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

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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

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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