Cryopreserved HepaRG™ Cells: An Alternative *In Vitro* Screening Tool for Human Hepatic Drug Metabolism, Induction of Metabolism, & Safety Applications

life technologies™

Jonathan P. Jackson, James Lovgren, Stephen S. Ferguson, Life Technologies, Cell System Division, ADME/Tox Business Unit, 4301 Emperor Blvd., Durham, NC 27703

ABSTRACT/Introduction –

Primary hepatocyte model systems remain the "gold standard" for in vitro studies to assess human metabolism and induction of metabolism. However, the use of primary human hepatocytes (PHH) in screening applications is limited by hepatocyte availability, donor variability, high costs, and a relative short lifespan (≤10 days) in culture using standard methodologies (i.e. sandwich cultures). The use of HepaRG™ Cells in hepatic screening applications may solve these limitations without sacrificing critical mature hepatocyte phenotypes such as drug metabolizing enzymes, transport proteins, and functional xenobiotic sensing pathways (CAR, PXR, AhR).

The goal of this work is to demonstrate the utility and reproducibility of differentiated, cryo-preserved HepaRG™ Cells in hepatic screening applications. Cryo-preserved HepaRG™ Cells will allow researchers to thaw, plate, and use HepaRG™ Cells on demand. In this study, we show that cytochrome P450 metabolic activities in cryo-preserved HepaRG™ Cells (e.g. CYP1A2, CYP2B6, and CYP3A4) are comparable to historical data derived from PHH. Additionally, we show that induction responses of CYP1A2, CYP2B6, and CYP3A4 in response to prototypical receptor activators are comparable to historical PHH induction data. These data demonstrate that cryo-preserved HepaRG™ Cells are metabolically competent and maintain functional xenobiotic sensing pathways similar to those exhibited in PHH cultures. Reproducibility data (inter- and intra-plate) from induction studies indicate that cryo-preserved HepaRG™ Cells minimize assay variability, providing a robust P450 induction screening platform. Finally, we show the utility of cryo-preserved HepaRG™ Cells in the evaluation of intrinsic and metabolically-activated toxicity. We demonstrate for the first time in HepaRG™ Cells that co-treatment with the CYP3A4 inhibitor ketoconazole abolishes aflatoxin B1-mediated cytotoxicity assessed by ATP depletion assays.

HepaRG™ Cells Background

•HepaRG™ Cells were derived from a differentiated human hepatoma at the Institut National de la Sante' et de la Recherche Me'dicale (INSERM) of France (1).

•HepaRG™ Cells are bi-potent hepatic progenitor cells that differentiate into two distinct hepatic cell types, hepatocyte-like and biliary-like cells. A fully differentiated HepaRG™ Cell population is comprised of ~50% hepatocyte-like and ~50% biliary-like cells (2-3).

•Unlike other immortalize hepatic cell lines (e.g. HepG2 and Fa2N-4), HepaRG™ Cells (hepatocyte-like cells) maintain many key primary human hepatocyte characteristics including drug metabolizing enzymes (e.g. P450s), transporters, and signal transduction pathways (i.e. CAR). These pathways are known to play important roles in liver injury as a result of drug exposure and are necessary to evaluate a new chemical entities drug-drug interaction and/or hepatotoxicity potential (4-5).

•After differentiation, HepaRG™ Cells are cryo-preserved for convenience allowing researchers to thaw, plate, and use HepaRG™ Cells on demand to conduct drug metabolism, drug-drug interaction, or drug safety evaluation studies.

MATERIALS AND METHODS –

HepaRG™ Cells:

•**Materials:** Cryo-preserved HepaRG™ Cells, Williams Medium E (WEM), Collagen I Coated 96-well Plates, GlutaMAX™ Supplement, HPRG770 medium supplement, HPRG720 medium supplement, HPRG730 medium supplement, HPRG740 medium supplement were obtained from Life Technologies. ATP depletion assays were purchased from Promega and completed following manufacturer's instructions

•**Culture for Drug Metabolism:** HepaRG™ Cells were thawed and plated (1E5 cells/well) onto a 96-well collagen I plate using WEM supplemented with HPRG770 and GlutaMAX™ Supplement. Media was renewed next day with WEM supplemented with HPRG720 and GlutaMAX™ Supplement. To evaluate P450 baseline activity, HepaRG™ Cells were cultured for 4 days prior to conducting in situ incubations with P450 prototypical substrates.

•**Culture for P450 Induction:** HepaRG™ Cells were thawed and plated (1E5 cells/well) onto a 96-well collagen (type I) coated plates using WEM supplemented with HPRG770 and GlutaMAX™ Supplement. Media was renewed the day following plating with WEM supplemented with HPRG770 and GlutaMAX™ Supplement. To evaluate P450 induction properties, HepaRG™ Cells were maintained in WEM supplemented in HPRG740 and GlutaMAX™ Supplement beginning on Day three. Dosing with prototypical P450 inducers was initiated on day three and continued for 72 hours. Inducers were renewed with media daily for three consecutive days prior to evaluating P450 activity (in situ) and mRNA expression.

•**Culture for Cytotoxicity:** HepaRG™ Cells were thawed and plated (1E5 cells/well) onto a 96-well collagen I coated plates using WEM supplemented with HPRG770. Media was replaced the next day with WEM supplemented with HPRG730 or HPRG720 and GlutaMAX™ Supplement. Media was refreshed every two days thereafter. HepaRG™ Cells were treated with chlorpromazine or aflatoxin on day four. 24 hours after treatment, ATP depletion assays were performed.

PHH:

•**Materials:** Cryo-preserved Hepatocyte Recovery Medium (CHRM™), Geltrex™ Matrix, ITS+, and PHH were obtained from Life Technologies.

•**PHH Culture:** Cells were first thawed using CHRM™ and plated using WEM (serum-containing) at the predetermined optimal density of 0.8E6 cells/ml in a 24-well plate hand-coated with simple type I collagen. Cells were allowed to attach for 4-6 hrs before overlay with Geltrex™ Matrix (Invitrogen) in serum-free WEM containing ITS+. The medium was replaced daily with fresh supplemented medium or medium containing the inducers. 24 hrs after plating, cells were treated with the prototypical inducers for 48 or 72 hrs. P450 activity was evaluated (in situ) 72 hrs after treatment and mRNA expression was evaluated 48 hrs after treatment.

RESULTS -

Figure 1. Morphology

A. Hu1074 B. HepaRG™ Cells

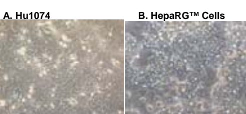
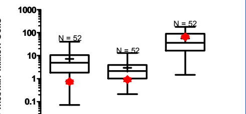


Figure 1– **HepaRG™ Cells and PHH share Similar Morphological Characteristics.**
Plated PHH and HepaRG™ Cells form a polarized 'epithelium' through cadherins/intergrins/tight junction protein interactions resulting in the formation of functional bile canaliculi-like structures. resembling the natural architecture of the liver.

Figure 2A. Baseline P450 Activity



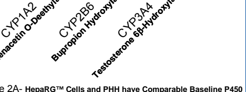
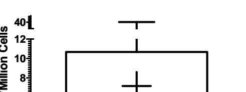
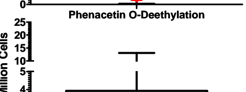


Figure 2A- **HepaRG™ Cells and PHH have Comparable Baseline P450 Activity.**
Comparison of baseline P450 activity in PHH and HepaRG™ Cells. Box and whisker plots were generated using data from multiple PHH preparations. P450 activity was evaluated in HepaRG™ Cells after 4 days in culture and is represented by red diamonds for each activity. All data plotted on a Log Y-axis scale to accommodate all three activities evaluated.

Figure 2B. Baseline P450 Activity





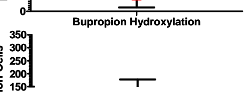
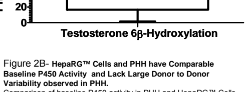


Figure 2B- **HepaRG™ Cells and PHH have Comparable Baseline P450 Activity and Lack Large Donor to Donor Variability observed in PHH.**
Comparison of baseline P450 activity in PHH and HepaRG™ Cells. Box and whisker plots were generated using data from multiple PHH preparations (N=52). P450 activity was evaluated in HepaRG™ Cells after 4 days in culture and is represented by red diamonds for each activity. Same data from Figure 2A; however, data plotted using a linear Y-axis scale.



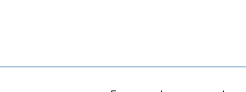


Figure 3. Induction of P450 Activity

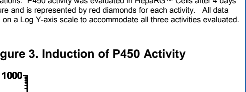


Figure 3- **HepaRG™ Cells™ and PHH P450 Activity Induction Responses are Equivalent.**
P450 enzyme activity induction responses in HepaRG™ Cells as compared to those observed in multiple preparations of PHH. All cells were treated for 72 hrs in culture prior to evaluating P450 activity in situ.

Figure 4. Induction of P450 mRNA

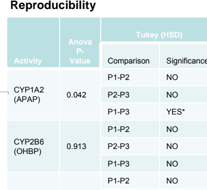
Figure 4- **HepaRG™ Cells and PHH P450 mRNA Induction Responses are Similar.**
P450 enzyme mRNA induction responses in HepaRG™ Cells as compared to those observed in multiple preparations of PHH. HepaRG™ Cells were treated for 72 hrs in culture prior to evaluating P450 mRNA expression. Activity and mRNA data were generated from same treated HepaRG™ Cells . PHH were treated for 48 hrs in culture prior to evaluating P450 mRNA.

Figure 5. Intra- and Inter-Plate Reproducibility

CYP1A2 Activity / CYP2B6 Activity / CYP3A4 Activity / CYP3A4 Activity

Figure 5- **P450 Activity Induction Responses are Remarkably Consistent in HepaRG™ Cells .**
P450 activity induction responses in HepaRG™ Cells were compared across three 96-well plates for each activity evaluated . Activities were evaluated after 72 hrs of treatment in culture with 50 µM OMP (phenacetin O-deethylation), 1mM PB (bupropion hydroxylation), or 10 µM RIF (midazolam 1-hydroxylation and testosterone 6β-hydroxylation) .

Table 1. Intra and Inter-Plate Reproducibility



Activity	Anova P-Value	Tukey (HSD) Comparison	Significance
CYP1A2 (APAP)	0.042	P1-P2	NO
		P2-P3	NO
		P1-P3	YES*
CYP2B6 (OHBP)	0.913	P1-P2	NO
		P2-P3	NO
		P1-P3	NO
CYP3A4 (Mdz)	0.562	P1-P2	NO
		P2-P3	NO
		P1-P3	NO
CYP3A4 (6βT)	0.265	P1-P2	NO
		P2-P3	NO
		P1-P3	NO

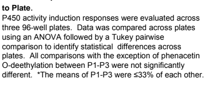


Table 1- **HepaRG™ Cells are Consistent from Plate to Plate.**
P450 activity induction responses were evaluated across three 96-well plates. Data was compared across plates using an ANOVA followed by a Tukey pairwise comparison to identify statistical differences across plates. All comparisons with the exception of phenacetin O-deethylation between P1-P3 were not significantly different. *The means of P1-P3 were ≤33% of each other.

Figure 6. P450 Enzyme Induction Regulatory Pathways

AhR ARNT → CYP1A Xenobiotic metabolism
PAHs, Xenobiotics, Steroids (Prototypical Inducer: OMP)

CAR RXR → CYP2B Xenobiotic, Steroid metabolism
PB, Steroids, Xenobiotics (Prototypical Inducer: PB)

PXR RXR → CYP3A Xenobiotic, Steroid metabolism
Bile Acids, Steroids, Xenobiotics (Prototypical Inducer: RIF)

Figure 6- **All Major P450 Enzyme Induction Regulatory Pathways are Functional in HepaRG™ Cells unlike Fa2N-4 Cells.**
HepaRG™ Cells were treated with specific agonist for each of the three major P450 regulatory pathways to demonstrate that each pathway is functioning properly. Cells were treated for 72 hrs in culture with each compound at three concentrations prior to evaluating P450 activity in situ. HepaRG™ Cells exhibited a dose dependent increase in the respective P450 activity for each agonist used suggesting that each regulatory pathway is functioning appropriately.

Figure 7. Cytotoxicity

Figure 7- **HepaRG™ Cells Exhibit both Metabolism-Dependent and -Independent Toxicity Mechanisms.**
HepaRG™ Cells were cultured in either 730 (tox) or 720 (metabolism) media for 3 days prior to dosing with chlorpromazine or aflatoxin . Cells were dosed in culture for 24 hrs prior to evaluating ATP concentrations. Aflatoxin exhibited a more potent toxic response in 720 media as indicated by a 2-fold decrease in the estimated EC_{50}. 720 media supports higher P450 activity than 730 suggesting that a metabolism-dependent toxicity mechanism is involved. However, similar dose response curves were generated in either media for chlorpromazine suggested that a metabolism-independent toxicity mechanism was involved.

Figure 8. Inhibition of Metabolism Dependent Cytotoxicity

Figure 8- **HepaRG™ Cells Supports Metabolism-Dependent Toxicity Mechanisms.**
HepaRG™ Cells were cultured in 720 (metabolism) media for 3 days prior to dosing with aflatoxin, ketoconazole, or both. Cells were dosed in culture for 24 hrs prior to evaluating ATP concentrations. Ketoconazole, a CYP3A4 selective inhibitor, fully or partially inhibited ATP reduction in cells treated with aflatoxin. These data indicate that aflatoxin toxicity is metabolism-dependent and is consistent with previous observations in HepaRG™ Cells (Fig. 7)

CONCLUSIONS -

• Baseline P450 Activities in HepaRG™ Cells were comparable to those observed in the spectrum of PHH preparations

•Induction of P450 activity in HepaRG™ Cells was comparable to the induction responses observed in a range of PHH preparations treated with the prototypical hepatic inducers of xenobiotic metabolism

•Cytochrome P450 activity induction responses in HepaRG™ Cells were consistent and reproducible from plate to plate

•Data demonstrated that all three major P450 enzyme regulatory pathways (CAR, PXR, and AhR) were functional in HepaRG™ Cells, unlike Fa2N-4 Cells that lack liver-like CAR expression (6). Pathway activation in HepaRG™ Cells was monitored at both the enzyme activity and mRNA expression levels

•HepaRG™ Cells support both metabolism-dependent and –independent toxicity mechanisms.

REFERENCES -

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Material and Methods Continued-

•**PHH In Situ Incubations:** Medium was aspirated from plates, and the cell monolayers were rinsed with Hanks' Balanced Salts Solution (HBSS). HBSS containing the P450 marker substrates phenacetin (CYP1A2), bupropion (CYP2B6), midazolam (CYP3A4), or testosterone (CYP3A4) was added directly to the monolayers. Plates were incubated at approximately 37°C in a humidified chamber while mixing on an orbital shaker. At the end of the incubation periods, samples were collected and stored frozen at –70°C until they were processed for LC-MS/MS analysis.

•**HepaRG™ Cells In Situ Incubations:** Medium was aspirated from plates, and the cell monolayers were rinsed with PBS. WEM supplemented with HPRG720 containing the P450 marker substrates phenacetin (CYP1A2), bupropion (CYP2B6), midazolam (CYP3A4), or testosterone (CYP3A4) was added directly to the monolayers. Plates were incubated at approximately 37°C in a humidified chamber while mixing on an orbital shaker. At the end of the incubation periods, samples were collected and stored frozen at –70°C until they were processed for LC-MS/MS analysis.

•**Activity Analysis:** Metabolite formation was measured by standard biochemical assays using GLP-validated LC-MS/MS assays. At least 6 calibration standards and 12 quality control samples (at 3 different concentrations) were used to evaluate the quality of the analytical runs. The extent of induction was evaluated by comparing the normalized enzyme activities of the inducer-treated cells to those of the vehicle control (0.1% DMSO) and calculating fold induction.

•**mRNA Analysis:** At the end of the treatment period, RNA was isolated from dosed cells according to the recommended procedures for the ABI PRISM® 6100 Nucleic Acid PrepStation (Life Technologies). Relative mRNA was analyzed using TaqMan® Assay methodology utilizing primer/probe sets for CYP1A2, CYP2B6, and CYP3A4 target cDNA as well as an endogenous control.

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