A Translational 3D Spheroid Assay for Preclinical Assessment of Drug-Induced Liver Injury

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Introduction

Primary human hepatocytes (PHHs) are considered the "gold standard" in vitro tool for investigating liver function. Although hepatocytes are often the *in vivo* targets of DILI, PHHs are not suitable for hepatotoxicity tests under conventional 2D monolayer culture conditions, due to the rapid loss of their hepatic phenotypes, functions, and cell viability.

Recent advances have shown that 3D culture of PHHs as spheroids can significantly prolong the cell viability and extend hepatic function *in vitro*.

In the present study, we validated and characterized a 3D spheroid using PHHs. Furthermore, four-week hepatotoxicity assay of 110 testing compounds was carried out with 3D PHH spheroids. It is indicated that the 3D liver spheroid is a useful tool for long-term DILI prediction.

Methods

Cell Seeding and spheroid formation

- Human primary hepatocytes were seeded at a cell density of 4000 cells/well in ultra-low attachment plate.
- The cells were aggregated into a spheroid manually one day after cell seeding. The cells were then incubated for 7 days in 37°C, 5% CO_2 for the spheroid formation. The medium was changed twice per week.

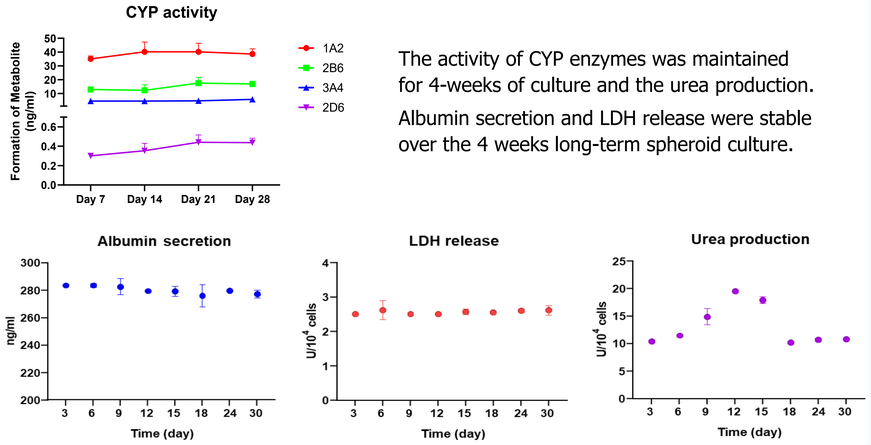
Functional characterization

- The spheroids were incubated for 28 days. The activity of CYP enzymes was detected by adding their substrates for 30 min and monitor the formation of metabolites by LC-MS/MS. LDH release, urea production and albumin secretion was detected using the relative assay kits.
- BSEP function: benzbromarone was added into the spheroid for 2 days and the fluorescence of cholyl-L-lysyl-fluorescein (CLF) was determined using high-content based instrument.
- P-gp function: The spheroids were labelled with calcein-AM in the presence and absence of PSC833 for 30 min and the calcein was analyzed by high-content based system.

Chronic Drug-Induced Liver Injury

- The spheroids were treated with 110 clinical drugs with known clinical DILI categories at 7 non-zero concentrations for 28 days. On day 7, 14, 21 and 28, the cell viability was determined.
- The spheroids were treated with chlorpromazine or buspirone for 14 days. The spheroids were labelled with biomarkers for GSH, DNA damage and mitochondrion function.
- The margin of safe (MOS) was calculated as the ratio of IC_{50} and Cmax.

1



The activity of CYP enzymes was maintained for 4-weeks of culture and the urea production.

Albumin secretion and LDH release were stable over the 4 weeks long-term spheroid culture.

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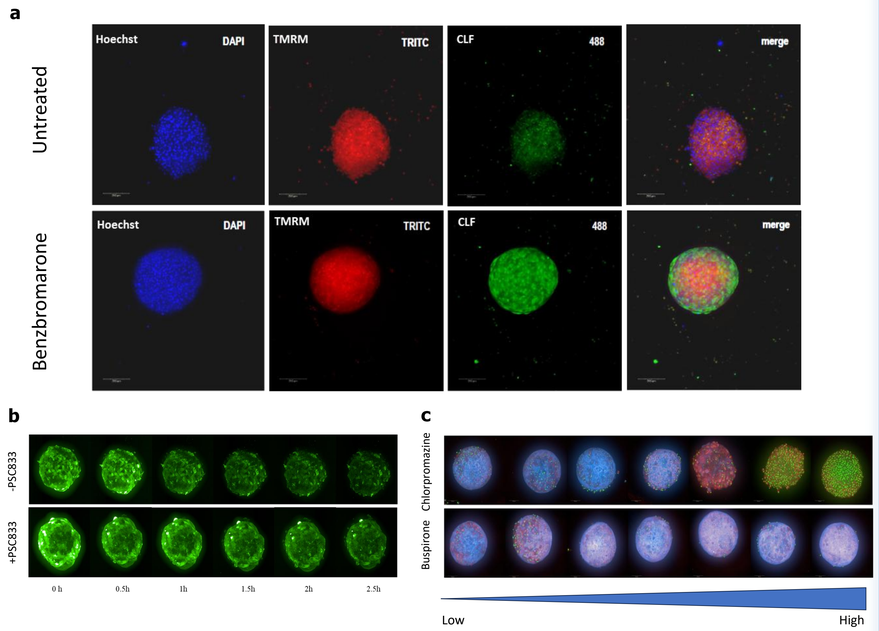
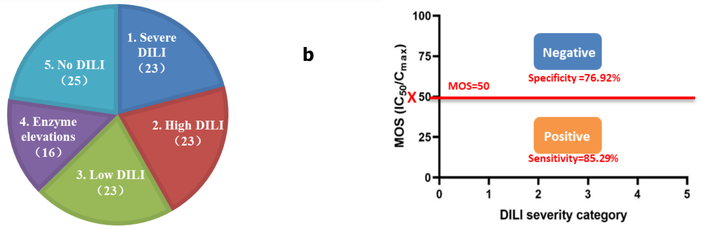


By using Operetta® CLS™ high-content analysis system, benzbromarone as a BSEP inhibitor was seen to increase the accumulation of the bile acid, and no mitochondrial toxicity was observed (a). The intracellular fluorescence of Calcein was accumulated much more amount in the presence of P-gp inhibitor PSC833 (b). It is shown that the BSEP and P-gp transporter function worked well in 3D spheroid.

After treatment with drugs, the spheroids were stained with mBCL (GSH content), Sytox Green (DNA damage) and Mitotracker Deep Red (mitochondrial) (c). The GSH content decreased, and the DNA damage increased after treated with Chlorpromazine, which is a well-known hepatotoxicity reference.

3



MOS at 50 was used as a cut off for DILI prediction with 76.92% accuracy for negative and 85.29% accuracy for positive using reference compounds (a and b respectively).

4

Hepatotoxicity test with 110 clinical drugs with different hepatotoxicity categories:
Using 3D spheroids enabled chronic exposure studies and facilitated screening for chronic toxicity drugs.

Compound	IC_{50} (μM)				C_{max}	DILI severity category
	7-day	14-day	21-day	28-day		
Asprin	> 200	> 200	> 200	> 200	133.00	No DILI
Alendronate	> 200	> 200	> 200	> 200	0.02	No DILI
Benserazide	> 200	> 200	> 200	> 200	3.89	No DILI
Flumazenil	> 200	> 200	> 200	83.59	1.12	No DILI
Flavoxate	132.00	106.00	71.81	35.22	1.79	No DILI
Cycloserine	> 200	> 200	> 200	> 200	333.00	Enzyme Elevations
Dexamethasone	> 200	> 200	> 200	> 200	0.22	Enzyme Elevations
Fludarabine	46.22	37.71	9.87	3.36	0.17	Enzyme Elevations
Felodipine	42.95	13.27	8.74	3.91	0.03	Enzyme Elevations
Nicardipine	35.24	10.17	5.20	3.81	0.08	Enzyme Elevations
Penicillin V	96.71	87.37	49.64	51.32	14.27	Low
Amitriptyline	54.68	22.84	17.12	16.13	0.72	Low
Desipramine	45.68	11.68	7.80	6.29	1.88	Low
Phenformin	44.79	19.88	11.33	6.58	0.63	Low
Tretinoin	25.72	14.54	4.51	2.16	1.33	Low
Naproxen	195.20	114.90	91.76	30.52	325.70	High
Nitrofurantoin	72.67	39.26	18.81	11.68	21.00	High
Ticlopidine	44.85	28.96	17.30	16.58	8.10	High
Rosiglitazone	43.78	30.03	21.86	14.90	1.00	High
Itraconazole	39.65	29.86	19.20	11.53	2.80	High
Bosentan	54.00	23.45	11.28	1.86	7.43	Severe
Flutamide	48.66	27.08	12.24	8.07	5.40	Severe
Amiodarone	32.71	26.03	18.46	12.86	5.30	Severe
Tolcapone*	32.55	11.86	7.41	1.75	47.60	Severe
Troglitazone*	19.36	13.44	0.66	0.65	6.40	Severe

*Data marked with an asterisk indicates drugs that have been withdrawn from the market.

Conclusion

3D PHH spheroids provide a stable and functionally relevant *in vitro* liver model that supports long-term culture, preserves metabolic activity, and maintains transporter function. Their ability to withstand four weeks of chronic drug exposure while revealing mechanistic toxicity signals demonstrates strong utility for DILI risk assessment. With high prediction accuracy across 110 clinically annotated drugs, this 3D platform offers a valuable improvement over traditional 2D hepatocyte models and enables more reliable evaluation of chronic hepatotoxicity in drug development.

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