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Expert Opinion

Human hepatocyte systems for *in vitro* toxicology analysis

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Abstract. Drug induced liver injury (DILI) is still the leading single cause of drug failure during clinical phases and after market approval. Currently, many laboratories aim to develop appropriate *in vitro* systems to predict drug hepatotoxicity. Primary human hepatocytes are still the gold standard, but they have substantial disadvantages such as rapid dedifferentiation *in vitro* and lack of cell proliferation. In addition to primary human hepatocytes, liver cancer-derived cell lines such as HepG2, cytochrome P450 (CYP450) overexpressing HepG2 cell clones and HepaRG were studied intensively. In contrast to HepG2, HepaRG show promising characteristics of differentiated primary human hepatocytes, but they represent only one donor. There is some hope that this lack of donor variability can be solved by the use of iPS-derived hepatocytes. However, iPS technology still seems to need some improvement to produce physiologically relevant hepatocytes. Upcyte hepatocytes represent the most recent technical advancement combining some features of primary human hepatocytes such as physiological activity and donor variability with the ability of cell lines for extended proliferation. Altogether, more work is needed to develop and validate appropriate *in vitro* systems for precise prediction of DILI risk.

Keywords: Biotransformation, CYP450, DILI, HepaFH cells, HepaRG, HepG2, iPS, pluripotent stem cells, upcytes

Abbreviations

DILI	drug-induced liver injury
ESCs	embryonic stem cells
iPS	induced pluripotent stem cells
pHHs	primary human hepatocytes

1. Background

With a worldwide estimated annual incidence of 13.9–24 per 100,000 persons, drug induced liver injury (DILI) remains an economical challenge for public health systems and is the leading cause of acute liver failure in US, Europe and industrialized Asiatic states [1]. Hyman Zimmermann (1914–1999), who was an US American pioneer of clinical hepatology, observed a general rule for detecting DILI in patients. This so-called "Hy's law" defines DILI as a combination of jaundice with hepatocellular injury detected by elevated serum levels of liver enzymes such as ALT and AST, but without existing cholestasis. In addition, patients must be negative for preexisting liver diseases that also can cause jaundice such as virus-induced hepatitis, liver damages by chronic alcohol abuse or non-alcoholic steatohepatitis (NASH). There are many studies showing that DILI patients have to face

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a mortality or liver transplantation rate of at least 5–10% [2, 3]. Moreover, in past 50 years DILI was the leading single cause of drug failure during clinical phases and after market approval [4, 5]. Therefore, regulatory authorities such as the Food and Drug Administration (FDA) recommended an industry guidance to detect DILI potentials of drug candidates as early as possible [6]. A major challenge is that DILI can occur in very small numbers of susceptible individuals at doses which normally are well tolerated. Very likely, this idiosyncratic DILI depends on individual genetic predispositions and multifactorial mechanisms.

In order to improve DILI prediction, numerous attempts have been undertaken such as to develop tools for risk assessment based on biomarkers [2, 7, 8] and to integrate experimental data into mathematical models [9]. Currently, many research groups are focusing on *in vitro* DILI models with human hepatocytes [10] because especially the risk for idiosyncratic DILI turned out to be almost undetectable in preclinical animal experiments [11, 12]. In order to assess the effect of liver biotransformation on drug hepatotoxicity, six different cytochrome P450 (CYP) isoenzymes are in focus. These are CYP1A2, –2C9, –2C19, –2D6, –2E1 and –3A4 which are responsible for more than 90% of oxidative biotransformation activity on human drugs [13]. Here we will compare the pros and cons of used hepatocyte models with respect to their biotransformation activity.

2. Primary human hepatocytes

Freshly isolated primary human hepatocytes (pHHs) are the gold standard for investigations on biotransformation of drugs and their potential hepatotoxicity. Most protocols for pHH isolation involve usage of collagenase preparations from *Clostridium histolyticum* which, however, might contain bacterial LPS endotoxin contaminations that potentially can cause activation of cytokines leading to an inflammatory response [14, 15]. This can be one reason for substantial and fast dedifferentiation processes observed during primary cultivation of human hepatocytes. Subsequently, enzyme mixtures with better defined collagenase preparations were developed [16]. Further factors that might cause observed dedifferentiation are (I) loss of normal cell polarity during tissue disruption [14, 17] and (II) downregulation of liver-specific transcription factors with consequent diminution of most phase I and phase II protein expressions [18, 19]. Consequently, strategies to improve pHH performance focused on the application of new cell culture surfaces to mimic extracellular matrix and to allow for repolarization [17, 18, 20]. In addition, better-defined cell culture media [21, 22] and cell exposure to chemicals that influence gene expression or epigenetic procedures [17, 23] were shown to further improve liver-specific characteristics of cultured pHHs. *In vivo*, hepatocytes embed into a complex 3D tissue structure and do show polarity with one cell side faced to the bile canaliculi and the other one oriented to liver sinusoidal endothelial cells. It is thus not surprising that pHH performance turned better upon co-culturing with endothelial cells [24, 25] and through formation of 3D organizations in spheroids [26] or by employing micro-patterned structures [27]. Despite all these attempts, use of pHHs still is limited by problems such as scarcity of tissue material, donor variability [28], and lack of cell proliferation [14].

3. HepG2 cells

Until now, there are far more than ten thousand scientific reports in PubMed on features and applications of HepG2 in combination with liver. HepG2 cells were established in 1979 from the liver tissue of a 15-year old Caucasian male [29]. In this original paper and in far most scientific reports thereafter, HepG2 cells were attributed as human hepatocellular carcinoma (hepatoma) cells. However, a reevaluation of the original histological data combined with new molecular genetic investigations

provided strong evidence that HepG2 cells indeed were derived from a hepatoblastoma [30]. In contrast to cell cycle-arrested non-transformed pHHs, HepG2 cells are highly capable for proliferation. Despite of their cancer origin, HepG2 cells retained some interesting features of differentiated hepatocytes such as albumin secretion [31], insulin-stimulated glycogen synthesis [32] and glutathione-based detoxification [33, 34], making them a popular tool for studies on liver functions. This includes *in vitro* study on DILI [35].

Unfortunately, some important metabolic activities of mature hepatocytes are missing such as urea formation due to lack of activity of certain enzymes of the ammonia detoxification cycle [36]. Because of this deficiency and because of their cancerous nature, HepG2 cells do not give promise as cell source for bioartificial liver devices. A further disadvantage of HepG2 cells is the very low or even the lack of functional expression of almost all relevant human liver phase I (except CYP2B6) and phase II enzymes [37–40].

Since expressions of most CYP450 enzymes are downregulated in HepG2 cells, there have been many attempts to overexpress human CYP450 cDNAs recombinantly. This had been accomplished by using stable plasmid transfection [41], or transient overexpression by using adenoviral vectors [42, 43]. Both approaches do have advantages and disadvantages: Using plasmid transfection, stable cell clones can be generated, but recombinant gene expression levels are largely dependent on plasmid integration sites and DNA interaction with the nuclear matrix [44, 45]. Since recombinant adenoviral genomes usually stay extra-chromosomally due to the virus' life cycle, gene expression initially is strong, but then attenuates along with cell proliferation [46, 47]. More recently, some groups employed lentiviral vectors for both strong and long-term gene expression of recombinant CYP450 enzymes [48, 49].

4. HepaRG cells

Using a selection process on isolated liver tumor cells from a Hepatitis C virus infected female, Gripon and colleagues established the bipotent cell line HepaRG [50]. The authors showed that HepaRG cells proliferate as progenitor cell type by using a fetal calf serum-containing growth medium, but can differentiate into hepatocyte-like cells upon incubation with growth medium plus 2% DMSO for two weeks. Differentiated HepaRG form colonies of cells with typical hepatocyte morphology and bile canaliculus-like structures surrounded by biliary epithelial-like cells [51]. Interestingly, differentiated HepaRG cells display activities of relevant phase I enzymes, most of them within the range shown for pHHs. It should be noted, however, that different laboratories obtained disparate results for CYP1A2 and –3A4 enzyme activities (Table 1), although HepaRG cells were derived from only one donor. Differentiated HepaRG cells also showed expression of relevant phase II genes which were only slightly lower (GSTA4, GSTM1) or even higher (GSTA1/2, UGT1A1) than that of freshly isolated pHHs. These observed biotransformation activities made the authors conclude that HepaRG cells should be "a reliable surrogate to human hepatocytes for drug metabolism and toxicity studies" [51]. Indeed, whole genome expression analyses upon exposure to known cytotoxic agents revealed that HepaRG resembled freshly isolated pHHs much more as was the case for HepG2 cells [52]. Interestingly, using 38 drugs with classified DILI risk according to FDA standard and a drug concentration for cell culture experiments set at 100-fold the therapeutic maximum plasma concentration, HepaRG were shown to predict the DILI risk with a sensitivity of 87% sensitivity and 87% specificity [53]. The following disadvantages are known for HepaRG: First, the cell line was derived from an individual with poor metabolizer alleles for CYP2D6, CYP3A5 and, to a lesser extent, also CYP2C9 [50, 54, 55]. Next to CYP3A4, CYP2D6 is the second most important phase I enzyme metabolizing about 30 % of all approved drugs [13]. Second, monolayer cultures of HepaRG cells have no detectable urea production [56]. Some ammonia detoxification function, however, could be rescued if HepaRG cells

Table 1
CYP450 enzyme activities of *in vitro* systems

Specific activity[1]	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A4	Reference[2]
Freshly isolated pHHs	9.2–68	283–395				212–1970	[73, 74]
Cryopreserved pHHs	2–20	1–2.6	1.8–46.6	0.8–2.5	0.18–0.9	10–104	[62, 66, 75, 76]
HepG2	0.75–1.8	0.6		0.01		0.015–4.9	[62, 73]
HepG2-1A2	120						JHK[3], unpublished
HepG2-2C19				85			(Steinbrecht et al., manuscript submitted)
HepG2-3A4						600	[48]
HepaRG	17.5–154.6	37.6–50	13.1–20	10	3.5	100–1160	[51, 73, 76]
iPS-derived hepatocytes	0.2–2	0.001–0.009	0.0075–4	0.04–0.13	0.15–0.4	0.5–5.5	[61, 62]
Human ESC-derived hepatocytes	0.15	0.0015	0.01			0.1	[62, 77]
Upcyte hepatocytes	0.7–3.4	20.9–161	4.8–29.1		0.1–16	21.4–225	[66, 71]

[1]pmol/mg protein/min. [2]data show values from optimal conditions of each reference. [3]Jan-Heiner Küpper, BTU Cottbus-Senftenberg.

are maintained as three-dimensional cultures [57]. Third, HepaRG do represent the liver metabolizing function of only one single donor. Last, HepaRG cells were derived from a hepatocellular carcinoma. Thus, they are cancer cells supposed to be genetically instable, which excludes some studies on malignant transformation of normal hepatocytes.

5. Pluripotent stem cell-derived hepatocytes

A hallmark of pluripotent stem cells is their intrinsic proliferation capacity and their potential to divide into cell types of all three germ layers. It is thus not surprising that there have been numerous attempts to generate functional hepatocytes from embryonic (ESCs) and induced pluripotent stem cells (iPS) [58–62]. ESCs represent a natural pluripotent stem cell type and their differentiation potential is thought to be superior to iPS cells [63]. Comparison of specific phase I enzyme activities reveals that hepatocytes derived from both pluripotent stem cell types perform much less well than HepaRG and Upcyte cells (Table 1). A major problem with ESCs is that for ethical reasons many countries released laws such as the so-called Embryo Protection Act in Germany which do not allow usage of ESC cell lines generated after a defined key date nor do they allow the generation of domestic ESC lines at all. Use of pluripotent stem cells generally is hampered by large cell line variabilities with respect to hepatic differentiation efficiency. An improvement of this limitation was that human pluripotent stem cell-derived hepatoblasts were shown to attach on recombinant human laminin-111 (LN111)-coated dishes to allow for their selective expansion [64]. Based on this procedure, Takayama and colleagues reproducibly reprogrammed pHHs into iPS which were differentiated into hepatocyte-like cells via hepatoblasts. They showed that pHH donor variability with respect to biotransformation capacity and hepatotoxicity was represented by the matched population of iPS-derived hepatocytes [65]. Since these authors did not disclose absolute enzyme activities, a quantitative comparison with the data shown in Table 1 is not possible. Altogether, it seems that up to date *in vitro* attempts failed to generate mature and fully differentiated hepatocytes from iPS.

6. Upcyte / HepaFH cells

In order to circumvent some limitations of above mentioned *in vitro* hepatocyte systems, an additional approach was developed recently. Using the Upcyte / HepaFH protocol it is possible to directly generate proliferation-competent human hepatocytes by lentivirus-mediated transfer of coding sequences for defined proliferation genes (Upcyte® factors) into normal primary hepatocytes [66, 67].

The authors could demonstrate significant expression levels of relevant phase I and phase II enzymes, their inducibility and biotransformation activity. In addition, Upcyte hepatocytes, which represent non-transformed cells genetically stable over many passages, should be an interesting tool to study hepatotoxicity and clearance of xenobiotics [68–70]. More specifically, a panel of 11 chemicals known to induce or non-induce CYP3A4 and CYP2B6 were run on second generation Upcyte hepatocytes which proved to be a good prediction model for those CYP enzymes [71]. Mechanistically, expansion of Upcyte hepatocytes is dependent on upregulation of oncostatin M receptor during the growth phase, and differentiation into functional polarized hepatocytes upon withdrawal of oncostatin M [72]. Low expression of proliferation genes and medium components seem to allow for successful inhibition of epithelial-to-mesenchymal transition during both growth and differentiation phases. The authors could further show that Upcyte hepatocytes can be used as a cell culture model for Hepatitis C infection.

As is the case for hepatocytes derived from iPS, Upcyte hepatocytes are genetically engineered which might limit their use for therapeutic applications. However, the Upcyte technology is a good step forward towards physiologically relevant substitutes of pHHs. Upcyte hepatocytes are derived from normal primary hepatocytes, which allows for the generation of cell banks with representative donor variabilities. Physiological activities of CYP enzymes were reported to be in the range of pHHs and HepaRG outperforming those of HepG2 and pluripotent stem cell-derived hepatocytes (Table 1). In contrast to HepaRG, monolayer Upcyte hepatocytes are capable for urea production [66, 67].

7. Conclusions

Freshly isolated pHHs are still the most physiological system to test hepatotoxicity of drugs. However, they have major disadvantages such as limited tissue availability, lack of proliferation and rapid dedifferentiation *in vitro*. As is known for human liver, pHHs show substantial donor variability. Use of liver cell lines such as HepG2 and, more recently, HepaRG circumvent the problem of limited availability. Especially HepaRG are a promising *in vitro* surrogate of pHHs as they display a good panel of liver-specific functions except urea formation. A disadvantage is that they represent only one donor and that they are cancer cells. The problem of donor variability can be solved by iPS-derived hepatocytes. This cell system, however, still seems to require improvement in order to catch up to pHHs, HepaRG and Upcyte / HepaFH cells. The latter cell system combines features of differentiated mature hepatocytes such as known for pHHs and HepaRG with the ability to proliferate as is the case for HepG2 and HepaRG. Furthermore, Upcyte / HepaFH cells can be generated from different donors as is the case for iPS-derived hepatocytes. Currently, Upcyte hepatocytes seem to be the most promising *in vitro* cell system for prediction of DILI.

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