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Cytotoxicity evaluation using cryopreserved primary human hepatocytes in various culture formats

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ABSTRACT

Sixteen training compounds selected in the IMI MIP-DILI consortium, 12 drug-induced liver injury (DILI) positive compounds and 4 non-DILI compounds, were assessed in cryopreserved primary human hepatocytes. When a ten-fold safety margin threshold was applied, the non-DILI-compounds were correctly identified 2 h following a single exposure to pooled human hepatocytes (n = 13 donors) in suspension and 14-days following repeat dose exposure (3 treatments) to an established 3D-microtissue co-culture (3D-MT co-culture, n = 1 donor) consisting of human hepatocytes co-cultured with non-parenchymal cells (NPC). In contrast, only 5/12 DILI-compounds were correctly identified 2 h following a single exposure to pooled human hepatocytes in suspension. Exposure of the 2D-sandwich culture human hepatocyte monocultures (2D-sw) for 3 days resulted in the correct identification of 11/12 DILI-positive compounds, whereas exposure of the human 3D-MT co-cultures for 14 days resulted in identification of 9/12 DILI-compounds; in addition to ximelagatran (also not identified by 2D-sw monocultures, Sison-Young et al., 2016), the 3D-MT co-cultures failed to detect amiodarone and bosentan. The sensitivity of the 2D human hepatocytes co-cultured with NPC to ximelagatran was increased in the presence of lipopolysaccharide (LPS), but only at high concentrations, therefore preventing its classification as a DILI positive compound. In conclusion (1) despite suspension human hepatocytes having the greatest metabolic capacity in the short term, they are the least predictive of clinical DILI across the MIP-DILI test compounds, (2) longer exposure periods than 72 h of human hepatocytes do not allow to increase DILI-prediction rate, (3) co-cultures of human hepatocytes with NPC, in the presence of LPS during the 72 h exposure period allow the assessment of innate immune system involvement of a given drug.

Abbreviations: DILI, drug-induced liver injury; NPC, non-parenchymal cells; 2D-sw, 2D-sandwich; SM, safety margin; MT, microtissue; TC, training compound.

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1. Introduction

Drug-induced-liver injury (DILI) continues to pose significant problems in drug development despite extensive screening during early development, suggesting that currently used *in vitro* models are not appropriate for effective screening or data not used effectively. Within the Innovative Medicines Initiative (IMI)-funded consortium 'Mechanism-based Integrated Systems for the Prediction of Drug-Induced Liver Injury' (MIP-DILI) an assessment of various cell types has been undertaken to determine the usefulness of simple 2D cell models, in the absence of prior human exposure data, for determining the hepatotoxic potential of a series of selected training compounds (Sison-Young et al., 2016). Eight of the 9 training compounds (TCs) incubated with either freshly isolated or cryopreserved primary human hepatocytes for 3 days in 2D-sandwich (2D-sw) monoculture were correctly identified when nominal *in vitro* concentrations were adjusted for *in vivo* exposure levels. Human hepatocytes exposed for 1 day or 3 days to TCs in 2D-sw monoculture, could not completely distinguish between established drugs with respect to their propensity to cause DILI in man. In particular, ximelagatran was not identified as a drug presenting a risk of inducing DILI in man, and entacapone was classified as a false-positive. However, assigning sensitivity and specificity of assays across such a small compound set is not useful.

Human hepatocytes undergo significant and irreversible modification in their transcriptomic profile at attachment to culture matrix (Richert et al., 2006), with down-regulation of phase I and II enzymes. Transporter mRNA levels are not affected by plating to the same extent as phase I and II enzymes. Instead down-regulation is moderate and, for some transporters, levels are even stable or up-regulated as also observed by others (Jigorel et al., 2005). Phase I metabolism rates are also significantly reduced following plating (Blanchard et al., 2004; Smith et al., 2012), whereas phase II metabolism rates are less reduced (Alexandre et al., 2002; den Braver-Sewradj et al., 2016). This has been partly attributed to the regeneration of co-factors in culture, suggesting a shift of phase I/phase II ratio towards phase II in cultured hepatocyes. Human hepatocytes in suspension, either freshly isolated or after cryopreservation, present a transcriptomic profile similar to liver *in vivo* (Richert et al., 2006), and their use in suspension has become a widely accepted model for prediction of *in vivo* metabolism (Jouin et al., 2006), drug–drug interactions through cytochrome P450 inhibition (Mao et al., 2012; Desbans et al., 2014) and have been used for assessment of drug and metabolite toxicity (Elaut et al., 2006). Although a major drawback of the use of hepatocytes in suspension is their short life span, *i.e.* up to several hours, limiting the contact time of TCs with cells, pools of cryopreserved primary human hepatocytes used in suspension have been recently described as useful for the screening of hepatotoxicants (Mennecozzi et al., 2015). The first aim of the present study was thus to compare the cytotoxicity profiles obtained with human hepatocytes in suspension exposed for 2 h to the mean data (partly from Sison-Young et al., 2016) obtained with 2D-sw monocultures exposed at day 2 after plating for 24 h. In order to do so, a pool of cryopreserved human hepatocytes was maintained in suspension under continuous shaking that has been shown to allow high viability and increase the metabolic performance of the cells (Simon et al., 2009).

The second aim of the present study was to compare the cytotoxicity profiles in established cultures of human hepatocytes exposed for longer periods of time (up to 14 days) to the mean data (partly from Sison-Young et al., 2016) obtained in 2D-sw mono cultures exposed for 72 h from day 2 after plating. Indeed, in recent years, hepatocyte culture systems allowing culture (and exposure) times up to several weeks have been developed, such as modified 2D-sw monocultures (Parmentier et al., 2013) as well as 2D co-cultures (Khetani et al., 2013) and 3D-monocultures and co-cultures (Darnell et al., 2012; Messner et al., 2013).

In the present study, cryopreserved plateable human hepatocytes were used since only minimal differences are reported in the phenotype between freshly isolated and cryopreserved primary human hepatocytes (Darnell et al., 2012; Smith et al., 2012), and in their direct response to toxicants (Sison-Young et al., 2016).

Sixteen TCs chosen by the consortium were tested. From their highest concentration without a clear cytotoxic effect, defined as inducing no more than 20% cell death (Bordessa et al., 2014), and their C_{max} *in vivo*, a safety margin (SM) was calculated. An SM <10 was set as identification of a DILI risk (Mueller et al., 2015). Finally, the effect of an inflammation stimulus on the cytotoxicity of specific TCs was evaluated.

2. Material and methods

2.1. Pooled cryopreserved human hepatocyte suspensions

The pool of cryopreserved primary human hepatocytes P0203T (n = 13 donors) was provided by KaLy-Cell (Plobsheim, France). The pooled hepatocytes were thawed in a water-bath (1–2 min) and diluted in 50 mL KLC-Thawing Medium (KLC-TM; proprietary formulation); centrifuged 170*g*; 20 min; room temperature, washed (KLC-Washing Medium (KLC-WM; proprietary formulation)); 100*g*; 5 min; room temperature and re-suspended in KLC-Suspension Medium (KLC-SuM; proprietary formulation). Cell number and viability were determined by the trypan blue exclusion method. After dilution to a concentration of 2×10^6 viable cells/mL in medium, the hepatocyte suspension was distributed into eight 96-well plates (50 μL/well). The plates were pre-incubated for approximately 15 min under shaking (900 rpm) in a humidified chamber at 37 °C with 5% CO_2.

2.2. Cryopreserved human hepatocyte cultures

2.2.1. 2D-sw monoculture

Cryopreserved primary human hepatocytes (list of donors in Table 1) provided by KaLy-Cell (Plobsheim, France) were thawed (1–2 min in water bath) and diluted in 50 mL KLC-Thawing Medium (KLC-TM; proprietary formulation); centrifuged 170*g*; 20 min; room temperature, washed with KLC-Washing Medium (KLC-WM; proprietary formulation); 100*g*; 5 min; room temperature and re-suspended in KLC-Seeding Medium (KLC-SM; proprietary formulation). Cell number and viability were determined by the trypan blue exclusion method. The cells were plated at a seeding density of 0.07×10^6 viable cells/well of a KaLy-Cell home-coated type I rat tail collagen (10 μg/well) 96-well plate. The cells were allowed to attach for 4–6 h (in a humidified chamber at 37 °C with 5% CO_2) after which the cells were overlaid with 0.25 mg/mL matrigel in KLC-SM for a sandwich like configuration culture (2D-sw) and left to incubate overnight (in a humidified chamber at 37 °C with 5% CO_2). Cells were used for analysis if the attachment efficiency was greater than 80%. Serum-free KLC-Maintenance Medium (KLC-MM) was used for compound treatment.

2.2.2. 2D co-culture

Cryopreserved primary human hepatocytes (donors JNB and 1307) obtained from Bioreclamation IVT (Baltimore, US) and Hepregen (Medford, MA) respectively, and cryopreserved 3T3 J2 mouse fibroblasts obtained from Howard Green at Harvard University (US) were used in the manufacturing of the human micropatterned co-culture platform (HepatoPac®, Hepregen Corporation). The HepatoPac cultures were prepared at Hepregen according to their own protocol (Khetani et al., 2013). In brief,