Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drug commonly used as analgesics and antipyretics, as well as for management of rheumatologic disorders. They are one of the most highly prescribed classes of drugs and are associated with adverse effects in a subset of patients. NSAIDs are known to induce the most frequent cause of drug-related liver injury (DILI) (Bosman et al., 2010). Diclofenac, in particular, frequently produces dose-dependent liver toxicity and is associated with dose-related increases in serum aminotransferase levels, leading to serious DILI. NSAIDs, such as diclofenac, may affect hepatic ATP content (Maudsley et al., 2001), and mitochondrial permeability transition (MPT) has also been shown to be important in DILI, liver injury, and the role that oxidative stress plays in MPT induction (Gómez-Lechón et al., 2003). Finally, according to work done by Schmitt et al., 1992, cytochrome P450 (CYP) related metabolic activation of the drug, and the formation of reactive metabolites is also related to diclofenac hepatotoxicity.

The combined hepatotoxic effects of these mechanisms usually occur within weeks of therapy commencement. This emphasizes the fact that in vitro safety testing should incorporate different utilization models to ensure clinical relevance. However, primary hepatocytes cultured in a two-dimensional (2D) manner on the bottom of a microtissue plate, following transfer of the P450-Glo and CellTiter-Glo 3D volumes, the wells containing the liver microtissues, in addition to primary hepatocytes cultured in 2D. Quantification of the various fluorescent and luminescent emissions via microplate reading and imaging was carried out by a novel cell imaging multi-mode reader.

Here we evaluate the suitability of 3D human liver microtissues for use in long-term toxicity studies. Primary human hepatocytes are re-aggregated into functional microtissues by hanging drop technique. The microtissues demonstrate in vivo like cell-cell and cell-matrix interactions and retained viability over weeks. A panel of assays was run to assess cell health and viability of different MOA evaluated by diclofenac using the 3D liver microtissues, in addition to primary hepatocytes cultured in 2D. Quantification of the various fluorescent and luminescent emissions via microplate reading and imaging was carried out by a novel cell imaging multi-mode reader.

2D/3D Long-Term Cell Health Analysis

A two week examination of hepatocytes cultured in 2D monolayers or 3D microtissues was conducted to assess whether cell health was maintained over the entire incubation period. This initial test was essential to validate whether cells cultured in 3D may serve as a reliable model for long-term cytotoxicity studies.

2D Assay Procedures

Cell Viability and Activity Assays CellTiter-Glo assay (Catalog No. G7571) and CellTiter-Glo 3D from Promega Corporation (Madison, WI) were used for cell viability measurement by means of cytotoxicity and metabolic activity assay analysis.

Material and Methods

Liber microtissues were obtained from InSphero, Inc. (Cambridge, MA). The microtissues were created using proprietary hanging drop technique with human hepatocytes from BioReclamationI VT (Baltimore, MD) using lot 10. Cryopreserved human hepatocytes were also provided to BioTek directly from BioReclamationI VT and from the CellTiter-Glo assay kit. The cytotoxic effect on hepatocytes in the liver microtissue model was then assessed using compound concentrations spanning 3 logs. Cell culture conditions and concentrations were the same as previously mentioned.

Figure 1 – Cellular health findings for long-term 2D/3D hepatocyte culture. (A) CYP3A4 enzyme activity, and (B) cell viability results for liver microtissues and 2D hepatocyte cell culture using three separate cell concentrations. Left side graphs display near luminescence values for each cell culture and concentration tested, while right side graphs exhibit normalized comparisons to Day 1 values. % CYP Activity and Viability calculated by the following formula: % CYP Activity = [LUC (Abs) / LUC (Abs) Day 1] × 100

Diclofenac Cytotoxicity Study – 2D Hepatocyte Model

Diclofenac Cytotoxicity Study – 2D Hepatocyte Model

Cytotoxicity assays were also performed using the CellTiter Green and Hoechst 33342 assays with hepatocytes cultured in 2D. This was done to further assess whether different induced toxicity levels could be seen between the two hepatocyte models. A single dosing of 10 µM diclofenac was used due to the fact that in vivo MPT levels remained the most constant over the two week dosing period, compared to lower cell concentrations.

Diclofenac Cytotoxicity Mechanism of Action Confirmation

Confirmation studies were then conducted to determine whether the different mechanisms of action (MOA) purported to be involved in diclofenac cytotoxicity, specifically effects on the mitochondria, resulted in hepatocytes cultured as 3D microtissues. Overall, the mitochondrial level and MPT pore opening were monitored on the same days in which cytotoxicity was assessed.

Figure 4 – Mitochondrial Oxidative Stress and MPT Induction Assessment. Mean pixel RFU values from the field of view (left y-axis) measured using MitosGX assay following a three day-dosing with diclofenac (A) or from image i-t scan signal following a seven day-dosing with diclofenac (B). % signal for diclofenac treated wells (right y-axis) calculated by comparing to untreated well signal using the following formula (Diclofenac Treated Well Signal/Untreated Well Signal)*100. Representative overlay images shown of liver microtissues after (A) 3 days dosing with 300 µM diclofenac and (B) 7 days dosing with 500 µM diclofenac using the DAPI or GFP (Cytochrome C imaging channel, respectively), and (C) 7 days after 7 day diclofenac treatment, stained with Image-iT Green (A) or Image-iT Blue (B) fixative (D). A. B. C. D.

Conclusions

1. 3D Human Liver microtissues retain viability and normal function over extended culturing periods, and provide a valid option for performing long-term dosing studies.
2. The CellTiter-Glo 3D and Pico-Glo luminescent assays, in addition to the CellTiter Green, MitosGX, and Image-iT imaging assays deliver accurate, reliable results when used with the microtissue 2D cell model.
3. The dedicated luminescence detection system on the CytoScan 3 is able to easily quantify the signal from the CellTiter-Glo, CellTiter-Blue, and CellTiter-Green.
4. Fluorescence microscopy of the liver microtissues, and cellular analysis, is also accomplished using the imaging module and Gen5 data analysis software.
5. Diclofenac mitochondrial injury and eventual cytotoxicity seen previously with other hepatocyte models are also seen in 2D liver microtissues having human hepatocytes cultured in 2D.
6. The differences seen in the induction of diclofenac-linked cellular events using liver microtissues, when compared to published results using other hepatocyte models, underscores the need for incorporation of the most relevant cell model when performing long-term cytotoxicity studies.