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Toxicogenomics-based prediction of acetaminophen-induced liver injury using human hepatic cell systems

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HIGHLIGHTS

- hHEP, HepaRG, HepG2 and hSPK-HPC are investigated for their capacity to predict hepatic toxicity.
- Cells exposed to acetaminophen are compared to clinical liver samples of acute liver failure.
- Transcriptomics analysis show comparable hepatotoxic functions in hHEP, HepaRG and hSKP-HPC.
- HepaRG shows the highest prediction of 'damage of liver', followed by hSKP-HPC and hHEP cells.
- HepG2 shows the slightest response to APAP and do not show activation of 'damage of liver' function.

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ABSTRACT

Primary human hepatocytes (hHEP), human HepaRG and HepG2 cell lines are the most used human liver-based *in vitro* models for hepatotoxicity testing, including screening of drug-induced liver injury (DILI)—inducing compounds. hHEP are the reference hepatic *in vitro* system, but their availability is limited and the cells available for toxicology studies are often of poor quality. Hepatic cell lines on the other hand are highly proliferative and represent an inexhaustible hepatic cell source. However, these hepatoma-derived cells do not represent the population diversity and display reduced hepatic metabolism. Alternatively, stem cell-derived hepatic cells, which can be produced in high numbers and can differentiate into multiple cell lineages, are also being evaluated as a cell source for *in vitro* hepatotoxicity studies. Human skin-derived precursors (hSKP) are post-natal stem cells that, after conversion towards hepatic cells (hSKP-HPC), respond to hepatotoxic compounds in a comparable way as hHEP. In the current study, four different human hepatic cell systems (hSKP-HPC, hHEP, HepaRG and HepG2) are evaluated for their capacity to predict hepatic toxicity. Their hepatotoxic response to acetaminophen (APAP) exposure is compared to data obtained from patients suffering from APAP-induced acute liver failure (ALF). The results indicate that hHEP, HepaRG and hSKP-HPC identify comparable APAP-induced hepatotoxic functions and that HepG2 cells show the slightest hepatotoxic response. Pathway analyses further points out that HepaRG cells show the highest predicted activation of the functional genes related to 'damage of

Abbreviations: ALF, acute liver failure; APAP, *N*-acetyl-*p*-aminophenol/acetaminophen/paracetamol; DILI, drug-induced liver injury; ESC, embryonic stem cells; hHEP, primary human hepatocytes; hSKP, human skin-derived precursors; hSKP-HPC, hSKP-derived hepatic progenitor cells; IPA, Ingenuity Pathways Analysis; iPSC, induced pluripotent stem cells; NAPQ, *N*-acetyl-*p*-benzoquinoneimine; NCE, new chemical entities.

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liver', followed by hSKP-HPC and hHEP cells that generated similar results. HepG2 did not show any activation of this function.

1. Introduction

At least 1200 drugs have been reported to exhibit potential hepatic toxicity (Biour et al., 2004). DILI is responsible for about half of the cases of ALF and is the main cause of liver transplantations (Mindikoglu et al., 2009; Prakash and Vaz, 2009). APAP (*N*-acetyl-*p*-aminophenol; acetaminophen) or paracetamol as it is mostly referred to in Europe, is a widely used over-the-counter analgesic and antipyretic drug. APAP overdose is the most common cause of acute liver injury leading to ALF (James et al., 2003; Blachier et al., 2013; Larson et al., 2005). At therapeutic levels, APAP is detoxified by glucuronidation and sulfatation and metabolised by cytochromes P450 enzymes to *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is subsequently neutralized by conjugation with glutathione. However, when taken in overdose, NAPQI production exceeds the clearance capacity of glutathione, leading to its hepatic accumulation and binding to DNA, lipids and cysteine groups in proteins (James et al., 2003). Ingestion of a single dose of 10 g can already induce levels of hepatic necrosis leading to liver injury (Whitcomb and Block, 1994).

Despite improvements in toxicological research, the overall frequency of drug hepatotoxicity has not decreased in the last 15 years (Blachier et al., 2013). One of the reasons for this problem is the poor detection of hepatotoxicity early during drug development. The discrepancies between preclinical studies with animal species and liver injury observed in humans, point to the fact that the current methodology applied by the pharmaceutical industry, does not allow to efficiently evaluate the potential hepatotoxicity of new chemical entities (NCE). It is believed that a shift from the use of animal testing towards alternative methods, such as application of human cell-based *in vitro* hepatic models, could result in a better evaluation of the hepatotoxic potential of NCE. Especially the relative low cost of *in vitro* experiments *versus* animal testing and the circumvention of interspecies extrapolation, make their use attractive. hHEP are today the gold standard for xenobiotic metabolism and cytotoxicity studies as they are thought to mimic the *in vivo* liver functionality most accurately. However, the availability of fresh human liver samples is very limited. Furthermore, isolated hepatocytes only have a short lifespan in culture (Guguen-Guillouzo and Guillouzo, 2010). Hepatic cell lines, such as HepG2 and HepaRG, are also extensively used in toxicological studies. HepG2 cells were originally isolated from a hepatocellular carcinoma and have shown to display several genotopic features of liver cells (Sassa et al., 1987). However, these cells have a much lower metabolic capacity compared to primary hepatocytes (Xu et al., 2004). HepaRG cells were also isolated from a hepatocellular carcinoma and at their most differentiated state they are composed of biliary-like and hepatocyte-like cells (Gripon et al., 2002). HepaRG display hepatic functions and express liver-specific genes at similar levels of primary hepatocytes. Therefore these cells are frequently used as an alternative to primary hepatocytes (Anthérieu et al., 2012; Guguen-Guillouzo et al., 2010; Guillouzo et al., 2007; Rodrigues et al., 2013). More recently, human stem cells were also considered as potential cell sources for hepatotoxicity testing (Rodrigues and De Kock, 2014; Szkolnicka et al., 2014; Chapin and Stedman, 2009; De Kock et al., 2012). Besides pluripotent embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) also multipotent postnatal stem cells are receiving increasing attention. Our group has previously shown that hSKP have the ability to differentiate into cells expressing relevant hepatic markers (hSKP-HPC) (Rodrigues et al., 2014; De Kock et al., 2009). A combination of both markers of immature and adult hepatocytes is expressed in the differentiated cells at the protein and gene level (Rodrigues et al., 2014; De Kock et al., 2009). Using a toxicogenomics approach, it was demonstrated that these cells could be employed in the evaluation of the hepatotoxicity potential of several hepatotoxic compounds (De Kock et al., 2009; Rodrigues et al., 2015a). In the current study, the response to APAP is evaluated in a set of commonly used human hepatic cell systems *i.e.* hHEP, HepaRG and HepG2 as well as in hSKP-HPC. The capacity to predict APAP-induced ALF is evaluated by comparing the hepatotoxic responses of the different cell types exposed to APAP to human liver samples from patients suffering from APAP-induced ALF.

2. Material and methods

2.1. Isolation, culture and hepatic differentiation of human skin-derived stem cells

hSKP were isolated from small skin segments obtained by circumcision of boys between 1 and 10 year old. Informed consent of the parents of the donors was obtained under the auspices of the Ethics Committee of the '*Vrije Universiteit Brussel*' and the '*Universitair Ziekenhuis Brussel*'. Cell isolation and culture were performed as previously described (De Kock et al., 2009). The cells were seeded at a density of 7.5×10^4 viable cells per mL (3×10^4 viable cells per cm^2) and cultured for 2 weeks in a 5% (v/v) CO_2-humidified air incubator at 37 °C. The culture medium used was composed of DMEM + GLUTAMAX/F12 Nutrient Mixture (3:1; Life Technologies) supplemented with 7.33 IU/mL benzyl penicillin (Continental Pharma), 50 mg/mL streptomycin sulfate (Sigma–Aldrich), 2.5 mg/mL fungizone, 2% (v/v) B27 Supplement (Life Technologies), 40 ng/mL basic fibroblast growth factor (FGF) 2 (Promega) and 20 ng/mL epidermal growth factor (EGF) (Promega). After 2 weeks, hSKP formed three-dimensional spheres that were passaged using 0.2 mg/mL Liberase DH solution (Roche Applied Science) and seeded at a density of 1.3×10^4 cells per cm^2 for further culture as monolayers. At this point, the cells were either differentiated to hepatic cells or cryopreserved for later use. All experiments with hSKP were performed in triplicate. Hepatic differentiation of hSKP was carried out as previously documented (Rodrigues et al., 2015) using a 24-day protocol in which subconfluent cells were exposed in a time-dependent manner to the following growth factors and cytokines: Activin A (Life Technologies), FGF4 (Biosource), bone morphogenetic protein 4 (BMP4), hepatocyte growth factor (HGF) (Life Technologies), insulin-transferin-sodium selenite solution (ITS) (Sigma-Aldrich), dexamethasone (Sigma–Aldrich) and oncostatin M (Life Technologies). The obtained differentiated hepatocyte-like cells are further referred to as hepatic progenitor cells obtained from human skin-derived precursors (hSKP-HPC).

2.2. Cell culture of hepatic cell lines (HepG2 and HepaRG)

HepG2 cells (ATCC; clone HB-8065) were recovered from liquid nitrogen and were cultured in 75 cm^2 tissue culture flasks (Falcon) in a humidified incubator (37 °C, 5% (v/v) CO_2). The cell culture medium was composed of DMEM (Lonza) containing 10% (v/v) bovine calf serum (Gibco). The cells were passaged at sub