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EDITORIAL

Judging the value of ‘liver-on-a-chip’ devices for prediction of toxicity

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1. Introduction

Despite a continuous increase in the application of *in vitro* tests for absorption, metabolism, distribution and excretion (ADME) and toxicity testing, conventional *in vitro* models have not been powerful enough to fully replace animal models to predict toxicity in human. In general, they lack sufficient functionality and predictivity for human ADME and toxicity. Organ-on-a-chip technologies have been proposed as a new generation of *in vitro* models for drug candidate screening in the preclinical phase of drug development. Most ADME and toxicity *in vitro* tests are liver-based as the liver is the most important organ in xenobiotic metabolism and is one of the major targets for drug- and chemical-induced toxicity. Liver-on-a-chip models, defined here as three-dimensional (3D) microfluidic cell or tissue culture systems that aim to mimic closely the anatomy, physiology, and functionality of the liver, are gaining increasing attention as *in vitro* alternative for *in vivo* testing. The chips on which cells are seeded are mostly made from optically transparent polymers and usually contain channels the size of which ranges between 50 and 500 µm. They can contain monocultures of hepatocytes, two-dimensional or 3D co-cultures of hepatocytes with several other hepatic non-parenchymal cells (NPC) or other stromal cells, hepatocyte spheroids, or organoids formed by mono- or co-cultures and intact tissue such as precision-cut liver slices (PCLS).

2. Required functionality of the liver-on-a-chip

Up to now, although there is consensus that liver functions and particularly biotransformation and transport functions as well as toxic responses should be reflected as close to *in vivo* as possible and be stable preferably for several days up to weeks, there is still discussion on how to reach this. In Table 1, we summarize the most important features generally considered as crucial for liver-on-a-chip systems for toxicity testing. The presence of hepatocytes and NPC of human origin, the mimicking of the liver microarchitecture, proper oxygenation, and the introduction of flow of medium are generally accepted now as important features.

Reproduction of the zonation and the microarchitecture of the liver may contribute to a proper functionality and thus toxicity prediction, but this has not been achieved up to now. In this respect, PCLS might seem to be the most appropriate model, as it contains all liver cells in their original sociology and matrix–cell and cell–cell interactions. However, its use in industrial settings is hampered by the limited availability due to the nonexistence of successful cryopreservation methods to date and by their relatively short lifetime. Progress has been made recently by showing that human PCLS remain fully functional for 5 days in static cultures.

3. Sources of hepatocytes and non-parenchymal liver cells

The main challenge is the use of fully functional human cells for the toxicity testing since animal primary cells or human cell lines like HepG2 or HepaRG make the extrapolation of the obtained results to the human *in vivo* situation hazardous due to the species-specific differences in metabolism and transport of drugs and the low/absent metabolic and transport activity of some of the enzymes and transporters, respectively. Primary human hepatocytes (PHH) are considered as the gold standard, although the maintenance of their metabolic competence during culture is one of the big hurdles [1]. However, recent advantages in co-culturing with NPC and application of 3D and flow conditions have resulted in major improvements. The availability of fresh human liver tissue as source for PHH is still limited, but the quality of cryopreserved cells is currently improving. The use of human-induced pluripotent stem cells (hiPSC) differentiated fully to hepatic cells will hopefully be possible in the near future as they are now widely available and promising results have been obtained with respect to their differentiation grade [2]. However, in the majority of the studies with PHH and hiPSC, it is impossible to evaluate the actual metabolic capacity due to incomplete description of the methods used, the variation in substrates and concentrations used and the lack of validation of the methods. Often only relative values to a benchmark are given, but the benchmark is not adequately characterized.

There is consensus that NPC are essential for proper liver functionality and they are known to be involved in many types of adverse effects in the liver. The source of these cells was mainly freshly isolated cells, but their differentiation from hiPSC is emerging. Standardization and validation of the hiPSC

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Table 1. Essential characteristics of a liver-on-a-chip.

Structural aspects	Multicellularity	Non-parenchymal liver cells are important for numerous hepatic functions and are involved in drug-induced adverse outcome pathways
	3D structure	3D cell structure mimics the *in vivo* situation better and improves functions of hepatocytes
	Flow	Flow mimics the *in vivo* situation better and improves functions of hepatocytes and EC *in vitro*, and in single-pass mode removes waste products, and allows *in vivo*-like concentration profiles
	Human cell source	Human cells predict human toxicity better
	Integration of biosensors	Integration of biosensors allows precise monitoring of intracellular and extracellular events involved in drug toxicity
	Physiological fluid-to-tissue ratio	Physiological fluid-to-tissue ratio helps to establish physiological concentration gradients between cells and medium
Functional aspects	Drug metabolism activity	Metabolic activity levels similar to those in liver *in vivo* are crucial for a reliable toxicity prediction
	Transporter expression	Expression of transporters at similar levels and localization as *in vivo* is crucial for a reliable estimation of the actual drug exposure
	Liver zonation	Liver zonation is important to identify primary target areas for toxicity in the liver and to reflect the balance between toxification and detoxification of drugs
	Long-term viability	Essential for drug-induced toxicities that require prolonged drug exposure
Requirements for application in drug testing	High sensitivity	Systems should be able to identify all the potential toxic candidates
	High specificity	Systems should preferably produce no false positive results
	Affordable and easy to handle	In order to be used in a routine drug testing, liver chip systems should be affordable to the majority of pharmaceutical companies and CROs and not require highly specialized experts
	Possibility for high-throughput drug screening	To allow fast analysis of multiple candidates

differentiation protocols for hepatocytes and NPC will help to provide a steady supply of well-characterized (co)cultures.

4. Technical aspects

Perfusion of the liver chips not only mimics the dynamic flow environment in the liver *in vivo* and stimulates hepatocyte functions, but it also may improve toxicity prediction of drugs [3,4] and provide control over the exposure to the toxin and removal of waste products. The application of flow to the chip in single-pass mode allows controlling the exposure to the toxin and to test time-dependent variations in the concentration of the toxic compound mimicking a repeated oral dose exposure, which is one of the most challenging issues in safety assessment using *in vitro* predictive toxicology. In cultures without flow or with recirculating flow, the concentration of the drug under study is only known at the start of the experiment and its decrease in concentration during culture is not controlled and seldom registered. Moreover, in those situations there is no removal of waste products and metabolites that are excreted by the liver basolaterally and *in vivo* cleared by the kidneys, or excreted into the bile, and they may accumulate in static cultures over time possibly to toxic levels. Direct connection of LC/MS or IM/MS to the single-pass flow system makes it possible to perform *on line* metabolism and proteomics biomarker analysis and to track changes in metabolic and signaling activities of the cells in time, which is of importance when instable toxic metabolites are formed [5]. However, nowadays non-flow-based liver models also show promising long-term viability and functionality and are technically much less complicated, which offers higher chances for application in high-throughput screening.

5. Toxicity prediction using liver-on-a-chip

Liver-on-a-chip systems have been shown to be able to predict clearance and toxicity of certain drugs, which were in line with *in vivo* clearance and toxicity data, and to correctly reveal their mechanisms of action [6,7]. However, the levels of sensitivity and specificity with respect to classifying drugs as liver toxins still need to be improved. Specificity of conventional culture models used by pharmaceutical companies to test drug toxicity is usually high (>95%); the sensitivity, however, is not higher than 50%. Inclusion of NPC to the cultures significantly improved the sensitivity of toxicity, reaching 70–75% [7]. Whether this is sufficient is still under discussion.

It is recognized that initiation and progression of liver injury is a dynamic process and cannot be properly characterized using end point measurements only. Most of the *in vivo* and *in vitro* studies provide little information on the kinetics of the biological response and therefore might miss important information on the drug's mechanism of action. Liver-on-a-chip technologies either with or without flow, on the other hand, can implement and provide direct *in situ* real-time monitoring of many cell parameters. For example, incorporation of fluorescent biosensors and live-cell 3D imaging allow direct assessment of cell morphology and behavior throughout the study. Furthermore, electrochemical sensors coupled to the bioreactors provide more information on the functional status of cells or specific organelles, such as mitochondria, and could provide real-time monitoring of oxygen uptake [4]. Overall, the readout of the existing systems can include a broad range of techniques, such as imaging, biochemical, and physical assays, which enable the detection of early signs of toxicity.

Drug–drug interaction is one of the important concerns during the drug development process as well as in the clinic. The suitability of liver-on-a-chip models for recapitulation of the *in vivo* drug–drug interactions has been shown in several systems [8,9].

6. Human-on-a-chip

So far, only *in vivo* animal or human models can cover all aspects of the disposition of a drug in the body, starting from absorption and metabolism in the gastrointestinal (GI) track, biotransformation in the liver, systemic exposure, and elimination by the kidney and the GI track. Development of a multi-organ chip for long-term culture might open a great possibility to perform repeated dose studies *in vitro*. Advances in the field of microengineering allow the construction and integration of multi-organ chips to a human-on-a-chip, stressing the importance of different organ interactions in drug-induced toxicity. Interaction between intestine and liver co-cultured in a biochip was shown for bile acids [9] and for nanoparticle uptake and toxicity [10]. *Maschmeyer*et al. developed a four-organ-chip that includes intestine, liver, skin, and kidney tissue connected to each other in a dynamic manner. It was possible to maintain the system viable and functional for 28 days (time required for repeated dose toxicity studies according to the guidelines) [11]. However, no toxicity studies have been performed yet to further validate this model.

7. Challenges on the road to validation and application in regulatory toxicity testing

There are still some shortcomings of the current liver-on-a-chip systems. One of them is the use of PDMS scaffolds for most of the models, which is hydrophobic and absorbs lipophilic compounds and their metabolites leading to the misinterpretation of the exposure and thus the test results. However, recently polymer materials, such as parylene, or polycarbonate have been proposed and used in a liver-on-a-chip production, which will eliminate the unspecific binding of molecules to silicone materials such as PDMS [12]. In addition, high costs of the current chip models and their relative complexity limit their use in a high-throughput setting [13]. Nevertheless, despite numerous obstacles on the way to the design of an ideal liver-on-a-chip model, significant progress has been made toward the development of non-PDMS pumpless systems based on primary human cells, which are somewhat less complicated and less expensive for a broad use in high-throughput drug screening, but are not fully characterized yet [12].

Commercially available liver-on-a-chip microfluidic systems that have been used for toxicity testing are summarized in Table 2. Even though some toxicity screening of certain model drugs has been performed, none of the systems has been fully validated with respect to toxicity prediction [6,14,15]. Before a liver-on-a-chip system will be accepted by the regulatory authorities as alternative for animal experiments and can be used for routine drug toxicity screening in the pharmaceutical industry, the requirements for this *in vitro* model will need to be standardized, since many technical aspects can influence the cell biology and response to the drug, such as culture duration, flow rate, cell density, scaffold material, and fluid-to-tissue ratio [15]. In addition, it has been recognized that in addition to characterization of phase I and phase II enzymatic activities, transporter activities have to be present and fully described. This is a crucial aspect, since transporters not only determine the actual drug exposure but also can be a direct target of drug toxicity.

The next step is to fully characterize human liver-on-a-chip systems and to determine their predictive capabilities for clinical outcomes based on the correlation between obtained *in vitro* data and available patient information and information from clinical studies. Models that are more predictive and efficient, widely available, and less expensive would be of great value for the prediction of toxicity and would represent an appropriate alternative to animal testing.

Table 2. Commercially available liver microfluidic perfused systems.

Perfused platform	Cell types	Validation for drug toxicity screening
3D perfused cell culture platform from Zyoxel	Hepatocytes, SEC	Tolbutamide, meloxicam, diclofenac, disopyramide, metoprolol, verapamil, theophylline, midazolam, methylprednisolone
Microliver chip from HµRel corporation	Hepatocytes, NPC	Buspirone, imipramine, sildenafil, timolol, nifedipine, diclofenac, indomethacin, carabamazepine, antipyrine
Microfluidic perfusion array from CellAsic	Hepatocytes	Diclofenac
Microfluidic 3D hepatocyte chip from Exploit Technologies	Hepatocytes	Acetaminophen, diclofenac, quinidine, rifampin, ketoconazole

8. Expert opinion

So far, existing *in vitro* models as well as *in vivo* animal models in many cases fail to predict correctly drug toxicity in human. The human liver-on-a-chip model is considered to be a better match for human and is expected to improve the preclinical developmental phase. This approach is fully compliant with 3R's requirements and has an excellent potential to replace animal studies.

The use of hiPSC as a hepatocyte source and possibly also as a source for NPC seems to be the most promising option, which makes it possible to design platforms which include hiPSC-derived cells from a large number of donors, thus better representing the variation in the human population and significantly increasing the possibility to detect idiosyncratic drug-induced liver injuries that represent one of the biggest challenges in drug development process prior to clinical trials. However, it is difficult to compare and appreciate the differentiation grade and liver functions obtained up to now, due to incomplete description of methods and data, which are often only presented as relative values to cells with unknown functionality.

It is possible to use PHH from a large number of donors to detect rare toxic responses; however, compared to hiPSC, which are in principle unlimitedly available, it is less feasible to test a large number of compounds on the PHH from the same donor, due to their limited availability. Moreover, the inclusion of NPC from the same donor to the hepatocyte culture is not always possible, whereas this is in principle possible by differentiating iPSC from one donor to both hepatocytes and NPC. The use of pooled hepatocytes (commercially available up to 100-donor pools) reduces variation and increases efficiency, but information on the variation in the human population is lost.

Taking into account the limited amount of test compound available at the early stage of drug development and given the liver-on-chip microscale size, it will be feasible to test the toxicity of a drug on much larger number of cell donors in microscale size chips and for lower costs compared to the currently used systems.

Even though liver-on-a-chip technology is still in its early phase of development and not many toxicity studies have been done to validate this model, it already demonstrates effectiveness in improving toxicity prediction and shows a great future prospective to improve the preclinical development phase. One of the biggest critics to all preclinical models is the use of concentrations that are sometimes much higher than therapeutic concentrations of the tested drugs. This issue is increasingly recognized by combining *in silico* exposure predictions with *in vitro* experiments. Even though modern liver-on-a-chip technologies cannot predict the clinical exposure, they might provide data on the kinetics of the interaction between drugs and the human cell/organ/body that might improve *in silico* predictions

Unfortunately, none of the models allows for separate collection of bile, which may introduce toxic responses to bile constituents accumulating in the medium. Only models with single-pass perfusion have the advantage that they remove the bile constituents from the chips, preventing high exposure of the liver cells to these, often toxic, compounds.

Medium composition is crucial for the maintenance of liver-specific functions. To obtain better insight into the optimal composition, it would be helpful if in each study the medium composition is explicitly described.

Implementation of various biosensors to organ-on-a-chip platforms that monitor changes in the cell environment will give a unique possibility to study the interaction between the drug and the tissue on the molecular level, followed by alteration in tissue physiological functions and to register all elements of adverse outcome pathways.

Modern organ-on-a-chip technology with medium flow allows to test *in vitro* the involvements of different organs in toxicity development. For example, apart from the liver itself, gut and gut microbiome as well as kidneys are known to affect the systemic availability of drugs and their toxicity. Therefore, platforms that include the intestinal and kidney compartments mainly using cell lines have been already developed and shown to be of great value. The next step is to include more differentiated cells or tissues.

Overall, we believe that liver-on-a-chip devices, after further optimization and standardization, can be implemented in the drug development process to identify drugs with the potential to cause liver toxicity. The currently achieved sensitivity of 75% might seem sufficient for initial drug screening at the early phase of preclinical studies, but drug candidates, which enter late stages of preclinical trials and clinical trials, should be characterized with higher sensitivity using models with better predictivity to minimize potential health risks and unnecessary costs. Undoubtedly, their implementation would lead to lower animal use, overall costs, and time needed for a new drug to reach the market and to better preclinical predictions.

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Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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