Human Liver Organoid Systems: Advancing Drug Toxicity Assessment

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Introduction

The development of new therapeutics typically requires 10–15 years and costs an estimated $2–3 billion per approved drug [1]. Despite this substantial investment, approximately 90% of compounds entering Phase I clinical trials ultimately fail to reach the market [2]. Drug-induced liver injury (DILI) remains a leading contributor to both preclinical and clinical drug attrition and is notoriously difficult to predict prior to human exposure [3].

Current *in vitro* hepatotoxicity screening relies largely on immortalized hepatic cell lines and primary human hepatocytes (PHH). However, immortalized models such as HepG2 cells exhibit limited cytochrome P450 (CYP) activity, while PHH rapidly lose metabolic competence in conventional culture systems, restricting the detection of metabolism-dependent toxicity [4,5].

Moreover, many forms of DILI arise only after repeated or chronic drug exposure. Standard assays, which often focus on short-term viability endpoints, primarily capture late-stage cytotoxicity and may fail to identify early mechanistic disturbances.

To create a more physiologically relevant *in vitro* system, we developed a novel differentiation medium that enables adult stem cell-derived liver organoids to mature into hepatocyte-like cells. Additionally, we developed a multiparametric assay capable of detecting early- and late-stage DILI in these hepatocyte-like organoids.

Methods

- Adult human liver organoids were expanded in expansion medium (EM) and differentiated into a hepatocyte-like state using differentiation medium (DM+).
- Hepatocyte-like identity and functionality were validated by RNA-sequencing, immunofluorescence, and cytochrome P450 activity assays.
- A multiparametric live-cell hepatotoxicity assay was developed and applied to matched structurally related toxic and non-toxic compounds associated with clinical DILI.

Generation of hepatocyte-like organoids

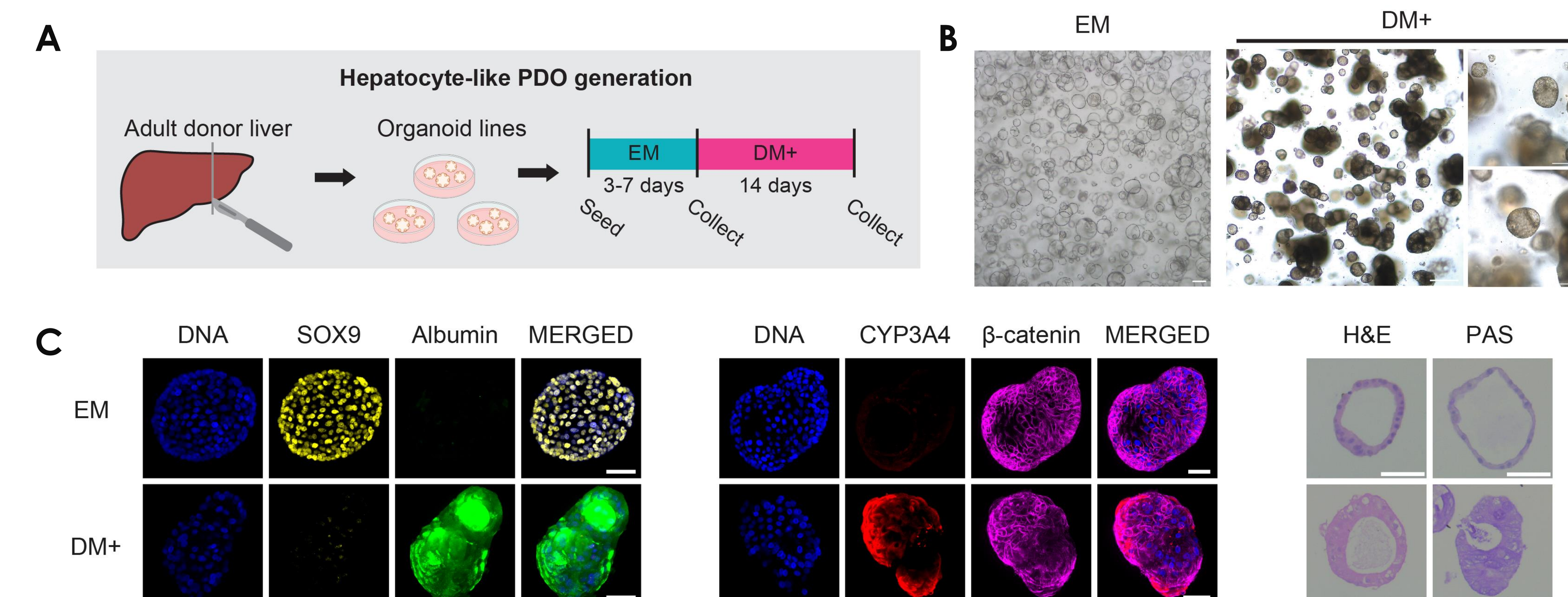


Figure 1A) Schematic overview of organoid generation and directed differentiation toward a hepatocyte-like phenotype. **B)** Differentiation is accompanied by distinct morphological changes, including increased compaction and darkening of the organoids. **C)** Differentiation induces expression of mature hepatocyte markers, including Albumin and CYP3A4, while reducing expression of progenitor and ductal markers such as SOX9. Functional maturation is further supported by increased glycogen storage capacity, demonstrated by positive PAS staining. Scale bar = 50 µm.

Differentiated organoids exhibit hepatocyte-like transcriptional signatures and functional P450 activity

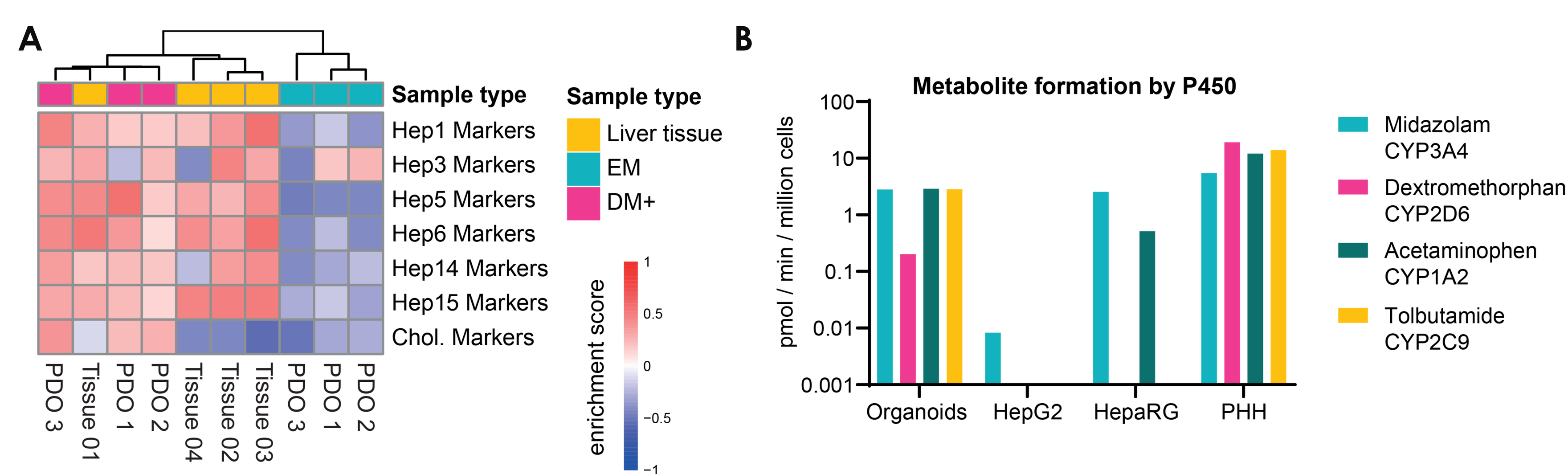
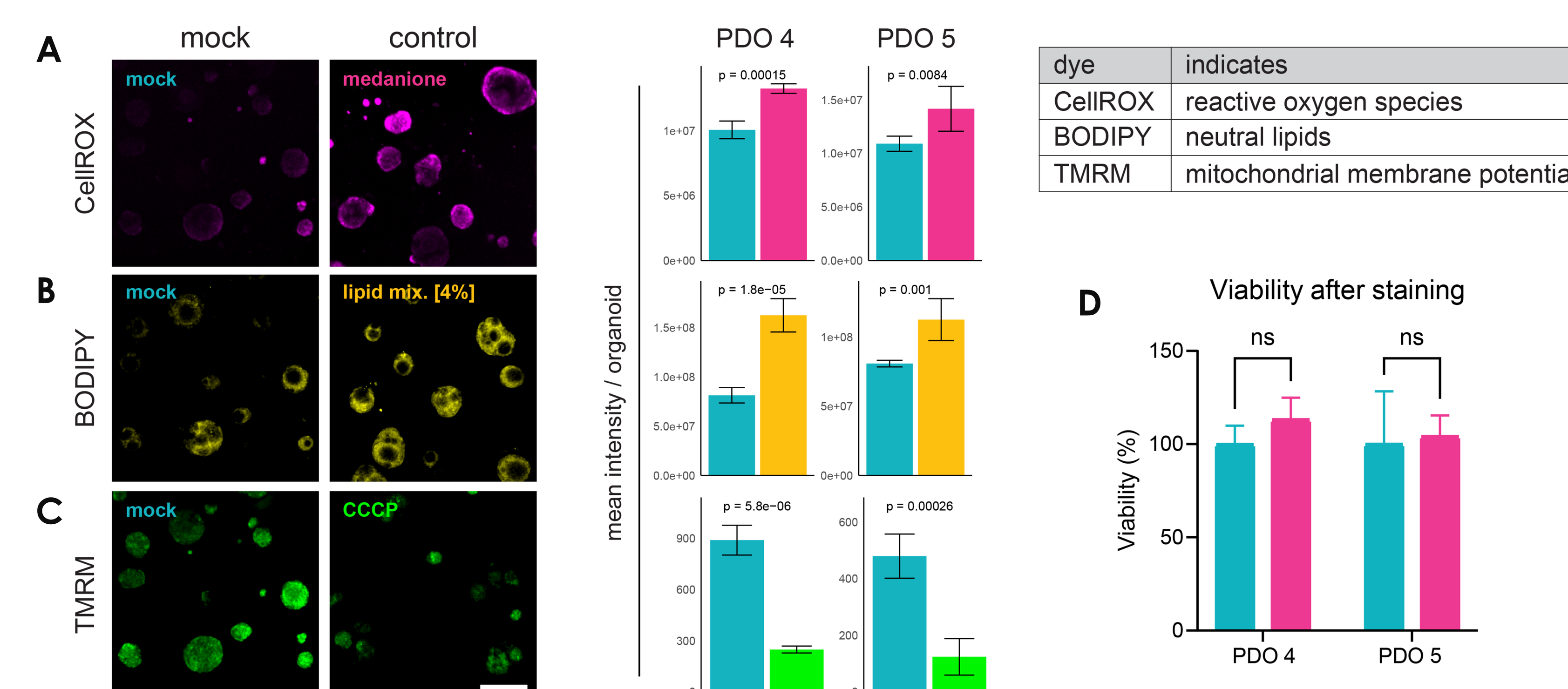


Figure 2A) Gene set variation analysis was performed using hepatocyte and cholangiocyte marker gene sets [6]. RNA-sequencing data from organoids cultured in EM and DM+ were compared with primary liver microarray data [7]. Differentiated organoids demonstrate enrichment of hepatocyte-associated gene signatures. **B)** Cytochrome P450 activity was assessed in differentiated organoids by LC/MS-based metabolite quantification following incubation with a probe drug cocktail for specific P450 isoforms. Enzyme activity was compared to commonly used hepatotoxicity models, demonstrating physiologically relevant metabolic capacity in differentiated organoids.

Development of a multiparametric hepatotoxicity readout



dye	indicates
CellROX	reactive oxygen species
BODIPY	neutral lipids
TMRM	mitochondrial membrane potential

Figure 3) To enable early and sensitive detection of hepatotoxicity, we developed a multiparametric assay combining conventional viability measurements with live-cell staining for early-stage toxicity markers. Functionality of the assay was validated using parameter-specific controls. **A)** Menadione [1mM], a superoxide-generating compound, induces oxidative stress and leads to increased total CellROX signal per organoid. **B)** Incubation with a lipid mixture [4% vol.] induces hepatic lipid accumulation, reflected by increased total BODIPY signal per organoid. **C)** CCCP [1mM], a mitochondrial uncoupler that collapses mitochondrial membrane potential, results in reduction of mean TMRM signal per organoid. Representative images of each staining are shown on the left, scale bar = 100 µm, followed by a quantification of mean signal / organoid in the right. **D)** Exposure to the complete staining protocol (**pink**) does not affect organoid viability compared to unstained controls (**teal**). All plots shows mean of technical replicates +/- SD, significance calculated using 2-way ANOVA.

Organoid-based drug-induced hepatotoxicity assessment

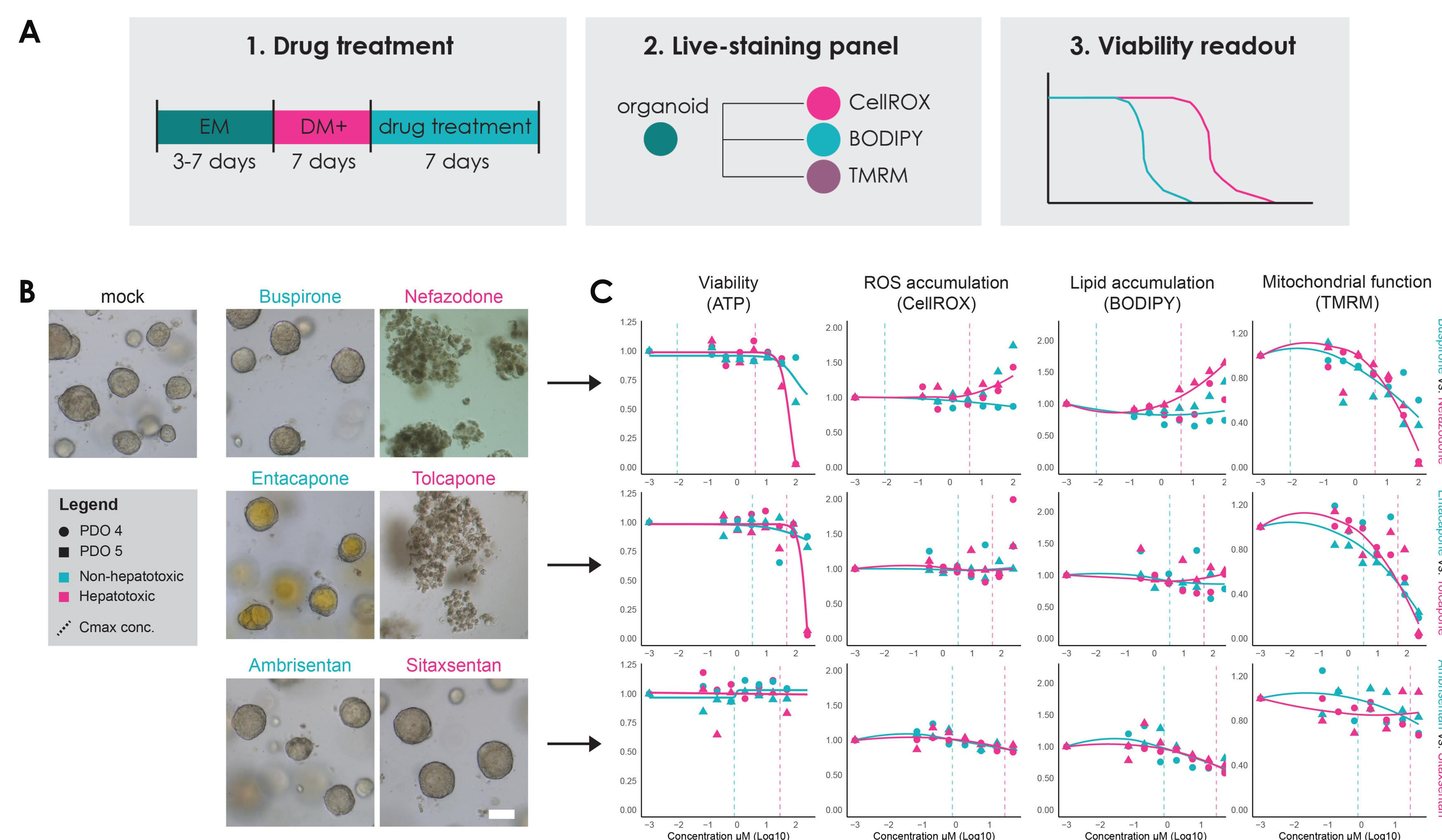


Figure 4A) Schematic overview of multiparametric hepatoxicity readout in context of toxicity screening. **B)** Brightfield images at day 3 show pronounced cell death in organoids treated with hepatotoxic compounds compared to non-hepatotoxic controls. Scale bar = 100 µm. **C)** Dose–response curves for viability, ROS, lipid accumulation, and mitochondrial membrane potential. Data are normalized to vehicle controls and shown as fold change; curves represent the mean across donors. Hepatotoxic (**pink**) and non-hepatotoxic (**teal**) compounds are compared, with physiological C_{max} indicated by dotted lines. Hepatotoxic compounds induce stronger reductions in viability and greater increases in ROS and lipid accumulation. Importantly, loss of mitochondrial membrane potential and increases in ROS and lipids precede viability loss.

Summary

- Hepatocyte-like organoids are a metabolically competent.
- Multiparametric profiling could improve sensitivity over viability-only assays.
- This platform could support improved preclinical safety screening at clinically relevant concentrations.

Conclusion

Here, we establish hepatocyte-like liver organoids and evaluate their predictive capacity using a multiparametric hepatotoxicity readout. For this purpose, a non-toxic, live-cell assay that enables simultaneous measurement of mitochondrial dysfunction, oxidative stress, lipid accumulation, and viability was set up.

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