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Bioartificial livers could replace the millions of animals used in drug testing and prove to be a reliable tool for hepatotoxicity assessment in preclinical drug development research.

Foundation review: Improved preclinical safety assessment using micro-BAL devices: the potential impact on human discovery and drug attrition

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Hepatotoxicity is often unpredictable in the early phase of drug discovery and leads to drug attrition in preclinical and clinical development. Here, we discuss the conventional preclinical liver models that do not mimic *in vivo* livers. We focus on key components such as new sources of hepatocyte-derived human stem cells, enhanced direct oxygenation, defined biocompatibility nanoscaffolds, organotypical cellular models, dynamic culture, and metabolite status inside and outside the cell for effective configuration for the development of a bioartificial liver (BAL) device to mimic the *in vivo* liver microenvironment. The potential for development of BAL devices could open up new avenues in: (i) hepatotoxicity assessment for selecting drug candidates during preclinical screening; and (ii) therapeutic approaches for liver cell therapy at the clinical stage.

Introduction

The pharmaceutical industry is facing an increased financial burden owing to a high attrition rate at the post-marketing stage. Despite technological and biological advances, the process of drug development from preclinical testing into the clinical setting remains lengthy (>12 years) and expensive (>US$800 million) for a single drug [1]. Moreover, it is an uncertain and inefficient process because only one drug out of ~5000–10 000 drug compounds reaches the market after preclinical testing [2]. Preclinical research costs ~US$16 million and takes in the region of two years for most pharmaceutical companies [1]. According to a 2006 survey of pharmaceutical companies, hepatotoxicity was ranked first in terms of adverse drug reactions [3] and for withdrawal of a drug from the market [4], probably because the liver is the central organ for drug metabolism. More than 50% of chemical entities that enter clinical trials fail because of efficacy or safety issues [5]. It is an ongoing challenge to reduce cost and time taken, and to increase the ability to predict hepatotoxicity in the preclinical phase to identify problems appropriately in these early stages before entering either clinical trials or marketing. More than 1000 marketed drugs with diverse clinical features are associated with hepatotoxicity [6]. Lee reported that 10% of liver disease is caused by drugs in the post-marketing phase [7]. Many approved drugs underwent post-market withdrawal within one or two years of approval because

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of hepatotoxicity. For an example, troglitazone, a drug for diabetics that has been widely used worldwide, was approved in 1997 and withdrawn from the market in 2000 with >90 cases of liver failure, 70 of which resulted in death or required liver transplantation [8]. Such results are a severe burden to drug companies, as well as a threat to 'patient safety'.

The pharmaceutical industry is focused on predicting hepatotoxicity from preclinical screening to the final approval stages. There are many reasons for the emergence of hepatotoxicity, such as idiosyncratic ones, age, genetic factors and nutrition. This present review focuses on new innovative approaches such as *in vitro* hepatotoxicity tests using reliable bioartificial livers that could offer alternatives to preclinical animal testing and provide better signals before clinical trials, reducing the number of drugs that fail late in clinical testing or even after approval.

Bioartificial liver

There is a fundamental difference between *in vivo* livers and the conventional dish- and/or plate-based *in vitro* culture. Worldwide routine lab-based toxicity assay studies provide information on static culture systems, such as 24-, 48-, 96- or 386-well plates with a 2D culture model, but liver tissue organization in *in vivo* livers is a dynamic process. The static culture system might not be able to predict human hepatotoxicity. That format suffers several limitations in mimicking the *in vivo* microenvironment. The complexity of the liver cannot be mirrored by such traditional *in vitro* static cellular models [9]. During cell isolation, when *in vivo* hepatocytes are transferred into *in vitro* milieu, 50% of liver-specific genes undergo downregulation in the first 72 hours [10], so it is essential to recapitulate liver functions, including expression of drug-metabolizing enzymes, by providing an appropriate human liver microenvironment. The bioartificial liver (BAL) could meet this demand and be a better match to *in vivo* livers. Figure 1 overviews the future prospective of BAL for hepatotoxicity assessment in early-stage drug discovery and development.

Over the past two decades, many BAL approaches based on different reactor geometry, flow parameters and nutrient supports have been introduced to support a failing liver [11–15], but the exploration of BAL in preclinical assessments of hepatotoxicity is very rare and still has several disadvantages in mimicking the liver microenvironment and complexity of *in vivo* livers. The main limitation of current BALs is the lack of a proper *in vivo* liver microenvironment, such as indirect and low oxygenation to liver cells, microscale biomaterials, short-term undesirable functions and lack of 3D interaction of growth factors and/or cytokines signalling with cells. When designing a hybrid artificial liver the organic components of the bioreactor must provide an architectural basis for reconstructing a proper *in vivo* liver microenvironment. The tissue architecture and individual cell organization pattern morphology reflect the functional differentiation of an individual organ that coordinates organ functions. Heart cells are arranged in muscle bundles that typically form from tissue organization that serves to develop and direct contractile strength. Kidney cells are arranged in glomerular or tubular architecture to serve excretory functions better. So, every organ has its own architecture of cellular structure that determines its function. In the liver, hepatocytes are arranged as monolayer plates. This form of tissue organization allows the cells to be highly vascularized. Each hepatocyte has two sinusoidal surfaces available for the exchange of nutrients, oxygen, toxins and xenobiotics, among other things. It can be assumed that this form of liver tissue organization is a qualification for full hepatic function, including hepatotoxicity prediction and mimicking a state-of-the-art liver. A controlled effective dynamic bioreactor system based BAL will be required to overcome these limitations.

Conventional static culture systems are characterized by a lack of mixing, control options for dissolved oxygen and waste removal from cells; therefore, dynamic cultivation in a bioreactor system is of great importance to overcome the existing limitations. Realistic and controllable dynamic bioreactor-based BAL device culture systems that more closely mimic *in vivo* human liver in terms of functionality as well as morphological and mechanical architecture could be applied during hepatotoxicity assessment to revolutionize the early stages of the drug discovery process. Most conventional models rely on animal testing but, unfortunately, these models lack human physiology and sometimes give false-negative responses. Therefore, we need *ex vivo* bioartificial livers such as the BAL device that should be very close to *in vivo* liver physiology. Many problems (i.e. time- and dose-response, hepatotoxicity assessments, pharmacokinetics) with drug testing on animals and the resulting imprecision in the prediction of the effectiveness of such drugs for humans can be overcome by utilizing devices such as BAL, which effectively functions as an actual liver.

The following novel approaches have been the focus in attempting to overcome the existing limitations, and it is recommended to use a 'current effective assemble bioreactor'-based BAL to support better human hepatotoxicity prediction and to minimize cost while maximizing patient safety at the preclinical stage and during early stages of drug development.

Key components for effective BAL configuration

- Opportunities and challenges of hepatocyte-derived human stem cells.
- Enhanced direct oxygenation.
- 3D scaffolding versus 2D scaffolding.
- Conventional versus organotypical cellular models.
- Static plate culture versus dynamic culture.
- Metabolite status both intra- and extra-cellularly.
- Interaction potential with cytokines and growth factors.

Opportunities and challenges of functional hepatocytes derived from human stem cells

Various regulatory agencies, such as the FDA, have declared and widely accepted *in vitro* primary human hepatocytes as the gold standard to use in preliminary predictive drug toxicity assays; however, owing to the scarcity of primary human hepatocytes, conventional non-human models are used during preclinical stages. These non-human models often miss the human drug response and provide much lower degrees of accuracy in predicting human hepatotoxicity. As an example, 50–60% of drugs were not hepatotoxic during animal testing but caused hepatotoxicity *in vitro* using human hepatocytes [16]. Non-human primary hepatocytes show significant differences when compared with human responses [16]. To fill this gap, there is presently a growing interest in using stem cells derived from adult human stem cells for

FIGURE 1

Illustration of major key assembly components of bioartificial liver for preclinical assessment of hepatotoxicity of drug discovery programmes. Bioartificial liver is usually a bioreactor loaded with suitable functional hepatocyte cells that perform functions of a normal liver. This device is widely used for temporary support to liver recovery or liver translation, but it is rarely used in hepatotoxicity assessment and differentiation and expansion of human hepatocyte-derived adipose or hepatocyte-derived iPS cells instead of conventional dish and/or plates for hepatic for upscaling generation of functional hepatic cells. Preclinical drug development (also termed as nonclinical drug development) is where the safety and therapeutic profile of a formulated drug can be monitored by *in vitro* and *in vivo* tests before progressing to clinical trials. Induced pluripotent stem cells (iPS cell), in the pluripotent state, can be achieved from a non-pluripotent cell such as adult skin fibroblasts by introducing the viral vectors Oct-4, Sox2, KLF-4 and C-Myc, and is also possible by direct delivery of proteins.

hepatotoxicity assessments. Stem cell researchers are highly placed to generate functional hepatocytes from adult human stem cells (Table 1) such as those from adipose tissue [17–25], including induced pluripotent stem cells (iPS) [26–31] and human embryonic stem cells (hESC) [32–44] – cells that could replace the primary human hepatocytes and provide a promising cell source. Generation of liver cells from skin cells using iPS technology is a new, challenging field that has ethical and practical advantages over employing embryonic stem cells. A patient-specific iPS cell line can be created for a broad spectrum of the population from multiple

TABLE 1

Drug-metabolizing enzyme expression.

Human organ part and/or cell line	Origin and/or source	Culture device	Time taken for generation of hepatocytes	Drug-metabolizing enzyme expression	References
Adipose tissue	Abdominoplasty patients	Collagen-I-coated six-well cell culture dishes	37 days	CYP7A1	[18]
Adipose tissue	Suction-assisted lipectomy patients	Culture flask	14 days	CYP2E1	[20]
Adipose tissue	Elective gynaecological surgery	Fibronectin-coated dishes	21 days	CYP3A4	[21]
Abdominal subcutaneous adipose	Elective abdominoplasty	Collagen type I dish		9–14 days	[22]
Human lipo-aspirates	Patients undergoing selective liposuction	Porous poly-lactide-co-glycolide (PLGA) scaffolds	14–21 days	CYP1B1	[23]
Abdominal subcutaneous adipose tissue	Six gastric-cancer patients	Collagen-type-I-coated dishes	40–50 days	CYP3A4, CYP1A1, CYP2C9, as well as NADPH P450 reductase	[17]
Excess adipose tissue	Gastro-omental artery during coronary artery bypass graft surgery and gastrectomy	Ultralow-attachment culture dish and/or collagen-type-I- or matrigel-coated dishes	41 days	P450 (CYP)1B1, CYP3A7, CYP3A4	[25]
iPS	Primary human hepatocytes	Collagen-I- and Matrigel-coated plates	20 days	CYP3A4, CYP3A2	[27]
iPS	Fibroblasts normal male caucasian	Six-well plate	14 days	CYP3A4, CYP3A2	[26]
iPS	Fibroblast from inherited metabolic liver disease	Fibronectin-coated plates and/or fetal bovine serum coated plates	7 days	CYP3A4 and display pathological cellular pathology feature seen in inherited liver disease	[30]
iPS	Mice	Implantation of iPS cells directly to early developing mice embryos		Efficient hepatic functional role to restore liver function in mice that lack the enzymes furmarylacetoacetatehydrolase (an example of human hereditary disease)	[31]
Fetal livers	Termination of pregnancy at 11–13 weeks' gestation	Fibronectin-coated plates	10 days	CYP7A1	[32]
hESC line, H9,		Collagen-I-coated plates	20–22 days	Phase I and II metabolizing enyzmes and Phase III transporters, nuclear receptors, CYP3A2, 2C9, 3A4 and 2D6	[33]
hESC lines SA167		Culture dishes	18–25 days	CYP1A1, 1B1, 2A6/2A7/2A13, 2B6, 2C8, 2C9, 2D6, 2E1 and 3A5	[34]
hESC lines SA002,		Culture dishes	18–25 days	CYP1B1, 2A6/2A7/2A13 CYP1A1, 2B6, 2E1, 3A5, UGT1A6, UGT2B7, NTCP, MDR1, MRP2, PXR, CAR and FXR	[34]
hESC lines SA001, SA002, SA002.5 and SA167		Collagen-I-coated 24-well plates	18–45 days	CYP1A, CYP3A and CYP2C	[36]
Human ESC lines H1 and H9		24-Well plates	8–18 days	CYP7A1, CYP3A4 and CYP2B6	[37]
hES cells (H1)		Low-attachment Petri dishes		CYP 1A1, 1A2, 2B6, 3A4 and 7A1	[38]

TABLE 1 (*Continued*)

Human organ part and/or cell line	Origin and/or source	Culture device	Time taken for generation of hepatocytes	Drug-metabolizing enzyme expression	References
pESC cells		6 cm gelatin dishes	22 days		[39]
hESCs (H1 and H9)		6-Well plate	14 days	CYP7A1, CYP3A4, CYP1A2	[41]
Human embryonic stem cells H1 and H7		6-Well plate	7–17	CYP3A (CYP3A4, CYP3A5, CYP3A7) and CYP2D6	[41]
hESC		Matrigel-coated dishes	30 days	P450 3A4/7	[43]
HESCs		Collagen-I-coated 24-well plates.	18–22 days	Presence of glutathione transferases (GSTs)	[35]
hESC lines WA09 (H9) and WA01 (H1)		Collagen-coated dish	15 days	CYP7A1, CYP3A4	[40]
hESC lines (SA002, SA167, SA 461)		Collagen plates	20 days	CYP7A1, CYP1B1	[44]

Drug metabolizing enzymes are expressed in hepatocytes derived from iPS cells, adipose tissue and human embryonic stem cells (hESC). UDP-glucuronosyltransferase 1–6 (UGT1A6) is an enzyme responsible for the conversion of small lipophilic molecule drugs into water-soluble molecules for excretion via the glucuronidation pathway. Human UDP-glucuronosyltransferase 2B7 (UGT2B7) is one of the major isoforms found in liver that have significant roles for metabolism of some clinical drugs, carcinogens and steroid hormones via glucuronidation. The Na^+-taurocholate cotransporting polypeptide (NTCP) is a major bile acid present at the basolateral (sinusoidal) membrane of human hepatocytes. Multidrug resistance 1 (MDR1) and multidrug resistance 2 (MDR2) are members of the family of ATP-binding cassette (ABC) transporters that are involved in transport of numerous compounds (e.g. bile salts, drugs, toxic and environmental agents). The pregnane X receptor (PXR) is a nuclear receptor, a sensor of a wide range of drugs or xenobiotics and induces a network of transporters and cytochrome P450 enzymes. The farnesoid X receptor (FXR) is also a nuclear receptor expressed at high levels in the liver and acts as an endogenous sensor for bile acids. PXR and the constitutive androstane receptor (CAR) also have significant roles in transcriptional regulators of cytochrome P450 expression.

patients, of varied genetic and disease backgrounds, to improve the understanding of disease mechanisms. Individual variability in drug response is also associated with adverse drug reactions, which are a vital problem in an effective drug discovery process [45]. The iPS technology could revolutionize the drug discovery process and boost the optimization for validation of drug candidate selection. It has been proved that iPS can be used to create a cell culture model for a limited number of rare neurological diseases, but some researchers [30] recently proved that iPS can also be used to study non-neurological diseases such as inherited metabolic liver disease.

Recently, two independent research studies have been carried out, providing outstanding experimental evidence about the potential of iPS to convert hepatocytes under certain conditions [30,31]. Roshid *et al*. developed a simple and effective chemically defined culture system to generate new iPS from dermal fibroblast cells of three patients with various inherited metabolic diseases of the liver [30]. They tested liver cells generated from skin cells of liver inherited patients, and the results interestingly showed the same properties and recapitulated the key features of inherited metabolic diseases in the patient's own liver cell but not in the control (i.e. a healthy individual). As an example, they examined iPS derived from an individual who had a mutation in a gene called A1ATD, which causes the accumulation of a protein called α1-antitrypsin in that individual's liver cells. Surprisingly, researchers found that this protein accumulated in the liver cells of patients and was not found in the liver cells of healthy individuals in the control group [30]. This study proved the potential of iPS to produce cell culture models of other inherited liver disorders, such as deficient low density lipoprotein (LDL) receptor-mediated cholesterol uptake and elevated cellular lipid and glycogen accumulation. This discovery provides an efficient methodology for the early stages of drug research and for the therapeutic screening of liver-targeted compounds that might be of potential relevance to the pharmaceutical industry. Another research team developed a method that generated iPS without viruses [31]. That study showed that hepatocytes, differentiated from mouse iPS, could repopulate the liver after transplantation and two-thirds partial hepatectomy [31]. Furthermore, the study tested the functional efficiency that iPS-derived hepatocytes restore liver function in mice lacking the enzyme fumarylacetoacetate hydrolase (FAH), an enzyme that is encoded by the gene mutated in hereditary tyrosinemia in humans [31].

Using iPS-derived hepatocytes, from the skin cells of either disease-specific patients or healthy individuals, it might be possible to conduct clinical trials in a dish which might significantly help optimize further research studies in humans. These trials could provide robust and scalable models for predicting human drug toxicity with safer more-customized pharmaceuticals, to improve the safety of clinical trials and reduce the drug attrition rate [46]. Although these research teams [30,31] differentiated functional hepatocytes from iPS using more-robust challenges than previously used, further optimization is needed to overcome the existing limitations [47]. Roshid *et al*. demonstrated that it is possible to obtain iPS-derived hepatocytes [30]. Furthermore, this study mentioned that these cells are not fully differentiated into mature hepatocytes because expression of α-fetoproteins also exists in differentiated hepatocytes [30]. Additionally, one limitation of the other group [31] is that it generates iPS without viruses and allows for the differentiation of hepatocytes by implanting them into an embryo; however, this strategy [31] might not be realistic when applied to human iPS. Therefore, further research needs to focus on replacing the differentiation site in an *in vivo* embryo, in order for *in vitro* hepatic differentiation to occur, before transplantation.

Most culture methods use a serum-based culture medium to conduct hepatic differentiation; however, this type of culture medium has a negative impact on the cells. Hannoun *et al*. compared a serum medium and a serum-free medium during human

embryonic stem cell differentiation and found that both cultures have the same potential for hepatic differentiation [48]. Huang *et al*. reported on the supportive role played by high-density human ESC-derived fibroblast-like cells (hESdFs) for the efficient generation of functional hepatocyte-like cells from primate ESCs and human iPS [49]. They mentioned the significant role of hESdF-secreted factors in inducing robust hepatic endoderm differentiation [49]. Li *et al*. identified hepatoblast-like progenitor cells derived from embryonic stem cells [50]. They noted that these cells could be clonally expanded, successfully, to repopulate the livers of fumarylacetoacetate hydrolase-deficient mice (which serve as a model of liver injury). Dalgetty *et al*. reviewed the generation of hepatic endoderm from different stem cell/progenitor cell populations, including iPS [51]. They explained the usefulness of new sources of patient-specific hepatocytes. They suggested that it might be useful to create a library of iPS samples, taken from men and women – from a variety of different ethnic groups – who also exhibit a diverse range of drug susceptibilities, resistances or diseases. This iPS library could provide an effective platform for drug screening and disease modelling, which could be used to individualize medical and drug treatment options. Roughly 70% of top pharmacy companies use embryonic stem cells and 10% of top biotech companies have started to use adult stem cells for toxicity models in a wide range of toxicity assays, including hepatotoxicity [52]. It is estimated that the use of embryonic stem cells as toxicity models between 1998 and 2006 increased by 200-fold during this time [53], and a further increase in the near future is likely [54].

Thalidomide, a drug used to treat morning sickness in pregnancy, was later found to cause birth defects in babies born to women who took this drug [55]; however, animal trials show no such teratotoxicity. Often, toxicity of a drug candidate is not discovered in preclinical stages until clinical trials are conducted. As an up to date example, TGN1412 (also known as CD28-SuperMAB) is an immunomodulator for the treatment of rheumatoid arthritis. In 2006 clinical trials on six volunteers were carried out using a 500-times lower dose than the dose found safe in animals [56]. Nevertheless, four volunteers suffered from multiorgan failure. Clearly, drug testing on animals is unrealistic and causes unforeseen reactions in human clinical trials.

Recently, Ebert and Svendsen discussed the opportunities and challenges for the use of stem cells (embryonic and iPS) for drug screening [57]. From 2003 to the present day, several optimized protocols have been developed for the generation of hepatocytes from hESC [57]. Expression of all major cytochrome P450s in hepatocyte-like cells derived from hESC [34] has been reported, and phase II enzymes, such as glutathione *S*-transferase (GST), were detected at levels comparable to human hepatocytes [42]. It has been reported that the expression of CYP1A activity in hepatocytes derived from hESC is the same level as expressed in primary hepatocytes, and after two years the same research group generated more-defined and efficacious cells where a relatively homogenous population of hepatocytes from hESCs reflected complete metabolic functions just like primary liver cells [33]. This group developed hESC-derived hepatocytes with a complete biotransformation system, including phases I and II metabolizing enzymes and phase III transporters [33]. Further, nuclear receptors, which are crucial for regulating the expression of metabolizing enzymes [33], are also expressed in hESC. Touboul *et al*. generated functional hepatocytes from iPS and showed the expression of cytochrome activity in a more defined condition without using feeder cells, serum, sodium butyrate or dimethyl sulfoxide [32]. These recent investigations could open a new window for pharmacology and toxicology studies. It is widely accepted that embryonic stem cells offer several advantages in drug biotransformation [53] but are associated with major ethical issues.

Basma *et al*. reported a simplified differentiation strategy to generate relatively homogeneous hepatocytes from hESCs, based on asialoglycoprotein receptors [38]. These generated cells exhibit the morphological and phenotypic properties of primary human hepatocytes. This simple differentiation method might be useful as an alternative source of primary human hepatocytes. Another research group proved the significant role of Wnt3a signalling for the efficient and scalable generation of human hepatocyte-like cells from hESC [41]. Furthermore, they demonstrated the synergistic effect of Wnt3a and Activin A on hESCs during human hepatic differentiation *in vitro*.

Owing to the major ethical issues of hESC-derived hepatocytes, many toxicology researchers are focusing on iPS to generate functional hepatocytes derived from human skin or fibroblast by iPS technology [58]. Very recently, Si-Tayeb *et al*. demonstrated the efficient generation of highly differentiated human hepatocyte-like cells from iPS [29]. Additionally, these researchers analysed a series of genes encoding phase I and phase II enzymes and found that many genes showed similar trends in expression, similar to mature liver cells, but that the levels of expression of these enzymes were lower in most cases, when compared with adult liver samples. They pointed out that hepatocyte-like cells derived from hESCs or iPS have differentiated to a state that only supports some hepatic functions, including expression phase 1 and phase 2 genes. However, the main limitation of this study is that these hepatocyte-like cells were unable to fully recapitulate an expression that mimics adult liver. Therefore, further investigation is needed. Sullivan *et al*. generated human hepatic endoderm from human iPS, obtained from men and women from two ethnicities, and found that all iPS lines showed a higher efficiency of functional hepatic endoderm formation [26]. These iPS-derived hepatic endoderms exhibited hepatic morphology and expressed hepatic functions, including CYP1A2 and CYP3A4 metabolism [26]. However, making complete hepatic differentiation with high efficiency with defined conditions is in the initial stage. If iPS could be developed in defined conditions, then it could be possible to create a wide range of hepatocytes for various populations worldwide, making them useful in predicting idiosyncratic hepatotoxicity. Differentiated cell types produced from a patient's iPS cells have been well-documented for therapeutic applications but their use in drug screening is rare. Ansari *et al*. raise issues about using the hepatic cell from pluripotent stem cells for drug screening [58]. If iPS technology proves robust, it could be a new platform for studying diverse samples of the human population, including individuals with particular disorders, and might help to reduce concerns over idiosyncrasy. Disease- or patient-specific iPS therefore has great potential in pharmaceutical companies, because current *in vitro* disease models are problematic. iPS cells could replace millions of animals currently sacrificed in preclinical testing. Generations of hepatocytes or other cell types from patient-

specific iPS will make it economically feasible to produce individualized drug candidates, but lack of efficient defined differentiation protocols is the main limitation that needs to be overcome. Banking of a wide variety of iPS and hESC from the human population has commenced [59,60] for future use to mimic the global genetic diversity in drug development. Drug metabolism is the sole explanation for hepatotoxicity. In fact, many studies have demonstrated other mechanisms of drug-induced liver injury. Genetic diversity is also one of the main parameters for hepatotoxicity, because drug metabolism varies among patients [61,62]. In fact, drug metabolism varies from person to person, influenced by individual genes [62]. This gap could be filled by a worldwide iPS cell bank in the near future, and it might resolve the ethical issues surrounding embryonic stem cells and idiosyncratic hepatotoxicity. Regarding iPS, human embryonic stem cells and adipose-derived hepatocytes, generation has been kick-started but there are still many hurdles to overcome to achieve optimization.

Stem-cell-derived hepatocytes could also contribute more in the place of primary human hepatocytes for human responses. Stem cells exist in gastrological and hepatological areas [63], but *in vitro* manipulation for toxicity models is scarce. Several hepatic cell lines have developed, but these are of limited interest owing to a low expression of drug-metabolizing enzymes. The main limitations are that primary hepatocytes are diploid, whereas immortalized cell lines are aneuploid and there are a limited number of gene expressions. Cell lines are not authentic diploids. Rather, they are aneuploids that might not represent the entire population, owing to their abnormal karyotype, causing them potentially to interfere with and significantly alter the extracellular signalling system [64].

Apart from hESC-derived hepatocytes, iPS and hepatic stem cells, it is widely accepted that generating hepatocytes from mesenchymal stem cells derived from human adipose tissue is a more reliable source for hepatotoxicity models. There is growing evidence reported regarding the generation of functional hepatocytes from human adipose tissue cells [17–25] that avoid the complications of surgical operations and have advantages over embryonic stem cells, iPS and hepatic stem cells. Virtually all individuals in all age groups (healthy and unhealthy) have high amounts of adipose tissue that is quickly and easily harvested without any major operation. All these mentioned cells could meet the gap created by various disease models that are species dependent. For example, many pathogens are species specific (e.g. hepatitis C) and a leading cause for the failure of new drugs in clinical trials is liver toxicity that was not predicted by animal *in vivo* or conventional *in vitro* models.

Although generation of hepatocytes from adult human organs and tissues, such as adipose, is an innovative approach, the main limitation is optimizing the defined culture condition differentiation, because many endogenous unknown substances in media have been used for hepatocyte differentiation. It has been reported that as much as 20–50% of commercial fetal bovine serum is virus-positive, containing such microbes as bovine enterovirus, bovine viral diarrhoea virus, parainfluenza virus type 3, bovine herpesvirus-1 (BHV-1), as well as other unidentified cytopathogenic agents [65] that could hamper the drug screening process. Some protocols for hepatocyte generation have been optimized without the use of serum and the efficiency for several functional hepatocytes is the same [48]. Sometimes, commercial vendors and researchers conduct their own pre-screening, quality-control assurance tests on serum. However, these activities are costly and time-consuming and the results could prove to be unreliable, owing to batch-to-batch variation. Hence, future optimization and medium development leading to a robust, serum-free, defined culture medium is absolutely necessary. This type of medium would provide a more clinically relevant culture system. To overcome the limitations of serum-related problems, Touboul *et al.*, among others, have optimized serum-free and chemically defined culture conditions for maintaining and differentiating hESC or iPS [29,32,48]. The cell culture industry is also standardizing defined culture media without serum and animal materials. Many companies have formulated serum-free culture media. Furthermore, they offer several media and defined reagents, with special reference to stem cell expansion and multilineage differentiation of hESC or iPS.

All of these above hepatocytes, derived from hESC–iPS, hepatic stem cells, human adipose tissue, are cultured in either undefined or less defined conditions in static dishes or plates that might be insufficient tools for efficient hepatic differentiation. It is our hypothesis that, if hepatic differentiation from expansion to functional differentiation is in a bioartificial liver (instead of dishes and/or plates), which is closer to an *in vivo* liver, there could be a greater chance of generating a large quantity of functional hepatocytes along with efficient liver-specific functions, including the expression of drug-metabolizing enzymes (Fig. 2). Further research is needed to test this hypothesis.

Enhanced direct oxygenation

The exploration of functional hepatocytes derived from adipose, iPS or embryonic cells in a bioreactor alone is not sufficient to mimic the human liver physiology. It is essential to provide a complete *in vivo* microenvironment, because liver cells need a unique microenvironment. Enhanced oxygenation is one of the most important concerns in the drug metabolism of a liver microenvironment. Hepatocytes consume >10 times more oxygen than other cells, and the liver consumes ~20–33% of the total oxygen used by the body [66]. Enhanced oxygenation is important for the *in vitro* liver cellular microenvironment [67], including the BAL model. Around half a century ago, it was reported that *in vitro* liver cells obtain only 4% of their oxygen requirement and therefore degenerate rapidly [68]. It is estimated that oxygen supply to an *in vivo* liver is >2000 nmol/ml of oxygen and in *in vitro* culture is <200 nmol/ml to the cells [69]. During the initial phase of culture, the oxygen uptake was 40% higher than the value observed in the stable phase in culture [70,71]. The high oxygen requirement of hepatocytes is essential during attachment and spreading on a scaffold or substrate after cell isolation from the *in vivo* liver [70,71]. The traditional cell culture method commonly used medium depths of 2–5 mm in Petri dishes and would rapidly produce hypoxia if hepatocytes respired at their physiological rate [72]. Tissue hypoxia is associated with inflammatory situations that are discussed later in this review.

It is an unfavourable situation when high-oxygen-demanding hepatocytes are generally maintained in Petri dishes where the plastic walls and culture medium are barriers to oxygen diffusion, create unrealistic oxygen-deficient culture conditions and force

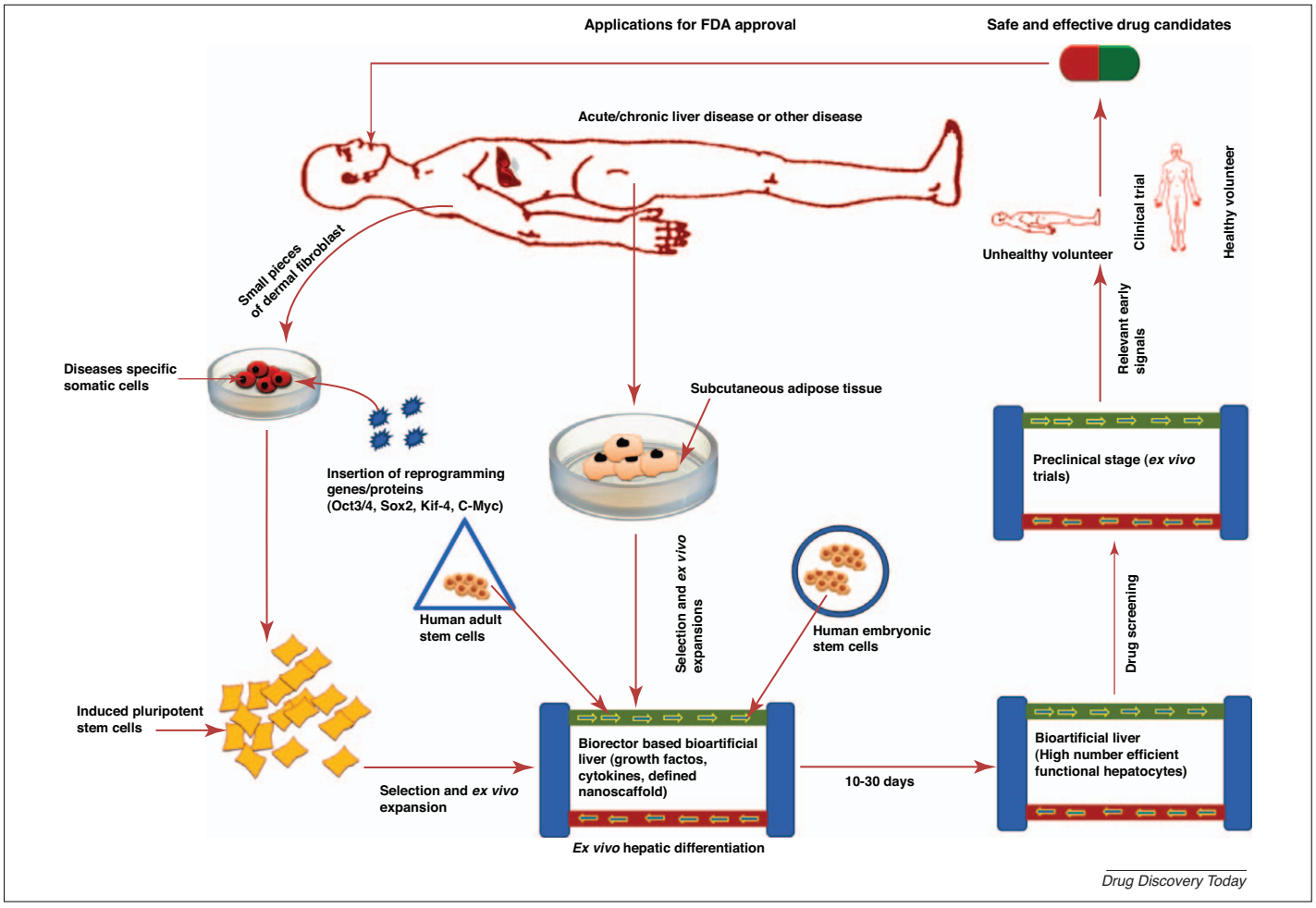

FIGURE 2

A hypothetical flow diagram for better hepatotoxicity predictions during drug development stages is created using bioartificial liver with appropriate cell sources that are hepatocyte-derived iPS cells or hepatocytes derived from mesenchymal stem cells from adult adipose tissue. Generation of hepatocytes from disease-specific somatic cells, particularly dermal fibroblasts by reprogramming methods, and generation of hepatocytes from subcutaneous tissue under certain conditions represent a new paradigm for drug-screening tests in the pharmaceutical industry. The disease-specific iPS cells or adipose tissue derived from hepatocytes from a wide variety of individuals (i.e. differing in terms of health, age, sex, race, and environmental and physiological factors) that mimic the donor's disease state or healthy state can be displayed as *ex vivo* preclinical and/or clinical trials to predict patients' responses to a drug candidate. Individualization of drug dose could be associated with idiosyncratic toxicity where wide inter- and intra-individual variability can occur in drug metabolism owing to environmental and physiological conditions, genetic factors and lifestyle (e.g. smoking, abusing alcohol and living in air polluted area). Another advantage of iPS cells is that a large number of single-polymorphism-based drug screens can be possible across wide geographical and ethnic populations with less cost and time. Variation in drug response is sometimes a major concern because adverse drug reactions in different individuals can be due to the variation in single nucleotide polymorphisms. CYP2C9 is one of the most important isomer-drug-metabolizing enzymes in human liver. It is estimated that 16 out of 170 genes are associated with genetic polymorphism. CYP2C9 expression is associated with genetic polymorphism that is highly polymorphic. More than 50 single nucleotide polymorphisms (SNPs) have been described in the CYP2C9 gene that metabolize >100 therapeutic drugs and many endogenous compounds. Patient-specific iPS cells might be an effective cell source to explore human polymorphisms associated with drug metabolism. There are >170 gene products (drug-metabolizing enzymes, membrane transporters, serum-binding proteins and transcription factors) known or expected to have a role in drug metabolism – and 16 out of 170 gene products are closely associated with genotype in drug metabolism [133]. So, using iPS or adipose-derived hepatocyte cells for hepatotoxicity assessment is extremely encouraging in drug discovery and development.

anaerobic metabolic states [73]. In these models, oxygen consumption depends on hepatocellular uptake rates, thickness of culture medium and ambient oxygen concentration. Despite these limitations, hepatocytes generally tolerate hypoxia situations because of their extraordinary capacity to satisfy energy requirements by anaerobic glycolysis. This, however, results in an inefficient utilization of glucose because of the conversion of glucose to lactate during oxidative phosphorylation [73]. Hence, hepatocytes generate less energy as a result of insufficient oxygen supply, which could interfere with the drug metabolism.

Oxygen is needed as a substrate for drug metabolism and also needed for energy production in the form of ATP. During drug metabolism, generally drugs are eliminated by the drug transport system via bile or the hepatic circulation, which are both energy-dependent and directly related to oxygen supply. During hypoxia ATP production falls when insufficient oxygen is available to maintain cytochrome oxidase. In addition, changes in the pattern of drug metabolites under hypoxia conditions might lead to the formation of new, more-toxic metabolites. Very few or no report has been documented on this subject. The oxygen requirement of

metabolism of different drugs varies greatly *in vitro*. Hepatic clearance and the half-life of drugs are increased several-fold, which might cause another problem. For example, the half-life of tolbutamide clearance is increased by 180-fold in hypoxia patients [74]. There are more examples *in vivo* but there are far less *in vitro* data. Much research is needed to know the signalling mechanism of drug clearance pathways that are affected by hypoxia.

Hepatocytes are extremely sensitive to hypoxia. Park investigated that there is a 5–10-fold reduction in the cytochrome 450 enzyme levels by culturing human hepatocytes in hypoxia conditions [75]. Very few studies have been conducted in patients who experience clinical hypoxia [76]. *In vitro* studies based on hypoxia are scarce. Hypoxia is frequently seen in critically ill patients [76,77]. Drugs entering our bodies undergo drug metabolism by either phase 1 or phase 2 enzyme systems, which are dependent on the availability of oxygen [76–78]. Several experiments support that both rate and pattern of drug metabolism can be altered or affected by altering the oxygen supply [76–78]. Phase I drug metabolism is more sensitive to hypoxia than Phase II metabolism. Therefore, during *in vitro* preclinical hepatotoxicity model testing, an adequate oxygen supply is as serious a concern as it is *in vivo* – to give an *in vivo* response during drug metabolism.

A BAL device can meet the adequate oxygen demand of cells to some extent. In the liver the problem of oxygen and nutrient supply to the cells is solved by arranging them in cell plates with sinusoidal structures located on each side. Every individual hepatocyte has its own membrane support and, therefore, its own oxygen supply position [10]. Various bioreactors [11] have been attempted to reflect this condition, some bioreactors are well designed for enhanced oxygen but, still, limitations exist (geometric construction can affect mass transport, hemoglobin-based oxygen carriers are a toxic concern) in every bioreactor design, which vary greatly with respect to the microenvironment and the means of oxygen and nutrient supply to cells.

Culture cells can suffer a lot or even die if the distance between the cells and blood vessel is >500 μm [79]. Cells must be within at least 100–150 μm of a blood supply for exchange of the required nutrients and oxygen, as well as to be able to expel metabolic waste and carbon dioxide [80]. The mean diffusion distance from the oxygen-permeable membrane to cells is ~20 μm in our clinical flat-membrane bioreactor [81], and in a few other bioreactors [11]. Taken together, enhanced direct oxygenation is crucial for drug metabolism, and an inadequate supply of oxygen will alter the drug metabolism and drug clearance.

3D scaffolding versus 2D scaffolding

A 2D traditional plastic culture system might not reflect liver tissue architecture *in vivo* and rarely predicts hepatotoxicity. Further, it is unable to do other valuable functions, such as cell–cell and cell–matrix interactions, and exchange of nutrients, oxygen, soluble factors and cytokines. Primary hepatocytes plated in 2D systems lose the biosynthesis of the drug-metabolizing enzymes [82]. Many researchers jump from *in vitro* 2D-cell-culture to 3D-cell-culture models [15,83] by using a wide range of synthetic scaffold or collagen, Matrigel® or alginate to bridge the gap between 2D *in vitro* and *in vivo* models. Many authors have mentioned multicellular spheroid culture, 3D-assembled sphere-shaped cell colonies and spheroid models, which are 3D culture models. Short-term survival potential of hepatocytes in these models, however, is less than two weeks, which is their main limitation. FDA rules require a survival potential of a minimum of 14 days and a maximum of 90 days for *in vivo* toxicological experiments [84]. Other limitations of animal-derived extracellular matrix containing unknown agents is that they can vary from batch to batch because of their endogenous signals, suffer from high lot-variability and have a high contamination potential [85], which creates undefined conditions. Nanorange or macrorange synthetic materials could be an option to avoid animal-derived materials where several nanofabrication scaffolds have been used for liver cell culture, although the solvents used for nanofabrication can be a toxic concern.

Virtually, *in vivo* hepatocytes exist in a 3D environment with a nanorange extracellular matrix [9]. In native liver tissue hepatocytes rely on a complex 3D network of blood capillaries to provide adequate oxygen and solution factor transport. In general, the scaffold size should be smaller than the cell, so that the scaffold can bind three-dimensionally [86]. The average size of a liver cell is ~10–20 μm. It is widely believed that when the size of the scaffold is larger than the cells the cells cannot be surrounded by the biomaterial scaffold. Most conventional biomaterials used for liver cell culture are in the microscale range. This means that upon attachment the cells still exist in a 2D topography, which is very common in conventional cell cultures in BAL devices and culture plates. It is actually a serious problem if the cells cannot be attached in an *in vivo* 3D topology. The signalling as well as diffusion is inherently asymmetric in traditional 2D culture [87]. Further, the liver lobule is a functional unit of a whole liver that consists of hepatocytes that are arranged into hepatic cords separated by the sinusoidal space. They are ~8–10 μm in diameter and lined with sinusoidal endothelial cells and Kupffer cells and plasma and proteins migrate through these lining cells via so-called fenestration into the space of Disse (100–150 nm). The hepatocyte has direct contact with the space of Disse for uptake of nutrients and oxygen. Taken all together, all types of liver cells (hepatocyte, sinusoidal endothelial cells, stellate cells and Kupffer cells) have either a nanometer or <10 μm range. Those cells also have a significant impact on drug metabolism. Therefore, a nanorange-defined scaffold is essential to create an authentic 3D microenvironment by holding liver cells in all dimensions.

Defined nanoscaffolds are essential for a bioreactor-based bioartificial liver to overcome the limitation of batch-to-batch variation and other complications of collagen, alginate, Matrigel®, and so on. Few defined nanoscaffolds have been introduced for liver culture of better drug-expression enzymes than conventional collagen or other microrange scaffolds. For example, Puramatrix™ is a defined, self-assembly nanoscaffold [88] that has been used in a wide range of clinical applications, including drug metabolism; however, assembly of such nanoscaffolds in existing relevant clinical bioreactors for hepatotoxicity assessment is rare. Recently, we have investigated the efficiency for drug-expression enzymes in such nanoscaffold-coated bioreactors and found a significantly higher expression of drug-expression enzymes than in a conventional collagen scaffold [89].

Nanoscaffold has another innovative potential – particularly in interaction experiments of small-molecule-like cytokines and growth factors that are routinely used for hepatic differentiation,

toxicity potential, therapeutic potential or maintenance of liver cell culture for the long-term. This interaction, as with *in vivo* conditions, might not occur in 2D cultures. In general, in a conventional scaffold one side of the cell body will be in direct contact with the substrate surface where induction takes place by surface receptors (Fig. 3). By contrast, the receptors for growth factors, cytokines and nutrients, and signals face toward the culture media. Thus, cells in a 2D conventional culture are partially polarized, inherently asymmetric and do not reflect an authentic *in vivo* environment (Fig. 3), which can seriously impair the predictive power of hepatotoxicity assays, cellular communication, transport of oxygen and nutrients, removal of wastes and cellular metabolism. By contrast, on a 3D nanorange microenvironment using the scaffold presented here, all functional motifs on the nanofibre scaffold encircle the whole cell body in all dimensions – where all growth factors, cytokines, nutrients and signals can interact three-dimensionally. In the 3D interaction these cytokines or growth factors freely search out their receptors three-dimensionally, rather than two-dimensionally. This type of defined nanoscaffold could be highly valuable for cytokine–drug interaction investigation. Hence, it is essential to use a defined nanoscaffold for hepatotoxicity assessment that gives a complete toxicity profile without interfering with any endogenous substance as like conventional scaffolds can. There is a considerable body of evidence growing with regard to the different behaviours in 3D and 2D cultures [82,83,90].

Conventional versus organotypical cellular models

Conventional cellular models rarely use such a long-term hepatotoxicity assessment as *in vitro* models. So, it is essential to keep these isolated cells functional for the long-term, at least 14–90 days as per the FDA guidelines, by providing an *in vivo* liver microenvironment where the cells can survive without losing liver-specific functions including drug metabolism potential. Cell culture models are a major challenge to comparing the *in vivo* physiology of human livers for the expression of cytochrome P450 enzymes during biotransformation of drugs for the pharmacological and toxicological fields [91].

The organotypical sandwich model is widely accepted for long-term maintenance along with liver functions. This sandwich culture model facilitates the preservation of certain liver characteristics, including the cuboidal morphology of hepatocytes with features such as bile canaliculi, tight junctions, gap junctions and prediction of metabolites [92]. The expression of CYP211 enzymes is generally lost in cell culture but we found, in our previous study, higher levels of metabolites in our organotypical model than in freshly isolated cells [93]. This indicates that there are many possible causes of high expression of other cytochrome P450 enzymes in an organotypical model. The organotypical sandwich model has better potential to show the higher expression of drug-metabolizing enzymes and perhaps these enzymes encourage restoration better in this model compared with monolayer models where expression is lost or is very low after isolation. We showed that the expression of phase 1 enzymes is significantly higher than the human hepatocellular carcinoma cell line (HEG2) in an organotypical model [94]. In another example, we investigated the sandwich model of porcine hepatocyte in a BAL model and were able to infect primary human hepatocytes with the porcine retrovirus [95], supporting other rare reports that porcine endogenous retrovirus (PERV) can infect human cells *in vitro* [96] and the SCID mouse *in vivo* [97], which highlights a potential zoonotic risk. But, many BAL models have been reported that suggest there is no risk in pig-to-human xenotransplantation [98].

Organotypical sandwich-cultured hepatocytes have a significant impact on investigating the hepatic accumulation and excretion of a wide variety of drugs based on the function and maintenance of hepatic transport protein expression and function [99,100]. Growing evidence suggests that hepatotoxicity is caused by compounds that inhibit one or more of the proteins responsible for bile acid excretion [99,100]. For example, troglitazone inhibits bile acid transport in the *in vitro* sandwich model [99], which might cause hepatotoxicity. It is widely accepted that the sandwich model is a fruitful model to find out if the inhibition of bile acid transport would be beneficial during drug development, allowing early prediction of drug candidates that cause cholestasis in humans [101]. Ansede *et al*. used sandwich-cultured rat hepatocytes as an *in vitro* model to assess the cholestatic potential of eight drugs for bile acid transport [99]. Further optimization of this sandwich model has been developed based on the influence of seeding density, extracellular matrices and days in culture [102]. Hence, this model is very promising for characterizing biliary excretion and the hepatic disposition of drug candidates. Despite such great potential, the conventional organotypical sandwich model can be hampered by complications, such as batch-to-batch variation and sensitivity of the upper layer of the flow model of the bioreactor owing to the undefined extracellular matrix used for this model. A few groups have used the synthetic sandwich model to analyse hepatic functions including bile excretion; however, further research is needed to make more-defined organotypical models.

Static plate culture versus dynamic culture

Virtually, hepatic oxygen supply is directed not only by the oxygen content of blood but also by the rate of blood flow in the liver. Conventional static culture is an insufficient model for proper prediction hepatotoxicity in the preclinical phase [91]. It is obvious that the liver *in vivo,* in contrast to a conventional Petri dish culture, is supplied with fresh nutrients more than once a day, which undoubtedly must lead to an accumulation of capabilities. Hepatic flow in a human liver is 1500 ml/min. It normally weighs 1500 g, so total liver flow is 1 ml/min per 1 g liver (equivalent to 10^6 hepatocytes). Therefore, to mimic the *in vivo* situation it is essential to make in the *in vitro* culture a flow model similar to this hepatic flow. Our flat-membrane bioreactor could be an option that consists of a multitude of stackable flat-membrane modules, each having an oxygenating surface area of 1150 cm^2. Up to 50 modules can presently be run in parallel mode. Each module is separated from the other and they are connected to a serum-free medium or plasma exposure or complement-inactivated human plasma reservoir individually with tubing from a multichannel peristaltic pump, which controls the flow rate (flow rate of 9 ml/h) in the inlet and outlet streams. However, it might be possible to control the wide range of flow rates by multichannel peristaltic pump in our flat-membrane bioreactor [81]. When a drug undergoes metabolism in an *in vivo* liver, the process is dynamic circulation of hepatic flow. The increased hepatic flow for higher

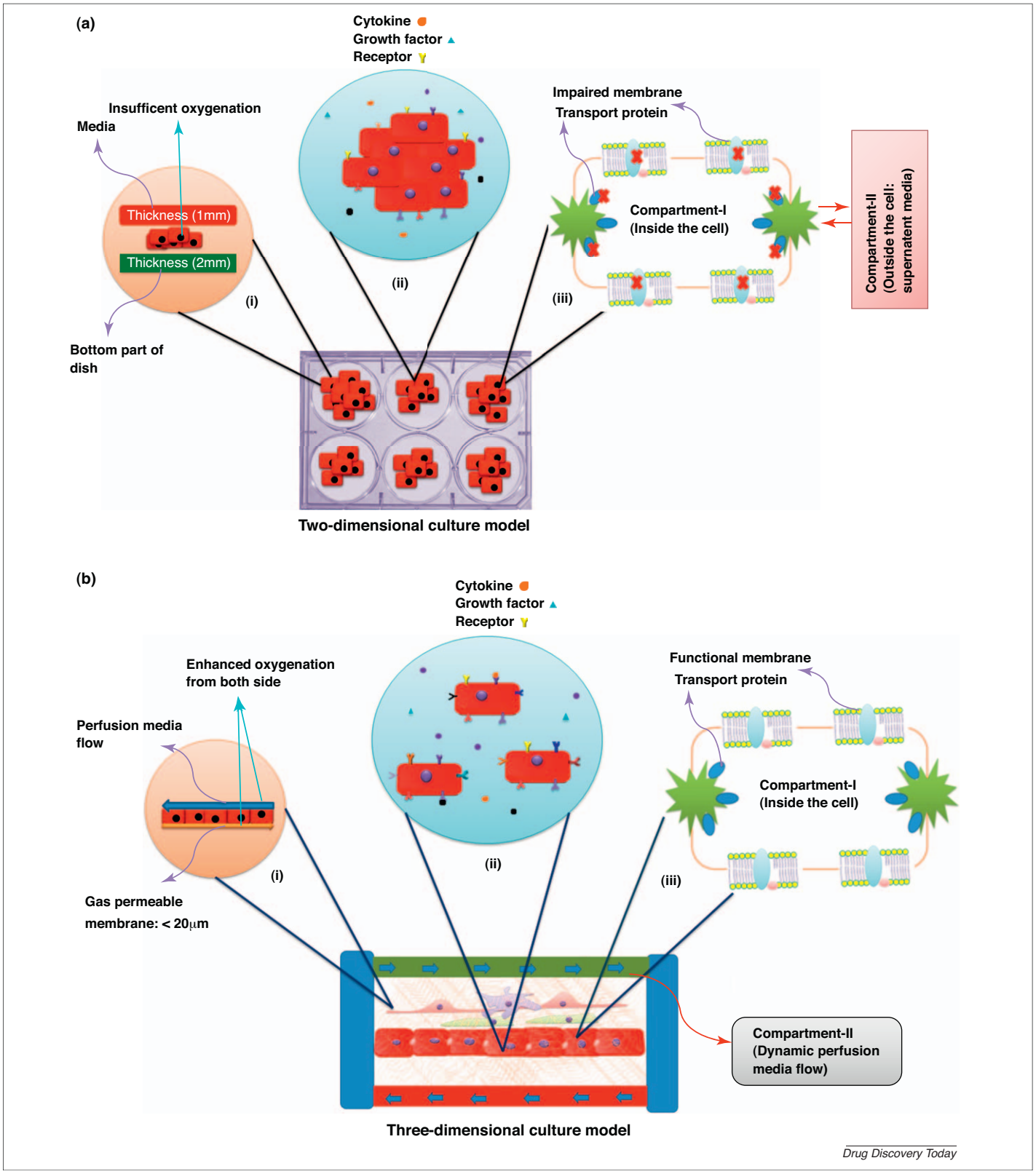

FIGURE 3

(i) Culture plate containing a discontinuous medium is predisposed to insufficient oxygenation, where the 2 mm bottom part of the culture dish and culture medium (1 mm) are the two major barriers. Hepatocyte cells consume ten-times more oxygen *in vivo* than *in vitro*. The distance between hepatocyte and hepatic plasma circulation – where hepatocytes exchange oxygen, nutrients, hormones, drug components and metabolites – is <20 μm. Under 2D culture conditions (A), hepatocytes can suffer from hypoxia, inflammation and lactate accumulation that all alter the drug metabolism. In a hypoxic situation, ATP formation from glycolysis is blocked because of insufficient oxygenation, resulting in impaired membrane transport proteins – because their formation is energy dependent. The distance for enhanced oxygenation to hepatocyte for exchange of the nutrients, hormones, drug and drug metabolites is the same for (B) 3D cultures (i.e. 20 μm). In this arrangement, the medium flows through culture cells that are attached to a nanoscaffold in a gas-permeable membrane (<20 μm thick), thus achieving

oxygenation during metabolism has been reported in several studies [103–105]. Drug clearance is not always directly related to hepatic blood. In the case of drugs that exhibit a low hepatic extraction ratio, drug clearance is related directly to the unbound fraction of drug and the intrinsic clearance of unbound drug. Hepatic blood flow is important for uptake into hepatocytes and determining presystemic drug elimination. Further, hepatic blood flow is different in old age and early adulthood [103–105]. Therefore, a controlled-flow bioreactor model is necessary to determine the drug response across a wide range of hepatic flow, depending on patients' conditions. These types of conditions might be possible in preclinical stages by using BAL where a wide range of flow rates can be controllable.

Hypoxia is directly associated with hepatic flow, which is also an important parameter in the preclinical stage of drug development. Acute hypoxia study in animals is difficult because of an increased chance of cardiovascular events that lead to death. Avoiding these difficulties is possible in BAL where several ranges of flow rate can be controlled precisely. Further research is needed for optimization of different hepatic flow models of the human diseased state of unhealthy individuals in a BAL model. A variety of hypoxia-based experiments can be designed where it is possible to measure oxygen consumption rate during culture conditions developed by using optical oxygen sensors in BAL. This could be an interesting approach to determine the profile of a drug candidate in a controlled environment before going to animal testing or hypoxia patients post-market. Ito *et al*. investigated age-related changes in the hepatic flow circulation in mice [104]. Hepatic flow is significantly reduced with increasing age along with reduction of liver mass, and in old age (above 65) there is a reduction in hepatic blood flow of about 35–40% [103–105]. Bile flow and bile salt formation are also reduced during the ageing process [106]. Reduction of liver mass, as well as a 30–40% reduction in blood flow, occurs during the human ageing processes [104]. Unfortunately, only a few *in vitro* studies have reported on age-related experiments. Viral hepatitis is associated with altered hepatic circulation [107]. Significant reduction in hepatic microcirculation in a human donor fatty liver and experimental model of hepatic steatosis has been reported [108]. Increased intracranial pressure (ICP) with decreased hepatic blood flow has been reported in liver patients [109]. Our flat-membrane bioreactor loaded with primary hepatocytes was able to maintain ICP in a pig model [110]. However, there is a lack of published reports about the effects of hepatic flow on drug responses in *in vitro* cultures. Therefore, a different hepatic flow model should be generated in the preclinical stages during drug development using a bioreactor.

Abnormal drug metabolism with prolonged elimination of drugs is often seen in critically ill patients. Among these patients, prolonged elimination of drugs is related to reduced hepatic flow, which might create a serious problem. Furthermore, hepatic flow can vary person-to-person, depending upon the condition of the patient's liver. No specific studies on different rates of hepatic flow have been conducted *in vitro* in the preclinical stages during drug development to mimic the hepatic flow of livers in ill and critically ill children or elderly patients, or in patients with fatty livers. It is essential to address these factors at the preclinical stage by creating various hypoxia environments and various rates of hepatic flow. These factors are better controlled in a bioreactor-based bioartificial liver.

Metabolite status inside and outside the cell

The safety testing of drug metabolites is of growing concern to the pharmaceutical industry and regulatory agencies alike [111] because it has been reported that >20% of the top classes of drugs have been associated with warnings or have been withdrawn shortly after receiving clinical approval owing to the toxicity of the metabolites [112]. Although many advanced publications of mechanism of wide range toxic model but lack of publication about the safety assessment of drug metabolites, even the hepatotoxicity of many metabolites has not been recorded in early preclinical stages and has only been detected once a drug has reached the market [113]. Recently, various regulatory agencies, including the FDA, have raised a serious concern regarding the need for complete drug metabolite profiles during the early clinical development of drug candidates and before the initiation of large-scale human clinical trials.

Toxic metabolites can accumulate in cells without any toxic effect on the liver. However, they might create a toxic effect by themselves or by interacting with the metabolites of another drug. Sometimes the drugs themselves are not toxic, but their metabolites might potentially be toxic. To detect drug metabolites in *in vivo* models or *in vitro* culture supernatants, samples, such as bile, urine and plasma, are collected from experimental

enhanced oxygenation of hepatocytes from both sides (perfusion media and gas-permeable membrane). (ii) Growth factors and cytokines have 3D structures that are widely used for three types of germ differentiation, including hepatic differentiation. Cytokine receptor/growth factor interaction is another challenging area for new drug development, as in 2D methods culture cell receptors might not be able to interact with cytokines and growth factors available in media. This could cause altered metabolism and declined functionality that affects their intracellular signalling pathways. For this reason, cells grown in 2D, either for differentiation or drug screening tests, continue to provide unsatisfactorily unclear and non-predictive data for *in vivo* responses. By contrast, in 3D culture conditions cytokines/growth factors interact with cell receptors three dimensionally, thus evaluating more accurately whether the drug metabolism is stimulated or blocked. (iii) A two-compartmental model can be used, where one compartment is supernatant/perfusion medium compared with *in vivo* hepatic blood circulation, and the other is inside the cell – termed cell matrix phase. A two-compartment model of a dynamic bioreactor based on bioartificial liver is a better simulator of an *in vivo* environment than a static 2D culture. Furthermore, many drugs rely on a hepatocyte membrane transport system, not only for uptake by hepatocyte but also for elimination from hepatocytes to hepatic blood circulation or bile. Membrane transport proteins are responsible for the removal and uptake of the drug and drug metabolites from cells. If a barrier exists, then drug or drug metabolites can accumulate inside the cell and cause necrosis by altering the drug metabolism (elimination or uptake). These barrier conditions are often present in conventional 2D cultures, causing the following cycle: low oxygenation to cell – hypoxia – anaerobic glycolysis – insufficient energy for drug elimination – delay or no elimination of drug–drug metabolites, oxidative stress, lactate formation, oxidative stress – membrane damage – impaired membrane transport system, due to unstable membrane stability. In 3D cultures, however, contrasting conditions occur, resulting in the following cycle: high oxygenation to cells – normal glycolysis – sufficient energy for drug clearance, no oxidative stress, no lactate formation – no membrane damage – good membrane transport system.

animals and humans. There is, however, no focus on whole-cell metabolite analysis in preclinical screening tests in the pharmaceutical industry. A few quick methods, such as the two-compartmental model and immunological detection, have been introduced recently to detect complete metabolite profiles in the preclinical stages. Drug metabolites inside the cell are rarely investigated using conventional methods, either in routine pharmacological research or in preclinical studies. We hypothesize a two-compartment model where one compartment (the culture medium) is hypothetically comparable to human blood or plasma in the liver circulation, whereas the other compartment (the cell matrix phase) compares the ability of the substance to bind inside the cell. A hypothetical *in vitro* two-compartment model compares the *in vivo* situation with special reference to drug metabolite detection during biotransformation. Cell matrix phase (cells + nanosaffold: compartment I) compares to the *in vivo* liver cells and supernatant (compartment II) compares to blood plasma because *in vivo* hepatocytes have direct contact with human plasma, where hepatocytes can extract oxygen and nutrients and detoxify chemicals in the plasma and their metabolites pass into the plasma. The two-compartmental model [114] and microplate culture systems [115] have been fast, reliable methods to detect drug metabolite profiles that are more realistic to human *in vivo* responses. Most pharmaceutical companies rely on animal experiments to detect all important circulatory and excretory metabolites, which ultimately provide less prediction of human responses. By contrast, the two-compartmental models and microplate culture systems are quicker and overcome this limitation, giving a more reliable drug metabolite profile. Generally, a drug undergoes a series of pathways during drug metabolism *in vivo*, whereas *in vitro* scenarios involve only one or two reactions. Therefore, these *in vitro* methods are faster, less expensive and potentially predict profiles earlier for all major and minor drug metabolites, because metabolites have therapeutic and adverse effective terms as active metabolites [116].

We hypothesize that it is of equal importance to understand the metabolite distribution in plasma and tissue because drug-metabolizing enzymes are equally distributed in the liver. In particular, two-compartmental models could help to detect the silent metabolites as well as reactive metabolites. This concept of the two-compartment model might provide adequate results rapidly before beginning large-scale clinical trials. Generally, *in vivo* metabolism studies of humans have been conducted relatively later on in drug development, but we recommend using the two-compartment model or microscale system to mimic the *in vivo* metabolite evolution in humans. This *in vitro* model could confirm *in vivo* results that are essential to the early stages of the drug development process. Bioactivation of metabolites to a reactive metabolite and its covalent binding to cellular macromolecules is believed to involve clinically adverse events, including idiosyncratic drug toxicity. Covalent binding is also an index for metabolite toxicity. As an example, diazepam is a compound that the liver should clear but, in our previous *in vitro* studies, we detected diazepam metabolites in the cell matrix phase, implying that, under certain conditions diazepam drugs might not be cleared by the liver. It is an innovative approach to do rapid screening for drug metabolites in the two-compartmental model in a BAL model to predict hepatotoxicity more accurately (Fig. 3).

Interaction potential with cytokines and growth factors

Generally, a drug is given to ill and/or critically ill patients (subacute, acute or hyperacute/chronic disease). All ill patients differ from healthy individuals with special reference to growth factors and cytokine levels in their blood plasma or liver circulation. Huan and Temple eloquently explained the drug exposure and responses of individual patients based on intrinsic factors (e.g. age, race, disease state, organ dysfunction, pregnancy, gender and genetic) and extrinsic factors (e.g. drug–drug interaction, environment, medical practice, regulatory, alcohol, smoking and diet) [117]. Multiple cytokines and growth factors are seen in diseased livers because of inflammation conditions that reduce drug clearance, reduce the cytochrome expression and diminish clinical efficacy [118,119]. It is well established that inflammatory processes affect the metabolism, distribution and elimination of certain drugs [118–122]. There is no such interaction of *in vitro* models during preclinical stages to determine the interaction potential of cytokines alone or in combination. Generally, cytokines and growth factors have roles that regulate immune responses to pathogens or cause pathological events; however, during various diseases levels of most of cytokines and growth factors are very often high. Tumour necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are secreted in acute and chronic liver-disease patients [123]. When a drug is used by acute or chronic liver-disease patients there is a greater chance of interaction with these cytokines and adverse reactions. Qato *et al*. reported that at least five prescription drugs are taken at any given time by the elderly (above 57 years) population in the USA, which constitutes 30% of the total US population [124].

Sometimes a drug itself causes inflammation and alters the cytokine levels. Cytokines can create hepatotoxicity or enhance the hepatotoxicity. For example, sulindac (SLD), a drug used for the treatment of inflammation and pain, has been associated with a greater incidence of idiosyncratic hepatotoxicity in human patients [125]. TNFα can enhance SLD sulfide-induced hepatotoxicity [126]. Human habits such as smoking increase the production of proinflammatory cytokines (IL-1, IL-6 and TNFα) and are believed to be involved in liver-cell injury [127]. In another example, a decreased clearance of theophylline in children caused toxicity after taking non-toxic doses of the drug for asthma [128]. Further, cancer patients experience the toxicity of anticancer drugs as a result of reduced hepatic metabolism and view that these drugs alter the drug-metabolizing enzymes by the release of cytokines [129]. Lee *et al*. surveyed the 68 recently FDA-approved therapeutic proteins and showed that 38 therapeutic proteins cause serious drug interactions, possibly caused by cytokine interactions [130]. As a part of hepatotoxicity assessment, there is also chance to get beneficial effects of cytokines and drug interactions. For example, interferon α (IFNα) has been used since 1986 for treating patients with chronic hepatitis C [131] but, very recently, Hoofnagle *et al*. discovered that the combination of the antiviral agent ribavirin and IFNα was significantly more beneficial when compared with treatment by IFNα alone [132]. However, *in vitro* studies using bioartificial livers to determine the therapeutic or hepatotoxicity potential in the preclinical stage reduce such a hepatotoxicity spectrum in the post-marketing phase.

It is hypothesized that many inflammatory cytokines could be produced that cause adverse reactions, either directly or indirectly,

by marketed drugs or by human lifestyle (alcohol, fast-food, air pollution, etc.). No such cytokine–drug interaction studies have been conducted or reported in the preclinical stages during drug development. Cytokines administered *in vivo* or incubated with culture hepatocytes could serve as good models for the *in vivo* effects of cytokines. Research experiments using bioartificial livers will increase the understanding of drug metabolism or regulation of cytokines in the near future. To date, there are no such examples during preclinical stages about this situation, but they are urgently needed. Many *in vivo* experiments have been conducted to determine cytokine interaction with a drug in different disease models. Some clinical hepatotoxicities are not predicted by rodent models, which have less predictability and lack such interaction studies as provided by *in vitro* models. However, it is rarely focused on in interaction experiments with these growth factors and cytokines in preclinical stages. *In vitro* studies using BAL urgently need to give early signals before either clinical trials or post-marketing stages, avoiding the adverse outcomes that have been observed in animal trials.

Concluding remarks

Hepatic differentiation of adult stem cells, including hESC or iPS, in appropriate devices, such as BAL, signals a potentially promising future for unlimited functional hepatocyte generation, which can be utilized for drug screening, disease modelling and liver therapy. However, additional optimization is required to improve the preclinical and clinical impact of this hepatic differentiation process with regard to adult stem cells. We expect that the explosion of disease-specific iPS will greatly speed up and revolutionize the drug discovery process, eventually leading to a better healthcare system. It is possible to predict a patient's individual response to a treatment approach that could lead to the development of personalized medical and drug treatments. These personalized treatments are far better at predicting the efficacy of drug candidates in humans than are drug tests conducted on animals.

Genetic manipulations of animal models (overexpression, knockdown, knockout and knocking strategies) might not be an authentic model for understanding the human disease. The iPS technology could offer a more appropriate system to overcome these limitations because the disease-specific iPS bear closer and better resemblance to the cells found in the patient's own system, without the need for genetic manipulation. Therefore, it is very possible that iPS could pave the way for unprecedented opportunities to recapitulate pathogenic and non-pathogenic diseases and to optimize validation of drug candidates. This approach could increase the relevance of the human drug response and increase the safety and accuracy of predicting toxicological outcomes, resulting in the reduction of drug attrition. Furthermore, the use of iPS could help lower the costs and the research and development time associated with drug development and discovery.

Although exact human hepatotoxicity predictions in the preclinical stage is difficult, a multiparametric controlled-based bioreactor system could help significantly to recapitulate more *in vivo* human liver response during drug development. We are focused on assembling a functional, efficient BAL based on recent innovative parameters, such as generation of functional hepatocytes derived from human stem cells, enhanced direct oxygenation, 3D scaffolding, organotypical cellular models, dynamic culture, the two-compartment concept, interaction potential with cytokines and growth factors. This robust model system could be a fruitful platform for better, faster and less-expensive hepatotoxicity assessment. Apart from hepatotoxicity assessment potential, this BAL might replace animal testing in toxicity assays in the >87 000 chemicals already manufactured for human use, of which 43% of these chemicals have no available toxicology data. Preclinical screening tests that utilize BAL models might facilitate the safe registration of new drug candidates and accelerate drug discovery and development by providing better predictability of hepatotoxicity.

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