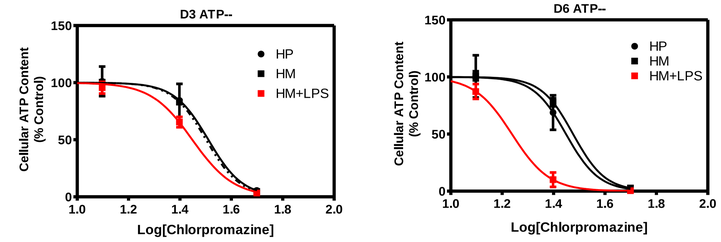

Figure 9. Chlorpromazine toxicity is potentiated in LPS- treated human HepatoMune co-cultures. (A) Stimulation of HepatoMune (HM) co-cultures with LPS for 72 hours resulted in a very mild exacerbation of Chlorpromazine-induced toxicity as seen above, **B**) Treatment of HepatoMune cc-cultures with LPS for 144 hours resulted in greater exacerbation of toxicity as shown by the larger leftward shift of the dose-response curve, IC50 values decreased from 29µM (day 3 post dosing) to 17µM (day 6 post dosing)

CONCLUSIONS

- Previously, we showed that rat and human HepatoMune™ co-cultures may be used to model inflammation-drug interactions using Trovafloxacin as a model compound with immune-mediated toxicity. We showed that Kupffer cells maintain their functionality in the HepatoMune co-cultures for up to 10 days in culture, exhibiting phagocytotic activity and secreting cytokines when stimulated by LPS. Here, we extend the studies to show that human HepatoMune co-cultures are able to model clozapine- and chlorpromazine immune-mediated toxicities.
- Consistent with literature reports, treatment of the cultures with cytokines down-regulated CYP3A4 activity and gene expression in both HepatoPac and HepatoMune cultures. The presence of the Kupffer cells intensified this effect. This shows that the Kupffer cells are needed to model the complex interactions that mediate immune mediated toxicities, which may not be modeled by hepatocytes alone
- Trovafloxacin toxicity was potentiated in LPS-treated human HepatoMune co-cultures replicating in vivo studies in rats and mice. Furthermore, our studies in rat HepatoMune cultures showing the abrogation of TVX-mediated toxicity in the presence of pentoxifylline was consistent with literature reports implicating TNF-α as a mediator of TVX toxicity
- We showed that the HepatoMune platform is highly specific, distinguishing between Trovafloxacin and its non-toxic analog, Levofloxacin.
- Clozapine and Chlorpromazine toxicities were potentiated in LPS treated human HepatoMune co-cultures after repeated administration of LPS and the compounds. This finding emphasizes the need to use a long-living platform such as HepatoMune that enables chronic dosing of compounds to model inflammation-drug interactions.
- Future studies on the HepatoMune co-culture platform will seek to evaluate the toxicity profiles of more compounds known to cause immune-mediated liver toxicities.
- The HepatoMune 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.

REFERENCES

1. Nguyen, T.V. et al. Establishment of a hepatocyte-Kupffer cell co-culture model for the assessment of proinflammatory cytokine effects on metabolizing enzymes and drug transporters. Drug Metab Dispos. Epub ahead of print March 4, 2015 doi: 10.1124/dmd.114.061317
2. Hewitt, N. J. et al. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev. 39(1), p159-234 (2007).
3. Khetani, S.R. & Bhatia, S.N. Microscale culture of human liver cells for drug development. Nat Biotechnol. 26(1), p120-126 (2007).
4. O'Brien, P. J. et al. High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. Arch. Toxicol. 80, p580-604 (2006)
5. Aitken, A.E. et al. Regulation of drug metabolizing enzymes and transporters in inflammation. Annu Rev Pharmacol Toxicol 46, p123-149 (2006)
6. Tukov, F. F. Modeling inflammation-drug interactions in vitro: a rat Kupffer cell-hepatocyte coculture system. Toxicol In Vitro. 20(8), p1488-1499 (2006)
7. Shaw, P.J. et al. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor alpha. Toxicological Sciences. 100(1), p259-266 (2007).
8. Brown, K.E. et al. Immunohistochemical detection of myeloperoxidase and its oxidation products in Kupffer cells of human liver. Am J Pathol. 15(6), p2081-2088 (2001)