Three Dimensional Hepatotoxicity Screening Using Corning® HepatoCells, Spheroid Microplates, and the SCREEN-WELL® Hepatotoxicity Library

Application Note

CORNING

Hilary Sherman, Hannah J. Gitschier, and David H. Randle, Ph.D.
Corning Incorporated, Life Sciences
Kennebunk, Maine

Introduction

Having the right model for drug screening is essential for predicting compounds that may cause drug-induced liver injury. Three dimensional (3D) hepatocellular models offer significant improvements over traditional two dimensional (2D) monolayer cell culture in terms of maintaining morphological and functional characteristics of tissue, and they may provide a better representation of *in vitro* drug toxicity[1]. Here we demonstrate how Corning HepatoCells, an immortalized alternative to primary human hepatocytes can be utilized for a 3D drug screen to discover potential hepatotoxins in conjunction with Corning Spheroid Microplates. Hepatospheres formed using Corning HepatoCells were compared to spheroids formed using alternative hepatocyte-like models. Cell viability was measured to assess hepatotoxicity after exposure to the SCREEN-WELL Hepatotoxicity Library from Enzo Life Sciences, a library consisting of 238 compounds with a variety of structurally and mechanistically different compound classes, as well as nontoxic controls. Selected hits identified in the 3D screen, which significantly reduced cell viability of the hepatospheres, were then assessed in a dose-dependent manner for potency analysis. These results demonstrate that Corning HepatoCells, together with Corning spheroid microplates, are powerful tools that can be used for reliable and reproducible 3D hepatotoxicity screening.

Materials and Methods

Corning HepatoCells (Corning Cat. No. 354881), HepG2 cells (ATCC® Cat. No. HB-8065), and HepaRG™ cells (Life Technologies Cat. No. HPRGC10) were seeded at various concentrations into 384-well spheroid microplates (Corning Cat. No. 3830) to optimize for hepatosphere size. HepatoCells and HepG2 cells were seeded using 50 µL per well of Corning Culture Medium for HepatoCells (Corning Cat. No. 354882) containing 10% fetal bovine serum (Corning Cat. No. 35-010-CV). HepaRG cells were seeded using William's E Medium (Life Technologies Cat. No. 12551-032) supplemented to 1x with HepaRG Thaw, Plate & General Purpose Medium Supplement (Life Technologies Cat. No. HPRG670) and 1x GlutaMAX™ Supplement (Life Technologies Cat. No. 35050-061). Medium was changed every other day, after the first 48 hours, using Corning Culture Medium for HepatoCells without serum for HepatoCells and HepG2, or William's E Medium supplemented to 1x with HepaRG Tox Medium Supplement (Life Technologies Cat. No. HPRG630), and 1x GlutaMAX for HepaRG. On day 7, spheroids were either lysed for nuclei enumeration via the ChemoMetec NucleoCounter® NC-200™ using Reagent A100 (ChemoMetec Cat. No. 910-0003) and Reagent B (ChemoMetec Cat. No. 910-0002) or assessed for viability using CellTiter-Glo® 3D (Promega Cat. No. 354882). Representative spheroids were fixed in 4% paraformaldehyde (Boston Bioproducts Cat. No. BM-155) for cryostat sectioning and H&E staining (assessed at the University of New England in Biddeford, Maine). The Enzo SCREEN-WELL Hepatotoxicity Library (Enzo Cat. No. BML-2851) and subsequent potency analysis compounds were added to the cells on day 7, followed by an additional 48-hour culture. Enzo library 10 mM stock plates were diluted ten-fold with Hanks Buffered Saline Solution (HBSS) to generate 1 mM dosing plates. Five microliters of the 1 mM Enzo library dosing plates, 2 mM chlorpromazine (positive hepatotoxic control), or HBSS containing equivalent DMSO (negative buffer control) were added to hepatospheres in 45 µL of medium per well, bringing the final concentration of DMSO in all wells to 1%. On the day of assay, 30 µL of CellTiter-Glo 3D were added to each well following Promega's protocol. Viability was quantified by measuring relative luminescence with the TECAN Infinite® 200. This screen was repeated 4 independent times. Selected hits were further analyzed for potency by repeating the screening protocol utilizing compounds administered at a range of doses.

Results and Discussion

To optimize seeding density, Corning HepatoCells, HepG2 cells, and HepaRG cells were plated at various concentrations for 7 days of culture. CellTiter-Glo® 3D was used to quantify ATP content from live cells, while lysing of the spheroids allowed for the enumeration of total cells (nuclei) in each spheroid. Taken together, the calculation of ATP per nuclei was used to determine the optimal seeding density for each cell type, leading to the highest luminescent signal without the formation of a necrotic core. As shown in Figure 1, 6,000 cells per well of HepatoCells and HepaRG cells and 200 cells per well of HepG2 cells resulted in a high ATP per nuclei ratio. These seeding densities were used for all subsequent studies. Bright field (Figure 2) and histological (Figure 3) images confirmed that all three cells types at the optimized density formed tight, uniform, spheres devoid of a necrotic core.

After 48 hours of culture with the Enzo SCREEN-WELL Hepatotoxicity Library or subsequent potency analysis compounds, the CellTiter-Glo 3D was used to assess hepatosphere viability. A hit was defined as any compound that elicited a luminescent signal that was greater than 5σ below the mean buffer control response. Figure 4 shows representative results from a single screen.

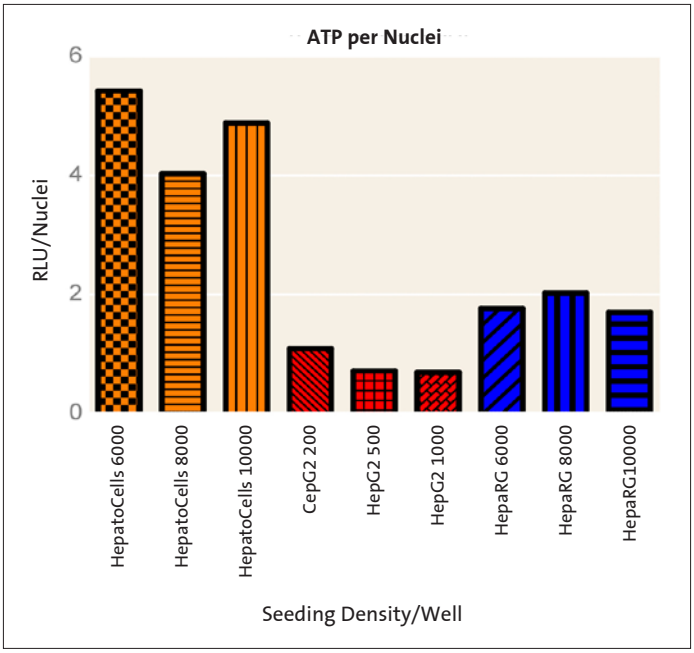

Figure 1. Ratio of relative luminescence units (RLU) to nuclei counts for seeding concentration optimization. Seeding densities were chosen that generated high luminescent signals for screening, while avoiding necrotic core formation. N = 3.

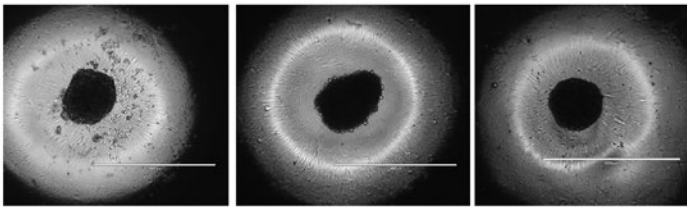

Figure 2. Representative photomicrographs of Corning HepatoCells, HepG2, and HepaRG spheroids after 7 days, with seeding densities of 6,000 cells/well, 200 cells/well, and 6,000 cells/well, respectively (40X total magnification).

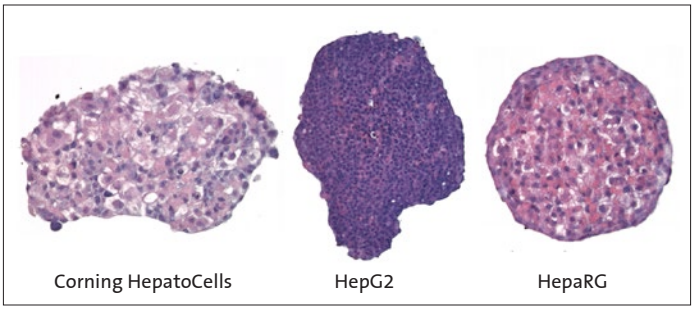

Figure 3. Representative H&E stained hepatospheres after 7 days of culture (5 µm slices, 200X total magnification).

When compared to HepaRG spheroids, the results indicate that, although there were many hepatotoxic hits detected in all three cell types, more hits and an overall larger assay window were observed with HepatoCells spheroids. Based on suggested mode of hepatotoxicity, hits were further classified into seven categories for mode of action: lipid accumulation, cholestatic effects, mitochondrial toxicity, toxic metabolite formation, mallory body formation, elevation of liver enzymes, and inhibition of the bile salt export pump (BSEP) (Figure 5). Hits were only categorized if they repeated in at least 3 of the 4 independent screens conducted. These screens demonstrate the detection of more known hepatotoxins with hepatospheres formed using Corning®

HepatoCells compared to HepaRG cells. In total, 64 hits were identified in this screen with good overlap between the 3 cell lines. Of those hits identified, 61% were shared by all three cell lines, with 26% shared between HepG2 and HepatoCells, and 13% shared between HepG2 and HepaRG cells.

Finally, to demonstrate the specificity of selected hits, potency was evaluated by repeating the screening protocol with known hepatotoxins (tamoxifen, troglitazone, and nicardipine) in a dose series (Figure 6). Toxicity (TC_{50}) values were calculated when possible and demonstrated that spheroids formed using HepatoCells displayed equal or greater sensitivity to the toxic compounds when compared to either HepG2 or HepaRG cells (Figure 7).

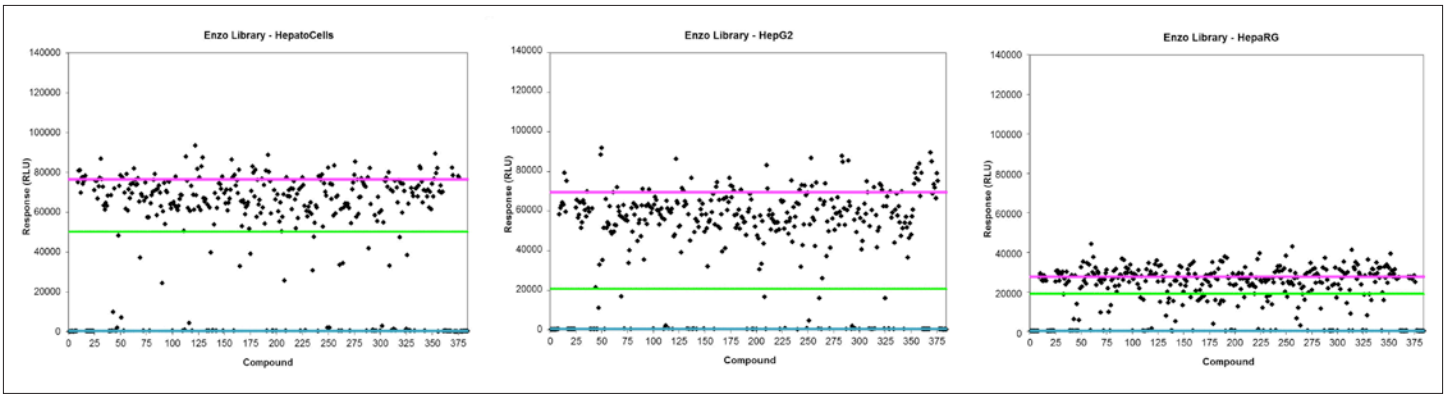

Figure 4. Representative SCREEN-WELL hepatotoxicity library results. Lines indicate the average response of non-responders (pink), average chlorpromazine (positive hepatotoxic control) response (blue), and 5σ below buffer (negative control) background (green). Anything below the green line was considered a hit. Of note, Corning HepatoCells displayed the largest signal window. Z' = 0.79, 0.57, and 0.80 for HepatoCells, HepG2, and HepaRG, respectively, based on 200 µM chlorpromazine control and buffer response from 32 wells.

Figure 5. SCREEN-WELL hepatotoxicity library summary from 4 independent studies. Hits were quantified if compound elicited a response >5σ below buffer response in at least 3 of the 4 individual screens.

	Corning HepatoCells	HepG2	HepaRG
Lipid accumulation	7	7	7
Cholestatic effects	6	8	7
Mitochondrial toxicity	6	7	4
Toxic metabolites	3	5	5
Mallory body formation	2	2	1
Elevation of liver enzymes	22	25	16
Inhibition of BSEP	2	3	1
Total	48	57	51

Figure 7. Calculated toxicity (TC_{50}) values of tamoxifen, troglitazone and nicardipine in hepatospheres formed from HepatoCells, HepG2, and HepaRG cells.

TC_{50} Values	Tamoxifen (M)	Troglitazone (M)	Nicardipine (M)
HepatoCells	1.4e-005	3.2e-005	4.8e-005
HepG2	1.1e-005	2.1e-005	2.7e-005
HepaRG	4.3e-005	–	0.013

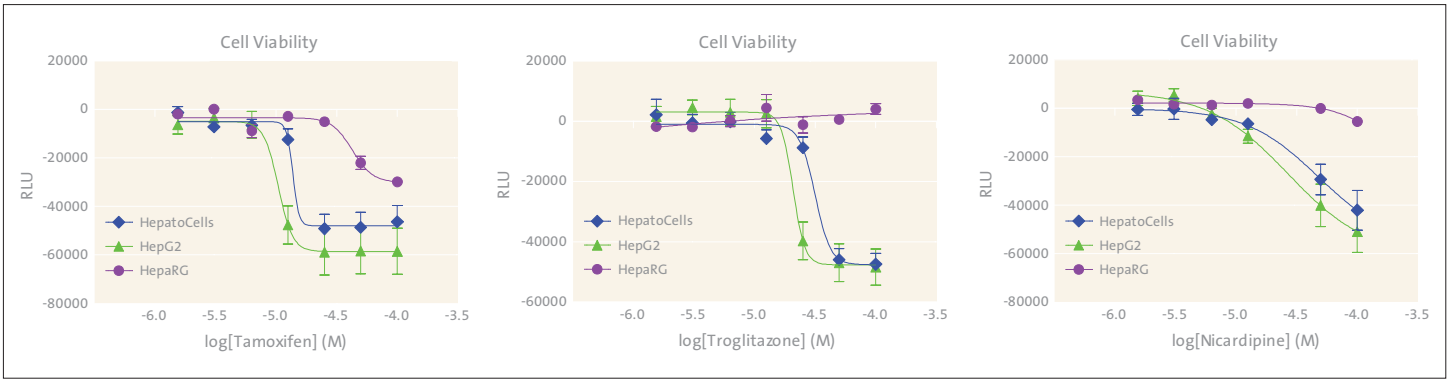

Figure 6. Potency analysis of selected compounds from the SCREEN-WELL hepatotoxicity library as assessed by CellTiter-Glo® 3D. N = 16 wells per concentration from 2 independent studies.

Conclusions

- Corning® spheroid microplates allow for the formation of consistently sized, single hepatocellular spheroids in each well. They are available in both 96- and 384-well formats, making them useful tools for 3D hepatotoxicity screening.
- Hepatospheres formed using the Corning spheroid microplates are amenable to histological analysis.
- The opaque walls and clear, round well-bottom design of the spheroid microplates allow for luminescent assays to be conducted in the plate without the need for a transfer step.
- Corning HepatoCells provide an ideal hepatocyte-like cell alternative to commonly used HepaRG cells, offering a larger assay window for luminescent ATP assays that is amenable to screening.
- Corning HepatoCells displayed increased or equivalent sensitivity to known hepatotoxins (tamoxifen, troglitazone, and nicardipine) upon potency analysis when compared to HepG2 and HepaRG cells.

Reference

Ramaiahgari SC, den Braver MW, Herpers B, Terpstra V, Commandeur JN, et al. A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. Arch Toxicol. 2014 May; 88(5):1083-95.

www.corning.com/lifesciences/solutions

At Corning, cells are in our culture. In our continuous efforts to improve efficiencies and develop new tools and technologies for life science researchers, we have scientists working in Corning R&D labs across the globe, doing what you do every day. From seeding starter cultures to expanding cells for assays, our technical experts understand your challenges and your increased need for more reliable cells and cellular material.

It is this expertise, plus a 160-year history of Corning innovation and manufacturing excellence, that puts us in a unique position to offer a beginning-to-end portfolio of high-quality, reliable cell culture consumables.

For more specific information on claims, visit the Certificates page at **www.corning.com/lifesciences**.

Warranty/Disclaimer: Unless otherwise specified, all products are for research use only. Not intended for use in diagnostic or therapeutic procedures. Not for use in humans. Corning Life Sciences makes no claims regarding the performance of these products for clinical or diagnostic applications.

For additional product or technical information, visit **www.corning.com/lifesciences** or call 800.492.1110. Customers outside the United States, call +1.978.442.2200 or contact your local Corning sales office.

Corning Incorporated
Life Sciences
836 North St.
Building 300, Suite 3401
Tewksbury, MA 01876
t 800.492.1110
t 978.442.2200
f 978.442.2476
www.corning.com/lifesciences

Worldwide Support Offices

ASIA/PACIFIC

Australia/New Zealand
t 61 427286832

China
t 86 21 3338 4338
f 86 21 3338 4300

India
t 91 124 4604000
f 91 124 4604099

Japan
t 81 3-3586 1996
f 81 3-3586 1291

Korea
t 82 2-796-9500
f 82 2-796-9300

Singapore
t 65 6572-9740
f 65 6861-2913

Taiwan
t 886 2-2716-0338
f 886 2-2516-7500

EUROPE

France
t 0800 916 882
f 0800 918 636

Germany
t 0800 101 1153
f 0800 101 2427

The Netherlands
t 31 20 655 79 28
f 31 20 659 76 73

United Kingdom
t 0800 376 8660
f 0800 279 1117

All Other European Countries
t 31 (0) 20 659 60 51
f 31 (0) 20 659 76 73

LATIN AMERICA
grupoLA@corning.com

Brasil
t (55-11) 3089-7400

Mexico
t (52-81) 8158-8400

CORNING | FALCON AXYGEN GOSSELIN PYREX