Validation of a Novel 3D Cell Culture System to Perform in vitro Cytotoxicity Analyses using Human Primary Hepatocytes

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Introduction

Hepatocytes are the primary cell type of the liver providing the majority of the detoxification which may increase the potential for cell dysfunction and death. Though the source of the insult may be caused by several factors, exposure to drugs represents a significant concern warranting FDA guidance on drug-induced liver injury (DILI). In vivo studies are still the gold standard, however, in vitro screening has gained importance for reducing animal exposures, being invaluable to high-throughput platforms and better equipped to study cellular mechanisms of action.

Typically, in vitro screening has incorporated primary hepatocytes cultured in a two dimensional (2D) format where the cells form a monolayer across the bottom of a microplate well. However, when cultured and applied in the 3D format, there is an increase in cell-cell and cell-extracellular matrix interactions, which better mimics the in vivo environment. The 3D structure of the liver is critical for its function and for the study of hepatocyte biology. As such, high-quality hepatocyte lines have been developed, characterized, and adapted to 3D cultures with characteristics and functionality more similar to those of primary liver cells.

RAFT™ 3D Cell Culture System

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RAFT™ is a customizable 3D cell culture system that represents a significant step forward in the area of cell culture technology. The system provides the versatility to perform successively more complex studies in a variety of applications. RAFT™ technology allows for the isolation, expansion, and characterization of primary hepatocytes cultured in 3D. The RAFT™ system is a modular system that can be configured to meet the specific needs of the user. The RAFT™ system includes the following components:

- RAFT™ 3D Cell Culture System
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Materials and Methods

Cell Viability and Activity Assays: CellTiter-Glo® (Catalog No. G7371) from Promega Corporation (Madison, WI) was used to assess levels of cellular ATP. ATP activity was assessed using CellTiter-Glo® assay with succinate (Catalog No. G9092), also from Promega Corporation, was used to measure cellular viability via cytochrome P450 (CYP450) enzyme activity analysis.

Cytotoxicity Assays: Cytotoxicity was determined with the CellTiter™ Green Cytotoxicity Assay (Catalog No. G8731) from Promega Corporation. Kinetic measurements of reactive oxygen species (ROS) activity and external lipid droplets were measured using CellROX® Deep Red Oxidative Stress Reagent and CellEvent™ Caspase-3/7 Green Detection Reagent, respectively. The results were analyzed using the Gen5 data analysis software.

Cell Health Assessment: Medium was removed from cells and replaced with 50 µL of pre-warmed medium containing 20 µM Camptothecin and 20 µM Camptothecin (Catalog No. C10422) and CellEvent™ Caspase-3/7 Green Detection Reagent (Catalog No. C10422). Assays were performed within 1 hour of addition to each compound.

Kinetic Oxidative Stress and Apoptotic Activity Monitoring

Cytotoxicity Activity: Cytotoxicity was determined with the CellTiter™ Green Cytotoxicity Assay (Catalog No. G8731) from Promega Corporation. Kinetic measurements of reactive oxygen species (ROS) activity and external lipid droplets were measured using CellROX® Deep Red Oxidative Stress Reagent and CellEvent™ Caspase-3/7 Green Detection Reagent, respectively. The results were analyzed using the Gen5 data analysis software.

Cytostatic Activity: Cytostatic activity was determined with the CellTiter™ Green Cytotoxicity Assay (Catalog No. G8731) from Promega Corporation. Kinetic measurements of reactive oxygen species (ROS) activity and external lipid droplets were measured using CellROX® Deep Red Oxidative Stress Reagent and CellEvent™ Caspase-3/7 Green Detection Reagent, respectively. The results were analyzed using the Gen5 data analysis software.

Cell Health Assessment: Medium was removed from cells and replaced with 50 µL of pre-warmed medium containing 20 µM Camptothecin and 20 µM Camptothecin (Catalog No. C10422) and CellEvent™ Caspase-3/7 Green Detection Reagent (Catalog No. C10422). Assays were performed within 1 hour of addition to each compound.

Conclusion

A seven day dosing experiment was then performed to ascertain whether the improvement in cell health observed from the results of the viability and CYP3A4 analysis study caused hepatocytes cultured in 3D to be impervious to the effects of camptothecin and pyocyanin, or delayed the ROS induction and apoptotic activity seen previously with 2D cultured hepatocytes. Cells were re-seeded with a range of compound concentrations on a daily basis. BioTek® Systems’ RAFT™ 3D Cell Culture System and CellTiter-Glo® CYP3A4 assay were then performed 1, 3, and 7 days after initiation of treatment.

Figure 1 – Creation of 3-Dimensional Hepatocyte/Collagen Hydrogel using RAFT System. (A) Cryopreserved hepatocytes were thawed and added to the prepared collagen solution. The mixture was then dispensed to the CellROX Deep Red Oxidative Stress Reagent (Catalog No. C10422) and CellEvent™ Caspase-3/7 Green Detection Reagent (Catalog No. C10422). Assays were performed within 1 hour of addition to each compound.

Figure 2 – Kinetic ROS Activation Analysis. (A-D) Total cell percentage exhibiting oxidative stress per captured 4x (2D) or 10x (3D) images. (E-H) 20x images of 2D cultured hepatocytes, and (I-L) 10x images of 3D cultured hepatocytes captured after 0, 6, and 16 hour treatments with 200 µM pyocyanin. Blue: Hoechst 33342; Green: DCFDA reagent; Red: MitoTracker Red Mitochondrial probe.

Figure 3 – Kinetic Apoptotic Activity Analysis. (A-D) Total cell percentage exhibiting apoptotic activity per captured 4x (2D) or 10x (3D) images. (E-H) 20x images of 2D cultured hepatocytes, and (I-L) 10x images of 3D cultured hepatocytes captured after 0, 6, and 16 hour treatments with 200 µM camptothecin. Blue: Hoechst 33342; Green: pSIVA reagent.

Figure 4 – Cell Viability and CYP3A4 Activity Estimation for Extended Culturing of Hepatocytes in 2D and 3D. (A) CYP3A4, (B) Cell viability findings for hepatocytes cultured in 3D or in 2D using the RAFT system. Left side graphs display raw luminescence values for each cell culture method. Right side graphs represent normalized percentages compared to the first day of analysis. % Viability and CYP Activity calculated by the following formula: R [%] = R/RO%.

Figure 5 – Long-term Oxidative Stress and Apoptotic Activity. Results of multi-day camptothecin and pyocyanin incubations on both ROS induction and apoptosis for cells cultured in 2D and 3D. % Unstained Wells calculated using the following formula: (A) pyocyanin incubations also show decreased 2D cell viability after 24 hour treatment with 200 µM pyocyanin. Blue: Hoechst 33342; Green: CellEvent™ Caspase-3/7 Green Detection Reagent. Red: Mitotracker Deep Red Oxidative Stress Reagent.

The results from the graphs in Figure 5A and B demonstrate that camptothecin and pyocyanin cause oxidative stress and apoptosis in 3D cultured hepatocytes. The incubation time eliciting the greatest response was found to be after a 3-day dosing, which can be seen by the increase in signal from the CellEvent™ Caspase fluororescent ROS, and caspase 3/7 probe, respectively (Figure 5C-D). These results contrast with the previous findings, as well as the 2D culture results seen in Figure 5E, indicating that induction of ROS and apoptotic activity occurs in less than 24-hours.

A long-term incubation experiment was then performed using a similar process, to determine whether the variations observed in early cytotoxicity marker induction also led to differences in final cytotoxic effects from the compounds. The number of total and dead cells was calculated following 1, 3, and 7 days dosing with equivalent compound concentrations.

Conclusions

1. Hepatocytes cultured in 3D using the RAFT system demonstrated improved cell health over extended culturing periods, when compared to cell cultured in 2D, allowing for use in long-term cytotoxicity analyses with reduced hepatocyte numbers.

2. The incorporation of cell health and cytotoxicity assays are capable of being used with cells cultured in 3D using the RAFT system, and yield accurate, repeatable results when detected and imaged using the Cytation 3 and with the Gen5 data analysis software.

3. Hepatocellular damage from camptothecin, as observed in human, was also detected in 3D cultured hepatocytes.

4. A variation in degrees and times of cytotoxic effects from 2D and 3D hepatocytes were seen from 2D cultured hepatocytes highlighting the necessity to incorporate relevant 3D cell models when performing experiments to determine potential hepatotoxic effects from repeated dosing of lead compounds.