3D cell- culture system for DILI with fresh human hepatocytes

Tomoko Jomura[1)] , Yuji Ishida[2)]

1) Toyo Gosei Co., Ltd., 2) PhoenixBio Co., Ltd.

Introduction

Cell-able® is a next generation of 3D cell-culture system based on *in-vitro* assay. In this study, we have developed a method for prediction of DILI with 3D co-cultured human hepatocytes by using a novel micro-patterned 384well culture plate and high-content screening (HCS) system. Cell-able® enable to keep long term hepatocyte culture more than 2 months. 3D spheroid can maintain hepatocyte specific functions and enable us to make long term consecutive drug exposure. We performed this study with fresh-human hepatocytes from chimeric mice with humanized liver. We have predicted hepatotoxicity by measuring 5 bio-markers such as nuclei, intracellular glutathione (GSH), mitochondria membrane potential (MMP), reactive oxidative stress (ROS) and oil droplet after 2weeks consecutive drug exposures using with HCS system. We have selected 32 drugs by referring previous studies which had uncertain possibility of hepato-toxicity. Moreover, ABC transporters have been studied such as multidrug resistant protein2 (MRP2) and Bile salt efflux pump (BSEP).

Methods

Cell culture and drug exposures

Feeder cells were plated on Cell-able® plate one day before hepatocytes plating. PXB-cells™ were plated on Cell-able® plate (8,000 cells/well, 384well plate) in culture medium. On the third day, the cells were treated with medium containing a drug of interest or vehicle (0.1% dimethyl sulfoxide [DMSO]). Culture medium was replaced to fresh one containing compound or vehicle every 2 to 3 days. Hepatocytes were treated with drugs or DMSO containing medium for 14days.

Imaging assay with multiple fluorescent probes for DILI prediction

On the 14th day of drug exposure, the medium was removed and the cells were stained by fluorescent probes in Williams' medium E. Each fluorescent signals were analyzed with HCS system using 10x objectives, Z-stack acquisition.

Efflux transporter inhibition assay by imaging with CDFDA

On the 7th day of drug exposure, the medium was removed and the cells were exposed with CDFDA in HEPES buffer to analyze efflux transporter ABCC2 (MRP2) activity. CDF accumulation in canalicular space was monitored by time-lapse imaging with HCS system. Drug induced inhibition effect of CDF accumulation was analyzed at time point 10 minute as shown in lower images.

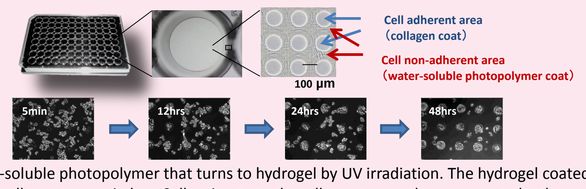
Expression of BSEP and MRP2 mRNA

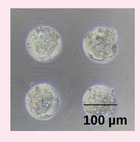
Total RNA was extracted from 2D and 3D cultured cells on day3 and day7. Complementary DNA was synthesized from total RNA. Relative mRNA expression of BSEP and MRP2 to beta-actin was analyzed by RT-PCR.

Materials and Equipment

The cells we used were fresh human hepatocytes from chimeric mice (PXB-cells™*, PheonixBio Co., Ltd.) and 3T3 swiss albino. Cells were cultured on Cell-able®* 384 plate (Toyo Gosei Co., Ltd.) with RM101 hepatocyte culture medium (Toyo Gosei Co., Ltd.). The fluorescent probes and filters used were monochlorobimane (mBCl) and DAPI filter for Glutathion (GSH), CellROX® and TRITC filter for reactive oxygen species (ROS), Tetramethyl rhodamine methyl ester (TMRM) and Texas Red filter for mitochondrial membrane potential (MMP), HCS Nuclear Mask™ Deep Red and Cy5 filter for nuclei , Bodipy® 493/503 and FITC filter for oil droplet , 5-(and-6)-carboxy-2',7'-dichloro-fluorescein diacetate (CDFDA) and FITC filter for MRP2 transporter activity. All HCS assays and analysis were performed with ImageXpress® Micro XL System and MetaXpress® Software (Molecular Devices, Inc.). Spheroids were detected using Multi Wavelength Cell Scoring Module and measured total Intensity or area. Real time PCR was performed with StepOnePlus™ (Thermo Fisher Scientific Inc.).

*Cell-able® plate

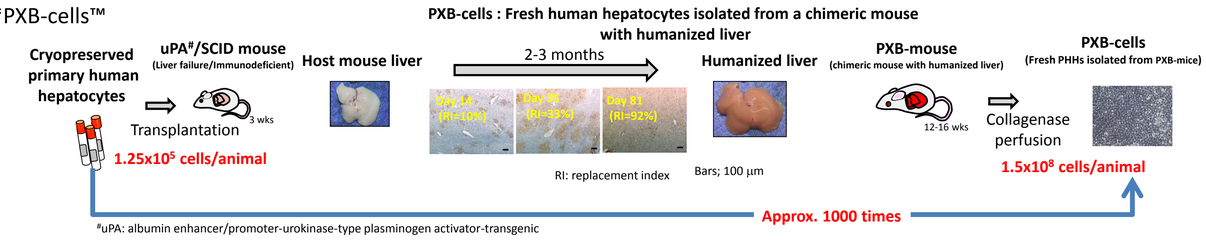




A microgram of day 5 after plating cryo-human hepatocytes on Cell-albe®plate.

Cell-able® plate is utilizing water-soluble photopolymer that turns to hydrogel by UV irradiation. The hydrogel coated area is not to allow cells to adhere. Through the use of this polymer, Cell-albe® plate has cell adherent area circles. Cells migrate to the adherent area, then aggregate by themselves.

*PXB-cells™



#uPA: albumin enhancer/promoter-urokinase-type plasminogen activator-transgenic

Results

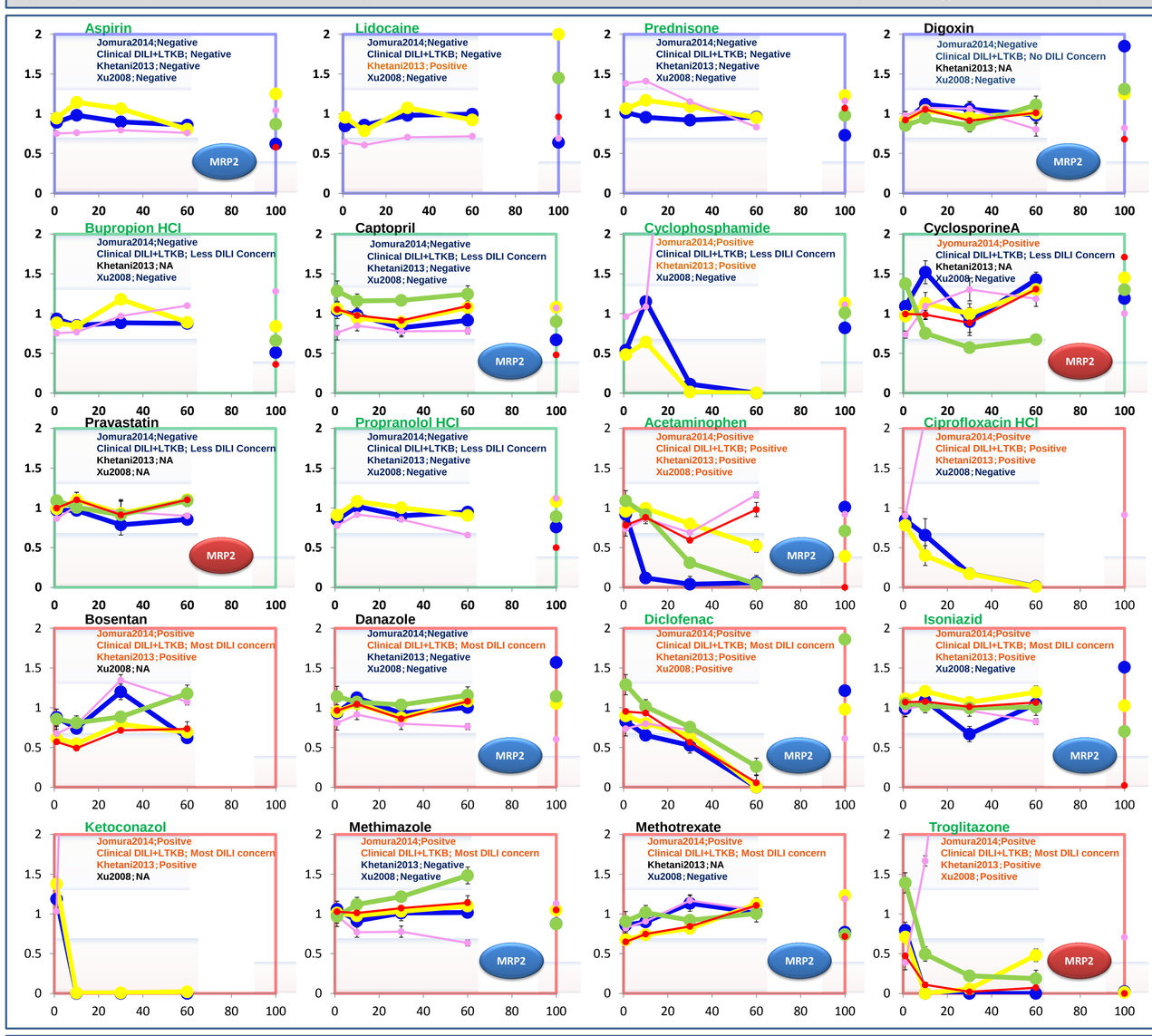
Imaging assay with multiple fluorescent probes for DILI prediction

HCS Imaging assay for prediction of DILI with PXB-cells™ 3D cultured on Cell-able® indicates good performance.

32drugs were studied through the method of Imaging assay with multiple fluorescent probes to predict DILI with PXB-cells™. 18drugs out of the 32drugs were also studied with cryo-human hepatocytes cultured on Cell-able®. The concordance rate of these two models was very high level (94%, data not shown). GSH, MMP and oil droplet signals showed their behavior depended on drug concentration, whereas neither nuclei nor ROS. Thus we selected GSH, MMP and oil droplet as bio markers to predict DILI. These data were compared with the result of matrigel sandwich culture model (Xu, 2008)[1)] and 2D co-culture model (Khetani, 2013)[2)] as shown in graphs in Fig. 1 and Table 1. Dobutamin-HCl, Paromomycin sulface, Cyclophosphamide, CycrospolineA, Piroxicam have been predicted as Positive concern. GSH has shown clearly down against Paromomycin sulface, Cyclophosphamide.

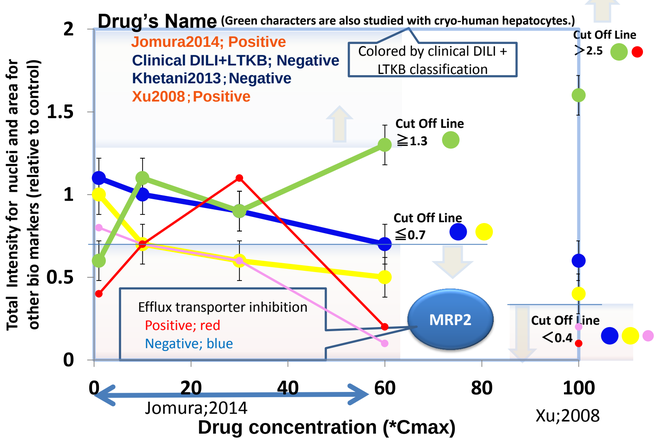
Table 1. Specificity and Sensitivity of each models

	Cell-able®		Xu, 2008	Khetani, 2013
	PXB-cells™	Cryo-Human hepatocytes	Cryo-Human hepatocytes	Cryo-Human hepatocytes
Specificity	75% (6/8)	100% (3/3)	100% (8/8)	67% (2/3)
Sensitivity	79% (11/14)	90% (9/10)	42% (5/12)	77% (10/13)



Note

- GSH
- MMP
- Oil droplet
- Nuclei
- ROS



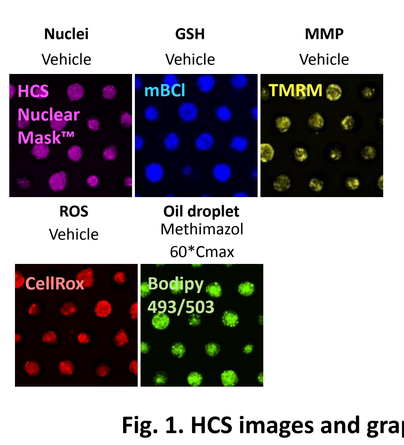


Fig. 1. HCS images and graphs

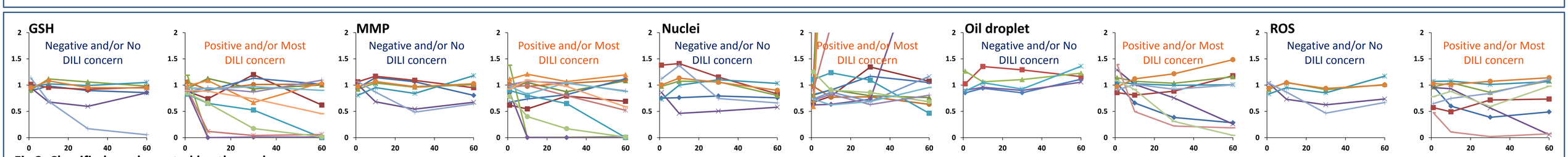


Fig 2. Classified graphs sorted by the probes

Efflux transporter inhibition assay by imaging with CDFDA

Inhibition of MRP2 activity by drug was analyzed with CDFDA as shown in Fig. 3. 17 drugs were tested and inhibition of CDF accumulation in bile canaliculi was observed in benzbromarone, pravastati, troglitazone and cyclosporinA. Aspirin images are shown as a negative example. Cyclosporin A images are shown as a positive example.

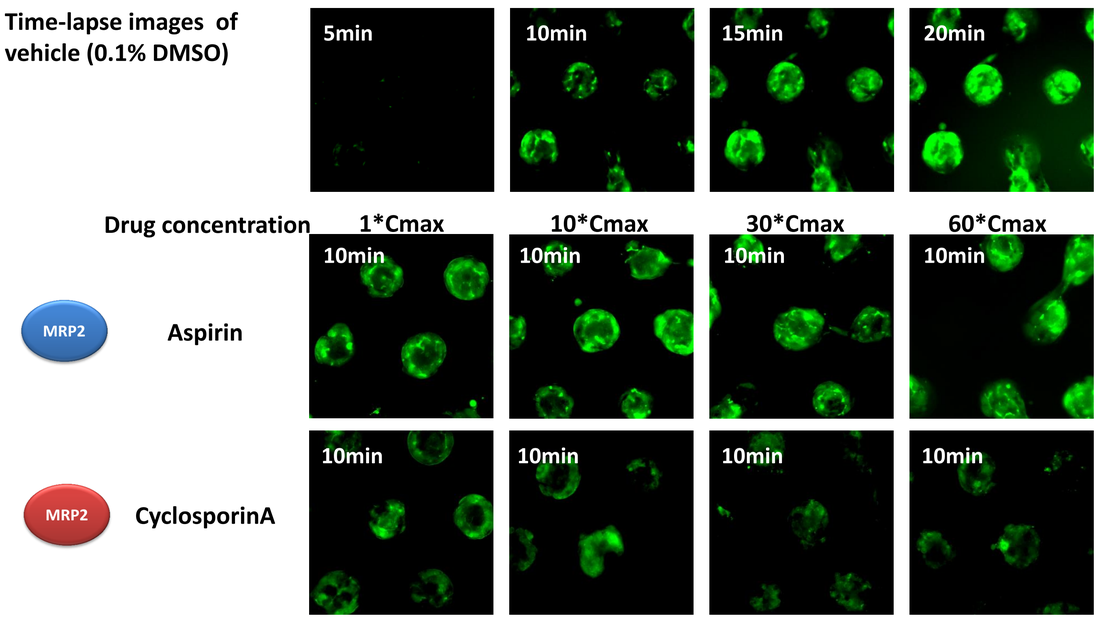


Fig. 3. Inhibition of CDF accumulation effect of drugs

Expression of BSEP and MRP2 mRNA

mRNA expression of BSEP and MRP2 were well maintained in Cell-able® 3D culture for 7days compare with 2D culture as shown in Fig. 4. Further improvement of BSEP mRNA expression was observed by matrigel over coat on culture cells.

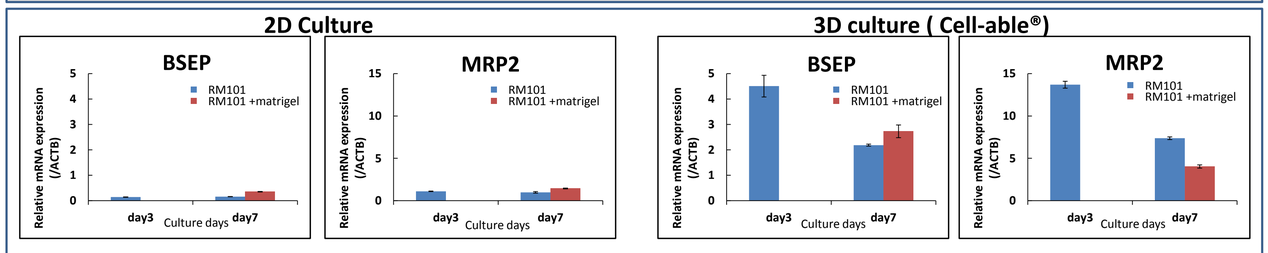


Fig. 4. BSEP and MRP2 (Comparison of mRNA expression between2D and 3D culture cells)

Initial relative mRNA expression levels of BSEP and MRP2 in PXB-cells on day 0 were 22 and 7.9 respectively.

Conclusions

Cell-able® 3D culture system shows good performance for DILI prediction.

- ✓ **It shows high sensitivity and specificity for long term drug exposures.**
- ✓ **384 well plate enables high throughput -imaging assay (5 probes x 384 wells /hr, 8,000cells/well, statistic consistent spheroids).**
- ✓ **It shows possibility of transporter inhibition assay by HCS.**
- ✓ **It maintains good expression of BSEP and MRP2 mRNA for a week.**

Acknowledgment

High content screening assay was performed by Ms. Mahomi Suzuki of Molecular Devices Japan. We would like to express our gratitude to her enormous contributions.

References

1) Xu et al., *Toxicological Sciences* 105, 97-105 (2008)
2) Khetani et al., *Toxicological Sciences* 132, 107-17 (2013)
3) Chen et al., *Drug Discovery Today* 16, 697-703 (2011)