Toxicology and Applied Pharmacology 336 (2017) 20–30

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Long-enduring primary hepatocyte-based co-cultures improve prediction of hepatotoxicity

Eric I. Novik[a], Jacquelyn Dwyer[b], James K. Morelli[b], Amit Parekh[a], Cheul Cho[a], Eitan Pludwinski[a], Anil Shrirao[a], Robert M. Freedman[a], James S. MacDonald[c], Zaid Jayyosi[b,*]

[a] Hμrel Corporation, North Brunswick, NJ 08902, United States
[b] Preclinical Safety, Sanofi, Framingham, MA 01701, United States
[c] Chrysalis Pharma Partners, LLC, Chester, NJ 07930, United States

ARTICLE INFO

Keywords:
DILI
Hepatotoxicity
Co-culture
Cytotoxicity

ABSTRACT

The failure of drug candidates during clinical trials and post-marketing withdrawal due to Drug Induced Liver Injury (DILI), results in significant late-stage attrition in the pharmaceutical industry. Animal studies have proven insufficient to definitively predict DILI in the clinic, therefore a variety of *in vitro* models are being tested in an effort to improve prediction of human hepatotoxicity. The model system described here consists of cryopreserved primary rat, dog or human hepatocytes co-cultured together with a fibroblast cell line, which aids in the hepatocytes' maintenance of more *in vivo*-like characteristics compared to traditional hepatic mono-cultures, including long term viability and retention of activity of cytochrome P450 isozymes. Cell viability was assessed by measurement of ATP following treatment with 29 compounds having known hepatotoxic liabilities. Hμrelrat™, Hμreldog™, and Hμrelhuman™ hepatic co-cultures were treated for 24 h, or under repeat-dosing for 7 or 13 days, and compared to rat and human hepatic mono-cultures following single-dose exposure for 24 h. The results allowed for a comparison of cytotoxicity, species-specific responses and the effect of repeat compound exposure on the prediction of hepatotoxic potential in each model. Results show that the co-culture model had greater sensitivity compared to that of the hepatic mono-cultures. In addition, "time-based ratios" were determined by dividing the compounds' 24-hour TC_{50}/C_{max} values by TC_{50}/C_{max} values measured after dosing for either 7 or 13 days. The results suggest that this approach may serve as a useful adjunct to traditional measurements of hepatotoxicity, improving the predictive value of early screening studies.

1. Introduction

Hepatotoxicity is one of the major reasons for drug attrition during clinical development and for withdrawal post-marketing (Russmann et al., 2009; van Tonder et al., 2013). In these cases, the predictivity of animal studies was insufficient to prevent these drug candidates from entering clinical trials. It is well accepted that there is a need for additional tools that will allow toxicologists to make a better assessment of human hepatotoxic risk. Currently, several different cell-based assays are used to evaluate potential hepatotoxic liabilities. Monocultures of primary hepatocytes are of limited value due to the loss of cytochrome P450 metabolic activity and a short experimental window, due to decreasing cell viability and de-differentiation (Hewitt et al., 2007). The HepG2 cell line is a human hepatoma cell line, but has low expression of metabolic enzymes, and reduced expression of nuclear receptors (García-Cañaveras et al., 2015). HepaRG cells can be differentiated towards hepatocyte-like cells showing a stable phenotype, and good CYP450 expression (Aninat et al., 2006). However, HepaRG cells are known to have limitations, for example, CYP2E1 expression is low compared to *in vivo* expression, and glutathione transferase A1 is not induced by phenobarbital treatment (Kanebratt and Andersson, 2008). The fundamental limitations of hepatocyte monocultures compromise the predictive utility of these *in vitro* assays (Smith et al., 2012).

In an effort to generate hepatic cultures with extended periods of metabolic competency, a number of co-culture models were developed over the past 20 years (Bhatia et al., 1997; Chao et al., 2009; Kidambi et al., 2009). This co-culture paradigm has been adopted to better predict xenobiotic clearance rate, metabolic profile and hepatotoxic liability earlier in the drug development process (Atienzar et al., 2014; Bonn et al., 2016). Here we describe a multi-species, hepatic co-culture model developed by Hμrel Corporation. The model is comprised of cryopreserved primary hepatocytes drawn from either human, dog, rat, or other pre-clinical species, and cultured in combination with a proprietary, supporting non-parenchymal stromal cell line. This co-culture

* Corresponding author.
E-mail address: zaid.jayyosi@sanofi.com (Z. Jayyosi).

http://dx.doi.org/10.1016/j.taap.2017.09.013
Received 20 April 2017; Received in revised form 25 August 2017; Accepted 14 September 2017
Available online 21 September 2017
0041-008X/

model allows hepatocytes to maintain viability and general cellular competency, including retaining hepato-specific morphology and the expression of CYP metabolic enzymes for two weeks. Therefore, the dosing regimen evaluated in this report was limited to 13 days.

In order to assess the value of the co-culture model for prediction of hepatotoxicity, a sample set of 19 compounds with known hepatotoxic liabilities were evaluated in conjunction with a second sample set of 10 compounds proprietary to Sanofi Pharmaceuticals. The first sample set includes well-studied compounds that represent a range of different indications and mechanisms of hepatotoxicity. The second sample set was comprised of 9 compounds that had been discontinued from active pharmaceutical development either during pre-clinical testing or during clinical trials in which hepatotoxicity was detected, and one compound discontinued for other reasons, in which hepatotoxicity was not detected. While compound development was canceled for a variety of reasons, *in vivo* liver toxicity was observed for all 9 compounds put forth for testing in the co-culture model.

Co-cultures comprised of cryopreserved rat, dog and human primary hepatocytes and non-parenchymal stromal cells were treated for 24 h, or repeat dosed every 48 h over 7 or 13 days. Cell viability was determined by measuring ATP levels. The co-culture results were compared to freshly isolated rat primary and cryopreserved human primary hepatic mono-cultures which had been treated for 24 h. Compared to the hepatic mono-cultures, the co-culture model appears to be more robust and less sensitive to hepatotoxicants following the administration of a single dose. However, equal or greater cytotoxicity was observed in the co-culture model following repeated exposure to hepatotoxicants. Although there were modest compound-specific differences in sensitivity across species in the co-cultures (and more pronounced species-specific differences with respect to certain compounds), the general increase in sensitivity compared to those obtained in the mono-culture models demonstrates the value of a repeat-dosing scheme using long-term co-cultures.

2. Materials and methods

2.1. Preparation and plating of freshly isolated rat hepatocyte monocultures

All reagents were obtained from Sigma-Aldrich Inc., St. Louis, MO, unless otherwise noted. All animals were housed in an American Association for Accreditation of Laboratory Animal Care accredited facility, and all procedures were reviewed and approved by the Sanofi Boston Institutional Animal Care and Use Committee. Rat hepatocytes were isolated following a two-step collagenase perfusion method from eight- to twelve-week-old male Sprague-Dawley rats (Green et al., 1983). Cells were re-suspended in William's E Medium supplemented with 0.025 μM dexamethasone (DEX), 1.24% insulin-transferrin-sodium selenite (ITS), 2.0 mM L-glutamine, 5% FBS and 1% Penicillin/Streptomycin (Hepatocyte Plating Media), and total cell count was performed using a hemacytometer and trypan blue exclusion dye. A viability level of 85% or greater qualified the cells for use in these assays. Freshly isolated hepatocytes were adjusted to a concentration of 1.5×10^5 cells/mL in fresh hepatocyte plating media and seeded at 200 μL per well into 96-well collagen-coated plates (Corning, NY, Cat. #354407), for a total of 3×10^4 cells/well. Cells were incubated at 37 °C, 5% CO_2 and 97% relative humidity for 3–4 h, at which time Hepatocyte Plating Media was removed and replaced with Hepatocyte Culture Media. Hepatocyte Culture Media had the same formulation as listed above, with the exception of 5% FBS, which was not included. Cells were incubated overnight prior to compound treatment.

2.2. Preparation and plating of cryopreserved human hepatocyte monocultures

Cryopreserved human hepatocytes were obtained from Bioreclamation IVT Inc. Lot/Donor #JGM was used for all assays performed in monoculture, and in co-cultures, as described below. Cells were thawed in a 37 °C water bath for approximately 2 min as described by the manufacturer. Once thawed, the hepatocytes were suspended in 6 mL of media per vial thawed, using 37 °C InVitroGro CP media, supplemented with Torpedo Antibiotic mix (Bioreclamation IVT Inc.). Total cell count was performed using a hemacytometer and trypan blue exclusion dye. A viability level of 80% or greater qualified the cells for use in these assays. Cell concentration was adjusted to 0.4×10^6/mL and seeded at 100 μL per well into 96-well collagen-coated plate (Corning, NY Cat. No. 354407) for a total of 4×10^4 cells/well. Cells were incubated at 37 °C, 5% CO_2 and 97% relative humidity for 3–4 h, at which time plating media was removed from the wells and replaced with 100 μL ice-cold InVitroGro HI Media supplemented with Torpedo Antibiotic Mix (Bioreclamation IVT Inc.), 5% FBS, and 225 μg/mL matrigel. Cells were incubated overnight prior to compound treatment.

2.3. Preparation and plating of hepatocyte co-cultures

All co-culture hepatocyte plates used for purposes of the predictive comparison described in this article were provided by Hμrel Corporation (North Brunswick, New Jersey). Hμreldog™ utilized beagle hepatocytes, Hμrelrat™ utilized Sprague Dawley hepatocytes, and Hμrelhuman™ utilized IVT Inc. Lot/Donor #JGM hepatocytes, the same lot used for the preparation of human monocultures described above. All co-cultures were plated on collagen coated 96 well tissue culture treated plates. Cryopreserved hepatocytes were removed from liquid nitrogen and thawed quickly in a water bath at 37 °C. Hepatocytes were transferred to a 50 mL conical tube containing 20 mL warm Hμrel PlatinumHeps™ medium (proprietary composition), 9 mL percoll, 1 mL of 10 × Phosphate Buffer Saline (ThermoFisher Scientific) and centrifuged at 500 × *g* for 5 min at room temperature. After removing the supernatant, the cells were re-suspended in PlatinumHeps™ medium and cell number and viability were determined using trypan blue exclusion. Non-parenchymal stromal cells (passages 10–20) were cultured at 37 °C in a 5% CO_2, 95% relative humidity atmosphere until used for experimental plating. On plating day, cells were detached from the plate surface using trypsin (0.25%), suspended in 15 mL DMEM medium and centrifuged at 1000*g* for 7 min at room temperature. After removing the supernatant, the cells were re-suspended in PlatinumHeps™ maintenance medium and cell number and viability were determined using trypan blue exclusion. Forty thousand, thirty thousand, and twenty-five thousand hepatocytes were seeded in each well of the 96-well dog, human and rat co-culture plates, respectively. The non-parenchymal stromal cells were added to each well the next day (proportion of non-parenchymal cells to hepatocytes proprietary). The cells were co-cultured at 37 °C in a 5% CO_2 for 6 days prior to shipment to Sanofi. Co-culture plates were carefully shipped in PlatinumHeps™ maintenance medium. Upon arrival at Sanofi, where the plates were unpacked and shipping media was replaced with fresh PlatinumHeps™ maintenance medium. The cells were then left to acclimatize overnight in an incubator at 37 °C in humidified atmosphere containing 95% air and 5% CO2, prior to compound treatment.

2.4. Bile canaliculi analysis

To detect functional bile canaliculi we incubated hepatocytes with 2 mg/mL of 5(6)-carboxy-20,70-dichlorofluorescein diacetate (C-DCFDA) for 10 min, washed with phenol red-free media, and imaged using fluorescence microscopy.

2.5. Enzyme activity and bioanalysis of test compounds

All experiments were performed in 96-well tissue culture treated plates with a compound incubation volume of 100 μl. On the day of the experiment, cultures were incubated with 100 μM of 7-hydroxycoumarin, 5 μM of midazolam or 5 μM of dextromethorphan

prepared in maintenance media at 37 °C and 5% CO2. Incubations were stopped after 30 min and metabolite formation was monitored. Metabolites assayed for were 1-OH midazolam, dextrorphan, 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate. These are indicative of CYP3A4, CYP2D6, UDP-glucuronyl transferase and sulfotransferase activity, respectively. The experiment was terminated by removing 100 µl of supernatants which were immediately frozen at − 20 °C.

Formation of metabolites was measured using LC-MS/MS at Hµrel's facilities. Samples were centrifuged at 1000*g* for 10 min before injecting 10 µl of each sample. The LC-MS/MS system comprised a Shimadzu LC-10ADvp pump (Shimadzu, Columbia, MD, USA), SIL-HTS auto-sampler (Shimadzu, Columbia, MD, USA), and an API 4000 mass spectrometer with a Turbo Ion Spray probe (Applied Biosystems/MDS SCIEX, Ontario, Canada). The separation of compounds was achieved using a reversed stationary phase (Advantage ARMOR C-18, 5 mm, 30.0 mm–2.1 mm, Analytical Sales and Services, Inc., Pompton Plains, NJ). A fast gradient using mobile phases of 0.1% formic acid in acetonitrile and water with 0.1% formic acid along with switching valves and pumps was used for analysis. Phenomenex C18 Synergi 50 mm × 2.00 mm was used as the analytical column.

2.6. Compound selection and experimental design

29 compounds were selected for assessment of their hepatotoxic risk in standard hepatocyte mono-culture following 24-hour exposure, and in the Hµrel co-culture model following 24-hour exposure or, alternatively, following repeat exposure for 7 or 13 days (Table 1). The compounds were grouped into two separate sample sets. Sample Set I consisted of 19 compounds, of which thirteen were classified as hepatotoxicants and six as non-hepatotoxicants based on the literature (O'Brien et al., 2006; Raja and Dreyfus, 2008; Xu et al., 2008). This sample set included two pairs of structurally similar drugs with the same clinical indication (troglitazone and rosiglitazone, bosentan and macitentan). One member of each pair had produced DILI in patients resulting in either drug withdrawal or a black box warning, and one had no known liver toxicity. Each compound was tested in 24 h rat mono-cultures to determine where 100% cell death was achieved, without precipitation of compound, so that a TC50 could be calculated. The concentration range for the subsequent assays was set based on these data. Sample Set II consisted of ten drug candidates proprietary to Sanofi, nine of which had been discontinued from development as a prospective pharmaceutical either during late preclinical (*in vivo*) toxicity studies or in clinical trials for reasons that included indications of liver injury. The tenth compound served as a negative control. One of these nine compounds generated markers of liver injury in rat and dog studies, but with large safety margins; however, progression of the compound was stopped in Phase I clinical trials due to elevation of liver transaminases. Two compounds generated a positive liver signal in preclinical animal studies and were discontinued before progressing to Phase I. Another compound had no indication of a liver toxicity in animal studies but liver toxicity was observed in Phase I trials, and was discontinued. The negative control compound had shown no indication of hepatotoxicity in pre-clinical studies and had never been progressed into clinical trials.

2.7. Compound dosing

Compounds were weighed and solubilized in the appropriate vehicle. For the majority of compounds, the vehicle was DMSO, however, some compounds were solubilized directly in culture media. Compounds solubilized in DMSO were dissolved at 400 × the final desired top concentration. Once solubilized, a 1:3 serial dilution series was prepared in DMSO, for a total of 6 concentrations, with a final DMSO concentration of 0.25%. In the case of compounds solubilized in culture media or for the media control, DMSO was added to a level of 0.25% prior to addition to cells. For mono-cultures, 100 µl of compound or control diluted in fresh hepatocyte culture media was added to cells and incubated for 24 h. For co-cultures, the same procedure was used for the 24 h time point, except that PlatinumHeps™ medium was used as diluent. For the 7-day co-culture incubations, 100 µl compound in media was added on Day 0 of treatment, then replaced on Days 2, 4 and

Table 1
Compounds employed in the study. A. Sample Set I: 19 well-studied reference compounds. B. Sample Set II: Proprietary compounds of Sanofi that were discontinued during either pre-clinical studies or clinical trials.

A. SAMPLE SET 1					B. SAMPLE SET 2	
Compound	**Clinical DILI**	**Therapeutic Category**	**FDA LTKB Drug Labeling for DILI Concern**	**Probable / possible mechanism of toxicity**	**10 proprietary compounds discontinued from drug development**	**DILI Category**
Troglitazone	Positive	Antidiabetic	Withdrawn- Most Concern	Complex – multifactorial	A	Positive - Preclinical
Nefazadone		Antidepressant	Withdrawn- Most Concern	Unknown – idiosyncratic	B	Negative - Preclinical
Benzbromarone		Antigout agent	Withdrawn- Most Concern	Reactive metabolite mediated	C	Positive - Clinical
Amiodarone		Antiarrythmic	Black Box Warning - Most Concern	Direct damage to lipid bilayers and disturbance of lysosomal and/or mitochondrial function	D	Positive - Clinical
Flutamide		Antineoplastic agent	Black Box Warning - Most Concern	Unknown – idiosyncratic and not clearly understood, may involve a toxic intermediate	E	Positive - Clinical
Bosentan		Antihypertensive	Black Box Warning - Most Concern	Alterations in bile salt transport	F	Positive - Clinical
Bicalutamide		Antineoplastic agent	Warning & Precaution - Most Concern	Unknown – idiosyncratic, may be caused by toxic metabolites	G	Positive - Clinical
Diclofenac		NSAID	Warning & Precaution - Most Concern	Idiosyncratic, possibly related to formation of acyl glucuronide	H	Positive - Preclinical
Tacrine		Cholinergics	Warning & Precaution - Most Concern	Unknown - may involve a toxic intermediate	I	Positive - Preclinical
Chlorpromazine		Antipsychotic	Adverse Reaction - Most Concern	Cholestatic – mechanism unknown, hypersensitivity likely, may involve a toxic intermediate	J	Positive - Clinical
Cylophosphamide		Antineoplastic agent	Adverse Reaction - Less Concern	Unknown – idiosyncratic		
Acetaminophen		Analgesics and Antipyretics	Not Included	Reactive metabolite mediated		
Propranolol	Negative	Beta Blocker	Warning & Precaution - Less Concern			
Rosiglitazone		Antidiabetic	Not Included			
Diphenhydramine		Antidepressant	Drugs of No Dili Concern			
Isoproterenol		NSAID	Drugs of No Dili Concern			
Kanamycin		Antibiotic	Drugs of No Dili Concern			
Macitentan		Antihypertensive	Not Included			
Primadone		Anti-epileptic	Drugs of No Dili Concern			

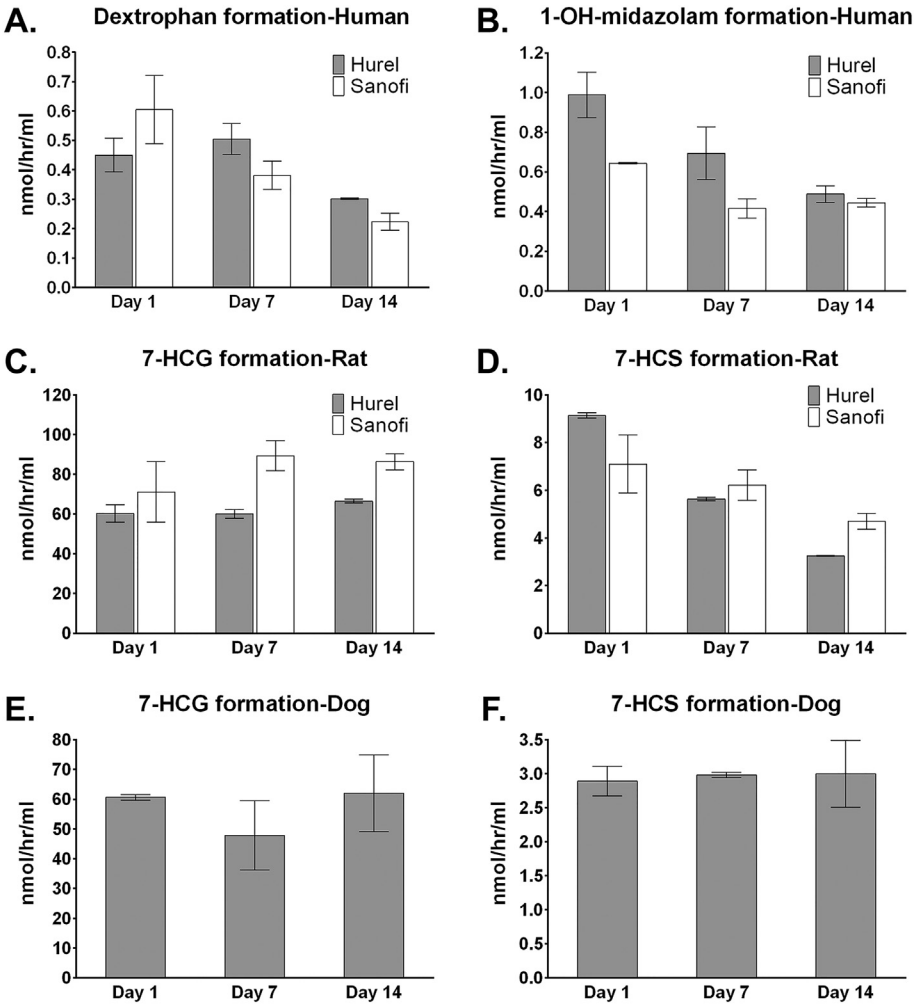

Fig. 1. Characterization of Phase I and Phase II metabolite formation of human, rat and dog co-cultures. All data is represented in nanomoles/h/million cells. 1A, 1B: 14 day time course of phase I metabolite formation of dextrophan (mediated by CYP2D6) and phase I metabolite formation of 1-hydroxy midazolam (mediated by CYP3A4) in human co-cultures at Hμrel and Sanofi. 1C, 1E: 14 day time course of phase II formation of 7-hydroxy coumarin glucuronide (7-HCG, mediated by UGTenzymes) in rat and dog co-cultures. Rat data was generated at Hμrel and Sanofi, while dog was generated at Hμrel only. 1D, 1F: 14 day time course of phase II formation of 7-hydroxy coumarin sulfate (7-HCS, mediated by sulfotransferase enzymes) in rat and dog co-cultures. Rat data was generated at Hμrel and Sanofi while dog was generated at Hμrel only.

6 of treatment. For the 13-day co-culture incubations, 100 μl compound in media was added on Day 0 of treatment, then replaced on Days 2, 4, 6, 8, 10 and 12 of treatment.

2.8. Cytotoxicity assessment and data analysis

All 96-well plates, both mono-culture and co-culture, were assayed in an identical manner. Following completion of the desired treatment period, Cytotoxicity was assessed using ATP content as a measure of cell viability, and was quantitated using the Promega Cell Titer Glo® (Promega Corp., Madison, WI) viability assay, performed 24 h after treatment or in the case of the 7-day and 13-day tests, (24 h after Day 6 and Day 12 of dosing respectively). For all compounds tested 3 technical replicates were performed per concentration, therefore biological replicate N = 1.

GraphPad Prism software was used to determine the compound concentration that achieves the death of 50% of the cultured hepatocytes exposed to the compound (the TC_{50} value). The data are represented as a ratio of a compound's TC_{50} value to its human average maximum plasma concentration (C_{max}) reported previously in the literature (the TC_{50}/C_{max} ratio) (O'Brien et al., 2006; Xu et al., 2008). A TC_{50}/C_{max} ratio score of 100 or below was defined to be a positive signal of hepatotoxicity (DILI). The TC_{50}/C_{max} ratio data was then used to generate an additional, time-based ratio, calculated by dividing each compound's TC_{50}/C_{max} value measured at 24 h after a single dose administration by the compound's TC_{50}/C_{max} value measured at 7 days or 13 days following repeat-dose administration. This time-based toxicity ratio therefore measures the degree of increase in toxic response after the repeat-dose exposure, compared to the strength of response observed after initial, 24-hour single-dose exposure.

3. Results

3.1. Hμrel co-culture model maintains hepatocyte functionality and differentiated morphology

The cellular morphology and canalicular formation of the co-cultures was characterized on the first day of dosing (Day 7) and had optimal morphology (Supplemental data). Fig. 1 indicates the metabolic competency and capacity of the co-culture to carry out Phase I and Phase II metabolic processes in hepatocytes of the human, rat, and dog species, respectively, with stable levels of enzyme activity spanning 14 days. Phase I enzyme activity was characterized in the human co-culture model concurrently at Hμrel and at Sanofi for a range of metabolic enzymes, including CYP450 isoforms 2D6 (Fig. 1A) and 3A4 (Fig. 1B), as a function of CYP-specific metabolite generation at a given time point. Phase II enzyme activity was characterized concurrently at Hμrel and Sanofi for the rat co-culture model, and at Hμrel for the dog co-culture model, for a range of transformations including glucuronidation (Fig. 1C and E) and sulfation (Fig. 1D and F), as a function of metabolite generation at a given time point. Phase II rat and dog enzymes were characterized as a function of 7-hydroxy coumarin and 7-hydroxy coumarin glucuronide formation, respectively. The co-cultures maintained hepatocyte morphology and functionality, including enzymatic activity, over the 14-day dosing period. Note that while metabolic enzyme levels were measured over a 14 day period, dosing of the co-

Table 2
Sensitivity and specificity of cytotoxic response to compounds of Sample Set I. A: Mono-culture of human cryopreserved primary hepatocytes after 24-hour single-dose treatment. B: Co-culture of human cryopreserved primary hepatocytes after 24-hour single-dose treatment and at Days 7 and 13 after repeat-dose treatment every 48 h. C: Mono-culture of rat fresh primary hepatocytes after 24-hour single-dose treatment. D: Co-culture of rat cryopreserved primary hepatocytes after 24-hour single-dose treatment and at Days 7 and 13 after repeat-dose treatment every 48 h. E: Co-culture of dog cryopreserved primary hepatocytes after 24-hour single-dose treatment and at Days 7 and 13 after repeat-dose treatment every 48 h.

			A	B			C	D			E		
Compound	Clinical DILI	Human C_{max} (μM)	Human mono-culture TC50/ C_{max}	Human co-culture TC50/C_{max}			Rat mono-culture TC50/ C_{max}	Rat co-culture TC50/C_{max}			Dog co-culture TC50/C_{max}		
			24 hr	24 hr	7 day	14 day	24 hr	24hr	7 day	14 day	24 hr	7 day	14 day
Troglitazone	Positive	6.39	25	15	9	7	14	13	13	17	13	12	14
Nefazodone		0.92	41	74	68	59	75	76	86	53	83	69	70
Benzbromarone		4.30	>104	44	8	9	12	29	12	7	37	9	9
Amiodarone		0.81	41	>215	15	11	38	>215	28	11	>215	30	22
Flutamide		6.00	25	>167	73	70	14	>167	75	69	132	77	73
Bosentan		2.00	382	807	104	41	407	469	158	50	721	102	63
Bicalutamide		1.97	>187	>187	>187	123	71	>187	>187	>187	>187	141	70
Diclofenac		4.20	212	250	109	75	75	213	74	56	184	59	59
Tacrine		0.06	2,016	>4,048	2,349	1,381	2,683	>4,048	1,937	635	>4,048	1,778	1,762
Chlorpromazine		0.84	25	83	36	33	37	83	30	31	88	81	86
Cylophosphamide		143.00	>84	>84	52	35	16	>84	14	4	>84	2	2
Acetaminophen		130.00	45	>308	60	43	105	>308	87	75	>308	52	37
	True Positive		6	4	8	10	9	4	9	10	4	9	11
	Total Positive		12	12	12	12	12	12	12	12	12	12	12
	Sensitivity (%)		50%	33%	67%	83%	75%	33%	75%	83%	33%	75%	92%
Propranolol	Negative	0.20	525	1,350	465	310	695	1,760	610	355	1,420	665	495
Rosiglitazone		1.04	432	678	408	327	309	620	517	351	723	170	150
Diphenhydramine		0.30	510	>1,667	580	547	690	>1,667	800	383	>1,667	>1,667	>1,667
Isoproterenol		0.006	>83,333	>250,000	>250,000	>250,000	>83,333	>250,000	>250,000	>250,000	>250,000	>250,000	>250,000
Kanamycin		72.20	>554	>554	245	169	285	>554	401	193	>554	>554	250
Macitentan		0.30	2,159	1,706	316	347	575	2,038	359	334	3,669	263	353
Primidone		4.67	>298	>298	>298	>298	>298	>298	>298	>298	>298	>298	>298
	True Negative		7	7	7	7	7	7	7	7	7	7	7
	Total Negative		7	7	7	7	7	7	7	7	7	7	7
	Specificity (%)		100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

cultures was done over a 13 day period.

3.2. Increased cytotoxicity with repeat-dosing scheme in the co-culture model

In all three species evaluated, increased cytotoxicity was observed in co-cultures following repeat-dose exposure to the majority of the hepatotoxic reference compounds, as compared to exposure for 24 h. This is indicated by the lower TC_{50}/C_{max} ratios observed at the later time points (Table 2). Although a decrease in TC_{50}/C_{max} ratios was also evident for several non-hepatotoxicants, in these instances the ratio values were > 100, correctly classifying these compounds as non-hepatotoxic. In all instances except for tacrine, the greatest decrease in TC_{50}/C_{max} values occurs between the 24-hour and the 7-day time points. For example, non-linear regression curves for both acetaminophen and cyclophosphamide demonstrated an increase in cytotoxicity between the 24-hour single-dose exposure and the subsequent, 7-day and 13-day repeat-dose exposures in the human, rat and dog co-cultures, with most of the increase occurring between 24 h and Day 7 (Fig. 2). Additional decreases in TC_{50}/C_{max} ratios occurred in some of the compounds between Days 7 and 13. The most significant decrease was observed for bosentan, where TC_{50}/C_{max} decreased from 104 on Day seven to 41 on Day Thirteen in the human, 158 to 50 in the rat, and 102 to 63 in the dog co-culture models (2.5-, 3.2-, and 1.6-fold shifts between Days 7 and 13, respectively).

3.3. Increased cytotoxicity detected in co-cultures with repeat-dosing as compared to single-dose exposure to mono-culture hepatocytes

Table 2 also compares the TC_{50}/C_{max} ratios achieved at 24 h with the human and rat mono-cultures, and at 24 h, 7 days, and 13 days with the human, rat, and dog co-cultures, when exposed to the reference compounds in Sample Set 1. Sensitivity and specificity scores based on the TC_{50}/C_{max} ratios were calculated for each model at the relevant time points (Table 2, dark grey boxes). All models correctly identified the non-hepatotoxic compounds of Sample Set 1 and achieved a specificity score of 100%, despite the decrease in TC_{50}/C_{max} values observed in the co-cultures with repeat-dosing.

The mono-culture of cryopreserved human hepatocytes identified six of the 12 compounds as hepatotoxic, resulting in a sensitivity score of 50%. Better sensitivity was observed with rat mono-cultures, which identified nine true positives for a sensitivity score of 75%. The highest sensitivity was achieved with the human and the rat co-cultures following 13 days' repeated exposure, each of which identified 10 True Positives out of 12, resulting in sensitivity scores of 83%; and with the dog co-culture at 13 days, which identified 11 True Positives out of 12 (sensitivity 92%). Overall, at 24 h the human and rat co-cultures did not demonstrate greater sensitivity to hepatotoxicants compared to the mono-cultures.

However, greater cytotoxicity was frequently observed in the co-culture models following repeat exposure over 7 and 13 days, compared to the toxicity observed after a single-dose, 24-hour treatment in either of the mono-cultures. Relative sensitivities of the human 24-hour mono-cultures and 13-day co-cultures are summarized in Fig. 3. Of the nineteen compounds in Sample Set I, it was possible in the case of thirteen compounds to compute a ratio of TC_{50} values comparing the sensitivities of the two models. Of these, 13-day incubation in the co-culture model demonstrated increased sensitivity (a lower TC_{50} value) compared to 24-hour incubation in the mono-culture in nine instances (69%), and decreased sensitivity in four instances (31%). For four other compounds of Sample Set I (benzbromarone, cyclophosphamide, bicalutamide, and kanamycin), it was not possible to compute a definitive ratio of the two models' TC_{50} values because toxicity was < 50% at the highest compound concentration assayed in the mono-culture. For these

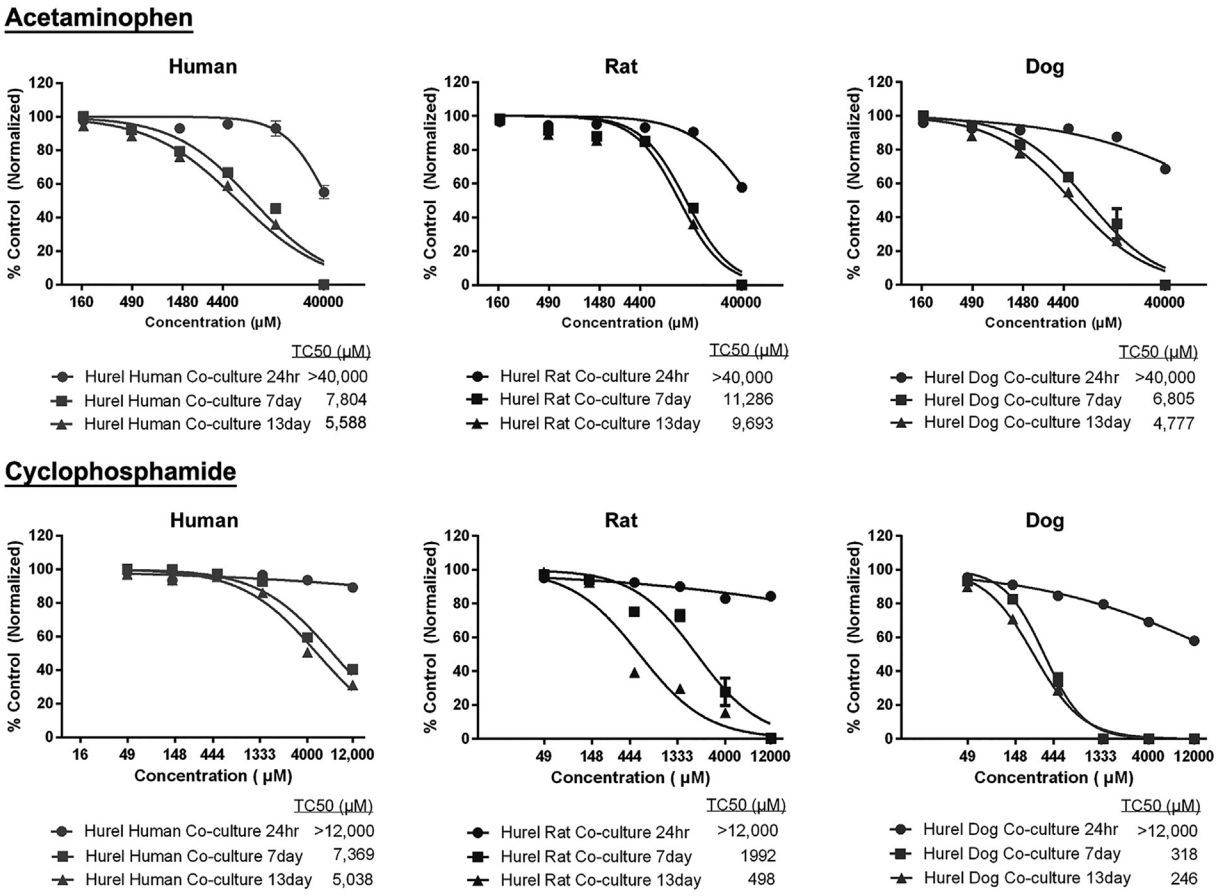

Fig. 2. Dose-dependent cytotoxicity after single and repeated treatments in primary cryopreserved hepatic co-cultures. Non-linear regression curves estimating dose-dependent cytotoxicity after single and repeated treatments of the Hμrel human, rat, and dog hepatic co-cultures with acetaminophen and cyclophosphamide, respectively. In each instance the greatest increase in cytotoxicity was measured between 24 h and Day 7, with some additional increase measured between Day 7 and Day 13.

four compounds, the ratio was computed on the assumption that the TC_{50} value achieved in the mono-culture model occurs at the highest concentration assayed for each respective compound. For the final two compounds of Sample Set I (primadone, isopreterenol), it was not possible to compute a ratio of the relative sensitivities of the two models' TC_{50} values because toxicity was measured below 50% at the highest compound concentrations assayed in both models.

For a number of the compounds in Sample Set I, the TC_{50}/C_{max}

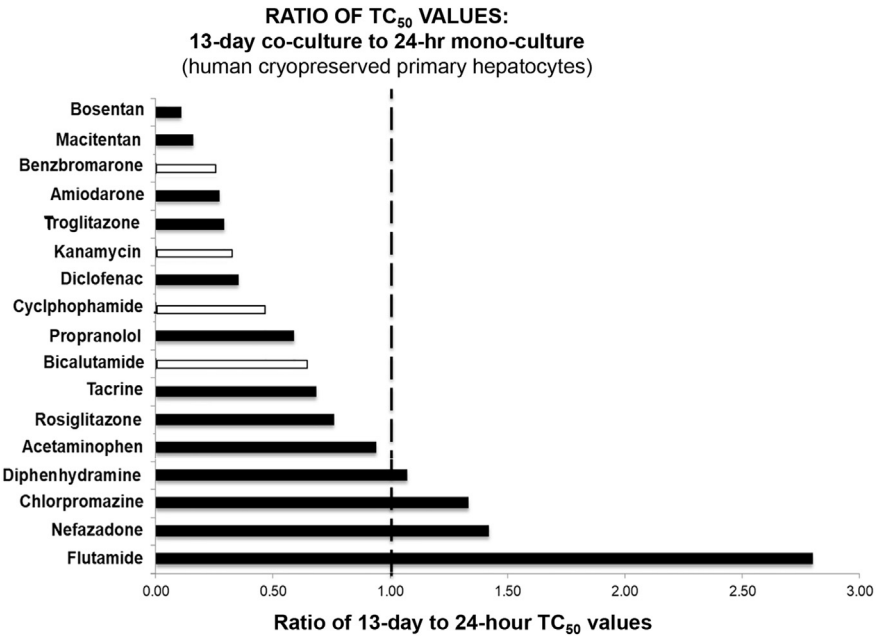

Fig. 3. Relative sensitivity of human 13-day co-culture and 24 h mono-culture to cytotoxicity. A ratio of the relative sensitivities was computed for thirteen compounds (solid bars). In four instances, it was not possible to compute a definitive ratio but the 13-day incubation demonstrated increase sensitivity compared to the 24 h mono-culture (clear bars).

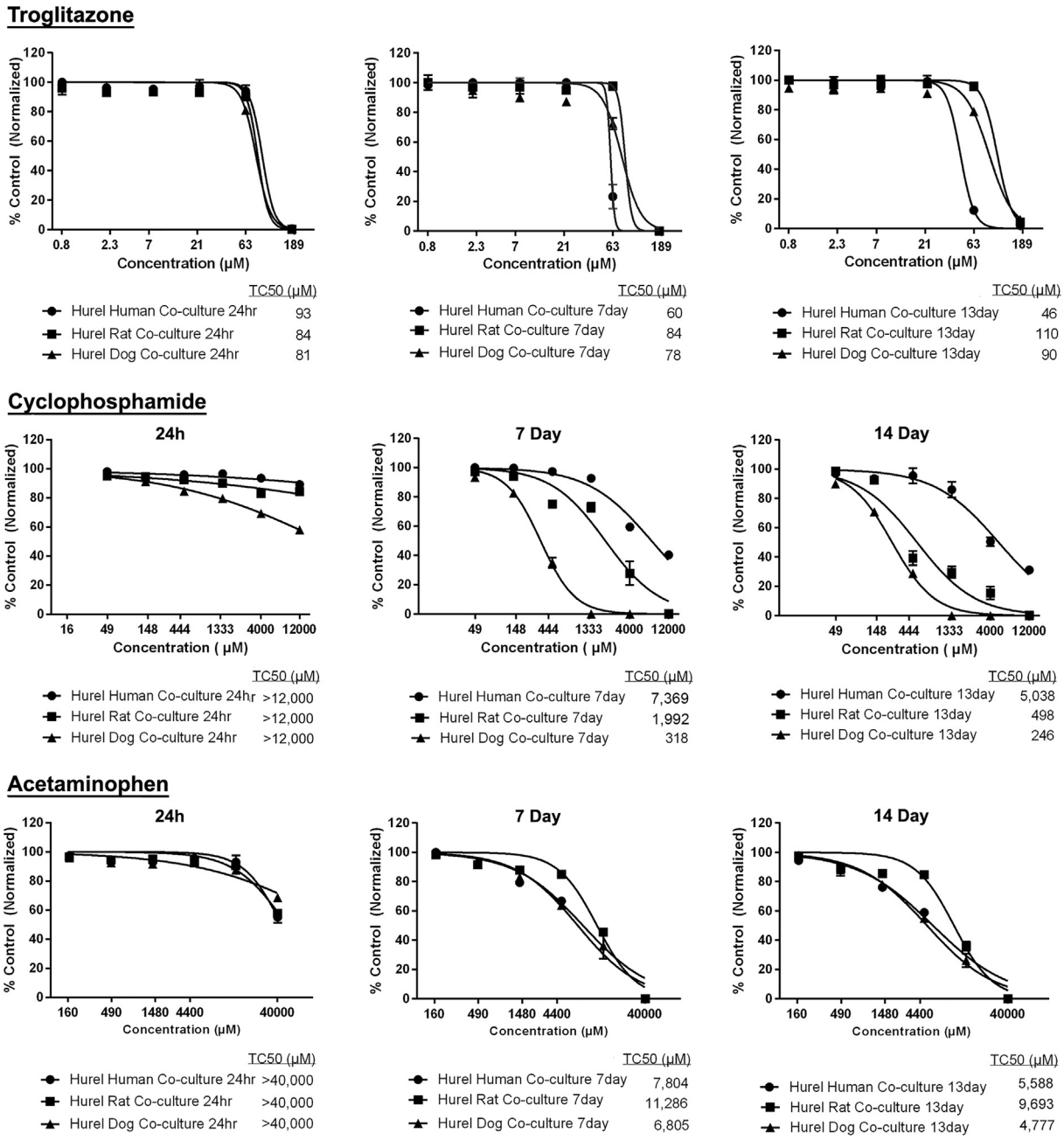

Fig. 4. Comparison of toxicity concentration curves in human, dog, and rat hepatic co-cultures after single-dose treatment at 24 h, and after 7-day and 13-day repeat-dose treatment every 48 h.

values observed in the co-culture model when measured at 24 h were higher than the equivalent measurements in the 24-hour mono-cultures, but lower when assayed after 13-day repeat-dosing. For example, with amiodarone, the human mono-culture exhibited a 24-hour TC_{50}/C_{max} ratio of 41, whereas the human co-culture ratio was > 215 following 24-hour single-dose exposure, but subsequently decreased to 11 on Day 13. Thus, a repeat-dose treatment with amiodarone in the human co-culture resulted in a TC_{50}/C_{max} value that is 3.7-fold lower than the equivalent value obtained after 24-hour exposure in the mono-culture, and that correctly identifies amiodarone as hepatotoxic. This trend appears to apply across all species. 24-hour exposure to amiodarone in the rat mono-culture resulted in a TC_{50}/C_{max} value of 38. In the rat co-culture model, a value of > 215 was observed for amiodarone following 24 h' single-dose exposure, which then decreased to a value of 11 on Day 13 following repeat-dose exposure, correctly identifying the compound as hepatotoxic in rat at a TC_{50}/C_{max} value 3.5-fold lower than the value obtained after 24-hour exposure in the mono-culture. Similar patterns of results were observed for diclofenac, chlorpromazine, benzbromarone (rat only), and bosentan (See Table 2).

3.4. Inter-species differences of hepatotoxic response

As presented in Table 2, a difference of sensitivity was observed in the identification of hepatotoxicants by the rat and human mono-cultures, with a lower incidence of True Positive predictions for human than for rat (50% *versus* 75%). Sensitivity improved at 13 days in the co-cultures of all three species. This pattern of results demonstrates the value of a repeat-dosing scheme using long-term co-cultures capable of improved over-all sensitivity.

Comparing among the three species' co-cultures, while the over-all degree of cytotoxicity predicted by the preclinical (rat and dog) and the human models was relatively similar for the majority of compounds (Table 2), troglitazone, cyclophosphamide and acetaminophen exhibited species-specific differences in levels and patterns of sensitivity

Table 3
Comparative cytotoxic responses of structurally related compounds with the same clinical indication. Sample Set I: troglitazone and rosiglitazone; bosentan and macitentan, measured by TC_{50}/C_{max}. Sample Set II: anonymized compounds A and B, measured by TC_{50}.

		Rat co-culture TC_{50}/C_{max}			Human co-culture TC_{50}/C_{max}			Dog co-culture TC_{50}/C_{max}		
Compound	**Human C_{max} (μM)**	**24 hr**	**7 day**	**13 day**	**24 hr**	**7 day**	**13 day**	**24 hr**	**7 day**	**13 day**
Troglitazone	**6.39**	**13**	**13**	**17**	**15**	**9**	**7**	**13**	**12**	**14**
Rosiglitazone	**1.04**	**620**	**517**	**351**	**678**	**408**	**328**	**724**	**170**	**150**
Bosentan	**2.00**	**469**	**158**	**50**	**807**	**104**	**41**	**721**	**102**	**63**
Macitentan	**0.30**	**2,038**	**359**	**334**	**1,706**	**316**	**347**	**3,669**	**263**	**353**

		Rat co-culture TC50/Cmax			Human co-culture TC50/Cmax			Dog co-culture TC50/Cmax		
Compound	**Human C_{max} (μM)**	**24 hr**	**7 day**	**13 day**	**24 hr**	**7 day**	**13 day**	**24 hr**	**7 day**	**13 day**
Cpd A--Sample Set 2	**N/A**	**178**	**105**	**79**	**289**	**168**	**150**	**197**	**125**	**115**
Cpd B--Sample Set 2	**N/A**	**>333**	**>333**	**>333**	**>333**	**>333**	**>333**	**>333**	**>333**	**>333**

(Fig. 4). Troglitazone had essentially identical sensitivity in the human, rat, and dog co-cultures at 24 h but at Day 13 greater sensitivity was observed in the human than in the pre-clinical models. Overall, cyclophosphamide demonstrated less toxicity in human and different patterns of toxicity in each pre-clinical species, requiring more time and repeat-dosing to emerge fully in rat, and with the greatest sensitivity being observed in dog. In the case of acetaminophen the human and dog models appear more sensitive than does the rat model (Fig. 2).

Table 4
Time-based toxicity ratio signals, 24 h to 7 days; 24 h to 13 days. Sample Set 1. Using a cutoff value of 4.0, two compounds classified as non-hepatotoxic also generated positive toxicity ratio signals: propranolol (24-hour-to-13-day ratio 4.4) and macitentan (24-h-to-7-day and 24-hour-to-13-day ratios of 5.4 and 4.9, respectively). Evaluated as a predictor of hepatotoxicity, the time-based toxicity ratio signals produced sensitivity and specificity scores of 33% and 71% when applied to the compounds of Sample Set I.

		Human Co -culture			
Compound	**Probable / possible mechanism of toxicity**	**24hr / 7day Toxicity Ratio positive (>4); negative (<4)**	**Time -Based Ratio Signal**	**24hr / 13day Toxicity Ratio positive (>4); negative (<4)**	**Time -Based Ratio Signal**
Benzbromarone	Reactive metabolite mediated	**5.5**	*positive*	**4.9**	*positive*
Amiodarone	Direct damage to lipid bilayers	**>14.5**	*positive*	**>19.3**	*positive*
Bosentan	Alterations in bile salt transport	**7.8**	*positive*	**19.7**	*positive*
Acetaminophen	Reactive metabolite mediated	**>5.1**	*positive*	**>7.2**	*positive*
Troglitazone	Complex – multifactorial	**1.6**	*negative*	**2.0**	*negative*
Nefazodone	Unknown – idiosyncratic	**1.1**	*negative*	**1.3**	*negative*
Flutamide	Unknown – idiosyncratic	**>2.3**	*negative*	**>2.4**	*negative*
Bicalutamide	Unknown – idiosyncratic – mild	**>1**	*negative*	**>1**	*negative*
Diclofenac	Idiosyncratic – probably related to acyl glucuronide formation	**2.3**	*negative*	**3.3**	*negative*
Tacrine	Unknown – no good animal model	**>1.7**	*negative*	**>2.9**	*negative*
Chlorpromazine	Cholestatic – mechanism unknown – hypersensitivity likely	**2.3**	*negative*	**2.5**	*negative*
Cyclophosphamide	Unknown – idiosyncratic	**>1.6**	*negative*	**>2.4**	*negative*
Propranolol	DILI negative	**2.9**	*negative*	**4.4**	*positive*
Rosiglitazone	DILI negative	**1.7**	*negative*	**2.1**	*negative*
Diphenhydramine	DILI negative	**>2.9**	*negative*	**>3.0**	*negative*
Isoproterenol	DILI negative	**1.0**	*negative*	**1.0**	*negative*
Kanamycin	DILI negative	**>2.3**	*negative*	**>3.3**	*negative*
Macitentan	DILI negative	**5.4**	*positive*	**4.9**	*positive*
Primidone	DILI negative	**1.0**	*negative*	**1.0**	*negative*

24h/7day tox ratio summary		**24h/13day tox ratio summary**	
True Positive Calls	33%	True Positive Calls	33%
False Negative Calls	67%	False Negative Calls	67%
True Negative Calls	86%	True Negative Calls	71%
False Positive Calls	14%	False Positive Calls	29%

Table 5
Time-based toxicity ratios' retrospective prediction of liver signals arising in pre-clinical animal studies or in Phase I clinical trials in the anonymized compounds of Sample Set II. All ten compounds were discontinued from development by Sanofi. While compound development was canceled for a variety of reasons, *in vivo* liver toxicity was observed in all compounds except negative control Compound B. Time-based toxicity ratio signals of 4.0 or greater were computed for compounds E, H, and I.

	A	B	C	D	E			F			G			
Sanofi Compound	Human Standard Culture TC50	Rat Standard Culture TC50	HμrelHuman™ TC50 Toxicity Ratio	HμrelHuman™ TC_{50} Toxicity Ratio	HμrelHuman™ Co-culture TC50			HμrelRat™ Co-culture TC50			Hμrel dog™ Co-culture TC50			Reason for withdrawal
	24hr	24hr	24hr - 7day	24hr - 13day	24hr	7day	13day	24hr	7day	13day	24hr	7day	13day	
A	165	62	1.7	1.9	289	168	150	178	105	79	197	125	115	Preclincal liver and kidney findings, did not progress to clinical trial.
B	>500	>500	1.0	1.0	>333	>333	>333	>333	>333	>333	>333	>333	>333	No preclinical liver findings
C	30	64	2.9	2.2	91	31	42	95	29	25	101	36	32	No preclinical liver findings, elevated liver enzymes in clinic trials.
D	47	38	2.0	1.3	81	40	63	75	38	32	109	45	38	Preclinical liver findings, elevated liver enzymes in clinical trials.
E	195	155	23.4	4.6	>375	16	82	>375	89	104	>375	92	140	Preclinical liver findings, elevated liver enzymes in clinical trials.
F	>1000	>1000	1.0	1.0	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	Preclinical liver findings, elevated liver enzymes in clinical trials.
G	81	78	2.2	N/A	158	71	**	88	75	74	131	72	**	Preclinical liver findings, elevated liver enzymes in clinical trials.
H	>100	>100	2.3	4.3	>300	132	69	>300	145	101	>300	91	74	Preclinical liver findings, stopped for lack of efficacy in clinical trials.
I	22	23	4.1	11.3	136	33	12	40	36	13	90	38	14	Preclincal liver findings, did not progress to clinical trial.
J	>250	>250	1.0	1.0	>750	>750	>750	>750	>750	>750	>750	>750	>750	Preclinical liver findings, elevated liver enzymes in clinical trials.

3.5. Differences in response between compounds within same therapeutic class

Another important indicator of a cell-based model's predictive value is its capacity to distinguish between structurally similar compounds within the same clinical indication but with differing hepatotoxic potential. Two pairs of chemically related compounds were included in Sample Set I and another anonymized pair was included in Sample Set II (See Table 3, compounds A and B). Troglitazone, an antidiabetic agent withdrawn from the market in 2000 due to severe adverse liver effects, was compared with rosiglitazone, an anti-diabetic that is still marketed with no reports of hepatotoxicity. Bosentan, which has a black box warning associated with moderate to severe elevations in serum aminotransferases, was compared with macitentan, which does not have a black box warning for hepatotoxicity. The co-cultures in all three species correctly identified troglitazone and bosentan as the hepatotoxic compound of each their respective, structurally related compound pairs, whereas neither the rat nor human mono-cultures correctly identified bosentan as a hepatotoxicant.

Following 13 days' repeat exposure, blinded compound A in Sample Set II, disclosed as hepatotoxic in rats *in vivo*, demonstrated greater cytotoxicity than blinded compound B, subsequently disclosed as structurally related to compound A but having shown no evidence of a hepatotoxic signal *in vivo* during preclinical studies.

3.6. Toxicity ratios afford a new, adjunctive time-based signal of hepatotoxicity

To study the degree of increase in cytotoxic response after repeat-dosing, time-based toxicity ratios were calculated by dividing each compound's TC_{50}/C_{max} value assessed at 24 h after a single treatment, divided by the TC_{50}/C_{max} value assessed after repeat-treatments, administered for either 7 or 13 days (Table 2; Table 4). Based on evaluation of the ratios produced by the non-hepatotoxicants in Sample Set I, a ratio value of 4.0 was selected as a cutoff, with values higher than 4.0 defined as positive time-based toxicity ratio signals and values lower than the cutoff classified as negative signals. Of the twelve hepatotoxic compounds of Sample Set I, four, benzbromarone, amiodarone, bosentan, and acetaminophen, generated a positive time-based toxicity ratio signal. These compounds also generated True Positive predictions of $TC_{50}/C_{max} < 100$ after repeat-dosing for both 7 and 13 days.

3.7. Retrospective application of time-based toxicity ratios to compounds discontinued from drug development

24-hour-to-7-day and 24-hour-to-13-day time-based toxicity ratios were computed for the ten compounds of Sample Set II; the results are displayed in Columns C and D of Table 5. Because average maximum plasma concentration data was not available for Sample Set II, the time-based toxicity ratios were calculated from TC_{50} values (TC_{50} 24 h/TC_{50} at 7 or 13 days) without reference to an arithmetic constant value for C_{max}. In this retrospective analysis, a positive ratio signal computed for a compound that Sanofi had discontinued from development due to observation of a liver toxicity signal is counted as a True Positive prediction, whereas a positive ratio signal for the negative control compound would be counted as a False Positive prediction.

The 24-hour-to-7-day toxicity ratio computations produced True Positive signals of 23.4 and 4.1 for compounds E and I in human, respectively. Compound E had produced liver signals in preclinical studies, but with large safety margins that allowed progression to clinical trials; however, compound E development was discontinued after producing liver toxicity in Phase I clinical trials. Development of compound I was discontinued due to the observation of liver toxicity in both dog and rat preclinical studies. The 24-hour-to-13-day toxicity ratio computations produced True Positive signals of 4.6, 4.3, and 11.3 in compounds E, H, and I, respectively. Compound H had produced preclinical liver signals, although this compound was discontinued during clinical trials primarily due to lack of efficacy. No False Positive signals were generated, as the time-based ratios computed for compound B, the negative control compound, were 1.0 for both time periods evaluated. By way of comparison, Columns A and B of Table 5 show the TC_{50} data for human and rat mono-cultures treated with the compounds from Sample Set II for 24 h.

4. Discussion

Drug induced liver injury (DILI) remains a primary cause of drug attrition in the clinic, manifesting through such diverse mechanisms as cholestasis, hepatocellular necrosis, and inflammatory reactions (hepatitis), among others. To address the need for better DILI prediction earlier in the drug discovery and development process, a number of hepatocyte-based *in vitro* models have been developed. These models include “2D” monolayers of primary hepatocytes or cell lines such as

HepG2 and HepaRG, 2D co-cultures, 3D spheroids and bioprinted cultures, perfusion systems and liver tissue slices (Lin and Khetani, 2016. Yet despite many years of academic and industrial research, there remains a distinct need to bridge the translational gap between *in vitro* DILI models and DILI as it appears *in vivo* (Usta et al., 2015).

In this study, the utility of mono-cultures of fresh rat and cryopreserved human hepatocytes to predict human hepatotoxicity was compared with that of a model consisting of cryopreserved primary hepatocytes from human, rat or dog co-cultured with a stromal cell line. A total of 29 compounds were studied, of which Sample Set I comprised 19 well-characterized molecular entities, 12 hepatotoxicants and 7 non-hepatotoxicants, for which human clinical data exists (Table 1). In addition, Sample Set II comprised 10 compounds for which Sanofi has proprietary data, 9 of which had been discontinued from drug development during pre-clinical animal studies or during Phase I or II clinical trials in which hepatotoxicity was detected. The compounds of Sample Set II were retrospectively studied to see if a long-enduring primary hepatic co-culture model might have identified the potential for hepatotoxicity that did not, until relatively late in the process, present liver signals severe enough to contribute to a decision to halt development (Table 5).

The data from Sample Set I show a concordance of 50–75% True Positives in the mono-culture models, with human mono-culture sensitivity being the lowest at 50% and greater sensitivity seen in fresh rat hepatocyte mono-culture at 75% (Table 2). Higher sensitivity was attained when these compounds were incubated in the rat or human primary hepatocyte co-culture system for 13 days, with both species' co-cultures scoring a sensitivity value of 83%. Sensitivity in the dog co-culture was the highest of all the models assessed, at 92%. The co-cultures' TC_{50}/C_{max} values measured at 24 h after a single treatment were frequently higher (less sensitive) than those observed in the mono-cultures. A possible explanation for this pattern of response is that the co-cultures are innately more robust than the mono-cultures, and therefore not only capable of maintaining their viability and cellular competency longer, but also less susceptible to initial insult by any given concentration of hepatotoxicant.

Fig. 2 shows the progression of toxicity over time for acetaminophen and cyclophosphamide in the co-cultures, with some species-specific differences evident with respect to both compounds. When exposed to cyclophosphamide, the human co-cultures were the least sensitive, rat co-cultures were intermediate, and dog co-cultures were the most sensitive. More subtle but significant patterns of differences in toxicity were seen over time when the three species were exposed to acetaminophen, with the rat co-cultures showing less sensitivity than either the human or dog co-cultures. Inter-species comparisons such as these are critical to determining the relative importance of the respective species' signals for assessing potential human risk. One study found a true positive human toxicity concordance rate of 71% for multiple species, with non-rodents being predictive for 63% of human toxicity and rodents alone for only 43% (Olson et al., 2000). Other reports have indicated species specific differences in transporter mediated uptake (Menochet et al., 2012) and metabolism (Drobna et al., 2010) of certain compounds and have linked these metabolic differences to the resulting hepatotoxicity. Having information of the type described here available early in the discovery process can influence the choice to move a compound into development, as well as provide additional insight into which pre-clinical species would be best suited for that compound's *in vivo* toxicology studies. Interspecies comparisons could also trigger additional investigations to aid in determining the mechanism of the differential response, further characterizing the liabilities of and aiding in decision making on progression of a chemical series.

The data in Table 3 show the ability of the Hurel co-culture systems to distinguish between structurally and functionally similar compounds with differing hepatotoxic potential. Macitentan and bosentan are both endothelin receptor antagonists, for the treatment of pulmonary arterial hypertension. Bosentan has a black box warning for hepatotoxicity, whereas macitentan is classified as a non-hepatotoxicant (Dingemanse et al., 2014). Macitentan was not called as a positive in any species of co-cultures, while bosentan was called as a hepatotoxicant in all species of the co-cultures after 13 days of repeat-dose treatment. Bosentan toxicity was detected only in the co-culture models and was not detected after 24-h treatment in either of the mono-culture models. It has recently been shown that bosentan produced cholestatic liver injury (Xu et al., 2015) in humanized mouse livers due to BSEP inhibition, which may also account for the results reported here. The longevity of the co-cultures may allow the necessary time for the hepatocytes to produce and accumulate bile acids in a sufficient quantity to produce a toxic outcome.

In an effort to explore whether additional, predictively useful information can be derived from the long-term repeat-dose methods described here, time-based toxicity ratios were calculated by dividing each compound's TC_{50}/C_{max} value measured at 24 h after a single dose by the TC_{50}/C_{max} value obtained after repeat-dosing for either 7 or 13 days (Table 4). In four instances (benzbromarone, amiodarone, bosentan, and acetaminophen) positive time-based toxicity ratio signals arose from Sample Set I compounds that were also correctly called positive by the TC_{50}/C_{max} scores produced in the 13-day human co-culture assay. In the other nine hepatotoxicants of Sample Set I, the time-based toxicity ratio did not predict hepatotoxicity. Positive ratio signals were also observed for macitentan and propranolol, two of the seven compounds listed as non-hepatotoxic in Sample Set I, reducing the specificity of this new metric when compared to the TC_{50}/C_{max} measurements applied to this Sample Set Thus, utilized as straightforward numerical predictors of hepatotoxicity, the time-based toxicity ratio generated sensitivity and specificity scorings significantly inferior to those afforded by the metric of TC_{50}/C_{max} applied to 13-day repeat-dose assays in the co-cultures.

The reasons for this pattern of results are not currently understood. However, all four instances in which positive time-based toxicity ratios were observed for hepatotoxicants in Sample Set I occurred with compounds known to exert their toxic effects through alterations in bile salt transport (bosentan) (Fouassier et al., 2002), reactive metabolite formation (benzbromarone) (Yoshida et al., 2017) and acetaminophen (Ramachandran and Jaeschke, 2017), or alteration of lipid metabolism (amiodarone) (Szalowska et al., 2014), phenomena generally not seen without repeat dosing over extended time periods in animals. In contrast, in the case of all the Sample Set I reference hepatotoxicants that produced negative ratio signals, the mechanism of action is classified in the literature as unknown, complex multifactorial, or idiosyncratic.

Two compounds not associated with clinical or non-clinical hepatotoxicity showed positive time-based ratio signals. Similarly to bosentan, examination of the literature on macitentan indicates the compound is capable of producing alterations in bile salt transport leading to cholestasis. Data has previously been generated which shows macitentan to be a potent inhibitor of BSEP, OATP, and other hepatic transporters, and therefore a compound capable of causing cholestatic hepatotoxicity; indeed, macitentan has been found to be a more potent inhibitor of those transporters than the structurally similar compound bosentan, which has a black box warning for hepatotoxicity (Raja and Dreyfus, 2008) and which produced high time-based toxicity ratio signals of 7.8 and 19.7 in the human co-culture after 7 and 13 days. Why bosentan is a hepatotoxicant and macitentan may simply be due to the higher potency, and therefore lower clinical dose requirement (~10-fold less) of macitentan compared to that of bosentan, which affords a greater safety margin in the case of macitentan (Dingemanse et al., 2014).

The time-based ratio signal of 4.4 calculated for propranolol was just above the chosen cutoff value of 4.0The reason for the positive time-based toxicity ratio signal being generated by this non-hepatotoxic compound is not known. Propranolol may be an example of a compound where additional studies conducted to enable a weight-of-evidence decision on toxic potential would outweigh this finding and lead to the correct conclusion of lack of relative risk. Propranolol's C_{max} of .20 μM is the lowest of any of the 19 compounds of Sample Set I, including macitentan's low C_{max} of .30 μM, suggesting that like macitentan, propranolol's high potency may be a factor contributing to its

non-hepatotoxicity (Table 2).

A further demonstration of the potential utility of the time-based toxicity ratio can be seen in the data from the evaluation of the ten proprietary Sanofi test compounds of Sample Set II. As shown in Table 5, three of the nine compounds that were discontinued and had liver toxicity signals produced positive time-based toxicity ratio signals that would have been sufficient to raise concern about the hepatotoxic potential of these molecules prior to or in parallel with the performance of pre-clinical animal studies. In a weight-of-evidence approach to decision-making on compound progression, these data may have enabled selection of other candidates without this potential for adverse effects on the liver, or informed the guidance provided to the medicinal chemists charged with minimizing the toxic liabilities associated with lead and pre-candidate compounds.

The available information suggests that time-based ratio signals may, if utilized as adjuncts to traditional TC_{50}/C_{max} or TC_{50} measurements, enhance the process of lead selection and advancement as a part of a comprehensive approach to early drug development. These ratios may signal the presence of a toxic mechanism that requires repeat-dosing over an extended time course in order to become evident; and they may therefore assist in a comprehensive data-based assessment of potential risk. Development decisions are seldom made on the basis of a single data point, but the availability of data from an assay with reliable predictive value can help to rank order compounds and flag compounds for further evaluation and/or structural modification before a decision to progress the compound is made.

In summary, the results of the present study indicate the potential of long-enduring co-cultured primary hepatocytes to enhance the predictive capability of *in vitro* screens for hepatotoxicity. Conventional screens using plated mono-cultures of freshly isolated primary hepatocytes from rats, or cryopreserved primary hepatocytes from humans, demonstrate less sensitivity in their ability to predict eventual outcome in an *in vivo* preclinical or clinical setting than do the results obtained from Hμrel co-cultures. Time-based toxicity ratios may further facilitate early decision making by indicating the presence of a mechanism of action that requires repeat-dosing to become evident. Availability of this data early in the discovery/development process has the potential to avoid late-stage failures in drug development programs.

While the data of the present study are interesting and suggest a potential improvement in the ability to predict human hepatotoxicity, there are clearly opportunities for further enhancement and improvement. The current data are based on the quantitation of ATP production. Although this endpoint is used routinely in cytotoxicity assays, additional endpoints could further enhance the predictive value of this assay system. The use of albumin secretion, for example, is one such potential improvement. In addition, microRNA's are becoming important biomarkers of cellular toxicity that can, in some cases, precede significant morphologic tissue damage and thus provide useful translational biomarkers. The use of miR122, for example, will be the subject of further exploration to determine if this endpoint can improve the overall predictive capability of the system.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2017.09.013.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouët, S., Morel, F., Guguen-Guillouzo, C., Guillouzo, A., 2006. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. Drug Metab. Dispos. 34 (1), 75–83.

Atienzar, F.A., Novik, E.I., Gerets, H.H., Parekh, A., Delatour, C., Cardenas, A., MacDonald, J., Yarmush, M.L., Dhalluin, S., 2014. Predictivity of dog co-culture model, primary human hepatocytes and HepG2 cells for the detection of hepatotoxic drugs in humans. Toxicol. Appl. Pharmacol. 275 (1), 44–61.

Bhatia, S.N., Yarmush, M.L., Toner, M., 1997. Controlling cell interactions by micropatterning in co-cultures: Hepatocytes and 3T3 fibroblasts. J. Biomed. Mater. Res. 34 (2), 189–199.

Bonn, B., Svanberg, P., Janefeldt, A., Hultman, I., Grime, K., 2016. Determination of human hepatocyte intrinsic clearance for slowly metabolized compounds: comparison of a primary hepatocyte/stromal cell co-culture with plated primary hepatocytes and HepaRG. Drug Metab. Dispos. 44 (4), 527–533.

Chao, P.-Y., Barminko, J., Novik, E., Han, Y., Cheng, K.-C., 2009. Development of a physiologically-based model using plated human hepatocyte for evaluation of metabolic clearance. Drug Metab. Lett 3, 298–306.

Dingemanse, J., Sidharta, P.N., Maddrey, W.C., Rubin, L.J., Mickail, H., 2014. Efficacy, safety and clinical pharmacology of macitentan in comparison to other endothelin receptor antagonists in the treatment of pulmonary arterial hypertension. Expert Opin. Drug Saf. 13 (3), 391–405.

Drobna, Z., Walton, F.S., Harmon, A.W., Thomas, D.J., Styblo, M., 2010. Interspecies differences in metabolism of arsenic by cultured primary hepatocytes. Toxicol. Appl. Pharmacol. 245 (1), 47–56.

Fouassier, L., Kinnman, N., Lefevre, G., Lasnier, E., Rey, C., Poupon, R., Elferink, R.P., Housset, C., 2002. Contribution of mrp2 in alterations of canalicular bile formation by the endothelin antagonist bosentan. J. Hepatol. 37 (2), 184–191.

García-Cañaveras, J.C., Jiménez, N., Gómez-Lechón, M.J., Castell, J.V., Donato, M.T., Lahoz, A., 2015. LC-MS untargeted metabolomic analysis of drug-induced hepatotoxicity in HepG2 cells. Electrophoresis 36 (18), 2294–2302.

Green, C.E., Dabbs, J.E., Tyson, C.A., 1983. Functional integrity of isolated rat hepatocytes prepared by whole liver vs biopsy perfusion. Anal. Biochem. 129 (2), 269–276.

Hewitt, N.J., Lechon, M.J., Houston, J.B., Hallifax, D., Brown, H.S., Maurel, P., Kenna, J.G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A.P., LeCluyse, E., Groothuis, G.M., Hengstler, J.G., 2007. 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), 159–234.

Kanebratt, K.P., Andersson, T.B., 2008. Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. Drug Metab. Dispos. 36 (7), 1444–1452.

Kidambi, S., Yarmush, R.S., Novik, E., Chao, P., Yarmush, M.L., Nahmias, Y., 2009. Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance. Proc. Natl. Acad. Sci. U. S. A. 106 (37), 15714–15719.

Lin, C., Khetani, S.R., 2016. Advances in Engineered Liver Models for Investigating Drug-Induced Liver Injury. 2016. pp. 1829148.

Menochet, K., Kenworthy, K.E., Houston, J.B., Galetin, A., 2012. Use of mechanistic modeling to assess interindividual variability and interspecies differences in active uptake in human and rat hepatocytes. Drug Metab. Dispos. 40 (9), 1744–1756.

O'Brien, P.J., Irwin, W., Diaz, D., Howard-Cofield, E., Krejsa, C.M., Slaughter, M.R., Gao, B., Kaludercic, N., Angeline, A., Bernardi, P., Brain, P., Hougham, C., 2006. 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 (9), 580–604.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., Dorato, M., Van Deun, K., Smith, P., Berger, B., Heller, A., 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul. Toxicol. Pharmacol. 32 (1), 56–67.

Raja, S.G., Dreyfus, G.D., 2008. Current status of bosentan for treatment of pulmonary hypertension. Ann. Card. Anaesth. 11 (1), 6–14.

Ramachandran, A., Jaeschke, H., 2017. Mechanisms of acetaminophen hepatotoxicity and their translation to the human pathophysiology. J. Clin. Trans. Res. 3 (Suppl. 1), 157–169.

Russmann, Stefan, K-U, G.A., Grattagliano, Ignazio, 2009. Current concepts of mechanisms in drug-induced hepatotoxicity. Curr. Med. Chem. 16 (23), 3041–3053.

Smith, C.M., Nolan, C.K., Edwards, M.A., Hatfield, J.B., Stewart, T.W., Ferguson, S.S., Lecluyse, E.L., Sahi, J., 2012. A comprehensive evaluation of metabolic activity and intrinsic clearance in suspensions and monolayer cultures of cryopreserved primary human hepatocytes. J. Pharm. Sci. 101 (10), 3989–4002.

Szalowska, E., van der Burg, B., Man, H.Y., Hendriksen, P.J., Peijnenburg, A.A., 2014. Model steatogenic compounds (amiodarone, valproic acid, and tetracycline) alter lipid metabolism by different mechanisms in mouse liver slices. PLoS One 9 (1), e86795.

van Tonder, Jacob John, S., V., G., M., 2013. Pre-Clinical Assessment of the Potential Intrinsic Hepatotoxicity of Candidate Drugs.

Usta, O.B., McCarty, W.J., Bale, S., Hegde, M., Jindal, R., Bhushan, A., Golberg, I., Yarmush, M.L., 2015. Microengineered cell and tissue systems for drug screening and toxicology applications: evolution of in-vitro liver technologies. Technology (Singap World Sci) 3 (1), 1–26.

Xu, J.J., Henstock, P.V., Dunn, M.C., Smith, A.R., Chabot, J.R., de Graaf, D., 2008. Cellular Imaging Predictions of Clinical Drug-Induced Liver Injury. Toxicol. Sci. 105 (1), 97–105.

Xu, D., Wu, M., Nishimura, S., Nishimura, T., Michie, S.A., Zheng, M., Yang, Z., Yates, A.J., Day, J.S., Hillgren, K.M., Takeda, S.T., Guan, Y., Guo, Y., Peltz, G., 2015. Chimeric TK-NOG mice: a predictive model for cholestatic human liver toxicity. J. Pharmacol. Exp. Ther. 352 (2), 274–280.

Yoshida, M., N. Cho, H. Akita and K. Kobayashi (2017). "Association of a reactive intermediate derived from 1′,6-dihydroxy metabolite with benzbromarone-induced hepatotoxicity.".