Rev. Toxicol. (2014) 31: 149-156

Cell-based models to predict human hepatotoxicity of drugs

Gómez-Lechón MJ[1,2], Tolosa L[1], Donato MT[1,2,3]

[1]Unidad de Hepatología Experimental. Instituto de Investigación Sanitaria La Fe (IIS La Fe). Avda. Fernando Abril Martorell, nº 106- Torre A. 46026 Valencia, Spain. [2]CIBEREHD, FIS, Spain. [3]Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Valencia, Spain.

Recibido 29 de Julio de 2014 / Aceptado 20 de octubre de 2014

Abstract: Drug-induced liver injury is a significant leading cause of liver disease and post-market attrition of approved drugs. Several hepatic cell-based models have been used for early safety risk assessment during drug development. Their capacity to predict hepatotoxicity depends on cells' functional performance. Cultured hepatocytes have contributed to increase knowledge of the metabolic patterns and mechanisms involved in drug toxicity. A major limitation of monolayer hepatocytes is that they undergo rapid loss of hepatic functionality over time, particularly drug metabolising capability. The sandwich culture model promotes polarised cell surface and stabilises hepatocyte functionality, particularly transport systems, better than monolayer cultures. As 3D spatial organisation and complex heterotypic cell interactions are essential for the functional homeostasis of the liver, hepatocyte models (3D cultures, co-cultures with NPCs and microfluidic systems) that mimic cell-cell, cell-matrix interactions and nutrient flow characteristic of the liver microenvironment have been shown to improve the metabolic competency of hepatocytes and have been proposed for better *in vitro* predictions of drug hepatotoxicity. In addition to hepatocytes, other cell-based models have been proposed for hepatotoxicity studies. Hepatoma cell lines are metabolically poor compared to hepatocytes, but offer key advantages, such as unlimited life span, reproducibility, high availability and easy handling, which make them useful for screening purposes. Alternatively, hepatic cell lines engineered for stable or transient expression of key drug-metabolising enzymes have also been used. Finally, stem cell-derived hepatocytes are emerging *in vitro* systems that would provide a stable source of hepatocytes from individuals with highly valuable particular polymorphic characteristics for preclinical drug metabolism and toxicity prediction of new drugs.

Key words: Co-culture, CYP-engineered cell line, hepatocytes, hepatoma cell line, microfluidic device, sandwich culture, spheroids, scaffold-based culture

Resumen: Modelos celulares para predecir la hepatotoxicidad humana de fármacos. La lesión del hígado por fármacos es una de las causas principales de enfermedad hepática y de retirada del mercado de fármacos autorizados. Son varios los modelos de células hepáticas utilizados durante el desarrollo de fármacos para la valoración temprana de su seguridad. Los estudios basados en hepatocitos cultivados han contribuido al conocimiento de los mecanismos implicados en la toxicidad por fármacos. Una limitación fundamental de los hepatocitos cultivados en monocapa es la pérdida temprana de funciones hepáticas, en particular la capacidad para metabolizar fármacos. El cultivo tipo sándwich mantiene la polaridad de los hepatocitos y los sistemas de transporte y estabiliza su funcionalidad mejor que el cultivo en monocapa. Puesto que la organización espacial 3D y las interacciones celulares heterotípicas son esenciales para la homeostasis funcional del hígado, los hepatocitos cultivados en sistemas que reproducen las interacciones entre células, célula-biomatriz y el flujo de nutrientes característicos del microambiente hepático (cultivos 3D, co-cultivos con células no parenquimales, sistemas microfluidicos) presentan mayor capacidad metabólica y han sido propuestos para mejorar la predicción *in vitro* de la hepatotoxicidad. Otras células hepáticas han sido propuestas como alternativa a los hepatocitos para evaluar la hepatotoxicidad. Si bien las líneas celulares de hepatoma tienen menor capacidad metabólica que los hepatocitos, presentan ventajas clave para el cribado de fármacos (vida ilimitada, reproducibilidad, gran disponibilidad, fácil manejo). También se utilizan células manipuladas para la expresión estable o transitoria de enzimas de biotransformación. Por último, los hepatocitos procedentes de células madre son sistemas *in vitro* emergentes que proporcionarían una fuente estable de hepatocitos, a partir de individuos con características polimórficas especiales, sumamente valiosa para la predicción preclínica de la toxicidad de nuevos fármacos.

Palabras clave: Co-cultivos, células manipuladas genéticamente que expresan CYPs, hepatocitos, líneas celulares de hepatomas, cultivo en sandwich, esferoides, cultivos en soportes tridimensionales.

Introduction

Drug-induced liver injury (DILI) is one of the most important issues in drug development as a leading cause of discontinuation of clinical trials and withdrawal or black box warnings of approved drugs [1]. DILI is a complex phenomenon which encompasses a spectrum of clinical disease ranging from mild biochemical abnormalities to acute liver failure. Hepatotoxicity can be induced by a drug itself or indirectly by the generation of reactive metabolites (bioactivation) (Figure 1). Toxic injury to hepatocytes is produced through multiple mechanisms involving damage to biomolecules, alteration of cell homeostasis/function and cell death [2].

Early safety assays during drug development are directed to reduce potential risk of toxicity to humans, however, preclinical testing in laboratory animals often fails to predict DILI. This poor predictivity is attributable to several reasons, including differences in drug metabolism and toxicity between human and experimental species [3,4]. In this scenario, different *in vitro* approaches have been explored to improve and accelerate the identification of hepatotoxicity induced by drugs. In particular, several hepatic cell-based screening protocols have been incorporated in drug

* e-mail: gomez_mjo@gva.es

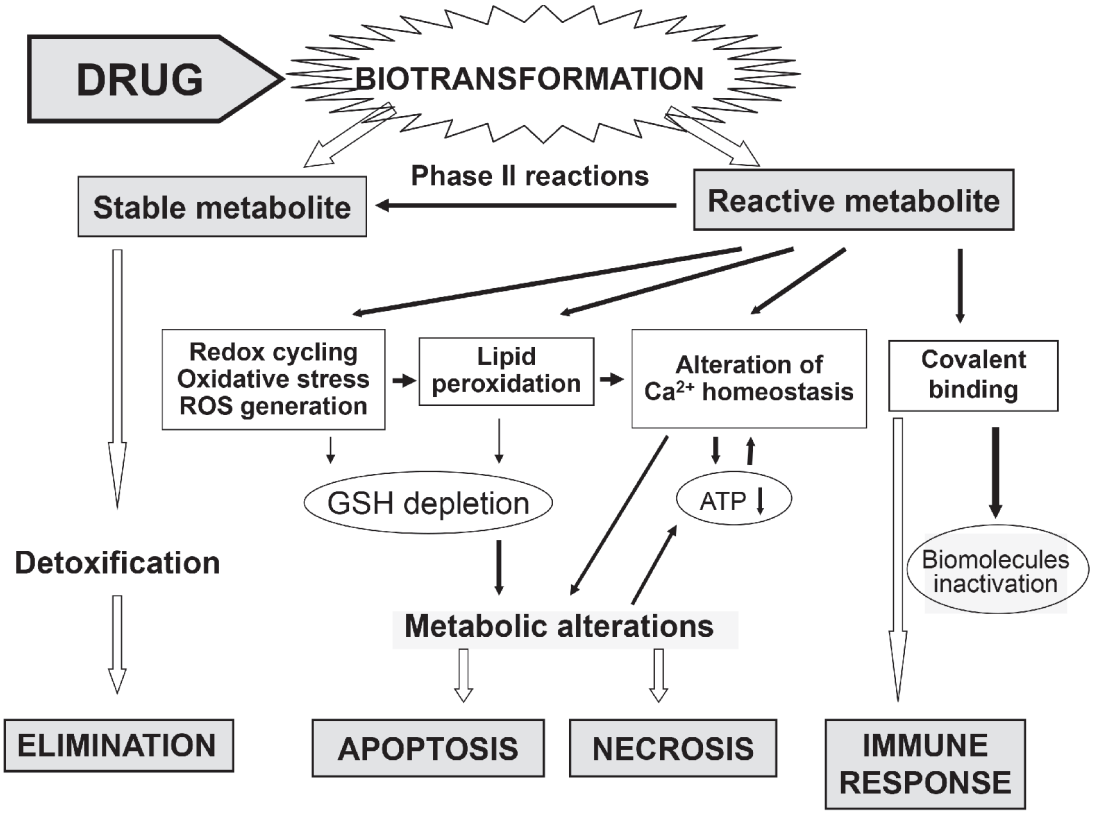

Figure 1. Molecular events leading to drug-induced liver cell damage and death. Drugs may act directly on cellular systems or after biotransformation by hepatocytes. In the latter case, toxicity is ultimately the balance between bioactivation and detoxification, which determines whether a reactive metabolite elicits a toxic effect or not. There are several processes known to play a role in the molecular events leading to irreversible cell damage and cell death by either necrosis or apoptosis.

development for early safety risk assessment [4-7]. Their capacity of predicting *in vivo* hepatotoxicity depends critically on the functional activities of the cell types used in each screening platform.

This paper presents the most valuable cell models for human hepatotoxicity predictions including cultures of hepatocytes in different 2D and 3D configurations as well as alternative cells to hepatocytes such as hepatoma cell lines, CYP-engineered cells and stem cell-derived hepatocytes. Major features, advantages and drawbacks of the different cell models are discussed.

2D culture models of hepatocytes

For decades, 2D-cultures of hepatocytes have been widely used for *in vitro* predictions of *in vivo* metabolic pathways and hepatotoxicity of drugs (Figure 2). Such cell models offer the advantages of being relatively inexpensive, reproducible, robust and convenient. Cultured hepatocytes from different experimental species, particularly rat and mouse, have been used. However, human hepatocytes have been considered the gold standard *in vitro* model for the prediction of drug metabolism and the assessment of hepatotoxicity [8-12], because qualitative and quantitative interspecies differences in drug-metabolising enzymes frequently make the extrapolation of drug metabolism and hepatotoxic effects from animal hepatocytes to man difficult.

Monolayer cultures involve plating cells on a rigid substratum pre-treated with extracellular matrix (ECM) proteins (collagen, fibronectin or Matrigel) [11,12], where they maintain key hepatic-specific functions [8-12]. However, one major drawback of monolayer cultures is that they undergo a rapid loss of hepatic

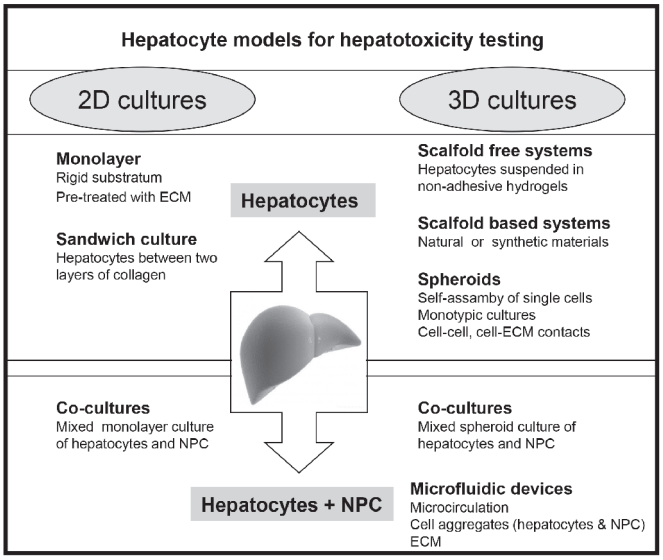

Figure 2. Hepatocyte models as tools for hepatotoxicity studies. The models extend from well-established hepatocyte culture models comprising a 2D monolayer and collagen-sandwich configuration, followed by emerging complex 3D hepatic cellular models including scaffold-based models, aggregates and microfluidic devices.

functionality over time, particularly drug metabolising capability, which confers them a short, limited sensitivity to drug hepatotoxicity detection [5,7,8,10,11] (Table 1).

Table 1. *Hepatocyte culture models for hepatotoxicity testing*

Models	Advantages	Disadvantages
2D Cultures of hepatocytes		
Monolayer cultures	Expression of CYP and Phase II enzymes Short-time inducibility of CYPs by xenobiotics Model of choice (human hepatocytes) for drug screening: metabolic profiling, drug-drug interaction, hepatotoxicity Good transferability of data to human data (human hepatocytes)	Short-term preservation of functionality Loss of cell polarity Limited availability (human liver) Donor to donor variability (human) Low resistance to cryopreservation Difficult transferability of data to human data (rodent hepatocytes)
Sandwich cultures	Higher cell viability, survival and expression of CYP and Phase II enzymes than monolayer cultures Inducibility of CYP enzymes by xenobiotics Retention of morphology, functional bile canaliculi and cell-cell contacts Good transferability of data to human data (human hepatocytes)	Difficult transferability of data to human data (rodent hepatocytes) Limited availability of human liver Donor to donor variability(human)
Co-cultures of several liver-derived cell types	Improved longevity/functionality of all cell types High expression of CYP and Phase II enzymes Inducibility of CYP enzymes by xenobiotics Retention of morphology, bile canaliculi and cell-cell contacts Good transferability of data to human data (human hepatocytes)	No standard as to which other cell type to use
3D Cultures of hepatocytes		
Scaffold- based systems/ Spheroids	Long-term maintenance of liver specific functions, CYP and Phase II enzymes and inducibility of CYP enzymes by xenobiotics High level of hepatic transporters Preserved liver-specific polarity Possibility of using for chronic toxicity and repeated dose Good transferability of data to human data (human hepatocytes)	In some 3D models there is formation of necrotic cores Techniques need to be improved to be applicable for highthrouput
Co-cultures of liver derived cell types	Longer expression of CYP and Phase II enzymes and inducibility of CYP enzymes by xenobiotics Intercellular interactions and communication Preserved liver-specific polarity Mimic liver cyto-organization	Variability of viability and differentiation status depending on culture conditions Techniques need to be improved to be applicable for highthrouput
Microfluidic devices	Incorporate shear flow Promote round cell aggregates more similar to *in vivo* morphology Sustained liver-like cell functionality Preserved liver-specific polarity Allow the possibility to precisely adjust flow rates and metabolite or drug concentrations in the medium Better correlation with *in vivo* data compared with static hepatocyte cultures and this correlation further improved in co-cultures Possibility of microscopic examination	Emerging technology Variability of viability and differentiation status depending on culture conditions Does not maintain viability/ functionality longer than other 3D methods

In an attempt to maintain liver-specific functionality over longer culture periods, a sandwich configuration was developed (Table 1). Hepatocytes are placed between two matrix layers, traditionally collagen or Matrigel. Maintaining hepatocytes in a sandwich culture prevents cell viability loss, enhances secretion of organic compounds, including urea and albumin, increases basal and induced drug-metabolising enzyme activities, and mimics *in vivo* biliary excretion rates [9,11,13,14]. Therefore, it has been suggested that the sandwich culture model is most useful for mechanistic studies of hepatobiliary toxicity [13,15-17]. This is important because biliary efflux activity is inhibited by various drugs that cause iatrogenic cholestasis, an important mechanism of DILI [18].

3D culture models of hepatocytes

As a result of the failure to predict hepatotoxic drugs in preclinical testing using traditional hepatocyte cultures, alternative models to phenotypically stabilise liver cell functions over a long period of time have been developed (Figure 2). They are based in recreating microenvironmental cues *in vivo*, such as a 3D architecture, multiple cell types, cell-cell and cell-matrix interactions, soluble factors, and dynamic nutrient flow which appear promising for drug screening and predicting drug efficacy and toxicity in humans. 3D liver cell models, which are amenable to routine use and high-throughput adaptation, are particularly desirable for industrial drug discovery to allow the realistic assessment of drug metabolism and adverse/toxic effects [5,19]. Advantages and disadvantages of the different models are summarized in Table 1.

Scaffold-based systems

3D cultures can be produced by embedding hepatocytes in scaffold-free and scaffold-based systems (for a review, see [5,19]). The former consists in suspending cells in non-adhesive hydrogels (i.e., alginate, Matrigel, collagen, self-assembling peptides) with subsequent polymerisation that aims to culture the hepatocytes encapsulated within a gel [20]. Scaffold-based systems involve seeding cells on 3D solid matrices; *e.g.*, derived from natural materials (decellularised liver-derived ECM) or synthetic materials (*e.g.*, alginate, polystyrene) [19,21]. While naturally derived substrates offer advantages in biocompatibility terms, and mimic cell-matrix interactions, synthetic scaffolds offer reproducibility and stability. Interconnected porous networks and the pore size of 3D scaffolds are very important for ensuring spatially uniform cell distribution, cell migration and cell survival, which all affect the diffusion of physiological nutrients and gases and the removal of metabolic waste. The currently available wide range of synthetic polymers opens up many opportunities for cell-specific tailored scaffolds. For example, the specific affinity of hepatocytes to the galactose residue has led to a range of synthetic scaffolds that present galactose on the surface for improved hepatocyte adhesion and function [5,22].

Multicellular spheroids

Hepatocytes can be re-aggregated by cellular self-assembly and by re-establishing cellular contacts to reform a 3D configuration. The fundamental concept is that suspended isolated hepatocytes are capable of reforming 3D tissue or spheroids if adhesion to a substrate

is prevented. Sustained cellular contacts are key for maintaining hepatic differentiation and functionality in spheroids [23] (for a review see [5]). In general, an intact actin cytoskeleton is required for the self-assembly and differentiation of liver cell spheroids [23]. The size of spheroids is critical since spheroids larger than 200-300 μm are at risk of having necrotic cores since oxygen diffusion is the most limiting parameter [5]. Spheroids can be created by various methods [5]: (1) spontaneous self-assembly in non-adhesive wells/dishes under static conditions; (2) agitation or microcavities; and (3) in a hanging drop. Several reports indicate an excellent long-term viability of human hepatocyte spheroids to preserve liver-specific polarity, the expression and activity of phase I and phase II drug-metabolising enzymes and induction. Thus, they appear to be a suitable model for discovering drug metabolites and long-term drug hepatotoxicity testing, such as the repeated-dose format and high-throughput systems [24].

3D co-cultures of hepatocytes and non-parenchymal cells

The liver comprises two major cell populations, hepatocytes and non-parenchymal cells (NPCs), including endothelial, stellate and Kupffer cells, among others. The cell-cell communication between hepatocytes, and between hepatocytes and NPCs, and also with the ECM, is a prerequisite for maintaining a differentiated phenotype and required for the *in vivo* functional homeostasis of the liver. Moreover, NPCs are considered important modulators of idiosyncratic hepatotoxicity. Thus, the use of co-cultures of hepatocytes and NPCs could further enhance the *in vivo*–like characteristics of a 3D culture device and provide more predictive results.

Spheroid systems that co-culture rat hepatocytes with hepatic stellate cells, the HSC-T6 cell line, HUVEC cells or Kupffer cells have been developed, and it has been underlined the relevance of these complex and long-lasting hepatic cell culture models [57]. More recently, a 3D scaffold co-culture of human hepatocytes, stellate, Kupffer and endothelial cells has been reported to maintain well-preserved composition and liver function for up to 3 months [25]. Therefore, presence of NPCs not only contribute to prolong the survival and to improve the function of hepatocytes in culture, but can also increase their sensitivity for DILI detection involving inflammatory mediators [25].

Microfluidic devices

In vitro microfluidic systems have been more recently developed to better mimic the *in vivo* situation due to better hepatocyte functionality [26]. Incorporating fluid flow into 3D culture systems is an important step for combating poor oxygen and nutrient diffusion issues through spheroids and aggregates of cells and ECM. The overall goal of many such efforts is to form a fully functional liver culture model that mimics the complex *in vivo* architecture of a liver lobule, and which can be used for toxicological and pharmacological research or can be modified in a bio-artificial liver for clinical use (for a review, see [5,19]). One real advantage is the possibility of precisely adjusting flow rates and metabolite or drug concentrations in the medium to mimic various physiologic conditions of blood, such as postprandial and starvation states or circadian cycles of hormone and metabolite concentrations. These devices preserve cell viability and the metabolic competency of human hepatocytes at higher levels than under static culture conditions [26-28]. The utility of these models for toxicity testing has been explored through the prediction of *in vivo* clearance rates. It has been demonstrated that the data from these systems are more correlative with *in vivo* data than those deriving from static hepatocyte cultures, and that this correlation improved further when co-cultures were used [28,29].

Hepatoma cell lines

Although human hepatocytes are the preferred cells for drug metabolism and hepatotoxicity studies, their scarce availability, inter-donor variability, short life span, and decreased metabolic capacity along culture time limit their routine use for screening purposes. Several human hepatoma cell lines (e.g., HepG2, Hep3B, Huh7, HepaRG) have been proposed as alternative cell models to hepatocytes [30]. These cells offer key advantages over hepatocytes such as their high availability, unlimited life span, stable phenotype, reproducibility, and easy handling (Table 2), which make them useful *in vitro* systems for drug safety assessment [30,31].

Table 2. *Alternative cell sources to hepatocytes for hepatotoxicity testing*

Models	Advantages	Disadvantages
Hepatoma cell lines	Highly proliferative, unlimited available cells Easy handling and relative low cost Standardized culture conditions Relative stable gene expression pattern Robustness and good experimental reproducibility Some cell lines retain certain liver-specific functions and drug-metabolizing capacity (i.e., HepaRG) Suitable to high throughput screenings Possibility of culturing in 3D configuration (enhanced functionality)	Undifferentiated phenotype characteristic of proliferative tumor cells Poor expression of some functions of adult human liver Altered expression of key transcription factors Scarce levels of certain CYP enzymes (depending on cell line) Absence on non-parenchymal cells Difficult transferability of data to normal (non-malignant) human liver
CYP-transfected cell lines	High activity levels of transfected enzymes Useful for metabolism-based toxicity studies High reproducibility and phenotypic stability (stably transfected cell lines) Identification of CYPs involved in the generation of toxic metabolites Controllable expression of functional CYPs (adenovirus-transfected cells) Tailored reproduction of metabolic phenotypes (multiple adenoviral co-transfection)	Unbalanced drug metabolism (cells over-expressing a single CYP) Potential altered expression of other hepatic functions More correlation studies to in vivo hepatotoxicity are required Uncontrollable risk of mutagenic effects (stably transfected cell lines) Transient expression systems require the generation of a new cell lot for each study (potential variability, time-consuming)
Induced pluripotent stem cells-derived hepatocytes	Stable genetic background High availability Defined phenotype Allow studies of inter-individual variability Possibility of culturing in 3D configuration (enhanced functionality)	Complex reprogramming steps Limited expression of liver-specific genes Variability in phenotype among preparations Few studies in toxicology yet

Most hepatoma cell lines express many liver differentiated functions; however, in general, they show a poor expression of drug metabolising enzymes (CYPs, conjugating enzymes) and transport proteins compared to primary hepatocytes [31-33]. Despite these shortcomings, hepatoma cells have been extensively used for cytotoxicity evaluations and to examine specific mechanisms of toxicity. In particular, HepG2, the best characterised human hepatoma, is one of the most currently used human cell models for hepatotoxicity screenings. As a result of this widespread use, exhaustive data on the effects of a huge number of compounds (model hepatotoxins, drugs, chemicals) on many parameters indicative of toxicity to HepG2 cells (viability, membrane integrity, cell proliferation, ATP level, etc.) are available in the literature [6,34,35]. Recently, multiplexed high content screening and automated assays adapted to HepG2 miniaturised culture formats (e.g., 96- or 386-well plates) have been proposed as valuable prioritisation tools during preclinical drug development [36,37]. These multiparametric assays have been applied to screen large series of compounds and have shown acceptable specificity and sensitivity to discriminate between hepatotoxic and non-hepatotoxic drugs.

HepaRG is a recently derived hepatoma cell line that is now considered the most promising cell model as a surrogate for human hepatocytes in *in vitro* assessments. Proliferating HepaRG are bipotent progenitor cells capable of differentiating into hepatocyte-like and biliary-like cells [38]. After several weeks of culture in the presence of DMSO, confluent monolayers of HepaRG cells differentiate towards a hepatocyte-like phenotype with bile canaliculi structures formation [38]. Differentiated HepaRG are now increasingly used in hepatotoxicity studies as they show important advantages over HepG2 and other hepatoma cells: 1) greater levels of phase I and phase II drug-metabolising enzymes, which enables the detection of toxic effects of reactive metabolites; 2) a polarised expression of the hepatobiliary membrane transporters required to identify toxicity due to the alteration of the normal function of hepatic uptake or efflux transporters; and 3) a stable metabolic competence for several weeks, which opens up the possibility of performing long-term repeated-dose studies for chronic toxicity assessment [32,39,40]. However, the demanding culture requirements, long-term differentiation protocols and high DMSO concentrations required to maintain differentiated HepaRG cultures are major drawbacks for their widespread use in hepatotoxicity testing [39,40].

Research efforts have been made to improve the functional capacity of hepatoma cell lines and to promote their performance for drug safety evaluation. Different 3D culture techniques (e.g., microencapsulation, cell spheroids or micro-space cell culture systems) have been explored to improve differentiation and the hepatic phenotype of HepG2 or HepaRG cells. Similarly to hepatocytes, hepatoma cells grown in 3D systems have exhibited better viability and functionality than in conventional 2D cultures [41-43]. Therefore, these 3D organotypic cultures have been proposed as relevant alternative systems for the more accurate assessment of human hepatotoxicity and for metabolism-mediated drug toxicity screenings [41-43].

CYP-transfected hepatic cell lines

Hepatotoxicity can be produced after bioactivation of the drug by biotranformation enzymes (mainly CYPs) into reactive metabolite(s) (Figure 1). The identification of bioactivable molecules requires the use of metabolic competent systems capable of generating toxic metabolites. Several cell systems based on liver-derived cell lines engineered to express high levels of CYPs (and other drug-metabolising enzymes) have been developed as *in vitro* tools for drug metabolism and hepatotoxicity studies [30]. These metabolically competent cells are generated by tranfection with vectors encoding for human CYPs resulting in stable or transient expression of the transgene [5,30]. In contrast to primary hepatocyte cultures, transfected cell lines show high levels of CYP activities along time in culture and offer the advantages of robustness and good experimental reproducibility; however important limitations of these cells is that transfection strategies can potentially alter the expression of other hepatic functions and overexpression of a particular enzyme may result in unbalanced metabolism and (Table 2).

Among cell lines manipulated for stable expression of drug-metabolising transgenes, those generated by the transfection of SV40 large T-antigen-immortalised human liver epithelial (THLE) cells or HepG2 cell line are the most widely used for hepatotoxicity assessment of bioactivable drugs [44,45]. Each CYP-transfected THLE or HepG2 cell line stably express high levels of an individual human CYP [45]. A study strategy based on the comparison of the effects of a particular drug to CYP-transfected cells and to parental non-CYP expressing cells has enabled the contribution of CYP-mediated metabolism to toxicity to be explored [44-46]. However, no more than one or two enzymes can be satisfactorily transfected into cells, and expression levels are often too high or low when compared to human liver/hepatocytes [46].

As an alternative, upgraded HepG2 cells generated by adenoviral-mediated CYP expression have been proposed for hepatotoxicity studies [30,47-49]. Adenoviral transduction has allowed the easily modulated and controlled expression of multiple transgenes (up to five CYPs) in host cells [5,48,49]. Then by selecting appropriate mixtures of adenoviral constructs, cells customised with a particular CYP profile (metabolic phenotype) can be produced [30]. The versatility of these cell-based assays opens up the possibility of making *in vitro* hepatotoxicity predictions to different population groups (e.g., extensive *vs* poor metabolisers). However, one limitation of this strategy is that transgene expression is transient and a new transfection must be performed for each experiment.

Pluripotent stem cells-derived hepatocytes

Human pluripotent stem cells-derived hepatocytes are emerging as cell-based systems that will potentially provide a stable source of hepatocytes for reliable and high-throughput screening for the metabolism and toxicity of candidate compounds. Different groups have developed protocols to isolate embryonic stem cells (ESCs) and induce them to form hepatocyte-like cells by mimicking the developmental pathway of the liver during embryogenesis [7,50]. However, the broad variability reported by distinct laboratories of the key enzymes implicated in drug metabolism in differentiated ESCs implies that the application of these cells in toxicity studies is still premature. Recent studies have focused on the 3D culture of ESCs for toxicity testing [51].

Human induced-pluripotent stem cells (iPSCs) are an attractive source of normal human cells because they possess self-renewing potency and pluripotency, and can differentiate into virtually any somatic cell type, like hepatocytes. They may provide a limitless supply of hepatocytes for high-throughput screening with minor batch variability from multiple individuals to improve reproducibility and to enable testing of individual-specific toxicity [7,52,53]. Hepatocyte-like cells differentiated from iPSCs recapitulate many hepatic functional properties. However, current hepatic differentiation protocols result in cells with lower levels of

enzyme activity and hepatic gene expression profiles than intact human liver or human isolated hepatocytes [54,55]. Perhaps in the future, iPSC-hepatocytes generated from individuals with different CYP polymorphisms would be of great value for the drug metabolism and toxicity prediction of new drugs in pre-clinical stages to enable more successful clinical trials [53,55,56].

References

1. Norris W, Paredes AH, Lewis JH (2008) Drug-induced liver injury in 2007. Curr Opin Gastroenterol 24: 287-297.
2. Gomez-Lechon MJ, Tolosa L, Castell JV, Donato MT (2010) Mechanism-based selection of compounds for the development of innovative *in vitro* approaches to hepatotoxicity studies in the LIINTOP project. Toxicol In Vitro 24: 1879-1889.
3. Lewis DF, Ioannides C, Parke DV (1998) Cytochromes P450 and species differences in xenobiotic metabolism and activation of carcinogen. Environ Health Perspect 106: 633-641.
4. O'Brien PJ, Chan K, Silber PM (2004) Human and animal hepatocytes *in vitro* with extrapolation *in vivo*. Chem Biol Interact 150: 97-114.
5. Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Böttger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CE, Gómez-Lechón MJ, Groothuis GM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Häussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzhütter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, LeCluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EH, Stieger B, Stöber R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM, Hengstler JG (2013) Recent advances in 2D and 3D *in vitro* systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. Arch Toxicol 87: 1315-1530.
6. O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, 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: 580-604.
7. Soldatow VY, Lecluyse EL, Griffith LG, Rusyn I (2013). Models for liver toxicity testing. Toxicol Res (Camb) 2: 23-39.
8. Gomez-Lechon MJ, Castell JV, Donato MT (2008) An update on metabolism studies using human hepatocytes in primary culture. Expert Opin Drug Metab Toxicol 4: 837-854.
9. Gomez-Lechon MJ, Donato MT, Castell JV, Jover R (2003) Human hepatocytes as a tool for studying toxicity and drug metabolism. Curr Drug Metab 4: 292-312.
10. Gomez-Lechon MJ, Donato MT, Castell JV, Jover R (2004) Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. Curr Drug Metab 5: 443-462.
11. Hewitt NJ, Gómez-Lechon MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG (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: 159-234.
12. LeCluyse EL (2001) Human hepatocyte culture systems for the *in vitro* evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci 13: 343-368.
13. Meng Q. Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity. Expert Opin Drug Metab Toxicol 2010; 6:733-46.
14. Kienhuis AS, Wortelboer HM, Maas WJ, van Herwijnen M, Kleinjans JC, van Delft JH, Stierum RH (2007) A sandwich-cultured rat hepatocyte system with increased metabolic competence evaluated by gene expression profiling. Toxicol In Vitro 21: 892-901.
15. Chatterjee S, Richert L, Augustijns P, Annaert P (2014) Hepatocyte-based *in vitro* model for assessment of drug-induced cholestasis. Toxicol Appl Pharmacol 274: 124-136.
16. De Bruyn T, Chatterjee S, Fattah S, Keemink J, Nicolaï J, Augustijns P, Annaert P (2013) Sandwich-cultured hepatocytes: utility for *in vitro* exploration of hepatobiliary drug disposition and drug-induced hepatotoxicity. Expert Opin Drug Metab Toxicol 9: 589-616.
17. Marion TL, Perry CH, St Claire RL, Brouwer KL (2012) Endogenous bile acid disposition in rat and human sandwich-cultured hepatocytes. Toxicol Appl Pharmacol 261: 1-9.
18. Trauner M, Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev 83: 633-671.
19. Mueller D, Heinzle E, Noor F (2013) 3D Hepatic *In Vitro* Models as Tools for Toxicity Studies. Current Tissue Eng 2: 78-89.
20. Lau TT, Lee LQ, Leong W, Wang DA (2012) Formation of model hepatocellular aggregates in a hydrogel scaffold using degradable genipin crosslinked gelatin microspheres as cell carriers. Biomed Mater 7:065003.
21. Kim BS, Park IK, Hoshiba T, Jiang HL, Choi YJ, Akaike T, Cho CS (2011) Design of artificial extracellular matrices for tissue engineering. Prog Polym Sci 36: 238-268.
22. Cho CS, Seo SJ, Park IK, Kim SH, Kim TH, Hoshiba T, Harada I, Akaike T (2006) Galactose-carrying polymers as extracellular matrices for liver tissue engineering. Biomaterials 27: 576-585.
23. van Zijl F, Mikulits W (2010) Hepatospheres: Three dimensional cell cultures resemble physiological conditions of the liver. World J Hepatol 2: 1-7.
24. Tostoes RM, Leite SB, Serra M, Jensen J, Bjorquist P, Carrondo MJ, Brito C, Alves PM (2012) Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. Hepatology 55: 1227-1236.
25. Kostadinova R, Boess F, Applegate D, Suter L, Weiser T, Singer

T, Naughton B, Roth A (2013) A long-term three dimensional liver co-culture system for improved prediction of clinically relevant drug-induced hepatotoxicity. Toxicol Appl Pharmacol 268: 1-16.

26. Chao P, Maguire T, Novik E, Cheng KC, Yarmush ML (2009) Evaluation of a microfluidic based cell culture platform with primary human hepatocytes for the prediction of hepatic clearance in human. Biochem Pharmacol 78: 625-632.
27. Goral VN, Hsieh YC, Petzold ON, Clark JS, Yuen PK, Faris RA (2010) Perfusion-based microfluidic device for three-dimensional dynamic primary human hepatocyte cell culture in the absence of biological or synthetic matrices or coagulants. Lab Chip 10: 3380-3386.
28. Novik E, Maguire TJ, Chao P, et al. A microfluidic hepatic coculture platform for cell-based drug metabolism studies. Biochem Pharmacol 2010; 79:1036-44.
29. Bhushan A, Senutovitch N, Bale SS, McCarty WJ, Hegde M, Jindal R, Golberg I, Berk Usta O, Yarmush ML, Vernetti L, Gough A, Bakan A, Shun TY, DeBiasio R, Lansing Taylor D (2013) Towards a three-dimensional microfluidic liver platform for predicting drug efficacy and toxicity in humans. Stem Cell Res Ther 4 Suppl 1: S16.
30. Donato MT, Jover R, Gomez-Lechon MJ (2013) Hepatic cell lines for drug hepatotoxicity testing: limitations and strategies to upgrade their metabolic competence by gene engineering. Curr Drug Metab 14: 946-968.
31. Donato MT, Lahoz A, Castell JV, Gomez-Lechon MJ (2008) Cell lines: a tool for *in vitro* drug metabolism studies. Curr Drug Metab 9: 1-11.
32. Kanebratt KP, Andersson TB (2008) Evaluation of HepaRG cells as an *in vitro* model for human drug metabolism studies. Drug Metab Dispos 36: 1444-1452.
33. Guo L, Dial S, Shi L, Branham W, Liu J, Fang JL, Green B, Deng H, Kaput J, Ning B (2011) Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. Drug Metab Dispos 39: 528-538.
34. Schoonen WG, Westerink WM, de Roos JA, Débiton E (2005) Cytotoxic effects of 100 reference compounds on Hep G2 and HeLa cells and of 60 compounds on ECC-1 and CHO cells. I mechanistic assays on ROS, glutathione depletion and calcein uptake. Toxicol In Vitro 19: 505-516.
35. Schoonen WG, Stevenson JC, Westerink WM, Horbach GJ (2012) Cytotoxic effects of 109 reference compounds on rat H4IIE and human HepG2 hepatocytes. III: Mechanistic assays on oxygen consumption with MitoXpress and NAD(P)H production with Alamar Blue. Toxicol In Vitro 26: 511-525.
36. Tolosa L, Pinto S, Donato MT, Lahoz A, Castell JV, O'Connor JE, Gómez-Lechón MJ (2012) Development of a Multiparametric Cell-based Protocol to Screen and Classify the Hepatotoxicity Potential of Drugs. Toxicol Sci 127: 187-198.
37. Garside H, Marcoe KF, Chesnut-Speelman J, Foster AJ, Muthas D, Kenna JG, Warrior U, Bowes J, Baumgartner J (2014) Evaluation of the use of imaging parameters for the detection of compound-induced hepatotoxicity in 384-well cultures of HepG2 cells and cryopreserved primary human hepatocytes. Toxicol In Vitro 28: 171-181.
38. 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: 75-83.
39. Rodrigues RM, Bouhifd M, Bories G, Sacco MG, Gribaldo L, Fabbri M, Coecke S, Whelan MP (2013) Assessment of an automated *in vitro* basal cytotoxicity test system based on metabolically-competent cells. Toxicol In Vitro 27: 760-767.
40. Klein S, Mueller D, Schevchenko V, Noor F (2013) Long-term maintenance of HepaRG cells in serum-free conditions and application in a repeated dose study. J Appl Toxicol 2013; doi: 10.1002/jat.2929.
41. Ramaiahgari SC, den Braver MW, Herpers B, Terpstra V, Commandeur JN, van de Water B, Price LS (2014) A 3D *in vitro* model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Arch Toxicol 88: 1083-1095.
42. Mueller D, Kramer L, Hoffmann E, Klein S, Noor F (2014) 3D organotypic HepaRG cultures as *in vitro* model for acute and repeated dose toxicity studies. Toxicol In Vitro 28: 104-112.
43. Gunness P, Mueller D, Shevchenko V, Heinzle E, Ingelman-Sundberg M, Noor F (2013) 3D organotypic cultures of human HepaRG cells: a tool for *in vitro* toxicity studies. Toxicol Sci 133: 67-78.
44. Dambach DM, Andrews BA, Moulin F (2005) New technologies and screening strategies for hepatotoxicity: use of *in vitro* models. Toxicol Pathol 33: 17-26.
45. Gustafsson F, Foster AJ, Sarda S, Bridgland-Taylor MH, Kenna JG (2014) A correlation between the *in vitro* drug toxicity of drugs to cell lines that express human P450s and their propensity to cause liver injury in humans. Toxicol Sci 137: 189-211.
46. Greer ML, Barber J, Eakins J, Kenna JG (2010) Cell based approaches for evaluation of drug-induced liver injury. Toxicology 268: 125-131.
47. Vignati L, Turlizzi E, Monaci S, Grossi, P.; Kanter, R.; Monshouwer, M (2005) An *in vitro* approach to detect metabolite toxicity due to CYP3A4-dependent bioactivation of xenobiotics. Toxicology 216: 154-167.
48. Tolosa L, Donato MT, Perez-Cataldo G, Castell JV, Gómez-Lechón MJ (2012) Upgrading cytochrome P450 activity in HepG2 cells co-transfected with adenoviral vectors for drug hepatotoxicity assessment. Toxicol In Vitro 26: 1272-1277.
49. Tolosa L, Gomez-Lechon MJ, Perez-Cataldo G, Castell JV, Donato MT (2013) HepG2 cells simultaneously expressing five P450 enzymes for the screening of hepatotoxicity: identification of bioactivable drugs and the potential mechanism of toxicity involved. Arch Toxicol 87: 1115-1127.
50. Baxter MA, Rowe C, Alder J, Harrison S, Hanley KP, Park BK, Kitteringham NR, Goldring CE, Hanley NA (2010) Generating hepatic cell lineages from pluripotent stem cells for drug toxicity screening. Stem Cell Res 5: 4-22.
51. Takayama K, Kawabata K, Nagamoto Y, Kishimoto K, Tashiro K, Sakurai F, Tachibana M, Kanda K, Hayakawa T, Furue MK, Mizuguchi H (2013) 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing.

Biomaterials 34: 1781-1789.

52. Katsuda T, Sakai Y, Ochiya T (2012) Induced pluripotent stem cell-derived hepatocytes as an alternative to human adult hepatocytes. J Stem Cells 7:1-17.
53. Yi F, Liu GH, Izpisua Belmonte JC (2012) Human induced pluripotent stem cells derived hepatocytes: rising promise for disease modeling, drug development and cell therapy. Protein Cell 3: 246-250.
54. Guguen-Guillouzo C, Corlu A, Guillouzo A (2010) Stem cell-derived hepatocytes and their use in toxicology. Toxicology 270: 3-9.
55. Medine CN, Lucendo-Villarin B, Storck C, Wang F, Szkolnicka D, Khan F, Pernagallo S, Black JR, Marriage HM, Ross JA, Bradley M, Iredale JP, Flint O, Hay DC (2013) Developing high-fidelity hepatotoxicity models from pluripotent stem cells. Stem Cells Transl Med 2: 505-509.
56. Anson BD, Kolaja KL, Kamp TJ (2011) Opportunities for use of human iPS cells in predictive toxicology. Clin Pharmacol Ther 89: 754-758.