ASSESSMENT OF A MICROPATTERNED HEPATOCYTE CO-CULTURE SYSTEM TO DETECT COMPOUNDS THAT CAUSE DRUG INDUCED LIVER INJURY IN HUMANS

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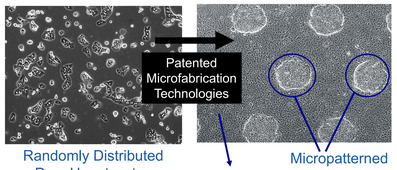
ABSTRACT

Liver Injury remains a major reason for late stage drug attrition. Therefore the pharmaceutical industry aims at developing predictive assays that can be deployed early in the drug discovery process when SAR (structure-activity relationships) approaches are still feasible. Xu *et al.*, 2008 [1] have shown that drug induced liver injury can be predicted to some extent using primary human hepatocytes in sandwich cultures and high content imaging of key cell injury endpoints (i.e. mitochondrial membrane potential, reactive oxygen species, lipid accumulation and nuclear stain). The sensitivity of this assay was approximately 50% with a 0-5% false positive rate. While high content imaging using sandwich cultures represents a key advance in the field, the sensitivity of the assay needs to be further improved. One can hypothesize that the low sensitivity could be due to the fact that the assay was only performed at 24 hours and that longer incubation with compounds could improve outcomes. However, the requirement would be that the hepatocyte culture would stay viable and metabolically competent for an extended period of time. In addition, a more complete set of phase I and II enzymes should be expressed at levels comparable to those found *in vivo* and transporters should be functional. Here, we use micropatterned co-cultures (MPCCs) of human hepatocytes and stromal cells in a 96-well format [2], also called HepatoPac™, to detect drug induced liver injury potential of 45 compounds previously utilized in the Xu assay. Bulk assay readouts such as albumin and urea secretion in supernatants, and glutathione and ATP levels in cell lysates were utilized along with dosing for 5-9 days for detecting adverse cellular effects. Our results indicate that *human* HepatoPac™ was able to accurately identify 26 out of 35 clinically toxic compounds (sensitivity of 74%) as opposed to 10 out of 35 in the Xu assay. Furthermore, *human* HepatoPac™ maintained the high specificity (80-90%) or low false positive rate seen in conventional cultures for 10 true negative drugs tested. HepatoPac™ created using *rat* hepatocytes, on the other hand, had a sensitivity of 49% and specificity of 80%, presumably due to species-specific differences in liver metabolism. Bridging the gap between animals and human *in vitro* will help reduce the need for costly *in vivo* animal studies. HepatoPac™ shows superiority over the conventional sandwich culture model, as well as the potential to help reduce costs associated with drug development by providing the industry with the necessary tools to help develop safer pharmaceuticals.

INTRODUCTION

Drug induced liver injury (DILI) is a leading cause of the pre-launch and post-market attrition of pharmaceutical compounds. The gold standard for toxicological evaluation of substances for regulatory submission are whole rodent and non-rodent models. However, species-specific variations between rodents, non-rodents and humans can be significant, especially in liver-specific metabolic pathways (i.e. CYP450). This, along with other factors, may severely limit the utility of animal models for predicting human-specific responses. Isolated primary human hepatocytes in adherent culture are widely considered to be the most suitable for *in vitro* testing [3]. They are relatively simple to use and maintain an intact cellular architecture with complete, undisrupted enzymes and cofactors. Conventional culture models utilized for industrial ADME/Tox screening expose hepatocytes to tumor-derived Matrigel™ and/or collagen-I gels (sandwich cultures). When utilized with near confluent monolayers, these models allow better retention of hepatocyte cyto-architecture and activities of specific CYP450s for 3-5 days over hepatocytes on rigid collagen substratum. However, sandwich cultures are inherently unstable in their phenotypic functions and their short-term functionality does not allow for clinically-relevant chronic drug metabolism and toxicity to be measured. Indeed, the current sensitivity of sandwich cultures, even with highly sensitive high content imaging readouts (Xu *et al*., 2008), is estimated to be approximately 50%. Accordingly, there is a need for better *in vitro* models of primary human liver tissue that are more predictive of clinical outcomes and can be used with existing industrial automation for high-throughput screening in industry-standard multi-well formats. We have utilized microtechnology tools to both optimize and miniaturize in a multi-well format (up to 96-well) *in vitro* models human and rat 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 liver genes, metabolize compounds using active Phase I/II drug metabolism enzymes [4], secrete diverse liver-specific products, and display functional bile canaliculi for 4-6 weeks *in vitro* (Figure 1 below). Furthermore, HepatoPac™ outperforms conventional culture models with respect to magnitude and longevity of liver-specific functions. In the current study, we wanted to assess the ability of HepatoPac™ to detect compounds that cause drug-induced liver toxicity in humans. We chose 45 compounds: 10 True Positives, 10 True Negatives and 25 False Negatives as classified by Xu *et al*., 2008 [1] and proceeded to dose Human and Rat HepatoPac™ cultures for up to 9 days. Albumin secretion, urea synthesis, glutathione (GSH) levels and ATP content were utilized as measures of adverse cellular effects.

The HepatoPac™ Platform



Miniaturized HepatoPac™

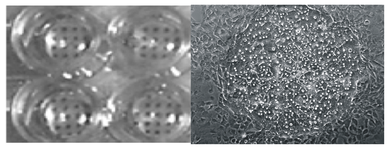


Figure 1. The HepatoPac™ platform miniaturized into an industry-standard multi-well format (4 well of a 96-well format shown here). Human HepatoPac™ cultures created using cryopreserved human hepatocyte donors retain *in vivo*-like morphology and long- term functionality (i.e. Phase I and II drug metabolism enzymes) for several weeks in vitro. Here, CYP3A4 activity via testosterone 6β-hydroxylation is shown; however, other enzymes such as CYP1A2, 2B6, 2D6, 2C9, 2C19 shown similar profiles. Phase II metabolism is shown here via glucuronidation and sulfation of 7-hydroxycoumarin.

MATERIALS & METHODS

- Cryopreserved human and Sprague-Dawley rat primary hepatocytes were obtained from commercial vendors. Compounds were purchased from Toronto Research Chemicals and Sigma-Aldrich.
- Human and rat HepatoPac™ were created using patented/proprietary methods developed at Hepregen Corporation, and then maintained for ~1 week to allow for stabilization prior to dosing with compounds. Sandwich cultured human hepatocytes (SCHH) were created using published protocols [1-2] and allowed 1 day to stabilize as typical in pharmaceutical practice.
- Cultures were exposed to 1, 10, 30, 60 and 100 times C[max] of a total of 45 compounds (10 True Negatives, 10 True Positives, and 25 False Negatives as shown below) over a period of 5 days (2 doses) or 9 days (4 doses) in serum-free medium for HepatoPac, while SCHHs were dosed twice over 3 days.
- Albumin secretion and urea synthesis were measured from culture supernatants as described previously [2]. ATP and GSH levels were measured using Promega assay kits per manufacturer's instructions.
- TC65/TC50 is defined as the drug concentration that caused at least 35%/50% reduction in signal as compared to vehicle control. A compound was called as toxic when a TC50 or TC65 could be calculated within the dose range tested for one or more assays. A compound was called as non-toxic when a TC50 or TC65 could not be calculated in the doses tested for any of the assays utilized here.

RESULTS

#	Compound Name	MW	C[max] µM	Clinical DILI	HIAT DILI, from [1]	SCHH DILI	Human HepatoPac DILI	Rat HepatoPac DILI
	True Positives in HIAT							
1	Benzbromarone	424.1	4.361	Positive	Positive	Positive	Positive	Positive
2	Clozapine	326.83	0.951	Positive	Positive	Negative	Positive	Positive
3	Diclofenac	318.1	8.023	Positive	Positive	Positive	Positive	Positive
4	Flurbiprofen	244.27	57.356	Positive	Positive	Positive	Positive	Positive
5	Mefenamic Acid	241.3	26.959	Positive	Positive	Positive	Positive	Positive
6	Mebendazole	295.3	0.126	Positive	Positive	Negative	Positive	Negative
7	Phenacetin	179.22	13.401	Positive	Positive	Negative	Positive	Negative
8	Phenylbutazone	308.37	486.772	Positive	Positive	Positive	Positive	Positive
9	Quinine	391.47	9.254	Positive	Positive	Positive	Positive	Positive
10	Trazadone HCl	408.32	5.065	Positive	Positive	Positive	Positive	Positive
	True Negatives in HIAT							
11	Aspirin	180.16	5.526	Negative	Negative	Negative	Negative	Negative
12	Buspirone	421.96	0.005	Negative	Negative	Negative	Negative	Negative
13	Dexamethasone	392.47	0.224	Negative	Negative	Negative	Negative	Negative
14	Dextromethorphan HBr	370.3	0.028	Negative	Negative	Negative	Negative	Negative
15	Fluoxetine	345.79	0.049	Negative	Negative	Negative	Negative	Negative
16	Lidocaine	288.81	36.298	Negative	Negative	Positive	Positive	Positive
17	Miconazole	479.1	0.024	Negative	Negative	Negative	Negative	Negative
18	Prednisone	358.43	0.068	Negative	Negative	Negative	Negative	Negative
19	Propranolol	295.81	0.201	Negative	Negative	Negative	Negative	Negative
20	Warfarin	308.34	4.868	Negative	Negative	Negative	Negative	Positive
	False Negatives in HIAT							
21	Acetazolamide	222.25	135.142	Positive	Negative	Pending	Positive	Positive
22	Betahistine DiHCl	209.12	0.004	Positive	Negative	Pending	Negative	Negative
23	Captopril	217.29	4.284	Positive	Negative	Pending	Negative	Negative
24	Chloramphenicol Palmitate	561.54	19.991	Positive	Negative	Pending	Negative	Negative
25	Ciprofloxacin HCl	331.34	11.476	Positive	Negative	Pending	Positive	Positive
26	Clomiphene Citrate	598.1	0.022	Positive	Negative	Pending	Negative	Negative
27	Clomipramine	351.3	0.191	Positive	Negative	Pending	Positive	Negative
28	Cyclophosphamide	279.1	265.359	Positive	Negative	Pending	Positive	Positive
29	Cyproterone acetate	416.94	0.656	Positive	Negative	Pending	Positive	Positive
30	Danazol	337.5	0.074	Positive	Negative	Pending	Positive	Positive
31	Dapsone	248.3	6.007	Positive	Negative	Pending	Positive	Negative
32	Estrone	270.37	0.022	Positive	Negative	Pending	Positive	Negative
33	Hydroxyurea	76.05	793.925	Positive	Negative	Pending	Positive	Positive
34	Imipramine HCl	316.87	0.087	Positive	Negative	Pending	Positive	Negative
35	Isoniazid	137.14	76.609	Positive	Negative	Pending	Positive	Positive
36	Maleic acid	160.04	1.000	Positive	Negative	Pending	Positive	Negative
37	Methimazole	114.17	1.868	Positive	Negative	Pending	Positive	Negative
38	Nifedipine	346.3	0.271	Positive	Negative	Pending	Negative	Negative
39	Norgestrel	312.45	0.009	Positive	Negative	Pending	Negative	Negative
40	Nortriptyline HCl	299.84	0.122	Positive	Negative	Pending	Positive	Negative
41	Phentolamine Mesylate	377.47	0.086	Positive	Negative	Pending	Negative	Negative
42	Piroxicam	331.37	5.135	Positive	Negative	Pending	Negative	Positive
43	Progesterone	314.46	0.193	Positive	Negative	Pending	Negative	Negative
44	Pyrazinamide	123.11	407.174	Positive	Negative	Pending	Positive	Negative
45	Tamoxifen	371.53	0.162	Positive	Negative	Pending	Positive	Positive

Table 1. Compounds tested and call in different models. Shaded compounds indicate differences in 'predictive' calls between one or more culture models.

RESULTS CONTINUED

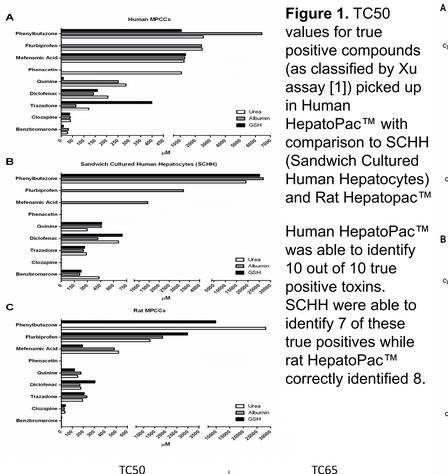


Figure 1. TC50 values for true positive compounds (as classified by Xu assay [1]) picked up in Human HepatoPac™ with comparison to SCHH (Sandwich Cultured Human Hepatocytes) and Rat Hepatopac™

Human HepatoPac™ was able to identify 10 out of 10 true positive toxins. SCHH were able to identify 7 of these true positives while rat HepatoPac™ correctly identified 8.

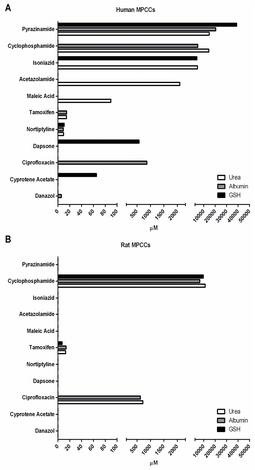


Figure 2. TC50 values for false negative compounds (as classified by Xu assay [1]) picked up in Human HepatoPac™ with comparison to Rat HepatoPac™.

Eight out of nine false negatives that were called toxic in rat HepatoPac™ were also called hepatotoxic in human HepatoPac™. Piroxicam was called toxic by rat but not by human. Eight additional false negatives, for a total of 16 (of 25), were called hepatotoxic in human HepatoPac™.

TC50

HUMAN

Actual DILI	HUMAN MPCC Predicted DILI (TC50)		
	Positive	Negative	Total
Positive	20	15	35
Negative	1	9	10
Total	21	24	45

Sensitivity = 57.1%
Specificity = 90%
Accuracy = 64.4%
Positive Predictive Power = 95.2%
Negative Predictive Power = 37.5%

RAT

Actual DILI	RAT MPCC Predicted DILI (TC50)		
	Positive	Negative	Total
Positive	13	22	35
Negative	2	8	10
Total	15	30	45

Sensitivity = 37.1%
Specificity = 80%
Accuracy = 46.7%
Positive Predictive Power = 86.7%
Negative Predictive Power = 26.7%

TC65

HUMAN

Actual DILI	HUMAN MPCC Predicted DILI (TC65)		
	Positive	Negative	Total
Positive	24	11	35
Negative	2	8	10
Total	26	19	45

Sensitivity = 68.6%
Specificity = 80%
Accuracy = 71.1%
Positive Predictive Power = 92.3%
Negative Predictive Power = 42.1%

RAT

Actual DILI	RAT MPCC Predicted DILI (TC65)		
	Positive	Negative	Total
Positive	17	18	35
Negative	2	8	10
Total	19	26	45

Sensitivity = 48.6%
Specificity = 80%
Accuracy = 55.5%
Positive Predictive Power = 89.4%
Negative Predictive Power = 30.8%

Figure 3. Confusion matrices for Human and Rat HepatoPac™ with either TC50 or TC65 Threshold

3 Assays (Alb, Urea, GSH)

HUMAN

Actual DILI	HUMAN MPCC Predicted DILI (TC65)		
	Positive	Negative	Total
Positive	24	11	35
Negative	2	8	10
Total	26	19	45

Sensitivity = 68.6%
Specificity = 80%
Accuracy = 71.1%
Positive Predictive Power = 92.3%
Negative Predictive Power = 42.1%

4 Assays (Alb, Urea, GSH, ATP)

HUMAN

Actual DILI	HUMAN MPCC Predicted DILI (TC65)		
	Positive	Negative	Total
Positive	26	9	35
Negative	2	8	10
Total	28	17	45

Sensitivity = 74.3%
Specificity = 80%
Accuracy = 75.5%
Positive Predictive Power = 92.9%
Negative Predictive Power = 47%

Figure 4. Confusion matrices for Human HepatoPac™ with or without inclusion of ATP (Promega's Cell-Titer-Glo Assay)

CONCLUSIONS & FUTURE DIRECTIONS

- For the detection of compounds that cause drug induced liver injury in humans, Human HepatoPac™ has a higher sensitivity (~74% vs. 50%) than the conventional sandwich culture model using either the high content imaging Xu assay [1] or bulk assays utilized here.
- Generally, all culture models tested here (Human and rat HepatoPac™, SCHH) have high (80-90%) specificity for true negative compounds.
- Selection of different thresholds/cutoffs (i.e. TC50 vs. TC65) and additional assays (i.e. ATP) can modulate the sensitivity of *in vitro* toxicity assessment.
- Rat HepatoPac™, as expected from known species-specific differences in liver metabolism pathways, proved to be less sensitive (by ~20%) in detecting human liabilities *in vitro* than human HepatoPac™.
- High content imaging readouts (i.e. nuclei, lipids, reactive oxygen species, glutathione) need to be explored alongside bulk assays used here to determine sensitivity differences for toxicity screening.
- The addition of other non-parenchymal liver cells (i.e. Kupffer macrophages, sinusoidal endothelia) to HepatoPac™ needs to be investigated to determine if sensitivity of the model could be improved further.

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