Assay Validation of Hepatotoxicity in Human Plateable Cryopreserved Hepatocytes using qHTS

Timothy A. Moeller[1], Sunita J. Shukla[2] and Menghang Xia[2]

[1]BioreclamationIVT (Baltimore, MD)

[2]NIH Chemical Genomics Center (Bethesda, MD)

ABSTRACT

The liver is the key organ for the biotransformation of endogenous and exogenous compounds to aid in their clearance from the body. In many cases, the metabolism of these compounds is critical to protect the body from toxic levels, but in some cases, the metabolites of parental compounds may incur hepatotoxicity. Taking the metabolism of compounds into account is essential not only to drug development, drug safety and environmental health issues, but also to evaluate the risks associated with injury to the liver. The ability to screen compounds to assess hepatoxicity is crucial, but has been traditionally limited to *in vivo* testing for accurate evaluation, which is not desirable for ethical and efficiency reasons. *In vitro* testing, which reduces animal use, has become a viable option with the advent of specific markers and sensitive reagents. The development of quantitative high throughput screening (qHTS) allows one to test an enormous number of potential toxins in relevant cellular systems, like primary human hepatocytes. For proof of concept, we have developed methods to utilize plateable cryopreserved human hepatocytes (PCHH) in a 1536-well format in order to screen known toxic compounds. PCHH were plated at two different cell concentrations in collagen-coated 1536-well plates. Test compounds, dosed over a four-log range, were added four hours after plating and incubated for an additional 40 hours. Intracellular ATP content was measured to determine IC_{50} for 12 compounds, including doxorubicin, tamoxifen, staurosporine, and phenylmercuric acetate. The signal-to-background ratio assay window is above 40-fold, which indicates that the 1536-well plate format is robust and can be utilized for assessing hepatotoxicity using primary human hepatocytes in a qHTS platform. The wide range of toxins identified from the qHTS approach illustrates the utility of this platform. Additionally, qHTS can be employed to further explore the susceptibility of hepatocytes to various mechanisms of toxicity using appropriate cellular death markers.

INTRODUCTION

Hepatotoxicity is a critical concern in the use and development of chemicals and drugs. As with compounds like troglitazone and potassium dichromate, the impact of hepatotoxicity can be significant, resulting in potential liver damage, liver transplantation or death. The risks to the liver from new and existing chemical entities need to identified. Traditionally, animal models have been used to assess hepatotoxicity; however, these studies are expensive, time consuming and may not be correlative due to species differences. Human *in vivo* studies are usually retrospective and, therefore, cannot predict new chemical entities risks. To screen for hepatotoxicity, various *in vitro* approaches have been described in the literature[1,2,3] and by regulatory authorities[4,5]. Quantitative high throughput screening (qHTS) is one approach to obtain IC_{50} values in order to assess toxicity risks[6]. Cell lines, such as HepG2, that have been used to assess hepatotoxicity lack the full expression of hepatocyte functions. Primary human hepatocytes represent the most correlative cell type to determine liver toxicity in an *in vitro* system and the use of these cells in 1536-well formats is just being realized. Herein, we describe the utilization of plateable cryopreserved primary human hepatocytes in a 1536-well microtiter plate format. The adherent hepatocytes are able to survive for at least 48 hours, allowing for long-term exposure to test articles across a wide dosing range in order calculate IC_{50} values.

MATERIALS & METHODS

Hepatocyte Preparation: PCHH (lots KQG and SCT) and medium were obtained from BioreclamationIVT. The PCHH were thawed at 37°C and diluted into 50 mL of *InVitro*Gro CP medium at 37°C. The cell suspension was centrifuged at 50 x g for 5 minutes. The supernatant was removed and the cell pellet was resuspended in *InVitro*Gro CP medium. The suspension was counted for viability and cell concentration using Trypan blue exclusion. Cell density was diluted to final concentrations of 0.8 x 10^6 viable cells per mL and 0.4 x 10^6 viable cells per mL.

Plating of Hepatocytes: Collagen coated 1536-well microtiter plates (Greiner Bio-One North America, NC and freshly coated collagen I) were loaded using a Flying Reagent Dispenser (Aurora Discovery, Carlsbad, CA) with 5 µL of cell suspensions to obtain 4000 (4K) and 2000 (2K) viable cells per well. The plates were incubated in 37°C, 5% CO_2 humidified incubator for the desired time.

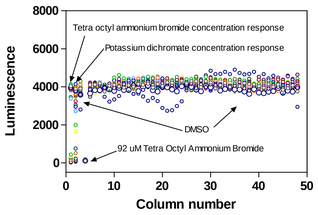
DMSO Plates: At four hours or 24 hours after seeding hepatocytes, plates were dosed via pin tool (Kalypsys, San Diego, CA) with control compounds tetra octyl ammonium bromide (92 µM – 2.8 nM) , potassium dichromate (92 µM – 2.8 nM) or DMSO (0.46%). The plates were returned to the incubator for an additional 24 hours. CellTiter-Glo® (Promega, Madison, WI) kit was utilized to measure ATP content as a measurement of cellular viability, where luminescent signal is proportional to the amount of ATP in metabolically active cells. CellTiter-Glo® (5 µL) was added to the cells and incubated at room temperature for 30 minutes. The luminescence of each well was determined with a ViewLux plate reader (PerkinElmer, Shelton, CT). Data was analyzed using Prism® 5 GraphPad for Z factor, a measure of the quality of an HTS assay[7].

Toxicity Determination: At four hours after seeding hepatocytes, plates were dosed via pin tool with test compounds dissolved in DMSO (Table 1) or DMSO only (0.46%). The plates were incubated for additional 40 hours. CellTiter-Glo® was utilized to measure ATP content as a measurement of cellular viability as described above. The IC_{50} of each compound was determined by using Prism® 5 GraphPad data analysis program.

RESULTS: DMSO Plates

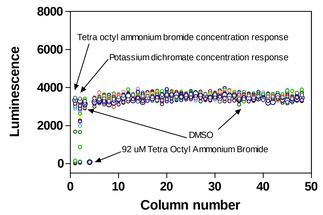
DMSO Plates:

Lot SCT was plated into 1536-well collagen I coated microtiter plates at 2K cells per well and dosed at four and 24 hours after plating with control test compound tetra octyl ammonium bromide, potassium dichromate or DMSO (0.46%) to assess well-to-well variation. The plates were incubated for an additional 24 hours after dosing. Variability was determined using Celltiter-Glo. Results indicate a consistent dispensing and adherence of the hepatocytes (Fig. 1 and 2) with a coefficient of variation of 4.9 and 4.0, Z-factor of 0.78 and 0.87, signal to background of 33 and 43 and relative luminescence units (RLU) of ~4000 and ~3200 for 28-hour and 48-hour incubations, respectively. This indicates viable hepatocytes with sufficient signal to measure cellular death using ATP as a marker.



S/B = 33; CV = 4.9; Z' factor = 0.78

Fig 1. DMSO plate 28 hours.



S/B = 43; CV = 4.0; Z' factor = 0.87

Fig 2. DMSO plate 48 hours.

RESULTS: Toxicity Determination

Toxicity Determination.

Toxicity of 12 compounds (Table 1) was assessed by determining IC_{50} values from a 16-point dosing range between 92 µM and 2.8 nM after 40-hour incubation. Two different cell densities of PCHH (lot KQG), 2000 (2K) and 4000 (4K) per well, were used to assess optimal cell density. 2K cells per well represented 40-50% confluence and 4K represented 80-90%. The RLU values were consistent at approximately 2000 and 3100 for 2K and 4K, respectively (Fig. 3), indicating consistent dispensing among wells and sufficient hepatocyte viability with greater than 40-fold signal to background. Both cell densities gave similar IC_{50} values for the panel of toxic compounds, demonstrating a robust and flexible range.

Test Article	*2K/well IC_{50} [µM] (R^2)*	*4K/well IC_{50} [µM] (R^2)*
Doxorubbicin	1.2 (0.6433)	2.3 (0.9835)
Tamoxifen	151.3 (0.9581)	104.2 (0.9412)
Staurosporine	11.2 (0.8646)	17.8 (0.9366)
Phenylmercuric acetate	4.0 (0.9920)	9.8 (0.9672)
Methylmercury chloride	14.2 (0.6346)	25.7 (0.8072)
Mercury chloride	51.1 (0.8317)	72.2 (0.9854)
Malachite green oxalate	3.3 (0.9537)	6.7 (0.9835)
Gentian Violet	9.4 (0.9632)	17.9 (0.9821)
Pararosaniline	44.0 (0.9095)	126.4 (0.9811)
Digitonin	16.6 (0.9883)	24.5 (0.9808)
Tetraoctylammonium	4.3 (0.9334)	4.1 (0.9935)
Potassium dichromate	5.4 (0.9492)	17.1 (0.9809)

Table 1. List of compounds, derived IC_{50} values and R^2 values for goodness to fit.

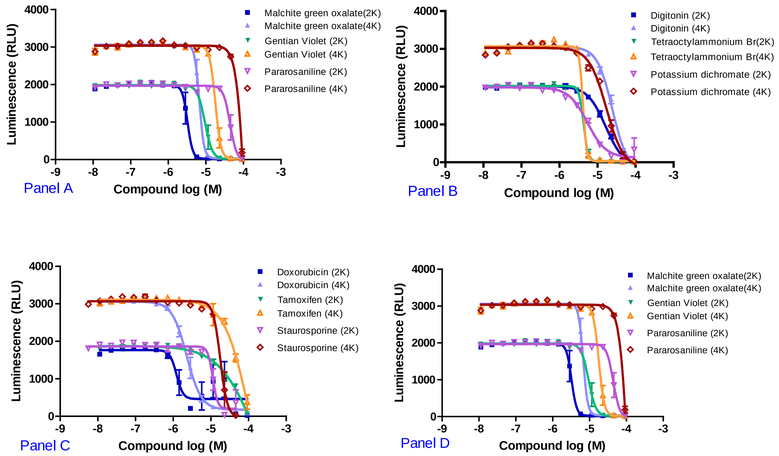


Fig 3. Graph of ATP levels measured by RLU versus concentration for 12 known toxic chemicals (Panels A-D) at 2K and 4K viable hepatocytes per well.

CONCLUSIONS

- PCHH are consistently dispensed in 1536-well collagen-coated microtiter plates and can be cultured for at least 48-hours without media changes, allowing use in qHTS.
- PCHH show a differential response to a wide range of chemical toxins as indicated through the determination of IC_{50} values from a 16-point dosing curve over four-log range as measured by ATP content in a qHTS format.
- qHTS screening with PCHH offers a system to directly measure human hepatotoxicity and may be expanded with other endpoints to further assess toxic events.

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