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REVIEW ARTICLE

Recent advances in three-dimensional cell culturing to assess liver function and dysfunction: from a drug biotransformation and toxicity perspective

Carlemi Calitz[a], Josias H. Hamman[a], Stephen J. Fey[b], Krzysztof Wrzesinski[b]* and Chrisna Gouws[a]*

[a]Pharmacen™, Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom, South Africa; [b]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M, Denmark

ABSTRACT

The liver is a vital organ fulfilling a central role in over 500 major metabolic functions, including serving as the most essential site for drug biotransformation. Dysfunction of the drug biotransformation processes may result in the exposure of the liver (and other organs) to hepatotoxins, potentially interacting with cellular constituents and causing toxicity and various lesions. Hepatotoxicity can be investigated on a tissue, cellular and molecular level by employing various *in vivo* and *in vitro* techniques, including novel three-dimensional (3 D) cell culturing methods. This paper reflects on the liver and its myriad of functions and the influence of drug biotransformation on liver dysfunction. Current *in vivo* and *in vitro* models used to study liver function and dysfunction is outlined, emphasizing their advantages and disadvantages. The advantages of novel *in vitro* 3 D cell culture models are discussed and the possibility of novel models to bridge the gap between *in vitro* and *in vivo* models is explained. Progression made in the field of cell culturing methods such as 3 D cell culturing techniques over the last decade promises to reduce the use of *in vivo* animal models in biotransformation and toxicological studies of the liver.

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1. Introduction

The liver, a powerhouse of metabolic processes, is a complex three-dimensional vital organ with a multitude of interrelated physiological and biochemical functions (Guillouzo 1998; Wolf 1999; Van Zijl and Mikulits 2010; Singh et al. 2011). Due to the livers involvement in various metabolic functions, in particular biotransformation of xenobiotics, it has become apparent that studying the physiological and pathophysiological condition of the liver is key to drug development (Van Zijl and Mikulits 2010). On the microscopic level, a myriad of cells within the liver is responsible for this maintenance of normal physiology and biochemistry (Nakamura et al. 2011; Wong et al. 2011). Hepatocytes, comprising 70–80% of the cytoplasmic liver mass, are the chief functional cells of the liver. These versatile somatic cells have a remarkable ability to regenerate and play a central role in the dynamic homeostasis of the liver (Vekemans and Braet 2005; Holt and Smith 2007; Ramadori et al. 2008; Nakamura et al. 2011; Wong et al. 2011). Functions performed by hepatocytes, and essentially the liver, include exocrine and endocrine functions, protein synthesis and storage, synthesis of cholesterol, bile salts and phospholipids, metabolism of carbohydrates and lipids as well as biotransformation of various endogenous and exogenous compounds (Holt and Smith 2007; Ramadori et al. 2008). The liver sinusoid, a capillary lined with sinusoidal endothelial cells and surrounded by hepatocytes, is the most basic functional unit of the liver. The endothelial cells are separated from the hepatocytes by a small space referred to as the space of Disse. Small channels referred to as bile canaliculi are formed between adjacent hepatocytes. The bile secreted by hepatocytes are collected into bile ducts and transported to the gall bladder for storage until it is needed in the intestines (Kang et al. 2013). Other non-parenchymal cells contribute to the remaining 20–30% of the liver mass, these include the stellate cells that help maintain the extracellular matrix (ECM), Kupffer cells that act like macrophages, natural killer cells and fibroblasts (Vekemans and Braet 2005; Kang et al. 2013). These various hepatic cell types working in a coordinated manner constitute the basic building blocks of the liver as a tissue. During dysfunction of the liver the 'normal' behavior of these cells is altered influencing growth, differentiation, marker secretion, invasion, migration or even death. It is these attributes that are helpful in the investigation of drug biotransformation and hepatotoxicity during drug development as well as in the assessment of normal and disease liver states (Gupta et al. 2016). However, current models to study biotransformation and hepatotoxicity hinder the elucidation of complex mechanistic liver functions, thus the development of novel experimental tools remain essential (Van Zijl and Mikulits 2010).

CONTACT Chrisna Gouws Chrisna.Gouws@nwu.ac.za Pharmacen™, Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa; Krzysztof Wrzesinski kwr@bmb.sdu.dk Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense M, Denmark
*Both authors share the position of last author for this publication.

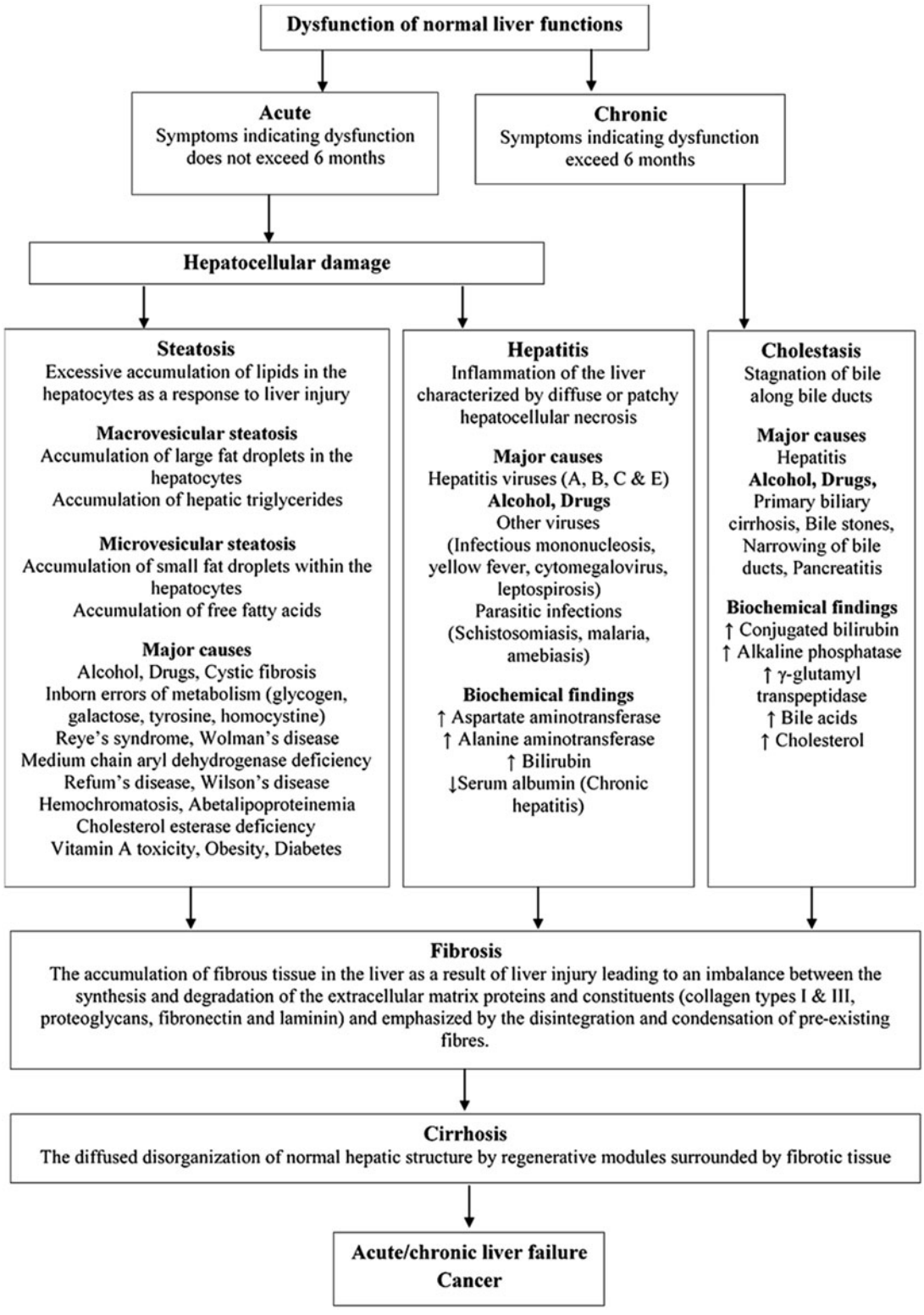

Figure 1. The progression from liver damage to liver disease as a result of liver dysfunction (Adapted from Wolf 1999; Heidelbaugh and Bruderly 2006; Featherstone 2007; Bernal et al. 2010; Hirschfield et al. 2010; Merrel and Cherrington 2011; Panqueva 2014; Privitera et al. 2014).

2. Liver dysfunction

Dysfunction and disease states of the liver results in devastating and often lethal consequences, as illustrated in Figure 1. Liver dysfunction presents as either hepatocellular damage and/or choleostasis and is classified as acute or chronic depending on the time of onset (Wolf 1999; Featherstone 2007; Privitera et al. 2014). Dysfunction in the form of hepatocellular damage occur due to various inflammatory responses including steatosis, hepatitis and/or cell death (necrosis), ultimately resulting in fibrosis and/or cirrhosis depending on the duration of the assault (Featherstone 2007). Steatosis (fatty liver or nonalcoholic fatty liver disease

(NAFLD)) is defined as the infiltration of hepatic triglycerides exceeding 5% by weight in the liver that occurs as a result of dysfunctional lipid metabolism. This accumulation of lipids may either be microvascular, macrovascular or both (Featherstone 2007; Merrel and Cherrington 2011). Microvascular steatosis is influenced by multiple factors and is also the result of drug toxicity from tetracyclines, while the development of macrovascular steatosis is attributed to alcoholic steatosis. NAFLD is the most common liver disorder in developed countries, is often related to the metabolic syndrome, insulin resistance and obesity. Persons suffering from NAFLD often show few symptoms apart from fatigue or general discomfort and so are often diagnosed only during routine blood tests. Since there is currently no approved treatment, acute liver failure due to NAFLD remains problematic (Featherstone 2007). Hepatitis or inflammation of hepatocytes due to cellular damage is attributed to the dysfunction of drug metabolism, alcohol metabolism, lipid metabolism and autoimmune diseases (Heidelbaugh and Bruderly 2006; Featherstone 2007). The most common cause of chronic hepatitis, however, remains viral infection (hepatitis C and B virus) (Featherstone 2007; Ramadori et al. 2008; Bernal et al. 2010).

During choleostasis there is an elevation of substances excreted by the bile and liver enzymes associated with the biliary tract as seen in Figure 1 (Featherstone 2007; Panqueva 2014). Accumulation of bile acids due to obstruction of the bile duct may also damage hepatocytes leading to the onset of fibrosis and cirrhosis if left untreated (Featherstone 2007; Hirschfield et al. 2010; Panqueva 2014). Choleostasis impedes drug biotransformation and metabolism due to sluggish or stagnant bile flow that impairs the biliary excretion of drugs causing a decrease in the solubility and absorption of fat soluble vitamins (Featherstone 2007).

The liver has remarkable regenerative capabilities and if disease states are addressed at an early stage, most of the hepatocellular damage may be reversed. However, if disease states are left untreated, then liver scar tissue can form during the restorative processes, which is a result of an imbalance between fibrogenesis and fibrolysis. This may lead to liver fibrosis, which disrupts blood flow and delivery of many essential substances (Featherstone 2007; Ramadori et al. 2008). Fibrosis is also the endpoint for most disease states resulting in chronic liver injury (Ramadori et al. 2008). Continued exposure to harmful substances, unhealthy lifestyle habits and disease states can ultimately lead to liver cirrhosis and liver failure or cancer (Heidelbaugh and Bruderly 2006; Featherstone 2007; Ramadori et al. 2008; Bernal et al. 2010; Merrel and Cherrington 2011). Thus, addressing possible liver dysfunction at an early stage may prove to enhance the life of a great many people.

3. Drug bio-transformation in the liver and its effect on liver dysfunction

The liver is seen quantitatively and qualitatively as the most essential site for drug biotransformation due to its ability to metabolize an almost endless selection of substrates (Wilkinson 2005; Liddle and Stedman 2006). A lack of biotransformation may result in an increase in the bioavailability and pharmacological activity of most drugs (by reducing the rate of their removal) (Tingle and Helsby 2006). Some drugs are inactive ('prodrugs') and need to undergo bio-activation to form the pharmacologically active metabolites. Hepatic drug biotransformation is governed by various factors including hepatic blood flow, plasma albumin binding, hepatocyte drug uptake, the functional integrity of hepatocytes as well as influences of the hepatobiliary system (George 1995; Ahmed and Siddiqi 2006). The biotransformation process can result in the production of hepatotoxins which may elicit interactions with cellular constituents, including proteins, lipids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), which eventually will cause hepatotoxicity and various liver lesions, most frequently steatosis (Guillouzo 1998; Singh et al. 2011). These interactions can result in a series of events in the liver, with a subsequent reaction from the liver as a result of injury. Liver toxicity due to pharmaceuticals and xenobiotics remain a concern as this is associated with distinct histopathological and clinical phenotypes, namely steatosis, choleostasis and hepatitis as illustrated in Figure 1 (Driessen et al. 2013; Sirenko et al. 2016). Currently, preliminary hepatotoxicity studies rely strongly on the extrapolation of data obtained from *in vivo* animal models and available *in vitro* cell culture models (Nakamura et al. 2011; Sirenko et al. 2016). Therefore, developing complex systems that can easily identify as well as effectively test potential hepatotoxicity remain an area of active investigation (Sirenko et al. 2016).

4. Current models used to study drug biotransformation and dysfunction in the liver

Due to the critical role of drug biotransformation in the development of new drug entities, as well as the potentially serious effects liver dysfunction may have on drug plasma levels and overall well-being of patients, numerous models and techniques have been developed to study dysfunction of the liver and its impact on drug biotransformation pathways. All these models and techniques have advantages and disadvantages, and the most suitable model must be selected for each application. The models and techniques frequently used for these various applications is briefly summarized in Table 1, along with some advantages and disadvantages. Animal models and the ethical considerations they involve, as well as the recent advances in three-dimensional cell culture techniques are discussed in more detail below.

4.1. Animal models: Ethical considerations and advances

In vivo animal models are able to take into account the combined effect of all pharmacokinetic parameters and pharmacological effects (i.e. multifactorial), while *in vitro* models can only measure a limited number of features (Zhang et al. 2012). The relevance of animal models may be questionable due to species differences, but the importance of these models in the development and testing of medicines, especially toxicity, is irrefutable. Historically, approximately 70% of

Table 1. Models used to study drug metabolism and dysfunction in the liver, with some of their advantages and disadvantages.

Model	Advantages	Disadvantages	References
In vitro			
Recombinant enzymes	• Effective and fast means to identify single CYP enzymes involved in drug metabolism • Various CYP enzymes are commercially available • Simplistic, well established, cost effective and high throughput screening assays • Identification of possible biotransformation pathways, CYP enzymes involved and mechanisms • Identification of substrates/inhibitors of metabolizing enzymes • Generating metabolites of interest	• Highly simplified specific assays which do not fully resemble the *in vivo* environment • Extrapolation process of data is complicated • Metabolic rates differ from those seen *in vivo* and in tissue • Phase I with phase II sequential metabolism is not addressed	(Ekins et al. 2000; Tingle and Helsby 2006; Lipscomb and Poet 2008; Zhang et al. 2012).
Subcellular fractions			
• Liver cytosol	• Drug enzyme activities preserved • High concentrations of N-acetyl transferase, sulfotransferase and glutathione S-transferase, present	• Short term studies • Only soluble phase II enzymes present • Full metabolic pathways cannot be investigated	(Guillouzo 1998; Brandon et al. 2003).
• Liver microsomes	• Well established, affordable and simplistic method • Most widely used subcellular fraction • Inter-individual variation can be studied • Drug enzyme activities preserved • Qualitative determination of metabolic identity • CYP reaction phenotyping • Provides information regarding drug-drug interaction and/or herb-drug interactions • Provides information regarding metabolic stability of the drug • Production of metabolites • Maintains higher levels of metabolizing enzyme activity than hepatocytes • Isolation of groups of enzymes possible	• Short term studies • Lack cell plasma membranes and drug transporters • Unsuitable for quantitative measurements • Incomplete representation of *in vivo* situation. • Do not maintain the balance between phase I and II enzymes • No cytosolic phase II enzyme reactions only CYP and UGT enzymes • Difficulty in studying multi-step metabolic processes • Co-factors required for activity	(Guillouzo 1998; Ekins et al. 2000; Brandon et al. 2003; Tingle and Helsby 2006; Lipscomb and Poet 2008; Alqahtani et al. 2013).
• S9 fractions	• Easy to prepare and reproducible • Potential for long term storage • Offers a more complete representation of the metabolic profile • Contains both phase I and II enzyme activity	• Overall lower enzyme activity compared to liver microsomes and cytosol fractions • Requires co-factors for activity	(Brandon et al. 2003; Lipscomb and Poet 2008).
Hepatocytes			
• Isolated hepatocytes	• Obtained from whole livers or wedge biopsies • Functions close to those of *in vivo* hepatocytes • Studies possible on several compounds at different concentrations • Interspecies studies possible • Representative of different lobular sub-populations • Drug transporters are present and functional • Sequential examination of Phase I and II pathways possible • Cryopreservation and preservation of phase I and II enzymes possible • Conservation of physiological co-factors • Well established and well characterized *in vitro* model • Viable for 4 weeks • May be incubated in suspension culture or plated • Study of mediators and enzyme inducers possible	• No bile canaliculi • Difficulty in obtaining fresh human cultures • Prolonged culture conditions result in complex data interpretation • Isolation process can be complicated and damage cells altering enzyme activity • Cellular interactions more difficult to study due to lack of non-hepatocyte liver cells • Inter-individual variation in CYP expression • Pathological status of donor and donor availability • Rapid decline in CYP activities (within hours or days): current supplier recommendations state that they should be used within 2 h of thawing.	(Guillouzo 1998; Brandon et al. 2003; Tingle and Helsby 2006; Lipscomb and Poet 2008; Alqahtani et al. 2013).
• Primary hepatocyte cultures	• Commercially available • Sequential examination of Phase I and II pathways possible • Partially differentiated cells, all enzymes and co-factors were initially present at physiological levels when the hepatocytes were isolated • Functions expressed for several days in certain conditions • Induction and inhibition of drug metabolizing enzymes possible • Inter-species studies possible • Sandwich culture and gel-immobilized techniques can be performed	• Early phenotypic changes • Altered bile canaliculi • Rapid decline in CYP activities	(Guillouzo 1998; Ekins et al. 2000; Brandon et al. 2003; Tingle and Helsby 2006; Alqahtani et al. 2013; Kanuri and Bergheim 2013; Ohkura et al. 2014).

(*continued*)

Table 1. Continued

Model	Advantages	Disadvantages	References
Liver cell lines HepG2; Huh7; HepG2/C3A HepaRG Fa2N-4 Transgenic cell lines	• Unlimited cell number • High availability • Low variability between experiments • Some functions preserved (and some can be recovered by 3D culture in systems which mimic *in vivo* conditions) • Co-culture with additional liver cell types (e.g. fibroblasts, endothelial, Kupffer cells or biliary-endothelial cells can induce additional functionality	• Various drug enzyme activities lost or decreased • Genotype instability • Lack phenotypical and functional characteristics of liver tissue	(Guillouzo 1998; Fey and Wrzesinski 2012b; 2013; Soldatow et al. 2013; Kanuri and Bergheim, 2013).
Stem cells	• Available in larger amounts than primary hepatocytes	• Currently, conditions are not known which can induce complete differentiation: the best can reach early fetal stages.	
• Embryonic stem cells	• Throughput depends on the application • Relatively unlimited supply • Defined phenotype	• Ethical concerns • Highly variable functionality within cell populations • May be useful for short term culture only (2-4 days) • Requires special media • Low expression levels of liver specific metabolism enzymes	(Soldatow et al. 2013; Ranga et al. 2014; Shearier et al. 2016).
• Induced pluripotent stem cells	• Throughput depends on the application • Not as controversial source of cells as embryonic stem cells • Relatively unlimited supply • Defined phenotype • Allows studies of inter-individual variability	• Complex 'reprogramming' steps • Low expression levels of liver specific metabolism genes • May be useful for short term culture only (2-4 days) • Variability among preparations • Limited literature for toxicology	(Soldatow et al. 2013; Zhang et al. 2014; Rookmaaker et al. 2015).
Liver slices	• Qualitative and quantitative data • Intact cellular architecture and lobular structure preserved • All enzyme and cell types preserved • Maintains zone-specific CYP activity • Cell-cell contact • Study of mechanisms of toxicity possible • Co-factors maintained close to physiological concentrations • Morphological studies possible • Selective intra-lobular effects detectable • Studies on human liver possible • Can be performed on various species making comparisons easy – standard procedure • Good *in vivo* - *in vitro* correlation • Studies possible on several compounds at different concentrations • Easy to prepare and avoids the use of harmful proteases or other enzymes	• Limited viability (6 h to 5 days) • Necrosis starts to develop after 48-72 h • No collection of bile possible • Poor correlation between intrinsic clearance rates and K_m values • Donor variability • Inter-assay variability (not all cells preserved similarly, damaged cells on outer edges and inadequate penetration of medium) • Need for specialized equipment and further development • CYP cannot be induced • Slight decrease in Phase II enzymes, albumin production and gluconeogenesis after 96 h • Metabolic enzyme levels decreases after 72 h • False prediction of metabolic rates • Uptake and metabolism lower in liver slices than hepatocytes • Cryopreservation has been demonstrated, needs optimization	(Guillouzo 1998; Ekins et al. 2000; Brandon et al. 2003; Tingle and Helsby 2006; Lipscomb and Poet 2008; Alqahtani et al. 2013; Soldatow et al. 2013).
Isolated perfused liver	Best representation of *in vivo* system (all enzyme functions preserved) • Three-dimensional architecture and lobular structure preserved • All liver cell types present, biotransformation studies of non-hepatocytes possible • Functional bile canaliculi • Collection of bile possible • Short term kinetic studies	• Short term viability (2-3 h) • Delicate model and difficult to handle • Study of one or a few compounds only • Bile excretion decreased after 1 to • 3 h • Suitable only for livers of relatively small animals • No significant reduction in the number of animals needed • Poor reproducibility	(Guillouzo 1998; Brandon et al. 2003).
In vivo Animal models: Rhesus monkey Macaque monkey Opossum Mini pig Rabbits Dogs Various rat and mice species Hepatitis C virus mouse models Zebrafish *Nematode (Caenahabditis elegans)*	• Ability to mimic human liver disease states (chemically or by means of cloning) • More complex and gives a holistic approach to elucidating drug metabolism and mechanisms	• Ethical considerations • Inter-species differences in drug metabolism and toxicology (worsening with evolutionary distance to man) • Labor intensive • Expensive • Time consuming • Requires skilled personnel (e.g. animal technicians, veterinarian) • Requires highly specialized facilities and equipment • Requires cloning in certain instances	(Rahman and Hodgson 2000; Tingle and Helsby 2006; Gunness et al. 2013; He et al. 2013; Kanuri and Bergheim 2013; Liu et al. 2013; Vercauteren et al. 2014).

human drug toxicities have been identified through animal testing; however, the development of better models for the prediction of human hepatotoxicity and liver dysfunction remains critically important (Davila et al. 2007; Freires et al. 2017). *In vivo* studies involving various animal species still serve as the gold standard in toxicology research, although a question raised more often than not is the moral legitimacy of animal experimentation (Paixão and Schramm 1999; Soldatow et al. 2013; Freires et al. 2017). From an ethical point of view, a strong need exists for the development of long term *in vitro* screening models, enabling the reduction of the number of animal subjects currently used in drug development in an attempt to abide by the three R's principle (i.e. reduce, replace and refine) (Baumans 2004; Hewitt et al. 2007; Freires et al. 2017). Furthermore, species differences between animals and humans can result in variances such as different levels of expression of various membrane transporters and metabolizing enzymes, relevant in areas of drug delivery, drug toxicity as well as drug interactions (Sabolić et al. 2011). The most important drawback relating to species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction have been extensively reviewed by Martignoni et al. (2006). The search for alternative models to address these shortcomings has increased in recent years, and the Zebrafish, a lower invertebrate *in vivo* model, has been found to be a valuable model for *in vivo* hepatotoxicity testing and drug discovery, overcoming many of the limitations mentioned (Driessen et al. 2013; Freires et al. 2017).

The Zebrafish (*Danio rerio*) is a tropical freshwater fish of the Cyprinidae family with an approximate length of 5 cm (Driessen et al. 2013; Freires et al. 2017). This *in vivo* model is well characterized, offering various advantages such as a completely sequenced genome with 71% of its genes homologous to humans', a short life cycle, accessibility and availability. Furthermore, the transparency of the zebrafish during larval stage allows for direct assessment of drug toxicity in a 96-well plate format, while the generation of high numbers of test subjects facilitates high throughput testing (McGrath and Li 2008; Driessen et al. 2013; He et al. 2013; Mesens et al. 2015; Freires et al. 2017). The assessment of drug induced hepatotoxicity in the Zebrafish is possible because of the similarities in the means by which the Zebrafish reacts to xenobiotic chemicals, including phase I and II biotransformation, exhibiting mechanisms of enzyme induction as well as oxidative stress when compared to these mechanisms in mammals (McGrath and Li 2008; Mesens et al. 2015). Zebrafish have been used successfully in enzyme reporter assays, cytochrome P450 assays, the visual assessment of liver necrosis and in evaluating histopathology (McGrath and Li 2008). Although this model still requires ethical approval it does address the three R's, since fewer animals are needed and because this is a lower invertebrate that can replace vertebrates (Redfern et al. 2008; Freires et al. 2017). However, there are still various limitations involved such as the need for specialized equipment, facilities and maintenance staff. To determine the effective compound concentration in the Zebrafish seems to be problematic, as it poses limitations when correlated and extrapolated to dosages administered to humans and rodents (Diekmann and Hill 2013; Freires et al. 2017). Other limitations include difficulty in achieving accurate oral dosages as most dosages are absorbed through the skin, and hepatotoxicity assessment is currently limited to acute studies (Hill et al. 2005; Redfern et al. 2008; Diekmann and Hill 2013; Flemming and Alderton 2013).

4.2. Three-dimensional cell culture models: Current advances in hepatotoxicity and biotransformation screening

In recent years, the efficacy of current two-dimensional *in vitro* cell culture models has increasingly come into question (Antoni et al. 2015). Efforts to improve the existing *in vitro* cell-based methods, used for pharmacokinetic and toxicity investigations as well as liver dysfunction, has shown that the physiological relevance of the system should be taken into consideration (Donato et al. 2008; Antoni et al. 2015). It is important to remember that the liver is a three-dimensional organ, established by cells *in vivo* that are continuously interacting with neighboring cells, as well as the extracellular matrix. These interactions by means of biochemical and mechanical signals are of utmost importance in normal cell and organ physiology (Lin and Chang 2008; Bell et al. 2016; Brajša et al. 2016). Some critical tissue-specific properties are absent in traditional two-dimensional (2 D) *in vitro* cell culture models, far removed from the natural *in vivo* state (Lin and Chang 2008; Antoni et al. 2015). Some of the important differences between cells cultured in 2 D and 3 D are presented in Table 2.

Three-dimensional (3 D) cell culturing systems are being explored in an attempt to establish novel *in vitro* models, capable of resembling native tissue and their normal functions more closely to ensure higher physiological relevance (See Table 3), while at the same time bridging the gap between current *in vitro* and *in vivo* models (Lin and Chang 2008; Wrzesinski and Fey 2013; Bell et al. 2016). In the last two decades, numerous advances have been made to produce high fidelity 3 D *in vitro* models using systems capable of long term maintenance, resulting in more accurate determination of drug activity, biotransformation and toxicology (Antoni et al. 2015). All the systems discussed offer various advantages and disadvantages which should be assessed individually before deciding on an appropriate method for a specific application.

4.2.1. Spheroid and organoid models

Spheroids are cell aggregates that are cultured in dynamic or static systems. Spheroid culture is seen as the best characterized model for 3 D culturing. Spheroid cultures as a model offers reproducibility, simplicity, as well as distinct similarities to the *in vivo* situation when compared to other models, and are also seen as a primary tool in drug discovery initiatives (Hirschhaeuser et al. 2010; Tung et al. 2010). The main difference between spheroids and other 3 D culturing techniques, such as scaffolds and hydrogels, is that spheroid assembly mimics the natural processes occurring during embryogenesis, morphogenesis and organogenesis with cell-cell

Table 2. Main differences between two-dimensional and three-dimensional cultured cells.

Two-dimensional (2D) cell culturing	Three-dimensional (3D) cell culturing
Cells can only be cultured for a limited period (days to one week) without intervention, allowing only acute toxicity studies.	Cells can be cultured for longer time periods (weeks to months) without intervention, allowing both acute and chronic exposure during toxicity studies.
Standardised and established assays are available to measure bio-transformation and toxicity.	Methods and assays to determine bio-transformation and toxicity are not standardized and are still being developed.
Altered cellular differentiation as cells are committed to cell growth, rather than differentiation.	Greater amount of cellular differentiation as cells within 3D constructs are at various stages of the cell cycle, resulting in *in vivo* tissue-like structures within aggregates.
Cells cultured in 2D present with unnatural cell shape, geometry and morphology, altering cellular communication and the cytoskeleton.	Cells cultured in 3D present with a more natural cell shape, geometry and morphology, better facilitating cell-cell communication, regulatory mechanisms and signaling networks.
Physiological relevance is questionable as most cells *in vivo* grow as aggregates rather than monolayers. Cells in the 2D environment adapt to the unnatural flat and ridged surfaces, which results in constructs with altered cellular metabolism, biochemical features and cell cycle kinetics.	More physiologically relevant as most organ and tissue structures *in vivo* consist of cell aggregates. Cells within 3D constructs adopt tissue-like structures with more physiologically relevant cellular metabolism, biochemical features and cell cycle kinetics.
Data collected from 2D experiments lack clinical relevance, since it provides misleading data in many instances, not representative of the *in vivo* response because of altered cellular responses	Provides clinically relevant data, more representative of the *in vivo* condition and, in many instances, comparable with animal studies.

Adapted from Khoruzhenko 2011; Breslin and O'Driscoll 2013; Wrzesinski et al. 2014; Edmondson et al. 2014; Antoni et al. 2015; Brajša 2016; Fang and Eglen 2017.

interactions dominating over cell-scaffold interactions (Hirschhaeuser et al. 2010; Tung et al. 2010; Achilli et al. 2012; Fang and Eglen 2017). This model effectively mimics avascular tumors through the formation of molecular gradients, namely oxygen, nutrient and growth gradients (Hirschhaeuser et al. 2010; Van Zijl and Mikulits 2010; Achilli et al. 2012; Fang and Eglen 2017). Spheroids can be cultured using hanging drop systems, rotating micro-gravity bioreactors, spinner flasks, ultra-low attachment plates, self-aggregation systems, microcarrier beads and pellet cultures (Lin and Chang 2008; Achilli et al. 2012; Breslin and O'Driscoll 2013; Page et al. 2013; Soldatow et al. 2013; Wrzesinski and Fey 2015; Fang and Eglen 2017).

4.2.1.1. Hanging drop cultures. Hanging drop spheroid cultures are the result of suspended cells at various seeding densities, which assemble through self-aggregation by means of gravitational forces at the air liquid interface of specifically designed 96-well plates (Messner et al. 2013; Kim et al. 2015). Messner et al. (2013) developed multi-cell liver spheroids for hepatotoxicity testing by co-culturing primary human hepatocytes with the non-parenchymal Kupffer and endothelial cells, to be used in inflammation mediated hepatotoxicity testing. Spheroids were allowed to form for three days after being seeded onto a 96-well hanging drop culture platform (GravityPLUS^{TM}) and were subsequently transferred to non-adhesive spheroid-specific GravityTRAP^{TM} 96-well plates. Spheroids were maintained for a duration of 5 weeks. Cell viability was measured by means of an ATP assay and morphological characterization was done by immunohistochemistry. Results obtained from the ATP assay indicated that the spheroids remained stable and functional over the 5-week period, with Kupffer and endothelial cells distributed throughout the spheroids.

Immunohistochemistry also indicated the presence of the broad specificity efflux pump (multi-drug resistance protein 1, MDR1) and bile salt export pump (BSEP). During hepatotoxicity testing over 14 days with acetaminophen and diclofenac, spheroids treated with acetaminophen presented with concentration dependant cell death, with a half maximal inhibitory concentration (IC_{50}) value of 754.2 μM, while spheroids treated with diclofenac presented with increased sensitivity and an IC_{50} value of 178.6 μM. Results indicated that this model allowed for the routine testing of compounds as well as determining chronic and inflammation mediated toxicity (Messner et al. 2013).

Gunness et al. (2013) explored HepaRG spheroids by means of the static hanging drop system and maintaining them in culture for a period of 3 weeks. Functionality was accessed by determining phase I enzyme and transporter activities as well as the expression of liver-specific proteins. To access if the model can be employed to predict hepatotoxicity and bio-transformation the model drugs acetaminophen, troglitazone and rosiglitazone were administered. All results where possible were compared to classic 2 D cell culturing techniques. Results indicated that the spheroid cultures maintained liver-specific functionality while expressing liver-specific markers such as albumin, CYP3A4, 2E1 and MRP-2 during the 3-week culturing period. With significantly higher production of albumin and CYP2E1 activity in the 3 D cultures compared to the classic 2 D cell cultures. Toxicity assessment indicated that the half maximal effective concentration (EC_{50}) value of 2.7 mM obtained for acetaminophen in 3 D cultures correlated with published *in vivo* data. Indicating that spheroids constructed from HepaRG cells can serve as a valuable *in vitro* tool for the assessment of incidences of acute and possibly chronic hepatotoxicity.

4.2.1.2. Microgravity cultures. Wrzesinski et al. developed and characterized a microgravity bioreactor spheroid cell culturing system using the immortal hepatic HepG2/C3A cell line, a subclone of the widely used HepG2 cell line (Fey and Wrzesinski 2012a; Wrzesinski and Fey 2013; Wrzesinski et al. 2013; 2014). In 2012, Fey and Wrzesinski investigated the median lethal dose (LD_{50}) of six drugs (acetaminophen, amiodarone, diclofenac, metformin and valproic acid), commonly used in toxicity studies, by means of microgravity bioreactors and HepG2/C3A cell spheroids while comparing data from to

Table 3. Three-dimensional (3 D) culture methods for liver cells designed to retain, recover or induce liver functionality *in vitro*.

Method	Advantages	Disadvantages	References
Microfluidic device	• Described as suitable for high throughput testing • Possibilities for multiple chambers with different cells in each	• Specialized equipment required • Expensive • Further analysis of 3D cultures produced may be difficult • Closed systems prone to blocking or air bubble problems • Lipophilic compounds tend to attach to devices • Reproducibility needs to be improved • Needs further toxicological validation	(Breslin and O'Driscoll 2013; Benam et al. 2015; Maschmeyer et al. 2015; Materne et al. 2015; Wrzesinski and Fey 2015).
Forced floating	• Relatively simple • Inexpensive • Suitable for high-throughput testing • Spheroids produced are easily accessible	• Variability in cell size and shape if not a fixed cell number per well • Plate-coating is relatively labor intensive	(Breslin and O'Driscol et al. 2014; Guo et al. 2014).
Hanging drop	• Inexpensive and simple to perform if using standard 96-well plate • Well-controlled homogenous spheroid size suitable for high-throughput testing • Fast spheroid formation and easily accessible • Co-culturing of different cell types possible • Easy to trace spheroid assembly	• Labor intensive if preparing plates in-house • More expensive if using specialized plates • Small culture volume makes medium exchange, without disturbing cells, difficult (proposed easier handling with commercially available formats) • Small spheroids (up to ~0.3mm)	(Lin and Chang 2008; Takahashi 2015; Wrzesinski and Fey 2015; Breslin and O'Driscoll 2016).
Non-adhesive surface (liquid overlay)	• Inexpensive • Simple to perform • Easy to scale up	• Variation in size/cell number/shape	(Lin and Chang 2008).
Ultra-low attachment plates	• Inexpensive in 96-well format • Simple to perform • Easy to scale up • Well-controlled spheroid size • Suitable for high throughput testing • Microscopic and HCA evaluation possible	• Part of spheroid has reduced access to media • Limited material for assay • Medium-sized spheroids (up to ~0.6mm)	(Ivanov 2014; Takahashi 2015; Raghavan et al. 2016).
Micro-moulding and printing	• Well-controlled spheroid size • Designed aggregate geometry • Co-culture of different cell types	• Require specialized facilities • Spatial resolution is currently limiting • Needs further validation before widespread toxicological application	(Lin and Chang 2008; Zarowna-Dabrowska et al. 2012; Murphy and Atala 2014).
Galactosylated substrates	• High preservation of viability and functions of primary hepatocytes	• Only suitable for cells expressing galactose receptors	(Lin and Chang 2008).
Pellet culture	• Simple to perform • Rapid aggregation of large number of cells	• Shear force • Mass production difficult • Small spheroids (up to ~0.3mm)	(Lin and Chang 2008; Zanoni et al. 2016).
Monoclonal growth	• Little work involved • Some are useful morphogenesis models	• Only occurs in certain cell types • Relatively long incubation periods • Require extra procedures to harvest the multicellular spheroids	(Lin and Chang 2008).
External force enhancement	• Rapid cell aggregation • Advantageous for working with stem cells	• Potentially undefined effects to cells • Require specialized equipment and culture conditions	(Lin and Chang 2008; Antonchuk 2013).
Agitation based approaches			
Spinner flasks	• Simple to culture cells • Mass production relatively easily achievable • Large spheroids possible (up to ~1 mm) • Long term culture (months) • Dynamic control of culture conditions • Co-culture of different cell types • Motion of culture assists nutrient transport • Spheroids produced are easily accessible	• Requires specialized equipment • Variation in size/cell number of spheroids (can be overcome by additional culture step; see forced floating methods) • Time consuming due to extra step required for homogenous spheroids • High shear force in spinner flask (may be problematic for sensitive cells)	(Lin and Chang 2008; Jiang et al. 2010; Miranda et al. 2010; Breslin and O'Driscoll 2013).

(*continued*)

Table 3. Continued

Method	Advantages	Disadvantages	References
Microgravity culture	• Simple to perform • Mass production possible • Large spheroids possible (up to ~1 mm) • Long-term culture (months) • Dynamic control of culture conditions • Better cell differentiation • Co-culture of different cell types possible • Recovery of some *in vivo* physiology • Good correlation with *in vivo* toxicity	• Requires specialized equipment • Variation in size/cell number (Well-controlled spheroid size can be achieved by the use of AggreWell™ plates to initiate spheroids • Cells require 18 days to recover from trypsination	(Lin and Chang 2008; Fey and Wrzesinski 2012a; 2013; Wrzesinski and Fey 2013; Wrzesinski et al. 2014; Zanoni et al. 2016; Aucamp et al. 2017).
Matrices and scaffolds	• Provide 3D support that mimics *in vivo* • Some incorporate growth factors	• Can be expensive for large-scale production • Can have difficulty in retrieving cells following 3D culture formation • Can limit cell-cell interactions	(Nakamura et al. 2011; Schutte et al. 2011; Breslin and O'Driscoll 2013; Ravi et al. 2015).
3-D scaffolds	• Provide 3D extracellular support	• Require specialized equipment for scaffold fabrication • Have not been tested on many cell types	(Lin and Chang 2008; Heffernan et al. 2015).
Collagen, hydrogel or Matrigel™ supports	• Often used in sandwich or raft cultures • Depending on application, presence of exogenous extracellular matrix material can enhance or exacerbate *in vivo* functionality	• Massive production difficult • Can be expensive • Potential batch to batch variation	(Miranda et al. 2010; Totok et al. 2011; Yip and Cho 2013; Wrzesinski and Fey 2015).
Supported cell sheet	• Prevent anoikis-induced cell death • Well-mixed co-culture	• Labor intensive and time consuming • Mass production difficult	(Lin and Chang 2008; Torok et al. 2011).
Primaria dishes	• Well preservation of viability and functions of primary hepatocytes	• Primarily used for hepatic-linkage cells	(Lin and Chang 2008).
Hollow-fiber reactor	• Moderate throughput • Counter-directional flow • Small cell numbers and media volumes • Microscopic evaluation is easy	• A complex system • Lack of physiological gradients	(Soldatow et al. 2013).
Single-well and multi-well perfused bioreactor	• High throughput • Cells from 3D tissue constructs • Sustained liver-like cell functionality • Physiological shear stress • Good correlation with *in vivo* clearance rates • Ability for microscopic examination	• Uses greater cell numbers and larger media volumes • Has been validated with rat and human hepatocytes	(Soldatow et al. 2013).
Ex vivo models			
Bio-artificial livers	• Low throughput • Microenvironment most similar to *in vivo* tissue • Allows for studies of functional heterogeneity • Ability to evaluate hepatotoxicity using human blood	• Requires large numbers of cells • Currently not in use with other cell types • Complex membranes needed for proper use • Does not maintain viability or functionality of hepatocytes longer than other methods	(Soldatow et al. 2013).

2 D cell cultures and available *in vivo* observations. To circumvent uncertainty experienced regarding cell numbers and population size, the spheroid data was normalized to amount of protein (μg) present within the spheroids. The latter allowed for dosages administered to spheroids to correlate with dosages administered to animal models during *in vivo* toxicity studies (mg/Kg). Spheroids were prepared using AggreWell®400 plates and cultured within resealable bioreactors (Fey and Wrzesinski 2012b patent). Microscopy, planimetry and protein content were evaluated, and they found the comparison of planimetric area and protein content of the spheroids to demonstrate a clear correlation with a relative standard deviation (RSD) of 21%. Intracellular ATP content was measured to determine cell viability for each of the drug treatments. Data from the later correlated better with published *in vivo* data than with LD_{50} values obtained using either primary hepatocytes or with cell lines in 2 D culture (correlation coefficient of 86% compared to either 75% or 55%, respectively), indicating the usefulness of this *in vitro* technique for determination of LD_{50} values (Fey and Wrzesinski 2012a).

Wrzesinski and Fey (2013) then went on to determine that 18 days of culture is needed for HepG2/C3A spheroids to reestablish physiological functions and ultra-structural traits after trypsination. Trypsination at regular intervals such as is needed for continuous 2 D cell culturing will result in a disruption of advanced cellular functions, signal transduction, gene expression as well as influencing ECM repair processes and natural cell structure (Page et al. 2013; Wrzesinski and Fey 2013). Wrzesinski and Fey found that cells recover after trypsination in 3 D cultures. However, in 2 D cultures cells must be trypsinised every 5 days, preventing them from recovering, placing 2 D cells in a continuous 'wound healing' cycle, whereas, with the 3 D spheroids this recovery continues up until 15–18 days, as suggested by changes in adenylate kinase, ATP, urea and cholesterol production. The latter corresponded well with published literature as several other cell lines, namely Caco-2, HT 29, MDCK, MCF-10 A and HepG2, are reported to have similar recovery, needing between 15 and 21 days. Wrzesinski and Fey thus proposed that this is a pervasive recovery process rather than differentiation, which may explain the physiological capabilities that more closely resemble *in vivo* conditions within 3 D spheroid cultures.

In 2013, Wrzesinski et al. continued to determine that the HepG2/C3A 3 D spheroids exhibited stable physiological functionality for a duration of at least 24 days after this recovery from trypsination. They found that 3 D spheroid culture provides a metabolically competent homeostatic cell model that reaches equilibrium within the culturing environment for a period of at least 24 days. Such a stable system permits determination of drug toxicity and mode of drug action, evaluation of biomarkers as well as the study of system biology, all of which requires metabolic functions to be stable over a long-term period (Justice et al. 2009; Wrzesinski et al. 2013; Antoni et al. 2015).

They further strengthened their argument by investigating the proteome of both exponentially growing 2 D cells and the 3 D spheroids at dynamic equilibrium. They concluded that there are significant changes within every aspect of cellular metabolism that serves as the foundation of architectural, functional and physiological differences within cells. Cells grown in 3 D constructs such as spheroids at dynamic equilibrium are focused on functionality, effectively mimicking the *in vivo* condition (Page et al. 2013; Wrzesinski et al. 2014; Antoni et al. 2015).

4.2.1.3. Spinner flasks. The human HepaRG cell line is a well characterized cellular model used to study the incidence and prediction of drug-induced hepatoxicity (Leite et al. 2012; Szabo et al. 2013; Nelson et al. 2017). Monolayer cultures show phenotypic characteristics similar to primary human hepatocytes, which include the expression of phase I, II and III liver enzymes, sensitivity towards protypical inducers as well as the possibility for several weeks of culturing with a stable phenotype (Leite et al. 2012; Nelson et al. 2017). Leite and associates (2012) investigated the long-term 3 D culturing of HepaRG employing spinner flasks. These HepaGR spheroids maintained liver-specific functions mimicking *in vivo* liver morphology for seven weeks, as well as a dose-dependent effect to acetaminophen exposure.

4.2.1.4. Ultra-low attachment plates. Janorkar et al. (2011) created a 3 D steatosis model using elastine-like polypeptide-polyethyleneimine co-polymer coated plates, resulting in the spontaneous aggregation and spheroid formation of a H35 rat hepatoma cell line that mimics the *in vivo* liver architecture and provides information on transcription regulation in fatty liver disease. The latter is seen in the ability of the model to promote the reuptake of fatty acids, accumulation of triglycerides, decrease in proliferation, depressed liver-specific functions and the accumulation of reactive oxygen species. This model thus provides a platform for the elucidation of relationships that exist during nonalcoholic fatty liver disease available in a 3 D model (Janorkar et al. 2011).

Wong et al. (2011) created sized controlled self-aggregating static spheroids termed hepatospheres, and co-cultured heterospheres from primary hepatocytes and hepatic stellate cells – both isolated from adult Sprague-Dawley rats. As a means to circumvent and address the challenge of producing large amounts of uniform sized hepatospheres and heterospheres, the spheroids were cultured by constructing concave microwell arrays with diameters between 300 μm and 500 μm, from poly(dimethylsilohexane) (PDMS) membranes and treating the membranes with 3% (w/v) bovine serum albumin (BSA) to prevent cell attachment. Both monoculture hepatospheres and co-cultured heterospheres were cultured for a period of 12 days. The cell viability and morphology of the outer and inner structures, namely bile canaliculi and cell-cell contacts, were determined on day three of culturing using scanning electron microscopy and transmission electron microscopy. Functional assessment relating to urea and albumin secretion, as well as CYP3A4 activity of both hepatospheres and heterospheres were conducted on days 1, 3, 5, 7 and 9 after culture initiation. Results indicated that hepatic stellate cells play an important role in the control and

organization of spheroid aggregates and in establishing cell to cell communication. The functional assessment showed an increase in albumin secretion of 30%, as well as an increase in CYP3A4 production in heterospheres compared to hepatospheres on day 8 and 9, respectively. Urea secretion of both heterospheres and hepatospheres remained unchanged. Based upon these findings, Wong et al. (2011) proposed that these heterospheres be used to create an artificial 3 D hepatic tissue construct to assess liver hepatotoxicity, regeneration and failure.

Bell et al. (2016) developed and characterized an easily scalable 3 D spheroid system. Primary human hepatocytes were allowed to self-aggregate within low binding 96-well plates, creating spheroid within 7 days. Spheroid phenotypes and molecular signatures were assessed and compared to 2 D cells by means of proteomic analysis. This self-aggregating static system was shown to be functional for a period of 35 days according to haemotoxylin and eosin (H&E) scanning, immunohistochemical staining and physiological parameters assessed. This spheroid system also proved to be an excellent model to study the incidence of drug induced liver injury, as five known hepatotoxins (amiodarone, bosentan, diclofenac, fialuridine and tolcapone) were evaluated and half maximal effective concentration (EC_{50}) results approached clinically observed concentrations. Co-culture with non-parenchymal cells is also possible in this system, to even more closely resemble the *in vivo* situation. By being able to chemically induce liver dysfunction in this system, it is a promising *in vitro* model to study liver dysfunction and disease (Bell et al. 2016).

Induced pluripotent stem cells (iPSC)-derived hepatocytes offers a hepatic model that closely represents primary hepatocytes due to comparable functionality and phenotypes, while overcoming batch to batch variability and limitations found within the latter (Sirenko et al. 2016). Sirenko et al. created a static three-dimensional spheroid model from iPSC-derived hepatocytes within a matrix suspension, cultured in 96-well GravityTRAP ultra-low attachment spheroid plates for a period of 72 hours. They attempted to develop and characterize confocal high-content imaging as a high-throughput screening technique, to establish drug induced hepatotoxicity of 50 known hepatotoxic drugs and comparing this to the HepG2 cell line and 2 D cell culturing. By employing fluorophores with the capacity to determine cell viability, DNA binding, incidences of apoptosis and mitochondrial markers in combination with high content screening, one can overcome the use of classic disruptive spheroid analysis techniques in high-throughput screening. The assays employed enabled characterization of hepatotoxicity by evaluation of spheroid size, cell number and spatial disruption, viability, nuclear characterization, apoptosis and mitochondrial potential. Their results indicated significant differences across the two cell types (iPSC and HepG2) and at different culturing conditions (2 D and 3 D spheroids), with regard to the pharmacological effects expressed by the model compounds. The latter indicated the potential of using spheroids from iPSC-derived hepatocytes in combination with confocal imaging techniques in high-throughput screening to determine of drug induced hepatotoxicity (Sirenko et al. 2016).

4.2.2. Hydrogel and scaffold supports

Hydrogels and scaffold supports are attractive methods for growing cells in three dimensions. This group of models refers to synthetic three-dimensional constructs produced from various materials, offering differences in porosities, permeability, surface chemistry and mechanical attributes (Hayward et al. 2013; Fang and Eglen 2017). The extra cellular matrix (EMC) plays an important role in the growth, differentiation, polarization, maintenance and signal transduction of cells growing *in vivo*, and typically the EMC is composed of various proteins, namely laminin, collagens, elastin, glycoproteins and proteoglycans (Hughes et al. 2010; Verhulsel et al. 2014). To mimic this *in vivo*-like environment *in vitro,* a variety of hydrogels and scaffolds can be used to create an artificial ECM. Hydrogels and scaffolds are biomaterials that can be grouped into two main categories, namely natural and synthetic (Hoffman 2012; Verhulsel et al. 2014; Fang and Eglen 2017). Natural hydrogels and scaffolds can be simple compounds, consisting of purified mixed proteins such as collagen, gelatin, fibrin and laminin; or they can be obtained from living cells such as the commercialized matrix Matrigel™, consisting primarily of laminin, collagen IV and enactin (Hughes et al. 2010; Hoffman 2012; Verhulsel et al. 2014; Fang and Eglen 2017). Other natural hydrogels or scaffolds include chitosan, alginate, agarose and silk fibers that are derived from natural materials. Conversely, synthetic hydrogels and scaffolds are produced from synthetic, non-biodegradable, porous polymers that are inert, reproducible and versatile (Hayward et al. 2013). The latter includes poly(ethylene-glycol), diacrylate, poly(acryl-amide) and poly(vinyl-alcohol) (Verhulsel et al. 2014). Hydrogels and scaffolds have been explored as biomaterial for many years offering a hydrophilic character, biocompatibility, chemical stability as well as being biodegradable in certain instances. These attributes make them valuable to the fields of three-dimensional cell culturing and tissue engineering (Hoffman 2012).

The two-week cultivation of HepG2 liver cells in 3 D constructs by encapsulating cells within two different sodium alginate-based hydrogels, SLM100 and SLG100 was also investigated and made possible. To understand cell-matrix interactions and systemic behavior on a micro-scale, cell viability, morphology and drug metabolism were quantitatively and qualitatively studied. All results, where possible, were directly compared to classic 2 D cell cultures. The results obtained by Lan et al. (2010) indicated that encapsulated HepG2 cells showed high cellular viability, albeit slight proliferation within 14 days of cultivation. The production of CYP1A1 and CYP3A4 liver-specific enzymes, as well as phase II glutathione production over the 14-day cultivation period indicated viability as well as functionality of the encapsulated cells. The encapsulated cells were also capable of the linear bio-transformation of the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) to HFC (7-hydroxy-4-trifluoromethyl).

To improve commercially available polystyrene-based scaffolds modifying surface properties was explored. Polystyrene-based scaffolds are produced by means of poly-HIPE technology (porous polymers derived from high internal

phase emulsions) and have the advantages of being highly porous, with controllable morphology and suitable mechanical properties. Hayward et al. (2013) attempted to overcome one of the major limitations of these polystyrene based scaffolds, namely surface chemistry. The extracellular matrix of cells *in vivo* allows for cell anchorage, cell-cell communication and normal functionality of cells. While carbohydrates and proteins provide biochemical cues to aid in the regulation of normal cellular function, the aforementioned are not provided by polystyrene-based scaffolds. The aim was to mimic these surface interactions by surface functionalization with galactose, a carbohydrate that is known to bind to hepatocytes by means of the asialoglycoprotein receptor (ASGPR). A parent formulation, SDE-polyHIPE, was constructed from the monomers styrene (STY), divinylbenzene (DBV) and 2-ethylhexylacrylate (EHA). A 26% pentafluorophenyl acrylate in a SDE-polyHIPE mixture was further modified by coupling either 2′-aminoethyl-β-D-glucopyranoside or 2′-aminoethyl-β-D-galactopyronoside to create Glu-SDE-polyHIPE and Gal-SDE-polyHIPE, respectively. HepG2 cells and cryopreserved primary hepatocytes derived from Sprague-Dawley rats were then seeded onto the three constructs and allowed to grow under normal culture conditions. The morphology of the 26PFPA-SDE-polyHIPE was characterized using scanning electron microscopy (SEM) and, using the HepG2 cell culture, histological analysis was performed. The results indicated that after 5 days of growth cells anchored and formed tissue-like layers in a similar fashion as commercially available Alvetex scaffolds that served as control. Image analyses indicated that cells appeared healthy and viable, with no signs of necrosis although less penetrations of the cells are noticed compared to the control due to the smaller void area of the 26PFPA-SDE-polyHIPE construct. After functionalization of the 26PFPA-SDE-polyHIPE with aminoethyl glycosides, the surface carbohydrates were characterized, primary rat hepatocytes were cultured onto the Glu-SDE-polyHIPE and Gal-SDE-polyHIPE constructs and albumin secretion was assessed. The results indicated that the addition of galactose to polystyrene based scaffolds proved to be advantageous in adhesion of hepatocytes to these scaffolds and improved cellular functionality.

4.2.3. Microfluidic, organs on chips and bioartificial liver models

Microfluidic technology provides an adaptable platform for biological applications, offering various advantages. It is a system of microscale dimensions corresponding to the cellular organization found in the *in vivo* situation, with chemical gradients able to mimic the dynamic micro-environment (Kim et al. 2015; Gupta et al. 2016). It is of the utmost importance that the micro-environment within 3 D cell cultures should mimic the *in vivo* physiological conditions. Microfluidic technology has the potential to replicate realistic fluid retention times and liquid-to-cell ratios (Kim et al. 2015). This multifaceted technology allows for the handling of several processes at once during cellular growth, in that it supplies nutrition, liquid flow, oxygen as well as removal of degradation products. The construction material of microfluidic devices is often permeable to oxygen (enhancing growth and proliferation), and as sample and reagent volumes are so small it also provides a very cost-effective platform. Typical substances used in the construction of microfluidic devices are glass, silicon, polymers (poly-dimethysiloxane (PDMS), polycarbonate (PC), polystyrene (PS) and poly-methyl methacrylate (PMMA)) and chromatographic paper. Cells are grown either with the support of hydrogels or in gel-free systems (Gupta et al. 2016).

4.2.3.1. Microfluidics. A dynamic microfluidic bioreactor as alternative *in vitro* hepatotoxicity testing method was created using the transparent polymer polydimethylsiloxane (PDMS). In this system, hepatic HepG2/C3A cells were seeded onto micro bioreactors coated with fibronectin. Viability experiments were conducted over 96 hours and all experiments, where possible, were related to static 2 D culturing conditions. Viability was investigated by means of lactate dehydrogenase, and bio-transformation by monitoring glucose, glutamine, ammonia and albumin concentrations as well as the expression of CYP1A1. Their findings indicated that cell proliferation was dependent on cell seeding density and flow rate, and the increased proliferation and metabolism within the dynamic conditions compared to the static conditions indicated the importance of a dynamic microenvironment for optimal cell metabolic activity (Baudoin et al. 2011).

Prot et al. (2012) then incorporated the use of integrated systems biology with microfluidics and biochip data for the use in the transcriptomic, proteomic and metabolomic profiles assessment during hepatotoxicity of HepG2/C3A cells exposed to 1 mM acetaminophen (APAP). Biochips where fabricated from PDMS and coated with fibronectin before seeding cells onto the biochips as described (Baudoin et al. 2011). All experiments were compared to normal 2 D cell culture, and untreated cells showed adaptive cellular responses to the microfluidic environment. However, cells treated with APAP resulted in perturbation of calcium homeostasis, lipid peroxidation and apoptosis due to the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) produced from APAP. Reported biomarkers of hepatotoxicity from APAP ingestion and glutathione depletion namely 2-hydroxybutyrate and 3-hydroxybutyrate, and the consumption of methionine, cysteine and histidine were observed in treated biochips. The later correlated well with literature and resulted in a more complete reconstruction of the APAP injury pathways, demonstrating a potential new approach to predictive toxicology (Prot et al. 2012).

4.2.3.2. Organs on chips. Organs on chips is an emerging and promising platform to study bio-transformation and drug toxicity, with the potential to study bio-threats and even chemical warfare. Bhise (2016) reported on a directly accessible liver on chip platform that can be employed for long term 3 D culture of human HepG2/C3A spheroids for four weeks, as a means of investigating drug toxicity comparable to *in vivo* conditions. Perfusable bioreactors were fabricated via a bioprinting approach, using polydimethylsiloxane (PDMS) and poly-(methyl methacrylate) (PMMA).

These bioreactors where then loaded with self-aggregating HepG2/C3A spheroids, encapsulated in photocrosslinkable gelatin methacryloyl (GelMA) hydrogel. The latter was incubated under continuous perfusion at 200 μl h^{-1} for 30 days, allowing evaluation of cellular functionality and response to an acute dosage of 15 mM acetaminophen (APAP). Secretion of the hepatic biomarkers namely: albumin, α_1-antitrypsin (A1AT), transferrin and ceruloplasmin, were used to determine cellular functionality. Immunostaining was used to assess the non-secreted cellular proteins cytokeratin 18, multidrug resistance-associated protein II (MRP2) bile canalicular transport proteins and tight junction protein ZO-1. Both the secreted and non-secreted proteins remained fully functional and stable for 30 days within the bio-printed constructs. However, this metabolic activity significantly decreased within the bio-printed constructs during the course of treatment with APAP, correlating well with data previously published by Fey and Wrzesinski (2012b).

As the human body is composed of interrelated tissue systems, microfluidic devices capable of mimicking the *in vivo* situation by simultaneously culturing various cell lines will be advantageous (Huh et al. 2011). Materne et al. (2015) have attempted just that by the 14-day long-term simultaneous culturing of NT2 neurospheres and co-cultured HepaRG and human hepatic stellate liver spheroids on a microfluidic organ on chip model. The microfluidic system was molded from apolydimethylsiloxane (PDMS) bonded to a glass microscope slide, consisting of two compartments for spheroid culturing and three pump membranes. Liver spheroids and neurospheres, created by means of the hanging-drop model, were loaded into the culturing compartments in HepaRG growth medium. Growth medium samples were taken daily to determine lactate dehydrogenase (LDH), glucose and lactate analyses, while drug biotransformation and toxicity of 2.5-hexanedione (16 mM and 32 mM) were assessed from day 6 by means of terminal deoxynucleotidyl transferase dUTP Nick-end labeling (TUNNEL) assay and physiological parameters. Results indicated that cells were viable for a period of 14 days, showing tissue specific expression of markers such as CYP3A4 and MDR2, and the ability to respond to the toxic onslaught of 2,5-hexanedione (Materne et al. 2015).

4.2.3.3. Hollow-fiber reactor. Shen et al. (2010) developed a polysulfone-g-poly (ethylene glycol) (PSf-g-PEG) hollow fiber to circumvent the instances of adsorption of hydrophobic drugs onto these hollow fiber systems. Two hollow fibers were produced and characterized, polysulfone (PSf) and polysulfone-g-poly (ethylene glycol) (PSf-g-PEG). Freshly isolated hepatocytes were imbedded in a type I collagen gel and then loaded into the hollow fibers by injection, cut into 2 cm sections and cultivated for 48 hours. To create a cylindrical gel, hepatocytes in collagen gel were loaded into hollow fibers and extruded after 10 minutes. After 48 hours of cultivation in various conditions, CYP1A2, 3 A and 2E1 activity and drug hepatotoxicity of model drugs (tetracycline, azathioprine, acetaminophen, salicylate, clozapine, rifampicin, chloroquine and aminodarone) were measured within PSf, PSf-g-PEG and cylindrical gels. The culturing medium of cell-free hollow fibers were also analyzed to determine drug adsorption to these model drugs after 48 hours. The results indicated that cells within the hollow fibers showed aggregate formation after 48 hours, compared to cells within the cylindrical gels which remained dispersed. Also, the activity of CYP enzymes were increased within the hollow fibers, with the PSf-g-PEG hollow fiber producing slightly higher CYP3A and 2E1 activity. Higher liver-specific functions were also noted for cells within hollow fibers compared to the cylindrical gel. The cells within the PSf-g-PEG hollow fibers also showed increased hepatotoxicity to the model drugs administered compared to the cylindrical gels. Furthermore, the PSf-g-PEG hollow fibers outperformed the PSf hollow fibers in terms of drug adsorption and accumulation greatly, reducing the number of drugs and proteins adsorbing to these surfaces indicating a promising tool for drug investigation *in vitro* (Shen et al. 2010).

4.2.3.4. Single-well and multi-well perfused bioreactors. It is well known that the incidence of nonalcoholic fatty liver disease (NAFLD) influences the efficacy of drug bio-transformation and ultimately drug toxicity. Current 2 D cell culturing techniques give limited information regarding molecular mechanisms of disease progression, and as there are major discrepancies between results obtained from humans and animal models of NAFLD it is necessary to investigate the possibility of more complex *in vitro* tissue organization systems. Thus, the contribution of Kostrzewski et al. (2017) *in vitro* 3 D perfused human NAFLD model is a step in the right direction. Cryopreserved human hepatocytes were seeded onto a multi-well collagen coated Liverchip® platform. Cells were cultured in lean or fat culturing medium containing physiological quantities of insulin (2 nmol/L) and glucose (5.5 mmol/L) with fat media containing 600 μmol/L free fatty acids. Cells were cultured in these conditions for a period of 14 days without the incidence of hepatotoxicity. Cells cultured in fat loaded medium, however, presented with changes in transcriptomics, proteomics as well as metabolic functionality changes such as reduced CYP3A4 and 2C9 activity. Also, cells cultured within the fat loaded medium were reactive to metformin, a known anti-steatotic drug serving as proof of concept that this could serve as an excellent steatotic liver model.

Creation of a bioartificial liver system was made possible by culturing primary rat hepatocytes from male Sprague-Dawley rats, using a 0.5% naturally self-assembling peptide nano-scaffold (SAPN) from PuraMatrix™ in 6-well and 24-well bioreactors, for a duration of 35 days. Gene expression was analyzed every 10 days employing semi-quantitative real-time polymerase chain reaction (RT-PCR), while liver functions (albumin secretion, urea metabolism, ammonia detoxification) and cell membrane stability from lactate dehydrogenase were determined on days 5, 15, 25 and 35. Mitochondrial structural status was determined using confocal microscopy and the liver specific markers Albumin and CYP 3A1 were analyzed by means of immunofluorescence. Drug biotransformation was investigated with the model compound Diazepam and its metabolites in a two-compartmental

model, and all results were compared with traditional 2 D cell cultures. The results indicated a physiologically stable 3 D system for a period of 4 weeks, capable of serving as an alternative to animal experiments and transforming current pre-clinical drug screening and drug development protocols with the ability to mimic native liver regeneration time. The results also indicated stable liver gene expression and biochemical functions, including drug biotransformation and detoxification, which were significantly better than traditional 2 D models employed during this study (Giri et al. 2012).

5. Conclusions

It is important to reflect on the current means of studying drug biotransformation and toxicity in both functional and dysfunctional liver. Animal models and primary hepatocytes still remain the golden standard in drug biotransformation and toxicity studies, but costs and ethical considerations make these methods increasingly problematic. Currently, scientists are challenged to reproduce human hepatic function sufficiently to study drug biotransformation and toxicity at multiple levels. Several challenges are facing *in vitro* models, such as the inability to maintain fully functional primary hepatocytes in culture for long periods of time. The recovery of the majority of liver functions in stem cell-derived hepatocytes still has to be demonstrated, while most established cultured cell lines are known to lack physiologically relevant levels of function. A renewable source of human non-parenchymal cells is lacking, and various cell types cannot yet be built into liver-like structures. It is important to note that, although 2 D cell cultures will always have its place, there are numerous advantages to culturing cells in 3 D. However, a general lack of understanding to this regard hinders the development and implementation of this technology to its full potential.

Despite these challenges, rapid progress is made at many levels. While intrinsically obvious, the importance of cultivating cells in a 3 D environment, similar to the one from whence they came, in order to obtain liver-mimetic tissue has only been realized during the last 10 years. Converting this realization to reality is the focus of much research in the field today. Diversification in this research illustrates that currently no one approach holds all the advantages. Furthermore, 3 D spheroids in low attachment plates will permit high throughput analysis of drug-drug or drug-compound interactions. Microgravity cultures allow for multifunctional and long-term repeated analysis in *in vivo* like conditions. Inclusion of additional cell types like Kupffer cells adds the 'immunological' and other angles for drug induced liver injury. Rapid transdifferentiation of adult cells (via induced pluripotent stem cells) into hepatocytes holds the promise to approach the human diversity of drug response and an approach to idiosyncratic drug response. Finally, the addition of other organs in chip-based systems, make progress towards simulating whole body toxicology. When all of these features coalesce, we will be standing with a powerful tool which will allow us to dispel the ethical shadow of using animals in toxicological studies and understand the biochemical and metabolic wizardry that is occurring every second in our livers. But there is still much to do.

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ORCID

Chrisna Gouws http://orcid.org/0000-0001-5747-8214

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