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

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ABSTRACT/Introduction –

Currently, primary human hepatocytes (PHHs) are used as the 'gold standard' *in vitro* hepatic model system due to their ability to support mature hepatic phenotypes (e.g. metabolism, transport, and induction) important in drug clearance, drug-drug interaction (DDI), and safety assessments. However, the use of PHHs for screening applications to identify potential DDI or safety liabilities earlier has been limited by availability, lot-to-lot variability, finite lot sizes and cost. Alternative model systems including HepG2 and Fa2N-4 have shown promise for certain screening applications (e.g. AhR-, PXR-mediated induction); however, these cell lines have failed to support the broader complement of PHH functionality (e.g. basal metabolism, biliary polarization, or lack of regulatory factors). Fresh HepaRG™ Cells have been shown to support mature hepatic phenotypes, but the perishable nature of fresh cells has severely limited their availability to the global community. Herein we report the characterization of cryopreserved HepaRG™ Cells for drug metabolism (e.g. CYP1A2, CYP2B6, CYP2C, CYP2D6, CYP3A4, UGT, SULT, and FMO) over multiple time points, induction (AhR, PXR, CAR), transport (uptake of taurocholate and estrone sulfate) and cytotoxicity (e.g. aflatoxin B1) with comparisons to multiple PHH preparations. Through these assessments, we demonstrate that cryopreserved HepaRG™ Cells have comparable functionality to PHH. We also show that cryopreserved HepaRG™ Cells maintain CAR responsiveness with specific activators, a critical pathway that is absent in Fa2N-4 cells and is generally lacking in lower quality lots of PHH. Furthermore, we provide induction data of CYP1A2, CYP2B6, and CYP3A4 enzyme activity in response to prototypical AhR, CAR, and PXR activators in multiple lots of both HepaRG™ Cells and PHH. These data show that induction responses in three different lots of HepaRG™ Cells were highly reproducible as compared to induction responses observed in 52 different lots of PHH. In conclusion, we present for the first time metabolism, transport, and induction comparisons between cryopreserved HepaRG™ Cells and PHH demonstrating the superior reproducibility of this model system with comparable functionality. These results coupled with demonstrating the longevity (≥22 days) of HepaRG™ cells in culture highlight this hepatic model system's potential in the study and prediction of xenobiotic clearance, DDI, and safety assessment applications.

HepaRG™ Cells Background

•HepaRG™ Cells were derived from a differentiated human hepatoma at the Institut national de la santé et de la recherche médicale (Inserm) of France (1).

•HepaRG™ Cells are bi-potent hepatic progenitor cell line that differentiates 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 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 DDI and/or hepatotoxicity potential (4-5).

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

MATERIALS AND METHODS –

•**Materials:** Cryopreserved HepaRG™ Cells, Williams Medium E (WEM), Collagen I Coated 96-well Plates, GlutaMAX™ Supplement, HPRG770 medium supplement, HPRG720 medium supplement, HPRG740 medium supplement, Cryopreserved Hepatocyte Recovery Medium (CHRM®), Geltrex™ Matrix, ITS+, and PHH were obtained from Life Technologies.
•**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™ supplements. Media was renewed next day with WEM supplemented with HPRG720 and GlutaMAX™ supplements. HepaRG™ Cells were cultured for 4, 10, or 22 days prior to conducting in situ incubations with prototypical substrates to evaluate drug metabolizing enzyme (DME) activity. Media was renewed every 2-3 days.
•**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™ supplements. Media was renewed the day following plating with WEM supplemented with HPRG770 and GlutaMAX™ supplements. To evaluate P450 induction properties, HepaRG™ Cells were maintained in WEM supplemented with HPRG740 and GlutaMAX™ supplements 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).
•**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 and GlutaMAX™ supplements. Media was replaced the next day with WEM supplemented with HPRG720 and GlutaMAX™ supplements. Media was refreshed every two days thereafter. HepaRG™ Cells were treated with aflatoxin on day four. 24 hours after treatment, ATP depletion assays were performed.
•**Culture for Uptake:** HepaRG™ Cells were thawed and plated (1E5 cells/well) onto a 96-well collagen I coated plates using WEM supplemented with HPRG770 and GlutaMAX™ supplement. Media was replaced the next day with WEM supplemented with HPRG750 and GlutaMAX™ supplements. Uptake studies in HepaRG™ Cells culture were performed on the 4 day of culture.
•**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 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.
•**PHH In Situ Incubations:** Medium was aspirated from plates, and the cell monolayer was 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 monolayer. 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.

RESULTS -

Figure 1. Morphology

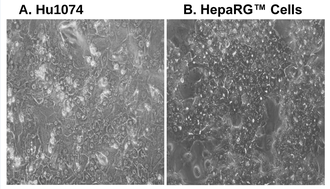


Figure 1– **HepaRG™ Cells and PHH share Similar Morphological Characteristics.**
Plated PHH and HepaRG™ Cells form a polarized 'epithelium' through cadherins/intergrins/tight junction protein interactions resulting in the formation of functional bile canaliculi-like structures. resembling the natural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

Figure 2. Baseline P450 Activity

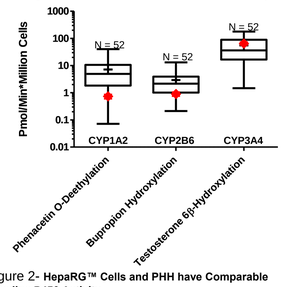


Figure 2- **HepaRG™ Cells and PHH have Comparable Baseline P450 Activity.**
Comparison of baseline P450 activity in PHH and HepaRG™ Cells. Box and whisker plots were generated using data from multiple PHH preparations (N=52). P450 activity was evaluated in HepaRG™ Cells after 4 days in culture and is represented by red diamonds for each activity. All data plotted on a Log Y-axis scale to accommodate all three activities evaluated.

Table 1. Baseline DME Activity

Enzyme	Substrate	Marker Metabolite	Donor	Specific Activity (pmol/min*million cells)	Std Dev
CYP1A2	Phenacetin	Acetaminophen	HepaRG PHH (N=52)	2.78 0.072-40.3	0.73
CYP2A6	Coumarin	7-Hydroxycoumarin	HepaRG PHH (N=9)	0.85 0.12-9.87	0.16
CYP2B6	Bupropion	Hydroxybupropion	HepaRG PHH (N=52)	17.9 0.21-13.1	2.21
CYP2C8	Paclitaxel	6α-Hydroxypaclitaxel	HepaRG PHH (N=7)	0.22 0.064-0.24	0.04
CYP2C9	Diclofenac	4'-Hydroxydiclofenac	HepaRG* PHH (N=3)	3.94 8.44-31.2	0.38
CYP2C19	Mephenytoin	4'-Hydroxymephenytoin	HepaRG PHH (N=13)	1.52 0.10-23.5	0.41
CYP2D6	Dextromethorphan	Dextrorphan	HepaRG** PHH (N=2)	0.40 3.93-14.0	0.06
CYP3A4	Testosterone	6β-Hydroxytestosterone	HepaRG PHH (N=52)	248 1.47-178	50.9
CYP3A4	Midazolam	1-Hydroxymidazolam	HepaRG PHH (N=0)	28.4 NA	2.39
FMO	Benzydamine	Benzydamine N-oxide	HepaRG PHH (N=0)	17.3 NA	1.74
UGT	7-Hydroxycoumarin	7-Hydroxycoumarin Glucuronide	HepaRG PHH (N=0)	346 NA	49.7
SULT	7-Hydroxycoumarin	7-Hydroxycoumarin Sulfate	HepaRG PHH (N=0)	9.23 NA	2.92

Table 1-**Characterization of Phase I and Phase II Drug Metabolizing Enzymes in HepaRG™ Cells.**
Drug metabolizing enzyme activities were evaluated in HepaRG™ Cells after culturing in 720 (metabolism) media for 10 days. PHH enzyme activities were evaluated after 4 days in culture. PHH data in this table is provided as a range showing the lowest and highest values observed within the sample population characterized. * HepaRG CYP2C9 Genotype = *2/*2; ** HepaRG CYP2D6 Genotype = *2/WT & *9/WT; NA = Not Available

Figure 3. Long-term Culture

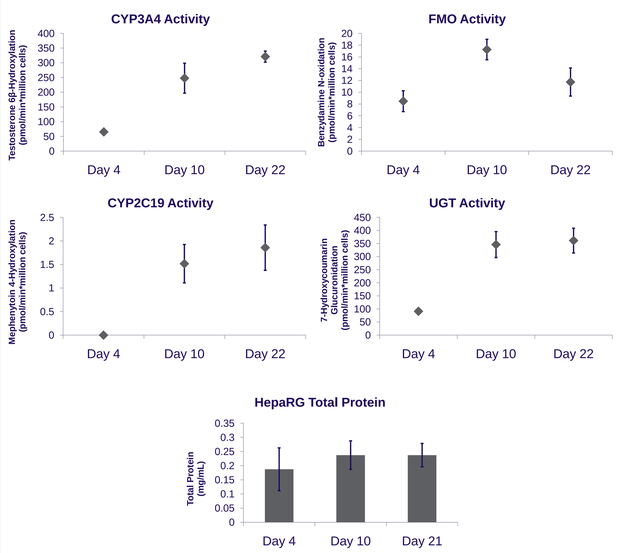


Figure 3 **HepaRG™ Cells Supports DME Activity in Long-term Cultures**
HepaRG™ Cells were cultured in 720 (metabolism) media for 4, 10, and 22 days prior to evaluation of DME activity *in situ*. In a parallel experiment, no significant changes in total protein were observed over time suggesting that increases in DME activity is not due to increase in cell number..

Figure 4 Inter-Lot Reproducibility

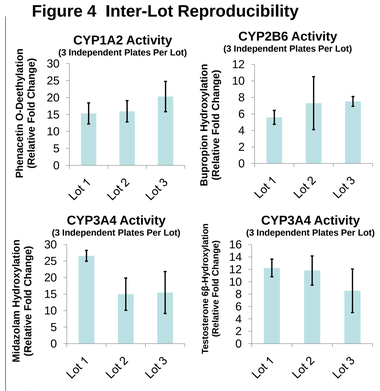


Figure 4- **P450 Activity Induction Responses are Remarkably Consistent in HepaRG™ Cells .**
P450 activity induction responses in HepaRG™ Cells were compared across three different lots for each activity evaluated . Activities were evaluated after 72 hrs of treatment in culture with 50 µM OMP (phenacetin O-deethylation), 1mM PB (bupropion hydroxylation), or 10 µM RIF (midazolam 1-hydroxylation and testosterone 6β-hydroxylation) .

Figure 5. Induction of P450 Activity

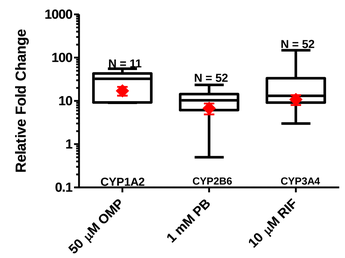


Figure 5- **HepaRG™ Cells™ and PHH P450 Activity Induction Responses are Equivalent.**
P450 enzyme activity induction responses in 3 different lots of HepaRG™ Cells as compared to those observed in multiple preparations of PHH. PHH data is represented by box and whisker plots using data from multiple PHH preparations (N=11 or 52). HepaRG™ data is represented by red diamonds. All cells were treated for 72 hrs in culture prior to evaluating P450 activity *in situ*.

Figure 6. P450 Enzyme Induction Regulatory Pathways

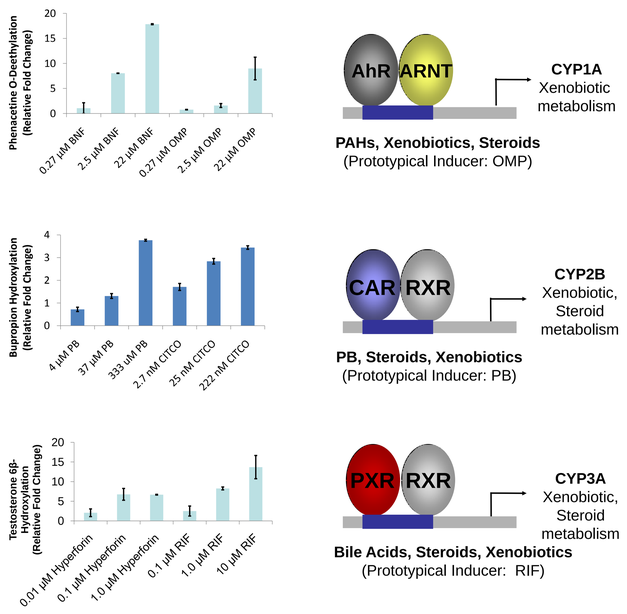


Figure 6- **All Major P450 Enzyme Induction Regulatory Pathways are Functional in HepaRG™ Cells unlike Fa2N-4 Cells.**
HepaRG™ Cells were treated with specific agonist for each of the three major P450 regulatory pathways to demonstrate that each pathway is functioning properly. Cells were treated for 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.

Table2. Inter-Lot Reproducibility

Activity	Anova P-Value	Tukey (HSD) Comparison	Significance
CYP1A2 (APAP)	0.212	L1-L2	NO
		L2-L3	NO
		L1-L3	NO
CYP2B6 (OHBP)	0.692	L1-L2	NO
		L2-L3	NO
		L1-L3	NO
CYP3A4 (Mdz)	0.124	L1-L2	NO
		L2-L3	NO
		L1-L3	NO
CYP3A4 (6βT)	0.247	L1-L2	NO
		L2-L3	NO
		L1-L3	NO

Table 2- **HepaRG™ Cells are Consistent from Lot to Lot.**
P450 activity induction responses were evaluated across three different HepaRG™ Cell lots. Data was compared across lots using an ANOVA followed by a Tukey pair wise comparison to identify statistical differences across lots. All comparisons between L1, L2, and L3 were not significantly different.

Figure 7. Bile Canaliculi Formation

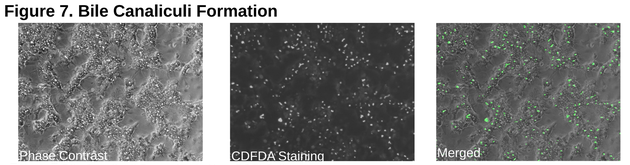
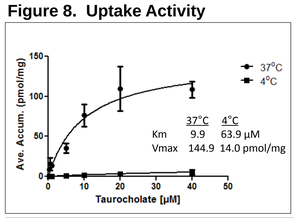


Figure 7 **Bile Canaliculi Formation in HepaRG™ Cells**
HepaRG™ Cells were cultured for 10 days prior to examination of bile canaliculi formation using CDFDA Staining. CDFDA staining demonstrates that bile canaliculi-like structures form exclusively within the hepatocyte-like cell population.

Figure 8. Uptake Activity



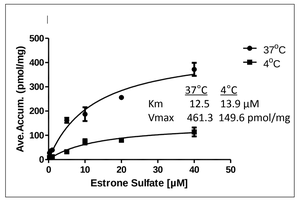


Figure 8 **Active Uptake in HepaRG™ Cells**
HepaRG™ Cells were cultured in 750 serum-free induction media for 3 days prior to evaluation of uptake activity. Radio labeled substrates were incubated with HepaRG cells for 30 minutes. After incubations ,cells were washed briefly and lysed for total protein and scintillation analysis.

Figure 9. Metabolism-Dependent Toxicity

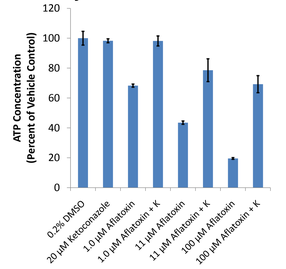


Figure 9- **HepaRG™ Cells Supports Metabolism-Dependent Toxicity Mechanisms.**
HepaRG™ Cells were cultured in 720 (metabolism) media for 3 days prior to dosing with aflatoxin, ketoconazole, or both. Cells were dosed in culture for 24 hrs prior to evaluating ATP concentrations. Ketoconazole, a CYP3A4 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.

CONCLUSIONS -

• Baseline P450 Activities in HepaRG™ Cells were comparable to those observed in PHH preparations
•HepaRG™ Cells support phase I and phase II enzyme expression and function
•HepaRG™ Cells support long-term expression and function of drug metabolizing enzymes for ≥ 22 days in culture
•Cytochrome P450 activity induction responses in HepaRG™ Cells were consistent and reproducible from lot to lot
•Induction of P450 activity in HepaRG™ Cells was comparable to the induction responses observed in PHH preparations treated with the prototypical hepatic inducers of xenobiotic metabolism
•Data demonstrated that all three major P450 enzyme regulatory pathways (CAR, PXR, and AhR) were functional in HepaRG™ Cells, unlike HepG2 and Fa2N-4 Cells that lack liver-like CAR expression (6)
•HepaRG™ Cells support bile canaliculi formation and the accumulation of CDF within these structures suggests that functional efflux transporters (i.e. MRP2) are present in HepaRG™ cultures
•Uptake activity studies demonstrate that functional uptake transporters (e.g. NTCP and OATP) are present in HepaRG™ cultures
•HepaRG™ Cells support metabolism-dependent toxicity mechanisms.

REFERENCES -

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Material and Methods Continued-

•**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.
•**Carboxy Dichlorofluorescein Diacetate (CDFDA) Staining:** HepaRG™ Cells were thawed and plated (3E5 cells/well) onto a 24-well collagen I coated plates using WEM supplemented with HPRG770 and GlutaMAX™ supplements. Media was replaced the next day with WEM supplemented with HPRG720 and GlutaMAX™ supplements. Media was refreshed every two days thereafter. After 10 days of culture, medium was aspirated from plates and the cell monolayer was washed with warm HBBS. HepaRG™ Cells were incubated for 15 minutes with cell culture media containing 5 µM CDFDA. After incubation, photomicrographs were taken of cell culture.
•**Uptake Analysis:** Cell culture medium was aspirated from plates and the cell monolayer was washed three times with warm HBBS. HepaRG™ Cells were incubated with the third wash at 37°C in humidified incubator with 95% air/5% CO2 for ten minutes. Buffer was then replaced with radio labeled compound dose solution prepared in HBSS buffer and incubated for 30 minutes. After incubation, dose solutions were aspirated and HepaRG™ Cells were washed four times with ice cold buffer and placed at -80C for a minimum of 15 minutes prior to cell lyses with 0.5% Triton X solution. Samples were analyzed using a plate scintillation counter.
Protein Analysis: Protein analysis was performed using Thermo Scientific Pierce BCA Protein Assay Kit following the manufacturer's instructions.
ATP Analysis: ATP depletion assays were purchased from Promega and completed following manufacturer's instructions