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The application of 3D cell models to support drug safety assessment: Opportunities & challenges ☆

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ABSTRACT

The selection of drug candidates early in development has become increasingly important to minimize the use of animals and to avoid costly failures of drugs later in development. In vitro systems to predict and assess organ toxicity have so far been of limited value due to difficulties in demonstrating in vivo-relevant toxicity at a cell culture level. To overcome the limitations of single-cell type monolayer cultures and short-lived primary cell preparations, researchers have created novel 3-dimensional culture systems which appear to more closely resemble in vivo biology. These could become a key for the pharmaceutical industry in the evaluation of drug candidates. However, the value and acceptance of those new models in standard drug safety applications have yet to be demonstrated. This review aims to provide an overview of the different approaches undertaken in the field of preclinical safety assessment, organ toxicity, in particular, with an emphasis on examples and technical challenges.

Contents

1. Introduction

Historically, drug safety assessment has been based on animal experiments and the data generated in those tests used to make a risk assessment of new medicines for humans. For most of these studies rodents (usually rats) are used, and because of regulatory requirements, non-rodent species, such as beagle dogs or cynomolgus monkeys are also used. The predictive capability of such animal experiments has repeatedly been shown to be limited, and rat or dog data insufficiently reflect the complete drug response in humans [1]. As numerous data confirm the limited predictive value of rodent tests for the situation in humans, a combined assessment based on rodent and the required second toxicology species (which by regulation has to be a non-rodent species) moderately increase translatability of the results obtained to be able to form a risk assessment for a drug candidate relevant for the

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human volunteer and patient [2,3]. Nevertheless there are still major gaps in our ability to make reliable predictions based on animal studies and this has triggered a constant debate on how to improve the situation [4]. Apart from a limited predictive strength, particularly ethical issues, but also budget- & time-constraints within the pharmaceutical industry, have triggered an intense demand over the last decades for in vitro assays which would improve the identification of potential complications in drug candidates. In this context, and to better address patient-specific requirements, a cellular model consisting of human cells could logically generate more relevant data and help reduce the use of animals in drug development. With the advantage of a technically simpler set-up, such assays can be applied at very early stages in the development of drug candidates and support medicinal chemists in the synthesis of lead compounds with fewer side-effects[5].

Particularly in the area of mutagenesis, a series of highly standardized and reproducible tests, able to detect with high precision the potential of a drug candidate to induce mutations in the DNA, are currently available. Among these assays, the AMES test, which is based on the growth of *Salmonella typhimurium*-bearing mutations in genes involved in histidine synthesis in the presence of the test substance, or the micronucleus test (MNT), which is used to detect structural or numerical changes to chromosomes in cells exposed to the drug, has been shown to be extremely valuable in screening out mutagenic substances at an early stage of development [6–8].

Another area where a reliable in vitro test exists is cardiac arrhythmia, where a single human ion channel, the *human Ether-à-go-go-Related Gene* (hERG), cloned into a cell line, is used to assess the potential of drugs to cause arrhythmic heart beats such as *torsade de pointes* [9,10].

Apart from these assays to detect acute effects in humans, a large battery of in vitro tests is available to assess drug metabolism. Most frequently, liver cells, or fractions thereof, are incubated with test compounds and metabolites formed by, for example, the cytochrome P450 enzyme system assessed by mass spectrometry [11–13]. Thus, a series of in vitro tests supporting drug safety assessment are available today and are complementary to animal studies or — ideally — could even replace animal tests. However, not all relevant, drug-induced, toxicities can be reliably predicted by in vitro tools and still many aspects of safety assessment rely on animal data. However, because of the recognized, limited, predictive ability of some animal tests and the pressure to reduce animal experimentation to a maximum, further research is under way to address the numerous open questions in this area [14,15].

As described above, a series of well-defined specific endpoints are well covered using cellular assays. The endpoint has been defined and validated over the years and thus such assays can be used for decisions on the way forward in a drug development program. What is still a major challenge for the pharmaceutical industry is the lack of relevant in vitro systems to identify organ toxicity [16]. For example, still the most frequent cause for drug attrition is liver toxicity followed by adverse effects involving the circulatory and hematological systems [17,18]. Even today, most of the published data on in vitro hepatotoxicity relates to such transformed cell lines as HepG2 or primary hepatocytes grown as monolayer cultures as a surrogate for the liver [19]. A range of investigations have revealed the limited predictive value of data derived from these simple systems to reflect the situation in the liver, where multiple cell types are present, including hepatocytes, Kupffer cells, stellate cells, cholangiocytes, and endothelial cells (Fig. 1; [20–23]). While a hepatocyte-only model can capture direct acute effects on this cell type, effects which are mediated by other liver cells which then indirectly affect hepatocyte viability may be missed. Indeed, a series of approaches have shown that incorporating additional cell types may increase predictive strength due to an improved depiction of liver physiology [24–27]. Similar ideas have been discussed in the areas of cardio- and kidney-toxicity. This review discusses current approaches in in vitro toxicity and potential applications of novel, more sophisticated, three dimensional (3D) tools which may be used to increase the ability to predict adverse effects and enhance the safety of novel drug candidates for patients.

2. Cellular tools in drug safety assessment

2.1. Predictive in vitro models

In the early stages of drug development, a series of in vitro tools intended to support the determination of uptake, metabolism, and, to a certain extent, excretion of a drug, as well as the detection of potential undesired effects, have been established. While a plethora of publications describing the establishment of various assays and cellular models addressing a wide variety of endpoints certainly exists, only a relatively small portion of these then become standard tools within industry. While the assay battery may vary from company to company depending on portfolio, capacities, and specific needs, there are cellular models which are used throughout pharmaceutical industry. Some of these assays and models are part of the set of studies required by regulatory agencies, while others have proven useful in reliably predicting safety profiles (Fig. 2). Regulatory authorities ask for standard assays including AMES, hERG, and assays to elucidate drug–drug-interaction potential and hepatic clearance. Together with other standard assays, such as those addressing, for example, teratogenic potential (embryonic stem cell test (EST) [28]), photosensitivity (3t3 NRU phototoxicity test [29]), or the activation of drug metabolism (CYP Induction [30]), these studies contribute to the selection of clinical candidates. In brief, these assays are based on the following in vitro models:

- The AMES test, required for FDA submission, provides with high precision near-certain results where a given drug candidate is genotoxic. As negative results do not absolutely rule out a genotoxic potential, additional well-known tests are usually performed in parallel — including in vitro as well as in vivo tests, such as MNT or Comet [31,32]. The AMES test uses bacteria which lack many of the genotoxin targets found in mammalian cells which are the reason why additional genotoxicity screens are required for regulatory purposes. The bacteria used lack capability to synthesize histidine which is required for growth. Thus, potential genotoxins are identified by growth of mutant bacteria colonies on histidine-free medium [33].
- Using High Content Screening (HCS) techniques, treatment-induced micronuclei are detected in mouse lymphoma cells in culture [34], while the Comet assay is based on electrophoresis of single eukaryotic cells [35].
- The hERG assay to detect compounds with a potential risk of inducing cardiac arrhythmias uses the human Ether-à-go-go-Related gene stably cloned into HEK 293 cells combined with Patch Clamp analysis [36].

In summary, these assays use a single cell type model in combination with a well-established, specific, readout which addresses one single endpoint relevant for in vivo physiology. The long-term history of these assays and the availability of large sets of historical data allow researchers in drug development to make decisions on potential in vivo-relevant issues in a drug candidate series.

Tests applied at very early stages usually allow identification of a hazard, i.e. the potential of the drug to cause a certain undesired effect, including, for example, mode of action. Only later, when more sophisticated tools are used, a risk assessment can be done, i.e. the probability that the drug causes a specific side-effect at a given dose in humans — and how this relates to the efficacious dose [37]. Apart from contributing to the risk assessment in the selection of a candidate series or of clinical candidates, considerable efforts are made to mechanistically understand any side-effects occurring during pre-clinical stages, i.e. in animal experiments. The goal of mechanistic studies is to understand the underlying mode of action and to provide

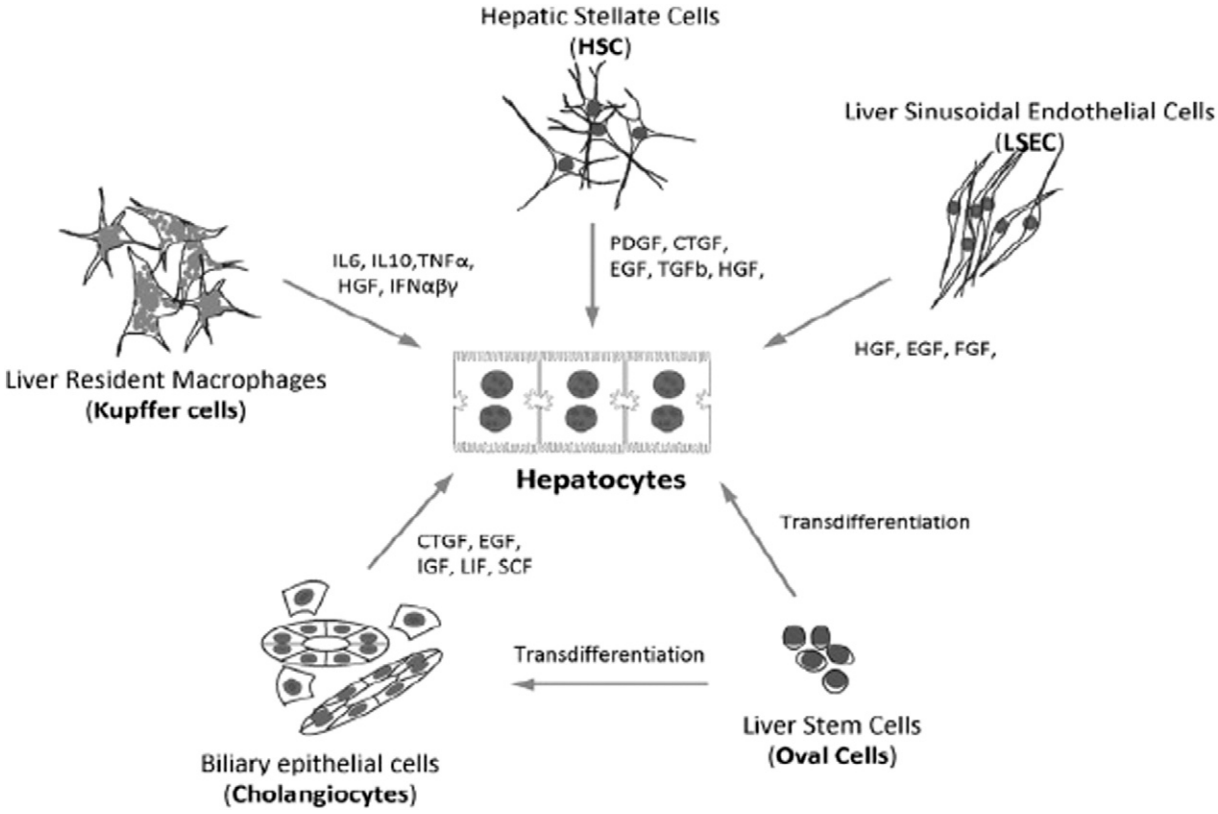


Fig. 1. Different cell types of the liver and the corresponding signals that are secreted in both health, disease or toxic challenge and which affect hepatocyte physiology (taken from LeCluyse et al., Critical Reviews in Toxicology, 2012; 42(6): 501–548).

an estimate of the human relevance of the observed effect. This is discussed in the following section [38].

2.2. Mechanistic in vitro models

Once a drug candidate has been selected and the molecule has entered the stage of in vivo testing, it is of key importance to increase the understanding of findings occurring during this phase in order to formulate risk mitigation strategies to design a way forward into clinical trials. It is not uncommon that during mid- and long-term animal studies, e.g. in rodents, clinical chemical or histopathological assessments provide indicators of potential treatment-related changes which could lead to organ toxicity. In many cases it is not known what the exact mechanism is and whether the effect observed has clinical relevance

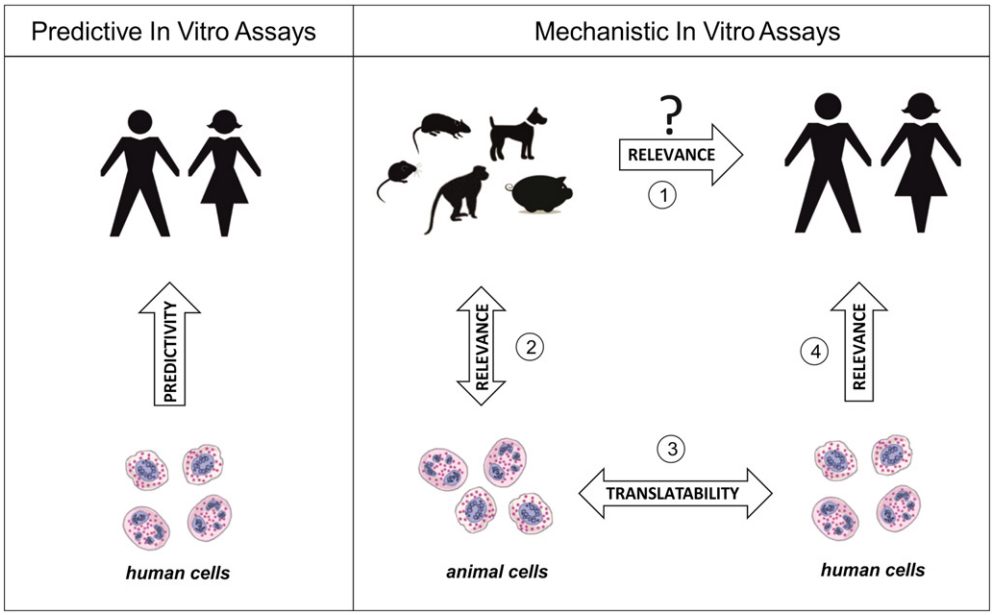


Fig. 2. In Vitro approaches for drug safety assessment: cellular assays directly predictive for the human situation (left) or a parallelogram approach (right): in the latter, potential human relevance (1) of an in vivo finding is first recapitulated in an in vitro system (2). If successful, transfer of the effect into a human cell system (3) helps in assessing human risk (4).

(Fig. 2, right panel, nr 1). For a drug development program to advance, it is therefore of key importance to address these questions with the help of mechanistic investigations. Ideally the outcome of such studies provides data and insight regarding:

- The dose at which the change occurs relative to the estimated clinically relevant (therapeutic) dose and thus to an acceptable safety margin
- The clinical relevance of a preclinical effect found, i.e. to provide evidence of a mechanism which is e.g. rodent-specific and thus not a risk for humans.

To be able to provide such guidance for a project team often requires a significant amount of exploratory research — both at the in vivo and the in vitro levels. While mechanistic in vivo studies serve to better characterize the effect and put it in perspective with respect to dose, in vitro models help to assess the potential species-specificity of a given effect. A typical approach to define human relevance or species-specificity is to first repeat the effect in an in vitro model of choice, reflecting the preclinical species where the effect was observed (Fig. 2, right panel, nr 2). Depending on the nature of the effect and the organ where it occurred, this can be technically challenging and often requires either a deep understanding of the mode of action or the use of physiologically relevant in vitro models — or both. Once the effect can be repeated in vitro in the preclinical species, a comparison to a similar human-related in vitro model is performed to see whether and how a treatment-related effect could be clinically relevant (Fig. 2, right panel, nr 3 & 4).

2.3. Limitations of today's cellular models for drug safety

It is clearly important to recognize the limitations of cell models used for predictive as well as for mechanistic approaches and to incorporate this knowledge into decisions. No individual cell-based screening or de-risking strategy is ever going to cover all in vivo-relevant situations; thus, a well-defined strategy, focused on established, well-known endpoints for predictive screening and on target organs for mechanistic studies, allowing the generation of sound and appropriate biological data, is likely to present the best prospect of improving drug safety.

The limitations of in vitro models currently used in drug safety are mainly that simple monolayer cellular systems do not accurately reflect the complex physiology of a target organ [16]. While cytotoxicity as such can be a very valuable parameter to estimate acute effects of a given molecule, the cell-type used does not seem to help in specifically revealing the target organ [16]. The complex nature of many known drug-induced toxicities cannot be captured accurately in a single cell-type often comprising short-lived monolayer in vitro systems [39]. The cellular microenvironment provided, for example extracellular matrix proteins and or scaffolds, is known to play a key role in maintaining physiological function of cells. This is not accurately reflected in standard monolayer cultures, nor is cell–cell communication and thus sub-acute drug effects, not directly inducing cell necrosis but rather depending on the interplay of cells, may be missed [40]. In particular, effects developing over longer time and requiring different cell types, as well as a cascade, often cannot be observed using standard cell cultures.

For example, trovafloxacin, a broad spectrum antibiotic marketed by Pfizer since 1997, can only be used with very stringent restrictions, after reports in 1999 stated that its use was associated with a number of cases of serious hepatic events, including acute liver failure [41]. The mechanism underlying trovafloxacin's liver toxicity still remains largely unidentified and testing the drug in primary human hepatocyte monolayers in vitro, for example, did not indicate any acute effect of the drug at therapeutic doses, when compared to other quinolone drugs [42]. Only recently published data suggest that inflammatory stress may play a role in trovafloxacin-induced hepatic toxicity [43]. Since inflammatory cells of the liver, such as Kupffer, endothelial, or stellate cells, are not present in cultures of isolated human hepatocytes, such effects may not be recaptured in vitro using current standard models (Fig. 3).

Similarly, in vitro models for the kidney, for example, usually use tubular epithelial cells only and thus are not able to mimic the complexity, either of a kidney tubule, or of the whole organ as such when cultured as a monolayer. Important aspects, such as membrane transport function, therefore cannot be reiterated in such a simple system so that the predictive value of such a tool is consequently very limited [44].

Thus, generally, unless a very well defined mechanism is assessed which can be related to an in vivo outcome, such as blocking of the hERG channel, in vitro systems used in drug safety assessment should ideally mimic the target organ as far as possible. To achieve this, many researchers use primary cells. While these cells certainly display many advantages over transformed cell lines, they often have other drawbacks, including, for example, a short culture life, where, in the case of the liver, formation of metabolites contributing to a toxic effect can be crucial.

2.4. Sandwich models

A well-established method to improve stability, functionality, and longevity of primary hepatocyte cultures is the overlay of extracellular matrix components resulting in so-called 'sandwich cultures' [45]. In this setup, after plating cells are covered with an additional layer of collagen, allowing the maintenance of some key cellular functions over a longer period of time. While such a system still consists of only one cell type and therefore does not fully represent the liver, sandwich cultures have proved useful for the study of drug transporters, for example [46].

2.5. How to improve safety prediction using in vitro models?

A wealth of different approaches to improve early safety prediction of drug candidates has been tried over the last two decades. After the establishment of basic in vitro technologies and cellular models, which can be applied to address drug toxicity issues in the 1970s and 1980s [47–50], the next two decades were characterized by the introduction of technologies such as genomics, proteomics, metabolomics, and high content imaging, which allow the capture of cellular events at a systems biology level rather than just at the single endpoint level [51–55]. Thus, the focus of the activities was driven by the idea that improving quality and the number of endpoints by capturing large sets of cellular events would ultimately improve prediction. The advantage of these technologies lies in the ability to generate a hypothesis and perform global screens without prior focus on a limited set of potential targets of drugs. Thanks to a deeper understanding of cellular events by measuring changes in gene, protein, or enzyme activity, safety assessment was thus improved by concentrating more on a mechanism-based assessment rather than entirely on phenomenological observations. While at an in vivo level, especially genomics — or 'toxicogenomics' — has proved very useful and highly predictive [56], at an in vitro level predicting organ toxicity remained a challenge despite clear technological progress. Nevertheless, new technologies have led to the establishment of mechanistic in vitro assays which allow molecular signals to be translated via chemo- and bioinformatics into disrupted pathways, consequently contributing to organ toxicity and ultimately clinical toxicity. A relevant example being the in vitro-detection of reactive metabolites formed in hepatocyte cultures or the functional assessment of mitochondrial impairment. These mechanistic endpoints can be assessed in cellular models and are used to identify drugs which have the potential to induce idiosyncratic drug-induced liver injury (DILI) in clinic [57–59].

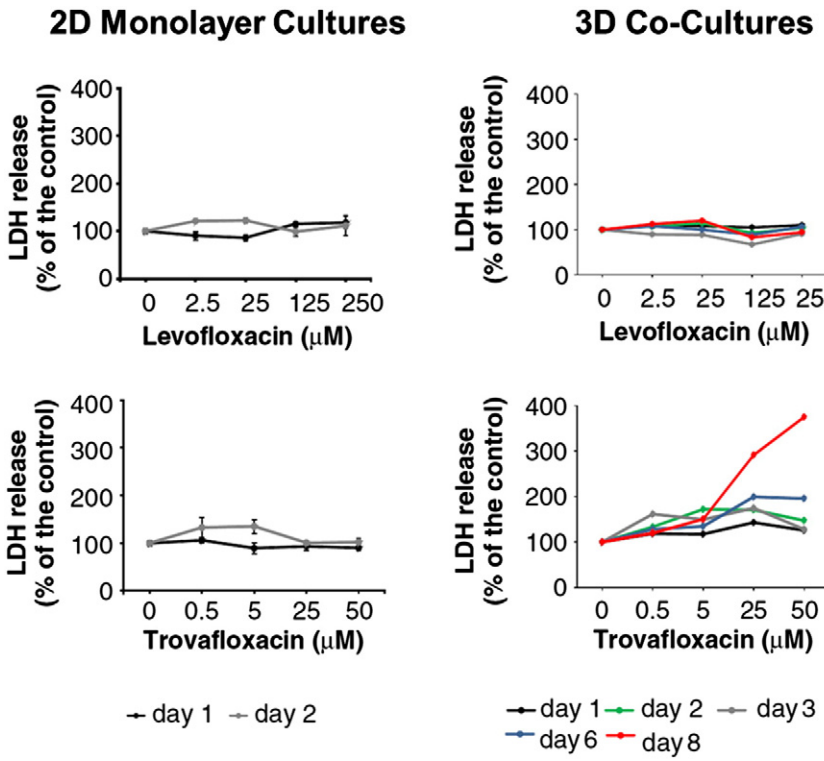


Fig. 3. The advantage of complex 3D systems for detection of drug-induced toxicities which require presence of different cell types: while trovafloxacin did not show any effect in conventional monolayer hepatocyte culture, in 3D hepatocyte-co-cultures an effect could be observed after prolonged compound exposure. The closely related but non-toxic drug levofloxacin showed no effect in either cell model (figure adapted from Kostadinova et al., Toxicol Appl Pharmacol 268(1):1–16 (2013)).

In recent years, there has been increasing awareness that not only are better readouts required to improve prediction of drug toxicity based on in vitro data, but also the models used to generate those data need reviewing. No matter how many aspects of cellular metabolism may be captured in parallel and analyzed using complex bioinformatics algorithms — the data can only be as valid for the in vivo situation as is the in vitro model chosen for a given test. It is therefore of great importance not only to update technologies for data generation but also to improve the models used. With the advent of stem cell-derived models, for example, and the availability of a wide variety of different primary cell types, research has begun into the development of novel approaches aiming to mimic organ physiology using more relevant cell types, mixing cell types together and creating scaffolds with the intention of creating in vitro models more relevant to the in vivo situation. In the next chapter, further examples of some of these developments, such as the addition of extracellular matrix to liver cells in combination with a 3-dimensional structure, are discussed.

2.6. Co-culture models

To represent the multicellularity of organs in vivo, several approaches exist for culturing a combination of cell types in the same well. Many years ago, researchers had already discovered that, for example, the co-culturing of primary human hepatocytes with non-parenchymal cells in a conventional 2D cell culture dish not only improved hepatocyte-specific functionality but also allowed long term cultivation of the cells [24]. Similar set ups have been proven useful for hepatocytes and Kupffer cells or hepatocytes and stellate cells [60,61]. Other approaches to 2D co-cultures used Transwells and the culture of cells in two separate compartments, allowing exchange of factors relevant for a given effect, while the different cell types had no direct contact [62]. An aspect not covered in those co-culture models is in vivo-like cellular architecture and direct cell–cell contact of different types of cells in 3-dimensions. In recent years and with the advent of nanotechnology, material science and micro-engineering, it has become possible to grow cells of different types in 3-dimensionally defined structures, allowing not only interaction of 2 cell types but also the growth of cells in a physiologically relevant context and contact to neighboring cells in all 3 dimensions.

3. The application of 3D models in drug safety today

3.1. 3D hepatocyte models

The recent development of more complex human liver in vitro systems has opened up a whole new field with a wide range of possibilities to be explored [63]. With the liver being the most important target organ for drug-induced toxicity, many approaches aimed at improving cell models in this area. For example, Khetani and Bhatia [64] created a 2D co-culture model of hepatic non-parenchymal cells with human hepatocytes. The model uses micro-patterned culture plates, where hepatocytes grow on defined areas as islands, surrounded by fibroblasts which serve as feeder cells. Thus, while not exactly embodying a 3-dimensional model, the approach demonstrates the value of co-culturing and a defined spatial arrangement of cells in a system. Initial experiments using this cellular setting revealed the maintenance of some key hepatocellular parameters over several weeks, demonstrating the relevance of the chosen approach. A recent validation study, using this specific model, revealed a noticeably improved performance for detecting hepatotoxic drugs when compared with recognized standard sandwich hepatocyte cultures [65]. Thus, while still a 2D model, the co-culture, permitting an extended culture time for hepatocytes, showed the advantage of introducing other cell types into an in vitro liver model. Earlier data have shown that drug-induced liver injury is often mediated by an interplay of different cell types residing in the liver, such as Kupffer cells, stellate cells or endothelial cells [66–68]. These cells play a key role in mediating and modulating

DILI, including idiosyncratic toxicity and hepatocarcinogenesis, probably via the release of, for example, inflammatory mediators, growth factors, and reactive oxygen species after their activation by drugs [69,63,70]. Consequently, over the years, researchers have invested in cell culture procedures which facilitate the long-term viability of different hepatic cell types in vitro, such as trans-well systems [39], bioreactors [71–73], and cultures grown as spheroids in hanging drops [74]. The models in some cases also included incorporation of continuous flow of cell medium through the system, mimicking blood flow through liver sinusoids [75]. These models can be maintained in culture for several weeks, but, due to their complexity and specific setup, may not all be ideally suited for routine drug testing under the specific conditions dictated by requirements within the pharmaceutical industry. At present only a few human 3D co-culture models are available for use in drug-safety assessment assays, so there is still an urgent need to further establish and validate them in order to assess their predictive relevance for drug candidate molecules, as has been done for a few systems available so far [39,65,74]. Examples of models which have been validated for their biological relevance, while at the same time ensuring that their technical set-up is compatible with industry testing, would the co-culture model established by Hepregen [65], the transwell-based system developed by Regenemed [39] or the spheroid model recently published by InSphero [74]. Novel liver models developed by Hemoshear, Zyoxel, and Hurel may soon be at a similar stage.

Most of the published work in this area describes proof-of-principle studies and indicates the potential ways forward, rather than proposing a model which, in its current state, would be applicable to today's industry testing regulations. The future will show which approach is the most promising and displays the best ratio between improved predictive ability and investment into more complex and potentially labor-intensive in vitro tools. The key to the application of a liver model in industry — apart from technical aspects, such as ease of use, automation, and reproducibility, is certainly a solid characterization of liver-specific functions over a time period of several days to weeks, including for example:

- Release of liver-specific factors such as albumin, urea, or transferrin
- Basal and inducible activity of key cytochrome P450s of the families 1A, 2B, 3A and 4A, ideally followed by demonstration of metabolite formation
- Demonstration of Insulin-responsiveness and bile acid synthesis
- Functionality of non-parenchymal cells, i.e. release of pro-inflammatory cytokines upon stimulus
- Morphological, quantitative & qualitative analyses of liver cell types over time to show stability of the system
- Cell viability over time.

After such a basic characterization, the validation with reference drugs for DILI, for example, based on standardized lists [76] and using relevant concentrations reflecting clinical exposures can be undertaken, depending on the intended use of the system.

3.2. 3D models for other major target organs in drug safety

Next to the liver, among the most frequent target organs affected by drug-induced side-effects are the heart and the kidney. It is therefore instrumental to have reliable and physiologically relevant in vitro tools in place to both predict and investigate liabilities in these tissues. As mentioned earlier, cardiac safety in vitro testing is restricted almost exclusively to the well-established hERG assay, and any functional or mechanistic follow-up is generally performed at the in vivo level — with few exceptions where, for example, rodent cardiomyocytes served as a model [77]. Only recently, with the advent of stem cells, novel in vitro models and assays have become available to identify drugs causing arrhythmias or direct damage to the cardiomyocytes [78,79]. With the advent of 3D models, some groups have applied this approach to cardiac cells, for example using micro-patterning cultures or 3D chitosan nanofiber scaffolds [80,81]. Interestingly, in the latter approach, ventricular cardiomyocytes isolated from cardiomyocyte–fibroblast co-cultures resulted in polarized cardiomyocyte morphology and retained their morphology and function for long-term cultures [81]. Similar to the liver, more complex cardiac in vitro models require characterization, including:

- Types of cells (atrial/ventricular, endothelial)
- Pulsation of cardiomyocytes
- Expression & function of relevant cardiac ion channels.

In vitro models of reconstituted skin have evolved as early attempts of researchers to form 3-dimensional cell cultures, starting from simple keratinocyte models, to models containing the different layers of cell types observed in human skin [82,83]. Those models have gained considerable interest, particularly in the cosmetics industry, where such in vitro tools have become part of the standard testing strategies to assess, for example, irritants and also genotoxicants [84,85]. Today, models are available not only to address effects induced by topical application of cosmetics, for example, but also skin changes arising from topical as well as systemic application of drugs to healthy [86,87] as well as diseased tissue [88].

Probably the first assay introduced into drug safety testing which makes use of 3-dimensional organ-like cultures was the so-called 'embryonic stem cell test' to assess the teratogenic potential of drug candidates on an in vitro level [28]. The test makes use of stem cells cultured as hanging drop spheroids, allowing the differentiation of stem cells into differentiated, pulsating, cardiomyocytes. A potential effect of a drug candidate interfering with the differentiation program in the cell is then assessed by quantification of pulsating areas in treated versus untreated cultures. Thus, the functional measurement serves as a measure of full maturation of embryonic cells into adult cells. The test has proved very useful for the early detection of developmental effects and has been validated in a large study by the European Centre for the Validation of Alternative Methods (ECVAM), as well as by individual pharmaceutical companies [89–91].

Renal toxicity accounts for 2% of failures in pre-clinical stages and 19% of all failures in Phase III [92] and the kidney is thus a frequently affected target organ for drug-induced toxicity. Due to the complexity of the kidney, in vitro approaches to address kidney liabilities early on are relatively rare and currently focus on recapitulating individual functional units within the kidney such as the tubules [93,94], which are one of the most common targets of nephrotoxic drugs and chemicals. To increase physiological relevance of such models, tubular cells growing as mono- or co-cultures have also been used in a 3D setting and tested for known kidney toxicants [95,96]. While still a relatively simple setup, these approaches indicated distinct improvements of cell models based on 3D devices over conventional 2D models. As for a kidney tubular model, most relevant features as well as parameters to be tested should include:

- Polarity of cells mimicking tubular structures, i.e. forming a lumen
- Transport of drugs/compounds from blood compartment to urine via epithelial cells
- Release of tubular toxicity biomarkers (e.g. Kim-1)
- Presence of transporters (PgP, OAT, OCT).

More recently, researchers have developed microfluidic-based cell models representing a series of human organs important for drug-induced adverse effects. These include the liver, heart, lung, kidney, and bone marrow [97,98]. These technically very elegant 'organs on chips' — albeit not yet comprehensively tested using large sets of validation compounds — are a promise for the future, since they bring together dimensionality, multicellularity, as well as flow. These features may be the key for in vitro systems to replace in vivo-relevant physiology as closely as possible, as exemplified by a lung model consisting of a polymer channel lined by human lung epithelial

and vascular endothelial cells that experience air and fluid flow, as well as continuous mechanical movement to mimic normal respiratory movement [99]. This in vitro lung device was further developed as a tool to predict and investigate drug-induced pulmonary edema — a side-effect seen in cancer patients treated with interleukin-2 [100].

Another promise for the future could be 3D models where the structure of cells in the model is not defined by a specifically designed scaffold, for example, but by means of a printer used to align the cells in the culture device [101]. However, these approaches need to mature first and to demonstrate utility under real life conditions. The great advantage of printed 3D cultures might be the absence of complex plastic scaffolds or other support devices which force cells to grow in a defined spatial pattern. The challenges and potential limitations of all these in vitro approaches for drug development in pharmaceutical industry are discussed in the following chapter.

4. Challenges and limitations of 3D models

4.1. Technical challenges

The advantages of 3D cellular models clearly lie in improved physiology, the ability to include different cell types in one model, and — mainly in the case of liver models — in longevity. These three aspects together have the potential to significantly improve biological relevance of an in vitro model and increase the chances that drug-induced organ toxicities requiring the interplay of factors from different cells and generation of a potentially toxic metabolite or accumulation-processes over time will be detected. Compared to a simple, single cell-type, system, these features render a 3D model very attractive. But the increase in complexity has a price, namely that different cell types require different growth conditions and different ratios between the cells, the need for complicated micro-engineered pumps, and expensive peripheral control-devices. Furthermore, different cell types need different quality checks and sourcing, which — depending on cell type — might be difficult. Moreover, seeding various types of cells will significantly increase variability in a cell culture well and thus entail a higher number of experimental duplicates and technical replicates in order to obtain reproducible results. Validation of functional parameters of each component in the system means that a larger set of tests has to be run before the main experiment can be performed. In many cases published so far, the in vitro model has been optimized for one or two key parameters and the chances are that overall, the system will produce misleading results due to this bias. In addition, long-term stability and functionality can be a challenge. While certain key parameters, for example, cellular viability, may remain constant for longer culture periods, it cannot be excluded that other parameters change, e.g. the ratio among the cells or cellular phenotypes. It is therefore crucial to measure quality parameters before, as well as at the end of, an experiment to guarantee that experimental conditions remain constant. An example is that of 3D liver co-cultures which could be kept constant for several months — including basic quality parameters such as cytochrome P450 enzyme levels. Because drug tests produced results difficult to interpret, gene expression of the cultures was performed across the experimental period (Fig. 4). Whole genome microarray analysis, at different time-points across the whole experiment, revealed that, after about 4 weeks, the cell model appeared to undergo physiological changes so that the pattern of expressed genes did not match that of an in vivo liver anymore (Fig. 4). This example was particularly intriguing and unexpected as the key liver functions, such as P450s, remained constant and thus a liver-like cellular environment and functionality could be expected. Without careful assessment of the gene expression pattern and comparison with benchmark data (in this case human liver samples), these physiological changes in the 3D culture would have remained undetected, thus probably rendering the interpretation of data from a drug effect experiment with this culture quite misleading. Thus, generally, complex multicellular 3D models will require extensive characterization and validation, as well as constant,

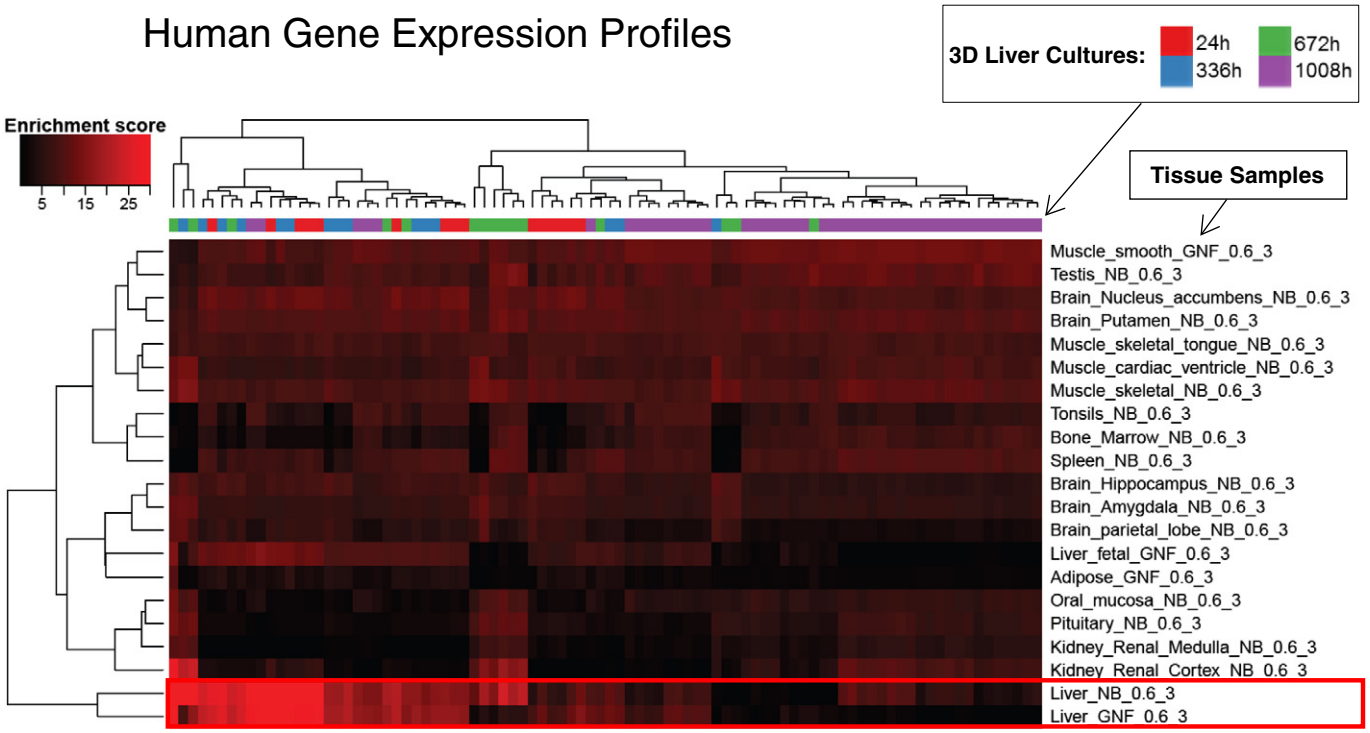


Fig. 4. Long-term culture of cells can introduce bias: A gene profile comparison was performed between 3D human liver cultures at different time-points in culture and data compared to human liver tissue profiles. While early time-points show the expected high similarity to human liver, changes occurring during prolonged treatment indicate cells in the 3D model are no longer representative of the liver (figure kindly provided by Jitao David Zhang, Laura Badi & Martin Ebeling; Roche Bioinformatics).

thorough, quality control before drug toxicity experiments can be performed.

Another example for a potential challenge is the material used to elaborate the devices, — i.e. the scaffold — but also the tubing material in the case of a microfluidic system. One such example was a microfluidic system allowing continuous monitoring of factors released from cells in a very elegant fashion. This system was tested for use in assessing hepatic drug clearance, i.e. quantification of the processes driving hepatic drug elimination (namely sinusoidal uptake and efflux, metabolism, and biliary secretion) using liver cells in vitro (N. Kratchovil, Hoffmann-La Roche Ltd., personal communication — unpublished data). A set of reference drugs, with established data on in vivo clearance and metabolites formed, was used to define a protocol for an in vitro drug clearance assessment. The unexpected outcome was that the recovery of both parent drug and the known metabolites was extremely poor. As a potentially too low number of cells could be excluded as a reason for lack of parent and metabolite in the outlet of the system, the test was repeated but without hepatocytes. This test revealed that more than 99% of dissolved compound applied did not reach the cells but was absorbed by the plastic material used to construct the device, and thus the commercially available tool in the version tested could not be used for generating meaningful data. This example emphasizes the necessity not only to build and validate cell culture devices for physiological parameters of cells to be grown in there, but also for practical use with reference drugs. In this case, the material chosen for the device was very well suited for the long-term culture of hepatic cells — but was unsuitable for experimentation using lipophilic drugs. Thus, to avoid non-specific binding, choice of material for creating the device (e.g. PDMS) needs to be carefully assessed. A promising approach to creating a more physiologically relevant tubing environment which at the same time does not include a non-specific binding bias has recently been published by Schimek et al. [102]. In their cell model, the micro channel system is fully covered by primary human dermal micro-vascular endothelial cells (HDMECs) thereby preventing drug molecule being absorbed by plastic material.

These and other technical difficulties need to be overcome before 3D systems can be applied in industry. Depending on the type of test for which the system is intended, simplicity of the technical setup and ability to run a series of experiments with relevant dose ranges in parallel will be crucial for pharmaceutical companies when choosing a 3D model for the regular testing of drugs.

4.2. Regulatory acceptance

It is a very long way from an exploratory technique to a proven and accepted tool in the field of drug safety and while researchers in industry will only use what functions and withstands exhaustive validation series, regulatory authorities will accept critical data using a novel cellular model only after joint cross-pharma validations and a substantial amount of published scientific evidence, as has been done for the EST, for example, which was validated by ECVAM [103,104]. A 3D system representing a target organ for drug toxicity will have to be validated using large sets of well documented and accepted reference drugs. An important example of a novel human-relevant in vitro model which has gained acceptance is the 3D skin models mostly used for testing cosmetics [83,105]. Thanks to thorough validation of the model using a series of test compounds with a known mode of action on the skin [86], the skin in vitro approach is now considered an accepted test for skin corrosion [106] and irritation [107,108], listed in a guideline of the Organization for Economic Co-operation and Development (OECD, see http://www.oecd-ilibrary.org/content/book/9789264071148-en; http://www.oecd-ilibrary.org/environment/test-no-439-in-vitro-skin-irritation_9789264090958-en). As such the 3D skin model serves as a role prototype for setting up, testing, and validating a new in vitro technique, designed to better predict drug effects relevant to humans, thereby reducing the need for animal testing. Next to such basic 3D test systems, which have gained wide application, 3D skin models are amenable to modification for the creation of disease templates and to ultimately elaborate a 'patient-in–a-dish' model or paradigm, as proposed by Groeber et al. [109].

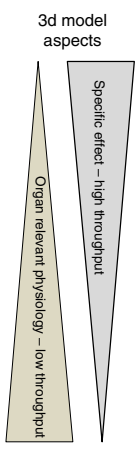
4.3. Outlook: the next steps

Recent years have seen growing interest from the drug safety community in cell models displaying improved physiology and there are hopes that, by using such systems, both pre-clinical as well as clinical failures during drug development can be greatly reduced, since such tools may better detect those cases which escape the testing procedures in force in pharmaceutical companies. While a series of approaches shows very promising results and raises hopes — there is still a lot of work to be done to fully exploit the value of culturing cells in 3D.

From the perspective of the pharmaceutical industry, the development of more in vitro models relevant to the human, including models involving multiple cell types growing in 3 dimensions, is of great interest. High hopes are placed in areas such as organ toxicity where current preclinical in vitro tests insufficiently capture drug effects. For example, a liver model capable of recapitulating the formation of reactive metabolites, hepatocyte–nonparenchymal cell interaction, and ultimately damage to liver cells after prolonged incubation, which is important for many known DILI drugs such as acetaminophen, [110] would be fundamental. Models addressing

Table 1
Prerequisites for 3D cell models in drug safety assessment.

Salient Points addressed in in vitro Models for Drug Safety	Requirements for an in vitro model in this Situation
– Predictive tests: early prioritization of drug candidates – Fast turnaround time – High/medium throughput – Low compound requirement – Qualitative assessment to guide medicinal chemistry	– Highly standardized, thoroughly characterized in vitro model with low technical & biological variability – Ease of use, simple handling – Amenable to automation – Readout allows quick evaluation and decision making
– Mechanistic tests: de–risking of in vivo findings in development stages – High biological relevance – Understanding of animal– versus human–relevant Mode of Action – Quantitative assessment to make «GO/NO GO» decision	– Mode of Action unknown: As close as possible recapitualtion of in vivo situation – in vivo phenotype displayed in in vitro system – Human relevant physiology – Ability to compare animal versus human at an in vitro level – Quantitative measurements possible



those issues by incorporating Kupffer macrophages, stellate, and endothelial cells next to hepatocytes are therefore considered most interesting [39,74,111] Furthermore, a cardiac system which can capture ion channel interactions beyond hERG, including structural damage-associated functional impairments [112] might be addressed using a combination of electrophysiological measurements and a multi cell-type cardiac model. The kidney remains a gap in in vitro toxicology, where structural complexity may only be captured by defined scaffold-based methods and semi-permeable membranes, recapitulating the exchange of ions and the transport of drugs. By and large, while a growing community of laboratories is working on the development of specific, tailor-made applications addressing specific questions, very promising work is in progress in terms of designing flexible platforms where organ-like cell cultures of different origin can be placed and allowed to grow in a more physiological environment [97,113]. The key advantage of a platform-based approach would be to have a basic setting which can be utilized for different cellular models — rather than having to establish cell models based on completely different approaches, depending on the organ of interest. Efficient cell seeding in defined areas might be achieved by use of 3D printing technology [114,115] and nutrient as well as oxygen supplementation enabled by microfluidics [114,116,117]. Such platforms may be used not only to grow different organoids in isolation but may even be interconnected to recapitulate liver-generated metabolites affecting heart or kidney, for example [118] — a novel concept endorsed recently by the Defense Advanced Research Projects Agency (DARPA), with grants supporting development of microphysiological systems mimicking organ-level biology of interconnected systems (see http://www.darpa.mil/Our_Work/DSO/Programs/Microphysiological_Systems.aspx). Such platform-based integrative approaches designed to address different organ-relevant toxicities in a flexible manner have great potential for application in the drug industry, once fully established and validated. Requirements important for a cellular model to address drug safety-relevant questions are listed in Table 1, including aspects where 3D models could play an important role. Therefore, while a series of promising approaches already exist — the full potential of complex 3D cultures for application in drug safety remains to be exploited in the future.

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