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may be dropped because of idiosyncratic-type liver toxicity with fatal outcome in patients and must subsequently be withdrawn from the market.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.

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
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.

---

**Table: Drug Development Stages**

<table>
<thead>
<tr>
<th>R&amp;D</th>
<th>Development</th>
<th>Market</th>
</tr>
</thead>
<tbody>
<tr>
<td>TI/TV</td>
<td>LO</td>
<td>LLO</td>
</tr>
</tbody>
</table>

- **Target I and V**
- **H2L**
- **Lead optimisation**
- **Toleration tox**
- **GLP tox**

**Industry benchmark milestones**

- NME
- Phase I
- Phase II
- Phase III
- Submission
- Launch

**Graph: Drug Development Progress**

- 10,000 compounds
- 250 compounds
- 1 approved drug
- 5 compounds

**Legend:**

- **Screening**
  - Hazard identification
  - Human DILI prediction

- **Mechanistic studies**
  - Risk assessment/de-risking
    - Translational studies
    - Pathway analysis
    - On/off target toxicity
    - Biomarker identification
    - HA queries

- **Competitive safety**
  - Marketing support
  - Competitive analysis

---

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.

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

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.

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

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 3D cultures for DILI prediction.

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.
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 IC50 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

www.samedanltd.com
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 (e.g., 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 (e.g., 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 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 underlying 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**

1. Alteri E and Guizzaro L, Be open about drug failures to speed up research, Nature 563(7,731): pp317-8, 2018