Assessment of Compound Hepatotoxicity Using Human Plateable Cryopreserved Hepatocytes in a 1536-Well-Plate Format

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ABSTRACT

Hepatotoxicity is a major concern for both drug development and toxicological evaluation of environmental chemicals. The assessment of compound-induced hepatotoxicity has traditionally relied on *in vivo* testing; however, it is being replaced by human *in vitro* models due to an emphasis on the reduction of animal testing and species-specific differences. Since most cell lines and hybridomas lack the full complement of enzymes at physiological levels found in the liver, primary hepatocytes are the gold standard to study liver toxicities *in vitro* due to the retention of most of their *in vivo* activities. Here, we optimized a cell viability assay using plateable cryopreserved human hepatocytes in a 1536-well-plate format. The assay was validated by deriving inhibitory concentration at 50% values for 12 known compounds, including tamoxifen, staurosporine, and phenylmercuric acetate, with regard to hepatotoxicity and general cytotoxicity using multiple hepatocyte donors. The assay performed well, and the cytotoxicity of these compounds was confirmed in comparison to HepG2 cells. This is the first study to report the reliability of using plateable cryopreserved human hepatocytes for cytotoxicity studies in a 1536-well-plate format. These results suggest that plateable cryopreserved human hepatocytes can be scaled up for screening a large compound library and may be amenable to other hepatocytic assays such as metabolic or drug safety studies.

INTRODUCTION

The liver is the key organ for the biotransformation of endogenous and exogenous compounds to aid in their clearance from the body. Paradoxically, these protective effects may increase susceptibility of the liver to toxicity from either the parental compound or its metabolites. Therefore, hepatotoxicity is a concern for the development and use of chemicals and

drugs in industrial, environmental, and pharmaceutical applications.^{1,2} With examples such as potassium dichromate^{3,4} and troglitazone,^{5,6} the impact of hepatotoxicity can be significant, resulting in potential liver damage, liver transplantation, or death.

Human *in vivo* toxicities are usually detected in late-stage development or postmarket release and, therefore, cannot predict risks from new chemical entities due to the lack of human exposure during discovery and early development.^{7,8} Traditionally, animal models have been used to assess hepatotoxicity with success for overt hepatotoxins, but they fail to accurately predict species-specific toxicities and idiosyncratic drug-induced liver injury (DILI).^{1,9,10} Additionally, animal models are expensive, time-consuming, and not amenable to large chemical library screens. With increasing numbers of new chemical entities for environmental and pharmaceutical uses, it is necessary to find a rapid and efficient method to screen chemicals for their potential toxicities. Since most severe DILI is due to hepatocellular injury,¹ alternative *in vitro* models are being used to estimate *in vivo* responses, to reduce and/or replace *in vivo* animal testing, and to increase the throughput of the evaluation of compounds screened and amount of data generated. Therefore, the ideal screening protocol would utilize human-derived cells in an *in vitro* assay to obtain human specific data while not endangering human volunteers.

As a result, there are several ongoing efforts dedicated to understanding chemical-induced toxicities in a variety of *in vitro* models. One key initiative is the National Toxicity Program (NTP), a U.S. federal government organization, started in 1978 to coordinate toxicological testing programs for the strengthening of toxicological sciences and development and validation of testing methods related to potentially toxic chemicals. Recently, the NTP, the NIH Chemical Genomics Center, and the U.S. Environmental Protection Agency initiated the Tox21 program for the development and validation of *in vitro* assays through the use of a high-throughput screening (HTS) platform.^{11–13}

Various *in vitro* approaches have been described in the literature to screen for hepatotoxicity.^{14–18} Recently, the advent of quantitative high-throughput screening (qHTS) has enabled researchers to obtain inhibitory concentration at 50% (IC₅₀) values directly from primary

ABBREVIATIONS: ATP, adenosine triphosphate; CV, coefficient of variation; CYP, cytochrome P450; DILI, drug-induced liver injury; DMSO, dimethyl sulfoxide; FRD, flying reagent dispenser; HCS, high content screening; HTS, high-throughput screening; IC₅₀, inhibitory concentration at 50%; LC/MS/MS, liquid chromatography tandem mass spectrometry; NTP, National Toxicity Program; qHTS, quantitative high-throughput screening; S/B, signal to background; SD, standard deviation; ST, sulfotransferase; UGT, UDP-glucuronosyltransferase; UPLC/MS/MS, ultra performance liquid chromatography tandem mass spectrometry.

screening, such as viability assays, to assess the toxicity potential of compounds in cell lines.^{19,20} Cell lines of hepatic origin, such as HepG2 cells, have been previously adapted to HTS formats^{21,22} and utilized to assess hepatotoxicity.^{19,20,23} However, HepG2 cells lack the full expression of hepatocyte proteins, such as phase I and phase II metabolizing enzymes and transporters, and thus may not correlate to *in vivo* hepatotoxicity.^{24–26} As an alternative to HepG2 cells, primary human hepatocytes represent the best predictive *in vitro* model to determine liver function for metabolism,^{27,28} drug–drug interactions,^{29,30} and potential hepatotoxicity of compounds.^{30–32}

Hepatocytes can be utilized in suspension for assays lasting a few hours or may be maintained in collagen-coated tissue culture plates for extended culturing. Traditionally, the use of hepatocytes has been limited to low density well formats such as 24-well for enzyme induction studies²⁹ or culture tubes for drug metabolism assays.³³ In addition, primary hepatocytes have been utilized as an *in vitro* model for determining hepatotoxicity and have shown strong correlation to *in vivo* hepatotoxicity.^{34–36}

In spite of the acceptance of hepatocytes in pharmaceutical research, they have had minimal use for short-term suspension assays or multi-day culturing protocols in HTS studies. Human hepatocytes in suspension cultures lasting several hours have been utilized in 96-well and 384-well formats for determining the metabolic clearance of drugs.^{37,38} Further, Wolff *et al.* have established a method for plated rat hepatocytes cultured for multiple days in 384-well format for high content screening (HCS) to monitor cellular functions.³⁹ However, no published reports have described employing cultured hepatocytes in 1536-well format. The low availability of freshly isolated human hepatocytes and plateable cryopreserved human hepatocytes had made them impractical for screening protocols and had limited assays to short-term incubations of six hours or less.³² However, recent improvements in availability and quality of plateable cryopreserved human hepatocytes have increased the opportunity for their use in HTS. Inclusion of primary hepatocytes in an HTS format would provide relevant data from the screening of large chemical libraries for the assessment of hepatotoxicity.

Herein, we describe the first reported multi-day culturing of plateable cryopreserved human hepatocytes from multiple donors in a 1536-well microtiter-plate format and its subsequent use in the determination of their hepatotoxicity potential of compounds by generating IC₅₀ values. Intracellular adenosine triphosphate (ATP) levels were measured to assess viability and consistency of plating and retention of hepatocytic function was confirmed through inhibition of CYP3A4 activity. To determine hepatotoxicity in a miniaturized format, the assay was validated during a 40 h exposure to a dozen of known toxic compounds, such as doxorubicin, tamoxifen, staurosporine, and phenylmercuric acetate.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma (St. Louis, MO). CellTiter Glo® and P450-Glo™ CYP3A4 Assay with Luciferin-IPA kits were purchased from Promega (Madison, WI). Collagen I-coated 1536-

well microtiter plates were purchased from Greiner Bio-One North America (Monroe, NC). Plateable cryopreserved human hepatocytes and medium were obtained from Celsis In Vitro Technologies (Baltimore, MD).

Determination of Enzymatic Activity in Cryopreserved Human Hepatocytes

Plateable cryopreserved human hepatocytes were characterized for cytochrome P450 (CYP), UDP-glucuronosyltransferase (UGT), and sulfotransferase (ST) enzymatic activities, as well as for their ability to be cultured *in vitro*. For enzymatic characterization, the cryopreserved hepatocytes were prepared according to instructions for use provided by Celsis In Vitro Technologies. Briefly, cryopreserved human hepatocytes were thawed at 37°C for approximately 2 minutes and transferred into 48 mL of *InVitroGRO*™ HT medium at 37°C. The cell suspension was centrifuged at 50 *g* for 5 min. The supernatant was removed and the cell pellet was resuspended in *InVitroGRO* KHB medium. The hepatocyte suspension was counted for viability and cell concentration using Trypan blue exclusion. Cell density was diluted to a stock concentration of 2.0×10^6 viable cells per mL. The hepatocyte suspension was incubated for 60 min at 37°C with equal volume of specific substrates for individual CYP enzymes as recommended by the FDA guidance⁴⁰ at the following final concentrations: phenacetin [15 μ M] (CYP1A2), coumarin [8 μ M] (CYP2A6), tolbutamide [15 μ M] (CYP2C9), s-mephenytoin [20 μ M] (CYP2C19), dextromethorphan [5 μ M] (CYP2D6), chlorzoxazone [50 μ M] (CYP2E1), testosterone [50 μ M] (CYP3A4), and 7-hydroxycoumarin [30 μ M] (UGT and ST). The metabolites of the reactions were quantified using high performance liquid chromatography, liquid chromatography tandem mass spectrometry (LC/MS/MS), or ultra performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) methods. The data were converted to the final units of picomoles of metabolite per minute per million cells of hepatocytes (pmol/min/million cells). The hepatocyte functional data from 172 donors of cryopreserved human hepatocytes, characterized as described above, were provided by Celsis In Vitro Technologies.

Attachment Confirmation of Cryopreserved Human Hepatocytes

The ability of cryopreserved hepatocytes to attach to cell culture plates was confirmed by culturing the plateable cryopreserved human hepatocytes for 5 days and by inducing the hepatocytes for protein synthesis of CYP1A2 and CYP3A4 as recommended in the FDA guidance.⁴⁰ Briefly, cryopreserved human hepatocytes were thawed at 37°C for approximately 2 minutes and transferred into 5 mL of *InVitroGRO* CP medium at 37°C. The hepatocyte suspension was counted for viability and cell concentration using Trypan blue exclusion. Cell density was diluted to a final concentration of 0.7×10^6 viable cells per mL. Cells were dispensed into 48-well collagen I-coated culture plate at a final density of 140,000 viable hepatocytes per well. The medium was changed during days 1 and 2

of culture with *InVitroGRO* CP medium. On days 3 and 4, wells were incubated with the positive controls omeprazole [50 µM] and rifampicin [25 µM] in *InVitroGRO* HI medium to induce CYP1A2 and CYP3A4 protein levels, respectively. Vehicle control wells were incubated with 1% acetonitrile in *InVitroGRO* HI medium. On day 5, the medium was removed and metabolism was determined from a 3-h incubation with phenacetin [100 µM] and testosterone [125 µM] in *InVitroGRO* KHB medium for CYP1A2 and CYP3A4 activity, respectively. The metabolites of the reactions were quantified using LC/MS/MS or UPLC/MS/MS methods. The data were converted to the final units of pmol/min/million cells. Fold induction was determined by the following equation: Positive Control Activity [pmol/min/million cells]/Vehicle Control Activity [pmol/min/million cells].

Cell Viability Assay Using a 1536-Well Format

The preparation of the cryopreserved hepatocytes for culturing was modified from the instructions for use provided by Celsis In Vitro Technologies. Plateable cryopreserved human hepatocytes were thawed at 37°C and diluted into 48 mL of *InVitroGRO* CP medium at 37°C. The cell suspension was centrifuged at 50 *g* for 5 min. The supernatant was removed and the cell pellet was resuspended in *InVitroGRO* CP medium. The cell suspension was filtered through a 40 µm nylon cell strainer (BD Biosciences, Durham, NC) to prevent obstruction of the dispensing tubes. The hepatocyte suspension was counted for viability and cell concentration using Trypan blue exclusion. Cell density was diluted to final concentrations of 0.4 × 10⁶ viable cells per mL and 0.8 × 10⁶ viable cells per mL to provide hepatocyte suspensions. The stepwise procedure from plating the cells to assay readout is described in Table 1. The hepatocyte suspensions were dispensed at 2000 (2K) or 4000 (4K) cells/5 µL/well in collagen I-coated 1536-well microtiter plates using a Flying Reagent Dispenser (FRD) (Aurora Discovery, Carlsbad, CA). The assay plates were incubated in a 37°C, 5% CO₂ humidified incubator for 4 or 24 h to allow for cell attachment. Compound addition was performed via pin tool (Kalypsys, San Diego, CA), where 23 nL of compound or dimethyl sulfoxide (DMSO) was added into the wells. The assay plates were incubated for an additional 24 or 40 h, followed by the addition of 5 µL per well of CellTiter-Glo reagent. This assay measures intracellular ATP content as a biomarker for cellular viability, where luminescent signal is proportional to the amount of ATP in metabolically active cells. Similarly, cell viability was also tested in HepG2 cells after compound treatment. The cells were dispensed at 2K cells/5 µL/well in 1536-well white/solid-bottom assay plates using an FRD. The cells were incubated a minimum of 5 h at 37°C, followed by the addition of compounds using the pin tool. The assay plates were incubated for 40 h, followed by the addition of 5 µL/well of CellTiter-Glo reagent via FRD. After a 30-min incubation at room temperature, the luminescence of each well was determined with a ViewLux plate reader (PerkinElmer, Shelton, CT). Hepatotoxicity potential of 12 compounds was determined by deriving IC₅₀ values from 16-point titrations, with each point in quadruplicate for a 40 h exposure. A 40-h exposure timepoint was extrapolated from

Table 1. Assay Protocol

Step	Parameter	Value	Description
1	Plate cells	5 µL	2000 or 4000 hepatocytes
2	Incubation time	4 h	Cells adhere and acclimate
3	Compounds	23 nL	2.8 nM to 92 µM dilution series
4	Incubation time	40 h	Compound treatment
5	Reagent	5 µL	CellTiterGlo reagent
6	Incubation time	30 min	Luminescent signal generation
7	Assay readout	Luminescence	ViewLux plate reader

Step Notes

1. Dispense cells into collagen I-coated 1536-well plates.
  2. Tissue Culture Incubator 37°C, 5% CO₂.
  3. Pin tool transfer of DMSO or test compounds into the assay plates.
  4. Tissue Culture Incubator at 37°C, 5% CO₂.
  5. Dispense CellTiter Glo reagent into assay plates.
  6. Room temperature in the dark.
  7. 1 s exposure, gain high, and 2 × binning luminescent read on Perkin Elmer ViewLux.
- DMSO, dimethyl sulfoxide.

previous publications for assessing cellular toxicity of the 12 compounds as measured by ATP content.^{19,20}

Functional Assessment of Cryopreserved Hepatocytes in a 1536-Well Format

Retention of CYP3A4 activity was confirmed by inhibiting its activity with the specific inhibitor ketoconazole.^{41,42} Cryopreserved hepatocyte lots IZT, LHO, and LMP were plated at 2K cells/well as described above. At 4 and 24 h after plating, 14 ketoconazole concentrations with each point in quadruplicate (9.2 µM–1.1 nM) were transferred to the wells using the pin tool and incubated for 10 min. One microliter of the Luciferin-IPA substrate from the P450-Glo 3A4 assay was transferred to the wells using the FRD to achieve a 3 µM final concentration. The plates were incubated for 1 hour at 37°C. A volume of 5 µL of luciferin detection reagent with esterase was dispensed into each well using the FRD and the plates were incubated at room temperature for 30 min. Luminescence values were detected using the ViewLux plate reader.

Data Analysis

The IC₅₀ values were determined and statistical analysis was performed using GraphPad Prism® 5 (La Jolla, CA). For viability, signal-to-background (S/B) ratio was calculated using luminescence values from DMSO-treated wells divided by tetraoctylammonium (92 µM, positive control)-treated wells. For CYP3A4 activity, S/B ratio was calculated using luminescence values from uninhibited wells (DMSO 0.46%) divided by maximally inhibited wells (9.2 µM,

ketoconazole). Coefficient of variation (CV) was calculated by using the standard deviation (SD) divided by mean. Z' factor, a measure of the quality of an HTS assay,⁴³ was determined using the equation $Z' \text{ factor} = 1 - [3 \times (\text{SD of positive control} + \text{SD of basal}) / (\text{median of positive control} - \text{median of basal})]$.

RESULTS AND DISCUSSION

Characterization of Cryopreserved Human Hepatocytes

To ensure appropriate metabolic activity associated with the cryopreserved hepatocytes, we first characterized hepatocytes from five different donors for metabolic enzyme level in traditional formats. Seven enzymes (CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A4) representing phase I metabolism and two families of enzymes (UGTs and STs) representing phase II metabolism were evaluated for drug metabolizing activities (Table 2). Of the phase I enzymes, CYP3A4 is responsible for the metabolism of the majority of drugs on the market⁴⁴ and thus is critical to be present at adequate levels. CYP3A4 activity of the five lots ranged between 50 to 108 pmol/min/million cells. From a survey of 172 lots from Celsis In Vitro Technologies, the mean CYP3A4 activity was 100 pmol/min/million cells with an SD of 98 pmol/min/million cells, representing the diverse range of activity between individuals. CYP2E1 is of interest for occupational and environmental medicine⁴⁵ given its metabolism of a broad range of chemical classes, including ketones, halogenated alkanes, and monocyclic compounds such as benzene and *p*-nitrophenol.⁴⁶ The metabolic activity of CYP2E1 ranged between 13 and 48 pmol/min/million cells of metabolite formation of 6-hydroxychlorzoxazone. This compared well against an average of 39 pmol/min/million cells with an SD of 36 pmol/min/million cells from the survey of 172 donors. All phase I enzyme activities were within one SD of the average as characterized by Celsis In Vitro Technologies (Table 2).

The phase II enzymes for UGT and ST family were measured using the substrate 7-hydroxycoumarin, which is not specific for individual isoforms. Therefore, the metabolite 7-hydroxycoumarin glucuronide reflects multiple UGT isoform activities, whereas the metabolite 7-hydroxycoumarin sulfate reflects multiple ST isoforms. The phase II enzymes were well represented with UGT metabolism ranging between 191 and 558, and ST between 11 and 58 pmol/min/million cells. All lots were within one SD of the average as characterized by Celsis In Vitro Technologies (Table 2).

The ability for cryopreserved human hepatocytes to attach to collagen I-coated culture plates was further evaluated by maintaining the culture for 5 days and inducing CYP1A2 and CYP3A4 enzymatic activity in a traditional 48-well cell culturing format. The hepatocytes retained typical cuboidal morphology and a confluence of >70% on day 5 (data not shown). Induction of CYP1A2 and CYP3A4 by exposure to omeprazole and rifampicin was determined by measuring enzymatic activities using specific substrate phenacetin and testosterone, respectively. The fold induction was calculated by dividing the enzymatic rate of the induced hepatocytes by the uninduced rate. CYP1A2 was induced >6-fold and CYP3A4 >11-fold for the lots used (data not shown). According to the FDA guidance, a fold induction >2-fold should be obtained from potent inducers, and the results were within the expected range documented in the guidance.⁴⁰ Therefore, the plateable cryopreserved human hepatocytes performed as expected compared to historical and documented activities and represent a suitable cellular system to mimic liver functions.

Hepatocytic Activities in 1536-Well-Plate Format

The retention of critical hepatocytic characteristics in miniaturized well format was investigated. At 48 h, hepatocytes at 2K/well after 24 h DMSO treatment (0.46% final concentration in the wells) showed

a monolayer with typical morphology for a hepatocyte culture at approximately 40% confluence (Fig. 1) as expected from the cell density of 80,000 cells per cm². Hepatocytes at 4K/well showed 80%–90% confluence at 48 h incubation (data not shown). A cell density of ~200,000 hepatocytes per cm² would provide near 100% confluence, which is concordant with literature values.^{32,47}

Determining enzymatic activities using traditional methods as described above were not employed due to technical restraints of a 1536-well format and

Table 2. Characterization of Hepatocytic Functions

Lot	Drug metabolizing enzyme activities (pmol/min/million cells)								
	CYP1A2	CYP2A6	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4	UGT	ST
KQG	10	106	31	1	29	31	50	375	20
IZT	12	24	35	1	0.4	48	51	456	58
LHO	21	102	8	6	7	13	48	558	36
LMP	9	44	16	0.3	21	15	108	200	11
SCT	10	49	25	0.5	26	15	108	191	12
Average ± SD n = 172	26 ± 24	69 ± 45	36 ± 31	11 ± 38	22 ± 16	39 ± 36	100 ± 98	325 ± 169	29 ± 34

Demographics and characterization of plateable cryopreserved human hepatocytes as measured by cytochrome P450, UGT, and ST enzymatic activity and induction of enzyme activity. The average metabolism was determined from a survey of 172 donors of cryopreserved human hepatocytes from Celsis In Vitro Technologies as the mean ± SD.

UGT, UDP-glucuronosyltransferase; ST, sulfotransferase.

Fig. 1. Photographs of plateable cryopreserved human hepatocytes in collagen I-coated 1536-well microtiter plates at 48 h in culture. The photographs were taken using Hoffman Modulation Contrast (HMC) at (A) 10 $\times$ and (B) 20 $\times$. Approximate confluence of 40% was obtained using 2K cells per well (80,000 cells/cm²).

requirements of UPLC/MS/MS methods. The luminescent P450-Glo Assay with Luciferin-IPA is amenable to HTS formats and has been shown to be specific for CYP3A4 detection. Further, Luciferin-IPA has been used to determine inhibition and induction of CYP3A4 in human hepatocytes.⁴² Enzymatic activity of CYP3A4 was confirmed by calculating IC₅₀ values of the known specific inhibitor ketoconazole at 4 and 24 h after plating cryopreserved hepatocytes. The combined data from three of the donors provided average IC₅₀ values of 0.197 μ M at 4 h and 0.146 μ M at 24 h. Further, the CYP3A4 activity was sufficient to produce sigmoidal curves for the calculation of IC₅₀ values (Fig. 2) with S/B ratios of 11.9 ± 6.8 at 4 h and 5.4 ± 1.5 at 24 h. The reduction of the S/B over time was expected due to the known loss of CYP activity in cultured hepatocytes.⁴⁸ The data provided evidence that the hepatocytes retained activity of CYP3A4 in the miniaturized well format.

Assay Optimization for Cytotoxicity Studies in 1536-Well-Plate Format

To determine the potential of utilizing plateable cryopreserved hepatocyte culture in a miniaturized well format, hepatocytes were dispensed into collagen I-coated 1536-well plates at 2K/well and cultured for 4 or 24 h before treatment with DMSO (0.46%). A cell density of 80,000 hepatocytes/cm² (2K/well) was in line with HepG2 culturing conditions previously tested^{19,20} and therefore was the basis for testing compound cytotoxicity in the current study for IC₅₀ determination as well as assessment of S/B ratio and well-to-well variation.

To confirm the ability to utilize hepatocytes in a cytotoxic assay, a CellTiter-Glo luminescent assay was used to evaluate hepatocyte viability. For control plate assessment, hepatocytes at 2K/well were treated with DMSO only in 1536-well-plate format. The cell viability assay performed well (Fig. 3) with CV values of 4.9% and 4.0% in the DMSO-treated wells for 24 h after 4 h and 24 h plating, allowing hepatocytes attaching to the wells, respectively. To evaluate the assay performance, Z' factor values of 0.78 and 0.87 were determined in hepatocytes treated with the DMSO ($n=1408$) or tetraoctylammonium (92 μ M) ($n=32$) for 24 h after the hepatocytes attached to the wells at 4 and 24 h, respectively. The signal (DMSO wells)-to-

Fig. 2. Concentration-response curves of ketoconazole for the specific inhibition of CYP3A4 as measured by luciferin-IPA metabolism in plateable cryopreserved human hepatocytes individual donors at 4 h (HR) and 24 h in 1536-well collagen-coated plates. The data presented are means $\pm$ SD of quadruplicate wells ($n=4$). SD, standard deviation.

background (tetraoctylammonium 92 μ M) ratios of 33 and 43 were observed after 24 h DMSO treatment in the hepatocytes preculture for 4 and 24 h incubations, respectively. The concentration response of tetraoctylammonium bromide and potassium dichromate was determined from 16-point curve performed in quadruplicate. The IC_{50} values of tetraoctylammonium bromide were 3.8 and 3.3 μ M at 24 h treatment in the hepatocytes preculture for 4 and 24 h, and 5.2 and 3.2 μ M for potassium dichromate at 24 h treatment in the hepatocytes preculture for 4 and 24 h, respectively.

The plateable cryopreserved hepatocytes performed well for attachment efficiency and viability in culture for 48 h, and were responsive to concentration–response curves of two known cytotoxins. The assay performance was considered excellent as determined by Z' factor and warranted further evaluation.

Fig. 3. Assay optimization of cell viability assay in human hepatocytes in a 1536-well-plate format. The hepatocytes were treated with compound or dimethyl sulfoxide (DMSO) for 24 h after (A) 4 h or (B) 24 h plating. Column 1 represents a concentration–response curve of tetraoctylammonium bromide (92 μ M–2.8 nM). Column 2 represents a concentration–response curve of potassium dichromate (92 μ M–2.8 nM). Column 4 represents tetraoctylammonium bromide (92 μ M). Column 3 and Columns 5–48 are DMSO only.

To further optimize the test system, we chose 12 compounds that have been cited in literature as cytotoxins in both *in vitro* and *in vivo* systems. The 12 compounds, a subset of the NTP 1408 library that has been used in our laboratory to validate HTS screens, have been shown to be toxic to human- and animal-derived cell lines, including liver-derived HepG2 cells.^{19,20,49} To evaluate the effects of the 12 compounds, two cell densities, 2K/well and 4K/well representing 40%–50% and 80%–90% confluence, respectively, were dispensed in a 1536-well plate to assess any confluence dependent differences. The 12 compounds were tested for their cytotoxic effects on these hepatocytes after 40 h treatment and IC_{50} values were calculated from the concentration–response curve for 2K/well and 4K/well conditions (Fig. 4). There was good correlation ($R^2 = 0.95$) between the IC_{50} values of these 12 compounds for 2K/well and 4K/well conditions and, as well, the curve fit was good with most R^2 values above 0.9. At higher concentrations of compounds, decreased cell viability was observed with most compounds achieving viability near 0% with the exception of methylmercury chloride. The potencies of most compounds were slightly higher at 2K/well than 4K/well. The data demonstrate a robust and flexible range of responses, as well as consistency between the 2K/well and 4K/well cell densities. A cell density of 2K/well was used for subsequent testing.

Cytotoxicity of Chemical Compounds

Inter-donor variation was assessed between four of the five individual donors (Table 2). Lot SCT, though used for optimization of the test system, was not used for 12 compound toxicity validation due to lack of supply. We observed good correlation of the IC_{50} values from different donors with average R values of 0.92 (Table 3). Notably, lot LMP was more sensitive to the compounds tested with higher potencies for most compounds as compared to the other lots. However, the ranking order of the compound cytotoxicity values among the lots was very similar. Inter-assay variability was investigated by assessing the toxicities induced by the 12 compounds on lot IZT on different days. The IC_{50} values of these compounds are very similar between the experiments (data not shown) with an R value of 0.97. The averaged human hepatocyte responses were further compared to HepG2 cell line to confirm cytotoxicity equivalence (Table 3). The two cell types compared favorably across the compound toxicity panel. There was a good correlation of the IC_{50} values between HepG2 and human hepatocytes, with an R value of 0.78. Among the 12 tested compounds, doxorubicin was the most potent in both cell types with IC_{50} values of 1.8 μ M for averaged human hepatocytes and 1.6 μ M for HepG2 cells. The other compounds with IC_{50} values below 10 μ M were gentian violet, malachite green oxalate, and potassium dichromate. In contrast, tamoxifen was the least potent compound with IC_{50} values of 62 μ M for averaged human hepatocytes and 45.1 μ M for HepG2 cells.

The 12 compounds we selected for this study represent diversity in use and mechanism of toxicity, as well as having no known metabolic dependency for action. For example, malachite green oxalate, gentian violet, and pararosaniline are dyes and have been implicated as being a mitochondrial toxin.^{50–52} Doxorubicin and

Fig. 4. Concentration–response curves generated using plateable cryopreserved human hepatocytes at 2K/well or 4K/well in 1536-well collagen-coated plates as measured by ATP content at 40 h of exposure for 12 chemicals. (A) Digitonin, (B) doxorubicin, (C) gentian violet, (D) malachite green oxalate, (E) mercury chloride, (F) methylmercury chloride, (G) pararosanine, (H) phenylmercuric acetate, (I) potassium dichromate, (J) staurosporine, (K) tamoxifen, (L) and tetraoctylammonium bromide. The data presented are means $\pm$ SD of quadruplicate wells ($n=4$).

tamoxifen are cancer chemotherapeutics and have been cited for *in vivo* and *in vitro* hepatotoxicities, presumably due to oxidative stress.^{53,54} Methylmercury chloride, phenylmercuric acetate, and mercury chloride are mercury-containing compounds that have been well established as an environmental toxins and public

health risks.^{55,56} Despite the fact that no definitive primary mechanism of toxicity has been reported, a range of potential mechanisms have been proposed, such as mitochondrial dysfunction, lipid peroxidation, microtubule disruption, and oxidative stress.⁵⁵

Table 3. Test Compounds

Chemical name	IC ₅₀ (μM)					
	CASRN	KQG	IZT	LHO	LMP	HepG2
Digitonin	11024-24-1	16.6	22.5	21.0	5.8	23.4
Doxorubicin	25316-40-9	1.4	3.9	0.9	0.9	1.6
Gentian violet	548-62-9	9.4	7.3	4.6	3.4	4.4
Malachite green oxalate	2437-29-8	3.3	3.1	2.2	1.5	1.3
Mercury chloride	10112-91-1	51.1	24.2	17.1	12.0	41.6
Methylmercury chloride	115-09-3	14.2	18.3	6.5	6.8	15.0
Pararosaniline	25620-78-4	44.0	59.5	33.1	13.0	12.5
Phenylmercuric acetate	62-38-4	4.0	3.7	1.5	1.7	12.3
Potassium dichromate	7778-50-9	5.4	6.0	3.6	2.5	7.6
Staurosporine	62996-74-1	11.8	3.1	1.1	0.8	11.6
Tamoxifen	10540-29-1	98.4	81.3	50.3	21.5	45.1
Tetraoctylammonium bromide	14866-33-2	4.3	3.7	4.4	1.7	11.1

Potencies (IC₅₀, μM) of compounds tested with plateable cryopreserved human hepatocytes (Donors IZT, KQG, LHO, and LMP) and HepG2 cells.

In addition, other researchers have demonstrated toxicity potential of these compounds in cell lines, such as mercuric compounds in human fetal hepatic cell line WRL-68,⁵⁷ potassium dichromate in HepG2 cells,⁵⁸ and malachite green oxalate in HepG2 and Caco-2 cell lines.⁵⁹ Cytotoxicity has also been demonstrated with several of the compounds in primary cells. Staurosporine has been shown to induce apoptosis in murine hepatocytes⁶⁰ and has been used as a positive control in human hepatocytes for HCS of hepatocellular toxicity markers.⁶¹ Several compounds have been documented to induce *in vivo* hepatotoxicities. For example, malachite green oxalate and gentian violet cause liver damage in fish and mammals.^{51,62} In addition, doxorubicin and tamoxifen have been implicated in human liver injury.^{53,54,63}

CONCLUSION

We present here the first confirmed dispensing and culturing of plateable cryopreserved human hepatocytes in collagen I-coated 1536-well microtiter plates, and their survival up to 48 h without medium changes. The assay system was consistent and robust as measured by ATP content providing an appropriate HTS platform for measuring viability of hepatocytes, while retaining metabolic function as confirmed by the inhibition of CYP3A4. We validated the system for determining *in vitro* hepatotoxicity by deriving IC₅₀ values for 12 known cytotoxic compounds that represented diverse chemical properties. The combination of HTS and primary hepatocytes offers a new platform and potential use with qHTS to

screen for hepatotoxicity of large chemical libraries. Further studies with compounds dependent upon hepatocytic function, such as metabolism and transport to modulate cytotoxicity, are necessary to delineate the benefits between human hepatocytes and commonly used cell lines such as HepG2. This system may also provide a suitable platform for other endpoints such as metabolic or drug safety studies.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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