The Next Frontier

3D microtissue models are overtaking 2D cellular models as a more physiologically relevant means to predict human safety during clinical phases

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Drug development is an arduous process punctuated by go/no-go decisions related to the efficacy and safety of drug candidates. When a new preclinical drug candidate with a reasonable profile of efficacy and safety is identified, it is still uncertain whether this new therapeutic agent will ultimately make its way to the patient and advance human health. In fact, ~90% of all compounds entering clinical trials fail, largely due to safety issues in clinical phases or drug efficacy issues in patients (1).

This failure is because preclinical approaches that use *in vivo* animal models and *in vitro* cell models for discovery and development do not reliably translate to patients. In the preclinical development phase, drug safety is responsible for eliminating the majority of drug candidates from the drug development pipeline. In a worst-case scenario, depending on the chemical library of a pharmaceutical company, up to 50% of drug candidates in the discovery selection process may end up causing preclinical drug-induced liver injury (DILI) with an insufficient safety window and thus will not be further developed. Even the compounds that pass the preclinical battery of regulatory assays and make it through to the clinical phases may be dropped because of idiosyncratic-type liver toxicity with fatal outcome in patients and must subsequently be withdrawn from the market. Beyond this human tragedy, the whole R&D investment, which takes up to 10 years and costs US ~$1-2 billion, is wasted.

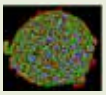
 **2D PHH model**	 **3D liver microtissue model**
Little co-culture compatibility, typically PHH only	Co-culture of hepatocytes with NPCs (eg, Kupffer cells, liver endothelial cells)
Loss of liver phenotype and cytochrome P450 (CYP) activity after a few days in culture; plastic adhesion effects change morphology	Preservation of liver phenotype, morphology, function, and metabolic activity for >3-4 weeks in culture
Models 'acute' hepatotoxicity testing only; not amenable to repeat-dose studies (high-concentration effects)	Enables long-term treatment and multiple dosings at low therapeutic concentrations (kinetic studies = 4D)
Fewer cell-cell contacts, no diffusion gradients	Native liver-like cellular contacts, no artificial scaffolds necessary

Figure 1: Unlike 2D PHH models, 3D liver models reflect the biology and physiology of human liver tissue more accurately

Just as preclinical animal models fail to predict human liver safety accurately, the current *in vitro* cellular models fail too. The application of *in vitro* safety studies has not changed significantly in decades. Using overly simplistic, 2D *in vitro* liver cell culture models from cell lines or primary human hepatocytes (PHH) as a filter for liver toxicity screening in frontloaded assays has only limited value.

3D microtissue models are produced from primary liver cells by scaffold-free tissue reformation and are the smallest functional unit of the liver that recapitulates structures and functionality observed in native liver. As such, 3D microtissues are more physiologically relevant and predict DILI more accurately than 2D cellular models (see Figure 1). These functionally robust microtissues, comprising hepatocytes, Kupffer cells, and liver endothelial cells (see Figure 2) are engineered for a broad range of experimental conditions and analytical methods, including:

- Long-term, stable co-culture (28 days versus seven days for typical 2D PHH monoculture)
- Multiple low-dose compound treatments that mimic patient treatment schedules in the clinic, which enables kinetic evaluations and opens the view into the fourth dimension
- Monitoring by continuous biomarker sampling (eg, aspartate aminotransferase, cytokines, albumin)
- Endpoint sampling for histopathology, immunohistochemistry, transcriptomics, proteomics, and lipidomics
- High content imaging, confocal microscopy
- High throughput screening capabilities in 96- or 384-well format
- Scalable mechanistic toxicity investigations

3D liver microtissues can be applied at critical junctures in pharma discovery and development (see Figure 3):

Discovery Phase

In the discovery phase, high-throughput, screening-type, and front-loaded assays for DILI hazard identification can be performed. As compounds are selected, efficacy and

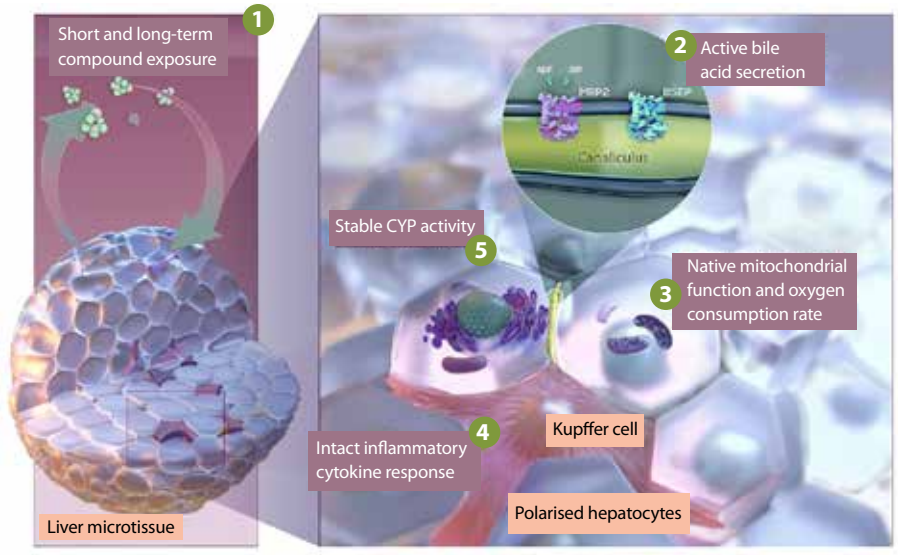


Figure 2: Physiologically relevant 3D *in vitro* human liver microtissue models mimic the structure and function of native human liver for more predictive drug safety testing

“Using overly simplistic, 2D *in vitro* liver cell culture models from cell lines or PHH as a filter for liver toxicity screening in frontloaded assays has only limited value”

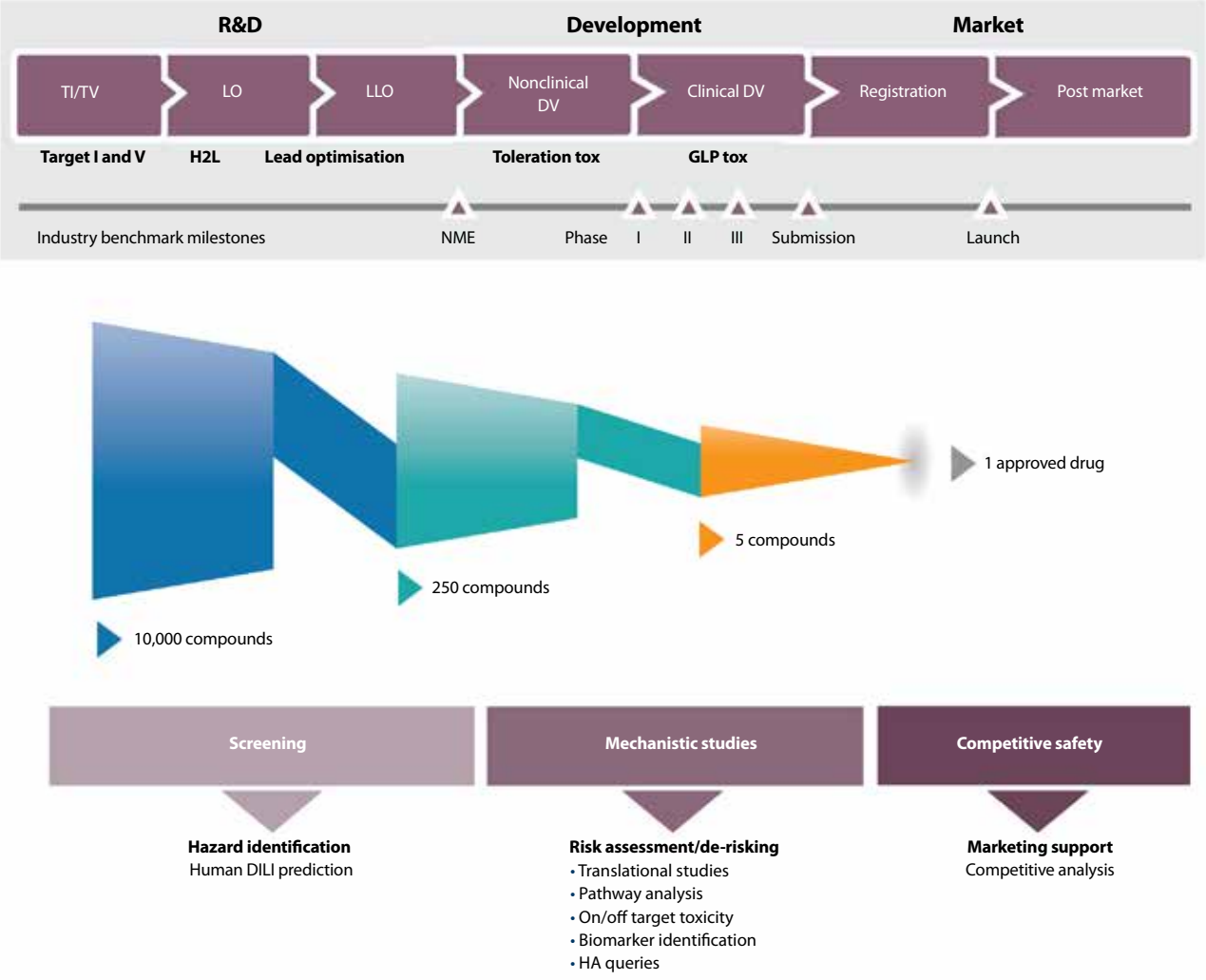


Figure 3: 3D liver microtissues deliver benefits across the discovery and development continuum to enable early decision-making and support health authority submissions

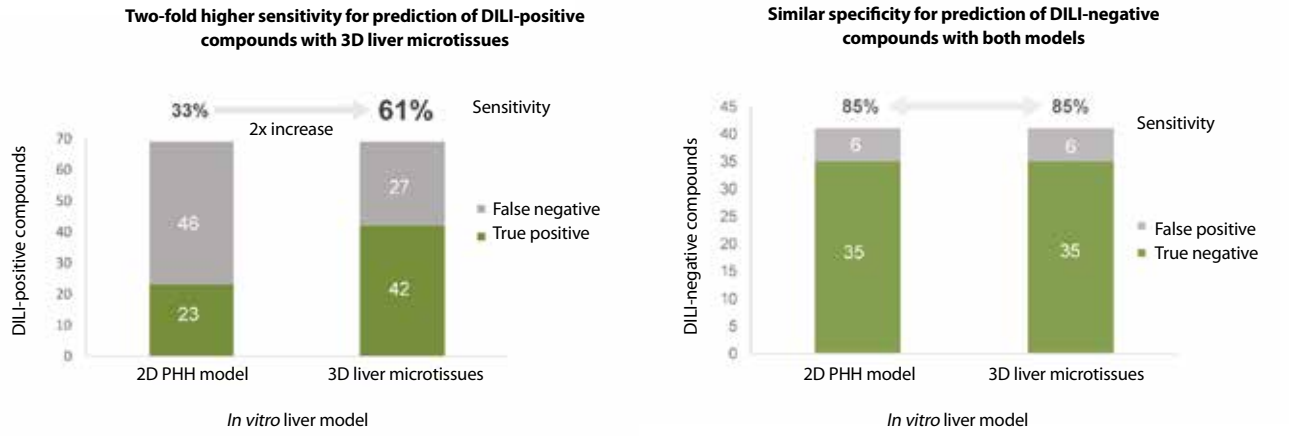


Figure 4: Comparison of 2D primary human hepatocyte cultures and 3D human liver microtissues using the same PHH lot, compound concentrations, and ATP-endpoint; study results confirmed 3D liver microtissues outperform 2D cultures for DILI prediction

potential to cause DILI are flagged in parallel. Frontloaded DILI hazard identification enables project teams to rank compounds according to their potential to induce DILI and allows medicinal chemistry groups to modify their molecules for quantitative-structure activity relationship investigation.

Development Phase

In the development phase, customised mechanistic toxicology studies are performed to evaluate specific questions that arise from regulatory toxicology studies. These studies investigate specific hypotheses on the mechanisms underlying the compound's abilities to trigger DILI.

> **"Risk mitigation strategies can be elaborated as part of the risk assessment and translation to human, facilitating the exploration of new biomarkers for possible clinical applications"**

Mechanistic translational studies enable decision-making by providing evidence on the translation of effects observed in animals to man. Elucidation of underlying human-relevant DILI mechanisms and pathway analyses supports back-up programs and investigation of potential on/off target liabilities and answers critical questions from medicinal chemistry. Additionally, risk mitigation strategies can be elaborated as part of the risk assessment and translation to human, facilitating the exploration of new biomarkers for possible clinical applications. Supplementing the Health Authority (HA) submissions by providing additional data with registration documents enables R&D teams to efficiently answer specific HA queries after submission.

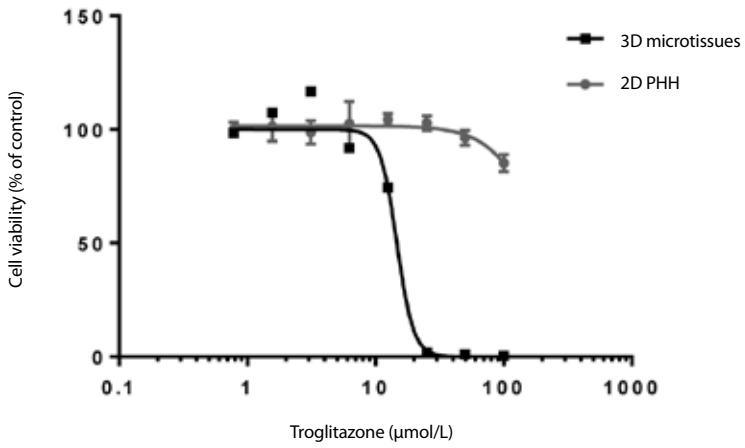


Figure 5: Troglitazone-induced toxicity was observed in the 3D human liver microtissue co-culture of Kupffer cell and liver endothelial cells after 14 days of exposure; no cytotoxicity was observed in the 2D PHH culture

Market Phase

In the market phase, competitive safety studies help differentiate new drugs from other comparable products on the market.

Recapitulating observed *in vivo* effects with 3D *in vitro* cross-species models
Examples of possible outcomes

A

Compound A	Rat	Dog	Human
Clinical/histopathology observation	ALT, centrilobular necrosis		
In vivo	–	+	?
Case 1 *In vitro**	–	+	–

GO

**In vitro* determination of leakage marker
ALT, LDH, or cell viability by ATP

B

Compound B	Rat	Dog	Human
Clinical/histopathology observation	ALT, centrilobular necrosis		
In vivo	–	+	?
Case 2 *In vitro**	–	+	+

NO GO

Mechanistic studies

Figure 6: Evaluating *in vivo* clinical endpoints *in vitro* to support go/no-go decision-making

Hazard Identification with a More Predictive Model

For DILI prediction, 3D cell-based liver toxicology assays unquestionably outperform comparable 2D assays. In a joint study, AstraZeneca, Genentech, and InSphero evaluated 108 clinical compounds with known DILI severity ranging from the most severe clinical DILI to no concern. DILI assessments using multicellular 3D human liver microtissues were compared with the 2D primary human hepatocyte monocultures historically used for evaluating DILI (2). The 3D liver microtissue co-culture model of PHHs, Kupffer cells, and liver endothelial cells was produced using the same donor source as the 2D PHH cell culture. Compound treatment of the 3D liver microtissues was conducted over 14 days, whereas in 2D hepatocytes culture, it was only for two days. Considerably more clinical DILI positive compounds were correctly identified by 3D liver microtissues as compared to using the 2D model. In fact, the sensitivity increased two-fold when moving from the 2D to the 3D liver assay. Only 33% of the true positive DILI compounds were identified by the 2D assays, whereas 61% were identified by the 3D assay (see Figure 4). Equally as important, the specificity for detection of clinical DILI negative compounds did not change in the 3D assay versus 2D assay, and no additional false positives were identified by the 3D assay.

Troglitazone, a compound strongly associated with severe clinical DILI, is one example from the set of 108 compounds of a DILI-triggering drug detected in 3D human liver microtissues, but not in the 2D PHH monoculture (see Figure 5). Troglitazone is an insulin-sensitising agent for Type 2 diabetes, introduced to the market in 1997 and ultimately withdrawn in 2000 due to the frequency of liver injury in patients. Prior to market launch of the drug, liver injury was not predicted in 2D *in vitro* assays or animal studies. The 3D human liver microtissues identified the toxicity as shown by the IC_{50} curve, whereas the 2D model gave no indication of DILI potential.

Supplementing Regulatory Toxicology

A sufficient therapeutic index is a key factor in the decision to move a drug into clinical studies. However, when the window between efficacy and toxicity is narrow, the decision to move ahead may be unclear or require additional data for pharma project teams and regulatory authorities. Additional data that explains the translation of animal-observed effects to man is needed. Translation

> **“When the window between efficacy and toxicity is narrow, the decision to move ahead may be unclear or require additional data for pharma project teams and regulatory authorities”**

can potentially be improved by the comparison of 3D *in vitro* cell models from desired preclinical species with a comparable human *in vitro* model. Cross-species 3D liver microtissue models, based on the same platform, can interrogate a compound's potential to induce liver toxicity across multiple different species (eg, human, monkey, dog, and rat). In the best-case scenario, cross-species evaluation is performed in parallel under identical conditions and validates that the toxicity encountered in a preclinical animal model is specific to that species only, not in humans. Alternatively, the results could show there is toxicity across all preclinical species and the human model, a clear no-go situation.

As a conceptual example, Figure 6A (page 67) illustrates that compound A showed no toxicity in rats, either *in vivo* or in the 3D *in vitro* model; however, toxicity was observed in dogs in both *in vitro* and *in vivo* models. The question is whether human clinical data is likely to be correlated either with the dog or the rat. The 3D human liver microtissue model would suggest that the human response would be

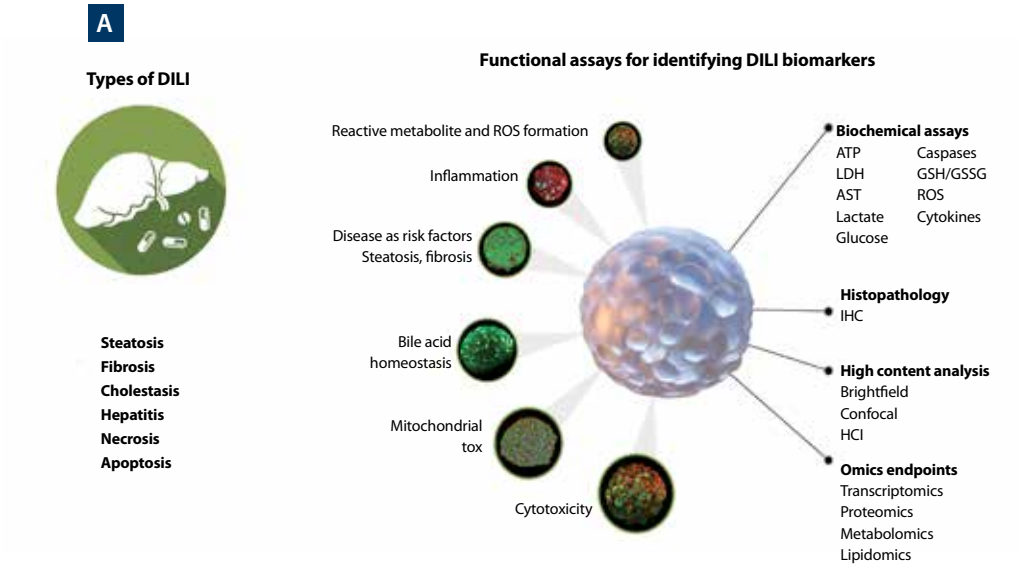


Figure 7: The functional characterisation of a DILI biomarker and the demonstration of the causal link to its cellular responses are essential for the validation of a hypothesis

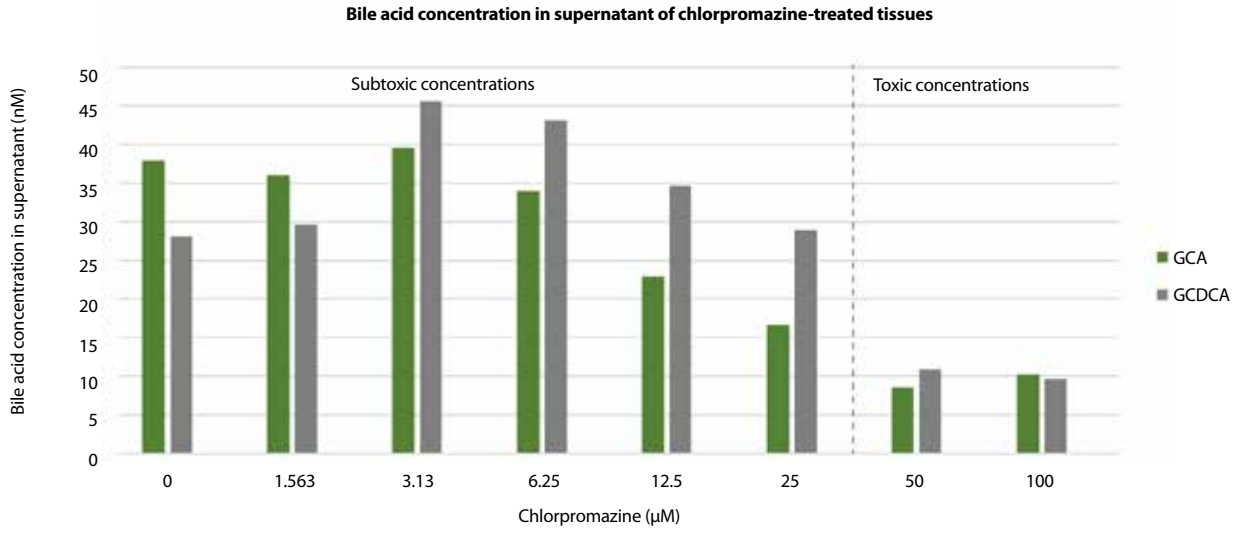


Figure 8: Chlorpromazine concentration-dependent inhibition of bile acid release from microtissues into the cell culture supernatant

more similar, in this case, to the rat models (no toxicity observed). A similar scenario is shown in Figure 6B (page 67), except that the translational study shows the same toxicity observed in dog and human and calls for additional mechanistic studies to better understand the toxic response.

Finding the Causal Link

Using the same 3D human liver microtissue models, multiple types of biomarkers may be investigated to elucidate translational properties ranging from histopathology to function and mechanism. Examples include the response of clinical biomarkers (eg, ATP, LDH, AST) to drugs and omics endpoints, such as transcriptomics, proteomics, lipidomics, and metabolomics (see Figure 7A).

Bile acid profiles are a new type of biomarker that can be effectively measured in 3D liver microtissues. The bile acid metabolites formed in hepatocytes and secreted via canalicular structures can be studied by analysing their concentrations in the cell culture supernatant. Figure 8 shows the chlorpromazine-impaired bile secretion into the cell culture supernatant. At non-cytotoxic chlorpromazine concentrations, three different bile acids: glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA) and glycodeoxycholic acid (GDCA) were inhibited compared to untreated controls as measured by liquid chromatography-mass spectrometry. This confirms the chlorpromazine-mediated mechanism of cholestasis due to inhibited inhibiting bile acid transport.

Demonstrating Causality Between Biomarker and Cellular Response

Physiologically relevant and functionally robust 3D liver microtissue models can be used to demonstrate the causality of the underlaying molecular pathways and the cellular event leading to DILI (see Figure 7B). For that, a specific modulator (either an enhancer/agonist or inhibitor/antagonist) of the pathway of interest is co-incubated with the investigational compound. Examples of enhancers are BSO (inhibitor of GSH synthesis) and LPS (inflammation, bile acids, etc). Inhibitors are antioxidants (ROS), specific enzymatic inhibitors, gene silencing, or knock-outs among others. The modulator can result in shifting the IC_{50} cytotoxicity curve, indicating a causal link to the addressed hypothesis.

The Way Forward

The availability of 3D liver microtissues with powerful translational capabilities is enabling a paradigm shift in our approach to drug safety assessment. These models are inherently more predictive than 2D primary human hepatocytes cultures, which have long been heralded as a gold standard. Physiologically relevant *in vitro* tissue models are better equipped to identify species-specific, unfavourable drug effects in the liver, interrogate underlying mechanisms of toxicity, and evaluate specific clinical biomarkers. Adoption of these models will undoubtedly reduce the attrition rates of drug candidates due to DILI, improving the situation for patients in terms of safety of better drugs, general productivity, and return on investment of our collective R&D efforts.

References

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2. Proctor WR *et al*, Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury, *Arch Toxicol* 91(8): pp2,849-63, 2017

About the authors

Judi Wardwell is a Senior Application Scientist at InSphero, with more than 25 years of experience working in the pharma industry. Prior to joining InSphero, she was the Manager of a functional genomic group at Bristol-Myers Squibb, where she developed physiologically relevant cell models and conducted phenotypic screens to identify new drug targets in developing integrated approaches to target identification. Judi has also held scientific research positions at Wyeth and Hoffmann-La Roche.

Dr Radina Kostadinova is Lead Product Manager for InSphero 3D InSight™ Liver Toxicology and Disease Platforms. She has more than 17 years of research experience in industry and academic settings, working in the fields of molecular and cellular biology and preclinical mechanistic toxicology. At InSphero, her focus is to develop and establish liver tissue models for detection of drug toxicity, as well as 3D liver disease models for the study of fibrosis, inflammation, diabetes, and obesity.

Professor Armin Wolf is Chief Scientific Officer at InSphero and Professor of Toxicology at the Technical University of Kaiserslautern, Germany. An accomplished pharma R&D executive and board-certified toxicologist with more than 30 years' cumulative experience at Janssen and Novartis, Armin offers a first-hand perspective on the challenges facing the pharma industry and the need for more physiologically relevant human models to improve efficiency in drug discovery and safety testing.
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