Abstract

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Evaluation of *in vitro* Toxicogenetic Models for Hepatotoxicity

(Under the direction of Ivan Rusyn MD. PhD.)

Numerous studies support the fact that a genetically diverse mouse population may be useful as an animal model to understand and predict toxicity in humans. We hypothesized that cultures of hepatocytes obtained from a large panel of inbred mouse strains can produce data indicative of inter-individual differences in *in vivo* responses to hepato-toxicants. In order to test this hypothesis and establish whether high-throughput *in vitro* studies using cultured hepatocytes from genetically distinct mouse strains are feasible, we aimed to: (1) determine whether the near-physiological maintenance of the cells isolated from different mouse inbred strains can be achieved, (2) evaluate whether viability and reproducibility of functionality be attained over subsequent isolations and (3) assess the utility of the model for toxicity screening. Our data suggest that cell function and expression of key liver specific genes of hepatocytes isolated from different strains is comparable. These experiments open new opportunities for high-throughput and low-cost *in vitro* assays that may be used for studies of toxicity in a genetically diverse population.

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List of Abbreviations

APAP- Acetaminophen

ATP- Adenosine triphosphate

EC_{50} – Concentration at 50% activity

CYP450 – Cytochrome P450

DILI- Drug induced liver injury

DMSO - Dimethyl sulfoxide

FBS- Fetal bovine serum

LDH- Lactate dehydrogenase

NIEHS- The National Institutes of Environmental Health Sciences

NAPQI- N-acetyl-p-benzo-quinone imine

NTP – National Toxicology Program

PXR – Pregnane X receptor

SL- Single layer

SNP- Single nucleotide polymorphism

Chapter 1

Literature Review

The unique characteristics of the laboratory mouse and its prominent role as a model organism in toxicology make this species a logical choice for testing new approaches. The mouse represents a unique model organism that is most helpful in addressing the potential drug-induced toxicities and their mechanisms, as well as aid in identification of the unique genetic determinants for inter-individual differences in susceptibility in humans (1). Unlike human populations, where experimental tests cannot be controlled, selective breeding of mice led to the development of unique strains that can be investigated under specific conditions (2). At the same time, different strains are representative of genetically diverse population and can be used as a model for human diversity (3). While mice are widely used in toxicology research, the exploitation of this genetic variation for experimental purposes has not been fully realized yet.

In parallel with a large effort to sequence the human genome, considerable effort is being put into re-sequencing mice used to model human diseases. A publicly-funded effort has sequenced the whole genome of the C57BL/6J inbred mouse strain and NIEHS Center for Rodent Genetics has re-sequenced 15 additional strains (4). In addition, several mouse SNP databases have been compiled that include data on up to 300,000 SNPs across about 70

mouse strains (5). SNPs are becoming the markers of choice for the study of complex genetic traits.

Our laboratory at UNC in the past 4 years have developed and validated an *in vivo* strategy using a mouse diversity panel (1;6) for a bedside-to-bench-to-bedside paradigm to identify genetic polymorphisms and novel mechanisms contributing to drug induced liver injury (DILI). The existence of DILI was verified in a subset of humans given the maximal recommended dose of acetaminophen. In the mouse diversity panel of 36 inbred lines, a whole-genome haplotype association analysis and targeted sequencing of the revealed polymorphisms identified four candidate genes that correlate strongly with acetaminophen-induced liver injury in mice. Furthermore, we validated these findings in two independent human cohorts. We hypothesized that a panel of inbred mouse strains is representative of a genetically diverse population and can be used as a model for human diversity.

Similarly Liu and colleagues, demonstrated that multiple genetic loci and an interacting network of metabolic factors affect susceptibility to acetaminophen induced liver injury in a panel of 16 inbred mouse strains (7). The authors conducted an integrated genetic, transcriptional and metabolic analysis to evaluate changes in hepatic gene expression and production of hepatic endogenous metabolites. The genetic factors responsible for betaine-homocysteine methyltransferase-2 (BHMT2) enzyme activity were identified as one of the factors contributing to susceptibility. It was also observed that BHMT's substrate, (S-methlymethionine), aided in the protection of acetaminophen induced liver injury in a strain-dependent manner (7).

Work published by Guo et al. showed the utility of a mouse diversity panel to elucidate strain-specific responses to treatment *in vivo* (8). The authors demonstrated that the

metabolism of warfarin, specifically the generation of 7-hydroxywarfarin, varied across a panel of inbred mouse strains, phenotypic differences were then computationally linked to the genomic region that encodes for CYP2C enzymes. To further validate this approach the group conducted a study *in vitro* in which the biotransformation of testosterone and irinotecan was investigated across 15 mouse lines. The results from this study revealed that underlying genetic variation within the CYP2B9 and UGT1A loci played a significant role in the metabolism of α-hydroxytestosterone and irinotecan glucuronidation (9).

These studies support the fact that a genetically diverse mouse population may be useful as an animal model to understand and predict rare adverse drug events in humans.

Chapter 2

Introduction

The growing list of chemical substances in commerce and the complexity of environmental exposures from agents of natural origin that are encountered in our daily lives represent an enormous challenge to the National Toxicology Program (NTP) and other health regulatory and research agencies that examine the toxic potential of exposures (10). Current chemical hazard testing procedures can only address a small fraction of these agents as single exposures in order to provide sufficient information to meet the extensive data needs under the current regulatory risk assessment paradigms (11). Options for a new toxicity testing paradigm, suggest a gradual transition from apical end-point testing to a system based largely on toxicity-pathway batteries (12). It is envisioned that data generated from high-throughput assays will provide a substantial proportion of the toxicity-pathway information needed for environmental decision-making (12). Therefore, a significant amount of attention should be directed towards the refinement and development of current high-medium throughput *in vitro* models for toxicity.

Isolates of primary hepatocytes constitute one of the most widely adopted applications in investigative toxicology as a method to evaluate hepatic drug metabolism and genotoxic potential of substances (13). Additionally, freshly isolated hepatocytes maintain expression and activity of enzymes and proteins, relevant to the metabolism and transport of drugs, and

arranged in a physiologically important context and regulated via cellular processes that occur within the liver *in vivo* (14). Cultures of primary hepatocytes have been established as valid *in vitro* toxicological models for many years and provide useful alternatives to intact animal testing (15). *In vitro* culture systems offer the potential to greatly reduce the number of animals needed to screen large numbers of compounds for adverse effects and can potentially provide extension to the human condition (16). Unfortunately, there are some inherent drawbacks to using primary hepatocytes, including the absence of organ-specific cell to cell interactions and the inability of cultured cells to maintain expression of key liver-specific genes related to metabolism in long-term cultures (14;17).

Studies that examine experimental exposures of toxicants in people are few for ethical reasons, making it difficult to understand genetic associations in human cohorts exposed in the work place or other environmental settings without validation in animal studies. Current approaches to toxicity screening and mechanistic studies do not take into account the genetic diversity present within populations and largely ignore uncertainties about how genetic variability might interact with environmental exposures to affect risk (18). These studies are generally carried out in a single strain in order to fix as many variables as possible. Additionally, conducting toxicity and mechanistic studies in a single strain provides the added benefit of standardizing the genotype across multiple chemicals.

Current approaches fail to capture the complexity of human toxicity with regard to diverse genetic backgrounds, uncontrolled environmental effects and duration of exposure. To address some of the current limitations in the interpretation of data with respect to population wide effects, toxicologists are using various mouse models. These models are being utilized to evaluate gene-environment interaction and assess genetic causes of inter-

individual variability in toxicity. Panels of genetically distinct animals are representative of a genetically diverse population and provide a fixed genotype for a given strain. Inbred mouse strains are well suited for systems biology approaches to enhance our understanding of modes of action, and furthermore, aid in the discovery of new biomarkers correlated with response to exposure. Each inbred mouse strain represents an unlimited supply of the genetically identical individuals, presenting the opportunity for repeat testing, which is important for demonstrating reproducibility of response.

Our goal is to establish whether high-throughput *in vitro* studies using cultured hepatocytes isolated from genetically distinct mouse strains can produce data that may be used for predictions of *in vivo* adverse effects of chemical agents in heterogeneous populations. First, we plan to establish whether standardized cell isolation and culture conditions are sufficient for the near-physiological maintenance of the cells isolated from different mouse inbred strains. Second, we plan to conduct a comparative *in vitro*/*in vivo* analysis of toxicant-induced responses to model toxicants.

Chapter 3

Methods

Chemicals: Acetaminophen, Phenobarbital, WY-14,643 and dexamethasome were purchased from Sigma Chemical (St. Louis, MO). Rifampin was kindly provided by Dr. David Dix (US EPA, Research Triangle Park, North Carolina). Calcein AM and ethidium homodimer-1 were obtained from Molecular Probes (Eugene, OR). Collagenase type I and fetal bovine serum was purchased from Sigma-Aldrich (St. Louis, MO). Insulin selenium transferrin was obtained from GIBCO (Grand Island, NY).

Animals: Primary mouse hepatocytes were isolated from 4-6 week old male mice (strains A/J, B6C3F1, BALB/cJ, C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/2J, FVB/NJ, BALB/cByJ, AKR/J, MRL/MpJ, NOD/LtJ, NZW/LacJ, PWD/PhJ and WSB/EiJ) from Jackson Laboratories (Bar Harbor, Maine). Strains were selected on the basis of their lineage and the extensive experience with some strains (i.e. B6C3F1/J, C57BL/6J and NOD/LtJ) was also taken into account. Mice were housed by strain in groups of three in a constant alternating 12-h light and dark cycle and allowed free excess to food and water. All procedures were approved by the Institutional Animal Care and Use Committee.

Preparation of culture dishes: One day prior to hepatocyte culture preparation, 24-well plastic culture plates were precoated with a collagen solution (PureCol^{TM} purified collagen),

to obtain a simple (rigid) substratum. Collagen solution (38.75μg/ml) was added to each well. Precoated plates were incubated at 37C and 5% CO_2 for 30 min. Following incubation the plates were washed with PBS and allowed to dry under UV light in a sterile hood. For the third experiment 24 and 96-wel plates pre-coated with SL collagen type I were purchased from BD Biosciences (Bedford, MA).

Isolation and culture of mouse hepatocytes: Primary hepatocytes were prepared and maintained by CellzDirect/Life Technologies (RTP, NC) for experiments 1 and 2. For experiment 3, primary hepatocytes were prepared by CellzDirect and maintained at the Laboratory of Environmental Genomics at UNC-Chapel Hill. Cell isolation and maintenance protocols were performed according to CellzDirect's standard operating procedures for mouse hepatocytes at the start of the study. Hepatocytes were isolated using a two-step collagenase perfusion method. In brief, the animals were anesthetized with a cocktail of xylazine and ketaset 10mg/ml, i.p. (2mg/10mg xlyazine Lloyd Laboratories, 2mg/10mg ketaset HCl Fort Dodge). Hepatocyte cultures were prepared at the same time of day throughout the study as diurnal effects have been shown to affect gene expression in rodent studies. The liver was perfused *in situ* with a saline solution without magnesium and calcium and a collagenase buffer (approximately 4.5 mg of collagenase type IV, Sigma), the solutions were warmed to 37-40C prior to use and remained warmed throughout the perfusion. A catheter was inserted into the atrium of the heart positioned in the superior vena cava just below the diaphragm. The portal vein was then cut. The liver was then perfused for approximately 12 min. (4 min. for the first perfusion solution and 8 min. for the second perfusion solution). Sterile q-tips were utilized to determine structural integrity, once it was

determined by the perfusionist that the liver had been fully digested it excised and fully disassociated in a supplemented culture medium containing 10% FBS (William's E culture media, Sigma). A filter (100 micron) was used to separate out debris from the desired cell fraction and a post filter yield and viability were obtained using a hemacytometer and the trypan blue exclusion method (Trypan Blue Sigma, 0.4%). Hepatocytes were purified by Percoll gradient centrifugation (Percoll®, Sigma), viable hepatocytes were then sequentially washed twice with WEM supplemented with 10% FBS and transferred to a tissue culture flask and placed on an orbital shaker (50 rpms) and allowed to incubate at 37C for 30 min. Subsequent wash steps were carried out to remove additional debris. Final yields and viability were calculated using the method describe above. Hepatocytes were suspended in a modified WEM in the presence of 10% FBS and added to the precoated dishes at a density of .150 x 10^6 cells/well of a 24-well plate and .02 x 10^6 cells/well for a 96 well plate, cells were allowed to attach for four hours at 37C and 5% CO2. Hepatocytes were cultured according to the workflow depicted in Fig. 1A and 1B. Four hours post plating the media was aspirated and .5 ml of fresh maintenance media without FBS was added to each well of a 24-well plate and 100µl per well for a 96-well plate and returned to the incubator for another 24h. Media was replenished on a daily basis.

Morphology: Phase contrast pictures were take of the hepatocytes in the single layer configuration at day 1,3,5 and 7 for the first two experiments, at a magnification of 200x with a (insert brand name of scope) inverted microscope. Cell morphology was assessed qualitatively by conducting intra and inter strain comparisons to day 1 cultures.

Photomicrographs were taken of hepatocytes on a daily basis for the third experiment at a magnification of 200x with a Olympus inverted microscope.

Fluorescence Microscopy: For the first and second experiments, hepatocytes were stained with calcein AM and ethidium homodimer-1 to qualitatively assess the proportion of live to dead cells (day 1,3,5 and 7). Each well received 1µl of dye per 1ml of supplemented WEM media and incubated at 37C for 15min. Subsequently, each well was rinsed three times with supplemented culture media to remove extracellular substrate before imaging at a magnification of 200x with a Olympus inverted microscope.

Cell and Media Harvest: Media was harvested on a daily basis. The first media harvest occurred 28h post-plating, subsequent media collections were performed every 24h. Media was pooled from two-wells on a culture plate, representative of one strain and time point, and transferred to a fresh eppendorf tube. Samples were then centrifuged for 3min. at 14000 rpm, supernatants were transferred to a 96-deep well block and stored at -20C. Immediately following media collections cultured cells were harvested in either 200µl of TRIzol™ reagent (Invitrogen) or PBS and stored at -80C at time points day 1, 3, 5 and 7.

Biochemical Assays: Harvested media samples were removed and allowed to thaw on ice for quantification of selected biochemical markers. Lactate dehydrogenase (LDH), pyruvate and lactate production was assessed by standard enzymatic procedures (Bergmeyer, 1970). Urea synthesis was evaluated using BioAssay Systems QuantiChrom™ Urea Assay Kit, this method uses a chromogenic reagent that forms a colored complex specifically with urea. To

account for pyruvate in the media all reported values have been subtracted from fresh media so that the data reflects the absolute pyruvate production by the cells.

Cytotoxicity: The stocks of reference compounds were prepared in DMSO at a concentration 200-fold higher than the exposure levels. On the day of exposure, working solutions were prepared by 200-fold dilution of the stocks in appropriate culture media, resulting in a final ratio of 2.5:500µl of stock solution to culture media. This method was employed to ensure a final vehicle concentration in the cultures was below .5%. Cytotoxicity of reference compounds to mouse hepatocytes was determined by measuring intracellular ATP content, GSH levels and caspase 3/7 activity. ATP and GSH levels were detected and quantified using CellTiter-Glo® Luminescent Cell Viability Assay and GSH-Glo™ Glutathione Assay (Promega) respectively as per the manufacturer's protocol. CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method that determines the cell viability based on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which signals the presence of metabolically active cells. GSH-Glo™Assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalyzed by glutathione S-transferase (GST). Caspase 3/7 activity was determined by Caspase-Glo® 3/7 (Promega), adding the caspase reagent results in cell lysis, followed by caspase cleavage of the substrate liberating free aminoluciferin, which is consumed by the luciferase, generating a luminescent signal.

The scheme for hepatocyte culture is described in Fig. 1C. Hepatocytes were prepared from 3 strains (B6C3F1/J, C57BL/6J and NOD/LtJ) and seeded in 24 and 96-well plates in a single layer configuration at 150,000 cell/well and 20,000 cells per well respectively.

Hepatocytes were incubated for 24h and 72h (two media changes at 24h and 48 h) before treatment with compounds. At treatment, culture media was replenished in each well with the appropriate volume of the working solution. After exposure to the reference compounds for 24h under normal incubation conditions, the recommended volumes of CellTiter-Glo®, GSH-Glo™ and Caspase-Glo® 3/7 solutions were added to each well of the 96-well plates and the cells were further incubated at room temperature as per the manufacture's protocol. Cultured cells and media were harvested from the 24-well plates as described above.

Protein: Protein quantification was performed using BCA™ Protein Assay Kit (Pierce) and the standard manufacturer's protocol, BSA was used as a reference standard.

RNA Extraction: Frozen samples were removed and allowed to equilibrate to room temperature. For cultured cells collagen layers were scraped with a sterile plastic scraper, 200μl of the TRIzol mixture was then transferred to a fresh eppendorf tube and homogenized using a 25 gauge needle and a 1ml syringe. The lysate was centrifuged for 15min. at 4C. From the resulting aqueous fraction, total RNA was extracted using the TRIzol reagent/Qiagen RNeasy Mini kit method and the standard manufacturer's protocol. All RNA samples are in a final elution volume of 50 microlitres of water. For assessment of total RNA integrity, samples were analyzed using RNA nano assay LabChips® (Agilent Technologies, Santa Clara, CA) and processed on a 2100 Bioanalyzer (Agilent Technologies) as per manufacturer's protocol. Quantification of RNA was also performed using the ND-1000 spectrophotometer (NanoDrop® Technologies).

cDNA preparation and RT-PCR Analysis: First-strand cDNA was prepared from 2μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as detailed by the manufacture. Transcripts encoding acyl-Coenzyme A oxidase 1 (ACOX1), albumin (ALB), carbamoyl-phosphate synthetase 1 (CPS-1), cytochrome P450, family 1, subfamily a, polypeptide 2 (CYP1A2), cytochrome P450, family 2, subfamily e, polypeptide 1 (CYP2E1), cytochrome P450, family 2, subfamily e, polypeptide 1 (CYP3A11), cytochrome P450, family 4, subfamily a, polypeptide 10 (CYP4A10), glutathione S-transferase, alpha 2 (GSTA2), hepatic nuclear factor 4, alpha (HNF4α), nuclear factor, erythroid derived 2, like 2 (NRF2), peroxisome proliferator activated receptor alpha (PPARα), UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1) and solute carrier organic anion transporter family, member 1b2 (SLCO1B2) were quantified by RT-PCR analysis (TaqMan Gene Expression Assays, Applied Biosystems). RNA samples, from selected time points, culture day 1 and 3, were analyzed for gene expression. Fluorescence intensity of the probes was quantified using the Roche Lightcycler 480 and manufacturer's software. Data analysis included the derivation of $2^{\Delta\Delta CT}$ values. The ΔC_t value for all genes were calculated relative to the average of C_t values for GUSB probes. The $\Delta\Delta Ct$ values were calculated using cultured cells ΔC_t values relative to mean ΔC_t values for strain-match whole liver samples.

Data and Statistical Analysis: The software package Graph Pad (version 4.0, Prism, La Jolla, CA) was used to plot the time-course and gene expression data, in addition to the dose-response curves and to calculate the EC_{50} values. Data are represented as mean values plus or minus the standard deviation for at least two replicate wells for each condition with a

minimum of 2 experimental repeats, with each experimental repeat representing cells from a different hepatocyte preparation. One-way ANOVA was used for statistical comparison with control (*). In cases in which more than one variable was compared (i.e. culture day and concentration) a two- way ANOVA with Tukey's multiple comparison test was utilized. A p value less than 0.05 was selected prior to the study to determine statistical significance between groups.

Chapter 4

Results

Assessment of cell viability and functionality in 15 strains

Conventional cultures from primary mouse hepatocytes were prepared from a total of 15 inbred mouse strains, using 3 different strains per week over 5 weeks. This study aimed to investigate basal differences in the functionality of isolated hepatocytes from distinct genetic backgrounds. Sample sizes were limited to n=1 due to logistical constraint of the study in collaboration with CellzDirect, subsequent experiments were conducted to test for reproducibility. We utilized standardized cell isolation and culture conditions to determine whether the near-physiological maintenance of the cells can be achieved across a panel of inbred strains. The experimental design for this study is depicted in Fig. 1a. At brief, the hepatocytes were isolated at t=0 and cultured out to day 7, culture media was harvested daily and the cultured cells were harvested on days 1, 3, 5 and 7 (Fig. 1a). Culture media was replenished on a daily basis. Total cell numbers, representing liver parenchymal cell yields, were compared between strains (Supplementary Table 1). The ability to achieve good cell yields was obtained across the panel of strains, with a mean and standard deviation of 59.3 ± 13.8 million cells per animal. Final viability, as determined by Trypan Blue exclusion, revealed little difference between strains, with a mean and standard deviation of 92.8 ± 3.5%.

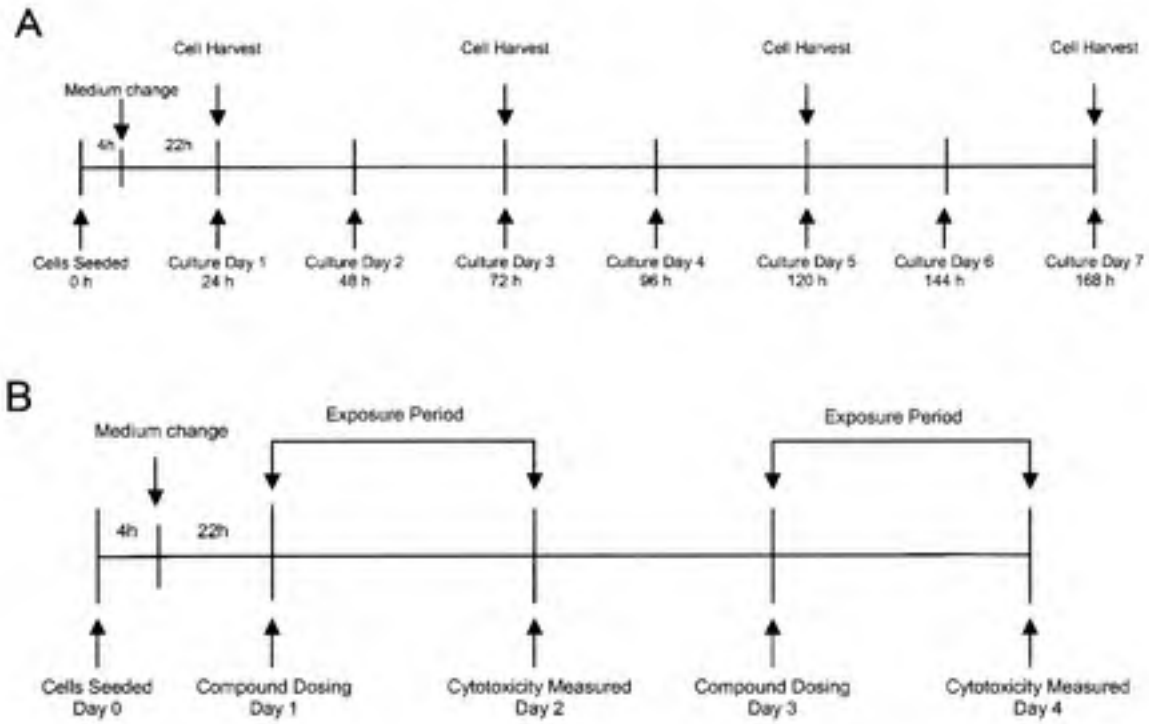


Figure 1: Schemes of primary cultures of mouse hepatocytes in a conventional monolayer for the measurements of basal level functionality and expression (A) and cytotoxicity (B). For functionality and expression experiments (A) handcoated collagen type I 24-well plates were prepared 24 h prior to seeding. Hepatocytes were seeded at t= 0 h with WEM supplemented with 10% FBS and allowed an attachment period of 4h after which fresh WEM without FBS was replaced. Media harvest was carried out daily and cells were harvest on culture day 1, 3, 5 and 7. To evaluate cytotoxicity cells were plated in precoated collagen type 1 96-well plates from BDTM. The cultures were treated with 0.5%(v/v) DMSO or the reference compounds (dissolved in DMSO) in 100μL of WEM without FBS, at dosing days 1 and 3 for a period of 24 h under normal incubation conditions. Cytotoxicity was measured at days 2 and 4.

Phase contrast microscopy was employed to assess morphological changes in the hepatocytes over time and evaluate monolayer confluency and cell health. Morphological assessment at culture day 1 revealed a healthy monolayer, as indexed by a typical configuration of cuboidal cells and confluency generally greater than 90%. Little differences were observed between strains at culture day 1. The progressive deterioration of the cultures starting at day 3 continuing on to day 7, marked by the loss of monolayer confluency and a high density of cells with irregular shape, was observed in cultures representative of all 15

strains. Fluorescence staining of calcein AM and ethidium homerdimer-1 was employed simultaneously in this study to qualitatively examine cell viability and death (data not shown). Images showed a greater number of dead cells on culture day 7 compared to culture day 1.

Samples of culture media were collected on a daily basis over a 7-day period and analyzed for LDH release, pyruvate, lactate and urea production to assess functionality. Following assessment, LDH, pyruvate, lactate and urea levels were normalized to the average release/production of each marker over the course of culture for each strain, this value is equivalent to 100% (Fig. 2). This method of normalization was chosen for an easy comparison of trends across the panel of strains. The overall trend of LDH release, a marker of cellular membrane integrity, was similar between strains typically stabilizing by culture day 2 (Fig. 2a). Pyruvate and lactate were measured as surrogate markers of cellular respiration in the aerobic and anaerobic states respectively. Pyruvate levels increased noticeably 48h after seeding, after which a plateau phase was observed in all 15 strains, whereas lactate levels stabilized immediately (Fig. 2c and 2d). The rate of urea synthesis, a surrogate marker for nitrogen metabolism, failed to stabilize over the course of the experiment. (Fig. 2b) Total protein was quantified in cells harvested from culture day 1, 3, 5 and 7 (Supplementary Fig. 2a). Protein levels remained stable over the course of the experiment.

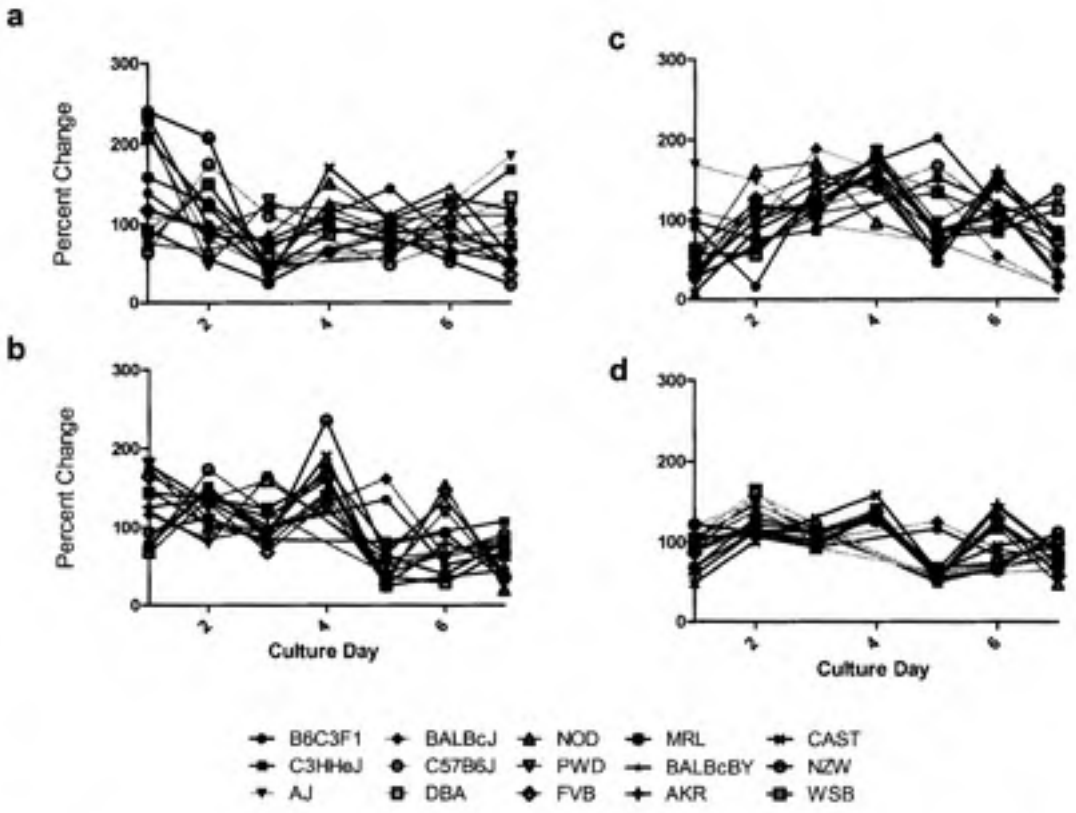


Figure 2: Functional characterization of cultured hepatocytes representative of 15 inbred mouse strains: A/J, B6C3F1, BALB/cJ, C3H/HeJ, C57BL/6J, CAST/EiJ, DBA/2J, FVB/NJ, BALB/cByJ, AKR/J, MRL/MpJ, NOD/LtJ, NZW/LacJ, PWD/PhJ and WSB/EiJ (n=1). Hepatocytes from male mice (4-6wks of age) were isolated by collagenase perfusion, purified by Percoll gradient centrifugation (final viability ≥ 90%) cultured on handcoated rigid collagen 24 well plates in a conventional monolayer up to day 7. Media was harvested daily, cell culture supernatants were stored at (-20oC) and assayed at a later date. Lactate dehydrogenase, pyruvate and lactate levels were quantified using standard enzymatic procedures (Bergmeyer, 1970). (a) Rates of LDH release. (b) Rates of urea production (BioAssay Systems BUN Urea kit) (c) Rates of pyruvate production (d) Rates of lactate production. Data was normalized to total protein (Pierce BCA kit) then mean centered to the average of daily (release/production) for each biochemical parameter.

Basal level of expression of key liver specific genes of hepatocytes was evaluated in the cultures (day 1 and 3) and whole liver samples for selected strains. RT-PCR analysis was conducted for comparison of key liver specific genes ALB, CYP4A10, HNF4α and UGT1A1 (Fig. 3). Transcripts were selected on the basis of function and significance in the cell relevant for the evaluation of drug metabolism and toxicity. Data was normalized to percent of matched whole liver samples (whole liver samples were unavailable for MRL/J). CYP4a10 and UGT1A1 were expressed at lower levels in hepatocytes from cultured cells compared to whole liver. Primary cultures were able to maintain expression levels of ALB and HNF4α compared to whole liver, which supports evidence that cells maintained phenotypic function past culture day 3 as indexed by previous biochemical analysis.

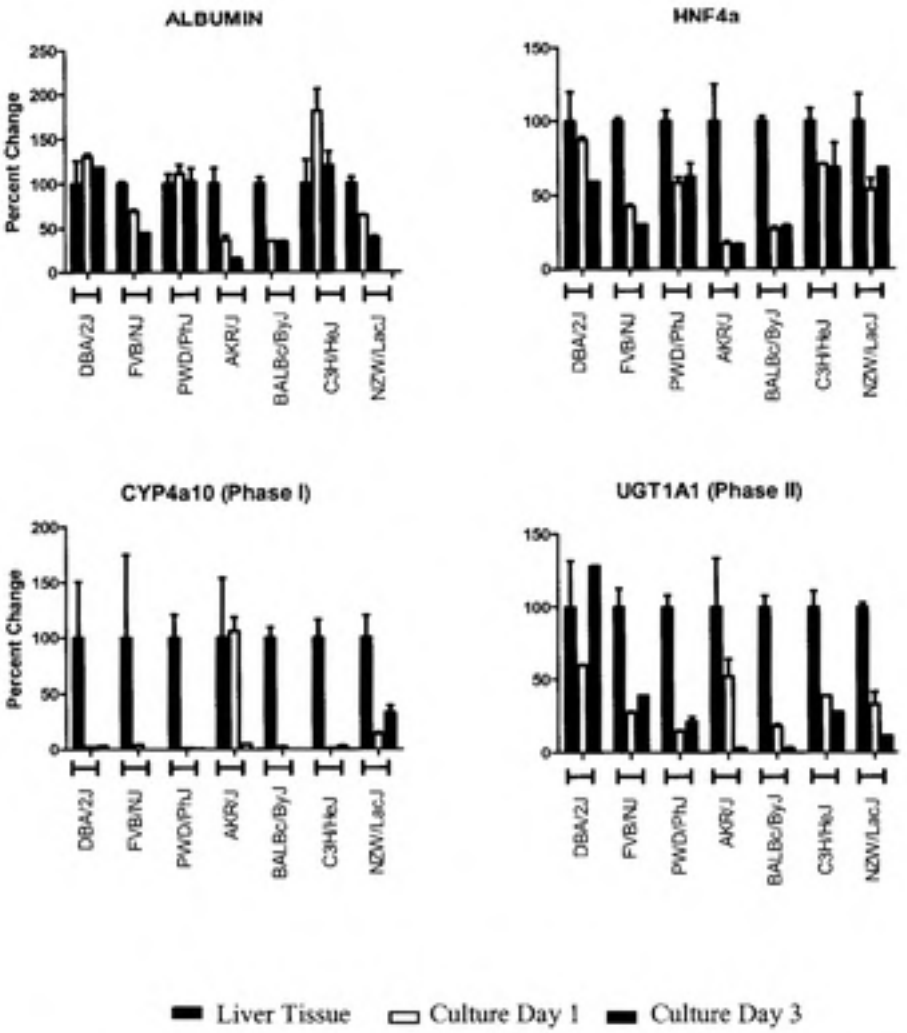


Figure 3: Gene expression analysis of hepatocytes in conventional monolayer. mRNA levels representative of liver-specific genes from a panel of inbred mouse strains (C3H/HeJ, CAST/EiJ, DBA/2J, FVB/NJ, BALB/cByJ, AKR/J, NZW/LacJ and PWD/PhJ). RT-PCR analysis of ALB, HNF4a, CYP4A10 and UGT1A1 in mouse liver tissue (black bar) and hepatocytes from cultured cells day 1 (white bar) and 3 (grey bar). All data was normalized to gene expression levels in whole liver samples.

Reproducibility of viability and functionality in 3 strains

Conventional cultures from primary mouse hepatocytes were prepared from 3 inbred mouse strains (n=3), in order to investigate reproducibility of viability and functionality. Male B6C3F1/J, C57BL/6J and NOD/LtJ were chosen for this study because of the extensive utilization of these strains in toxicological studies conducted by the National Institute of Environmental Health Sciences and the National Toxicology Program. The experimental design for this study mirrored that of the first study (Fig. 1a). Final cell yields and viability were similar between strains and could be reproduced in subsequent preparations. The mean values and SD for strain specific cell yields and viability are as followed: 66 ± 12.51 million cells and a final viability of 94.3 ± 1.54% for B6C3F1/J, 56. ± 3.5 million cells and a final viability of 93.7 ± 2.1% for C57BL/6J and 77.7 ± 18.1 million cells and a final viability 95.3 ± 1.2% for NOD/LtJ (Supplemental Table 1).

Phase contrast microscopy revealed similar morphological changes in the cultured hepatocytes over time. A reduction in monolayer confluency and a high density of disintegrated cells with irregular shape was observed in all cultures as time progressed. We measured LDH, pyruvate, lactate and urea levels to assess hepatocyte functionality over time. Following assessment, LDH, pyruvate, lactate and urea levels were normalized to the average release/production of each marker over the course of culture for each strain. The distinct trend for each biochemical marker mirrored what was observed in the first experiment, with the exception of urea synthesis. LDH levels stabilized by day 2, a significant spike in pyruvate levels was seen 48 h post plating after which steady state levels were reached, lactate levels stabilized immediately and a significant drop in urea synthesis was observed 48 h post plating after which stabilization was reached (Fig. 4). Total protein

was quantified in cells harvested from culture 1, 3, 5 and 7 (Supplementary Fig. 2b). Furthermore, there was great reproducibility between biological replicates and little observed difference between strains.

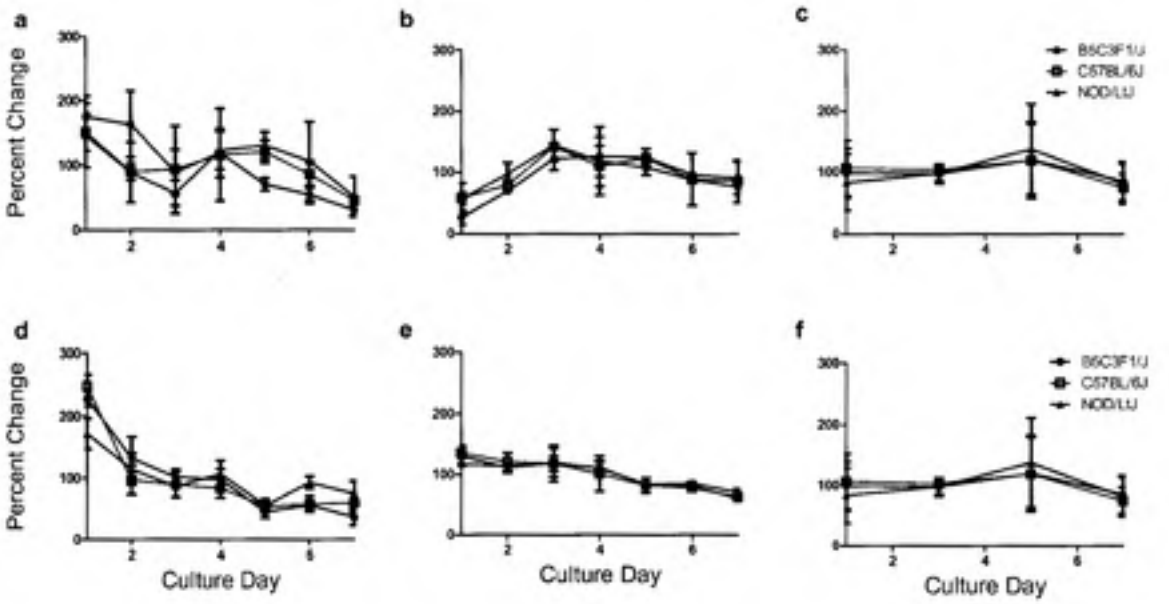


Figure 4: Functional characterization of cultured hepatocytes representative of 3 inbred mouse strains: B6C3F1/J, C57BL/6J and NOD/LtJ (n=3). Hepatocytes from male mice (4-6wks of age) were isolated by collagenase perfusion, purified by Percoll gradient centrifugation (final viability ≥ 90%) cultured on BD BiocoatTM 24 well plates in a conventional monolayer up to day 7. (a) Rates of LDH release. (b) Rates of urea production (BioAssay Systems BUN Urea kit) (c) Rates of pyruvate production (d) Rates of lactate production. (e) Rates of ATP production (Promega ATP kit). (f) Rates of GSH (unconjugated) production (Promega GSH kit). Data was normalized to total protein (Pierce BCA kit) then mean centered to the average of daily (release/production) for each biochemical parameter.

Baseline expression of key liver specific genes was evaluated in samples harvested from culture day 1 and 3. RT-PCR analysis was conducted for comparison of key liver specific genes ALB, CPS-1, CYP1A2, CYP3A11, CYP4A10, GSTA2, HNF4α, UGT1A1 and SLCO1B2. Transcripts were selected under the same criteria as described above. Samples from cultured cells were compared to matched whole liver samples and the data was normalized to the percent of whole liver. All liver specific phase I and II enzymes were expressed at statistically significant lower levels in cultured cells compared to whole liver (Fig. 5). Cultured hepatocytes were able to maintain expression of ALB, CPS-1 and HNF4α, transcripts relevant to hepatocellular function, compared to whole liver. The transporter SLCO1B2 was not expressed in the whole liver samples from NOD/LtJ. Lastly, we found that the expression levels of liver specific genes varied little between biological replicates.

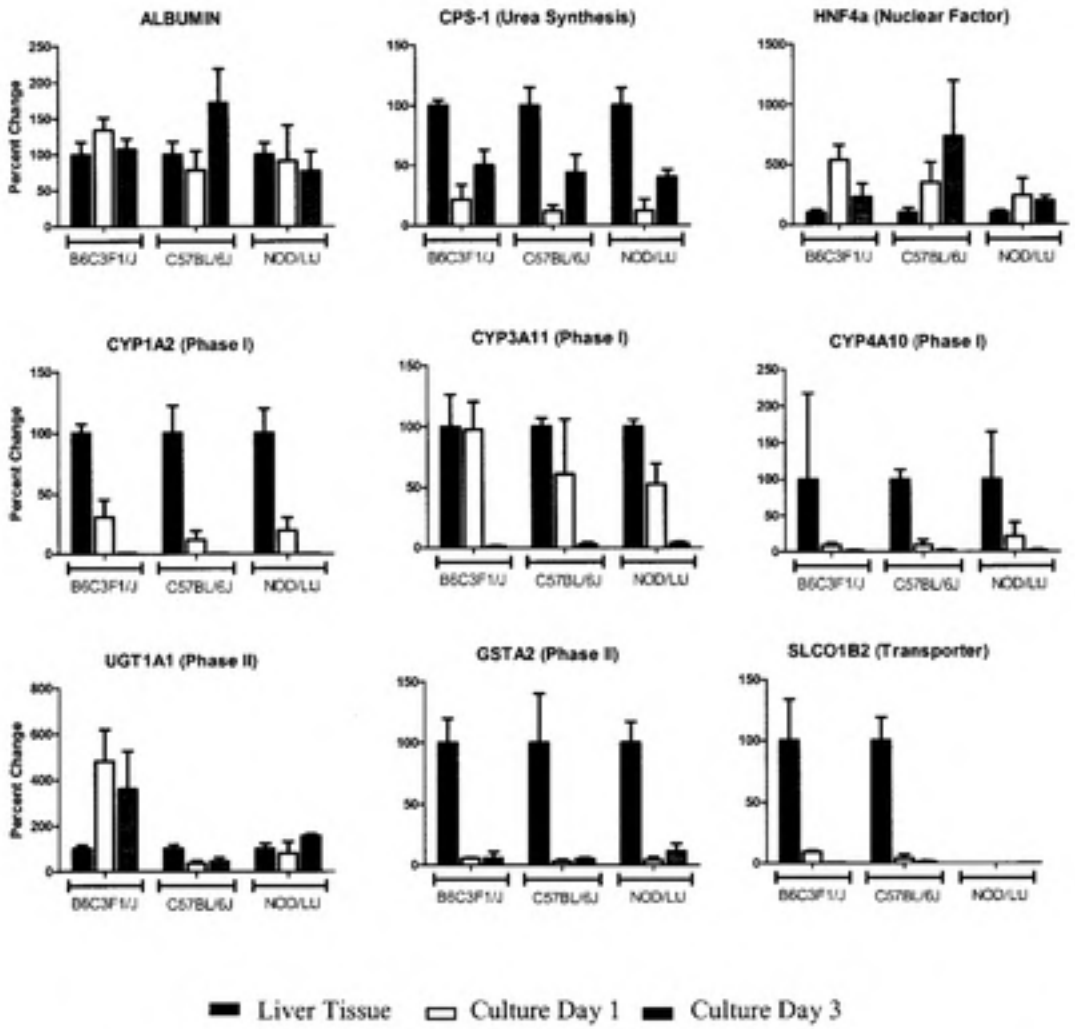


Figure 4: mRNA levels representative of liver-specific genes (ALB, CPS-1, HNF4a, CYP1A2, CYP3A11, CYP4A10, UGT1A1 and GSTA2). Comparison of gene expression levels of in mouse liver tissue (black bar) and hepatocytes from cultured cells day 1 (white bar) and 3 (grey bar). All data was normalized to gene expression levels in whole liver samples.

Toxicity screening in 3 strains

Conventional cultures of primary mouse hepatocytes were prepared from male mice, B6C3F1/J, C57BL/6J and NOD/LtJ (n=3), to assess the utility the model for toxicity screening. To investigate strain-specific sensitivity we quantified the acute toxicity to model toxicants. Hepatocytes were treated with five different concentrations of four reference compounds (0.3-30 mM acetaminophen, 1-100 μM phenobarbital, 0.1-10 mM WY-14,643 and 1-100 μM rifampin) for 24h. Treatment started 24h and 72h post seeding as depicted in (Fig. 1b). Toxicity was determined by ATP cytotoxicity assay.

As shown in Fig. 6a, hepatocytes originating from different strains responded similarly to treatment with 0.3mM, 1mM, 3mM, 10mM and 30mM acetaminophen at culture day 2, following a 24 h exposure period, as indexed by cell survival as a percent of control. A one-way ANOVA was performed to evaluate the significance of concentration in response to acetaminophen and was determined to be significant in cultures originating from the three mouse lines (p value ≤ 0.005), at culture day 2 and 4. The rate of GSH depletion surprisingly differed between strains at culture day 2. The EC_{50} values based off of GSH content at culture day 2 were compared between strains, 1.1mM for B6C3F1/J, 6.38 mM for C57BL/6J and 1.52mM for NOD/LtJ (Fig. 6a).

Culture day appeared to have a significant effect on strain-specific responses to treatment with acetaminophen. Marked differences were obtained in the viability of hepatocytes originating from different strains treated with 30mM acetaminophen at culture day 4, following a 24 h exposure period (Fig. 6a). Strains B6C3F1/J and C57BL/6J were far less sensitive to acetaminophen treatment compared to NOD/LtJ, as hepatocytes from B6C3F1/J and C57BL/6J maintained approximately 75% viability following 30mM

acetaminophen treatment, while hepatocytes from NOD/LtJ lost more than 55% viability. Furthermore, culture day appeared to have a significant effect on the sensitivity pattern for all three strains treated with acetaminophen, the sensitivity of the hepatocytes decreased significantly with the day of culture. In comparing the loss of intracellular ATP content, as measured by EC_{50} values, hepatocytes treated on culture day 3 revealed a significant decrease in sensitivity to acetaminophen toxicity. EC_{50} values for B6C3F1/J increased from 6.79mM to >30mM, for C57BL/6J it increased from 6.94 mM to >30mM and for NOD/LtJ it increased from 6.04mM to a value equal to 30mM. Culture day had a similar effect on GSH levels. Notably higher GSH levels were observed in cultures following 24 h treatment period with acetaminophen on day 4 compared to day 2 (Fig 6a).

RT-PCR analysis was conducted to evaluate gene interaction of key liver specific transcripts, NRF2 and CYP2E1, which are known to be involved in the molecular response to acetaminophen. Expression of NRF2 increased with culturing, however, there were no observed differences between concentrations and strains (Fig. 6b). Expression of CYP2E1 was not maintained by hepatocytes derived from any of the three mouse strains and decreased significantly from culture day 2 to 4 (Fig. 6b).

A different sensitivity pattern was observed in cultures treated with 0.1mM, .3mM, 1mM, 3mM and 10mM WY-14,643 compared to cultures treated with acetaminophen. Culture day appeared to have a milder effect on the sensitivity pattern for all three strains treated with WY-14,643 (Fig. 7a). A within strain comparison of the EC_{50} values from culture day 2 and 4, following 24 h of treatment, illustrates this pattern. The EC_{50} values for B6C3F1/J increased from 1.15mM to 1.85mM, for C57BL/6J it increased from 1.09mM to 1.94mM and for NOD/LtJ it increased from 1.68mM to 1.92mM.

As shown in Fig. 7a, there were marked differences in the viability of hepatocytes originating from the different strains treated with 1mM WY-14-643 at culture day 2, following 24 h exposure period. These strain-specific differences in cell viability are evidenced by differences in cell survival (percent of control). After a 24 h exposure period at culture day 2, hepatocytes from B6C3F1/J and C57BL/6J exhibited 55% survival while NOD/LtJ exhibited 75 % survival. GSH content was depleted in hepatocytes treated with WY-14, 643. GSH depletion was dependent on concentration. Concentration was shown to be a significant variable by one-way ANOVA in all cultures treated with WY-14,643 (p value ≤ 0.005). The rate of GSH depletion was fairly consistent between strains and treatment days.

RNA from hepatocytes treated with increasing concentrations of WY-14,643 was extracted and analyzed for mRNA levels of key genes involved in the cellular response to WY-14,643. Expression levels of ACOX1, PPARα and CYP4A10 were compared across treatment groups, culture day and genotype. Hepatocytes were able to maintain the expression of ACOX1 in culture, however, the cells failed to express CYP4A10. Furthermore, the expression of ACOX1 appeared to be influenced by treatment. Expression of PPARα was consistent across treatment groups, strains and day of culture.

Cellular response to treatment with 1μM, 3μM, 10μM, 30μM and 100μM rifampin was consistent between strains and treatment days. As shown in Fig 8A, small differences were obtained in cell viability and GSH content in the hepatocytes prepared from 3 mouse strains and treated with various concentration of rifampin at culture day 1 and 3. We assessed whether gene interaction correlated with response to treatment with rifampin, as determined by intracellular ATP and GSH. Expression of CYP3A11 and UGT1A1 was evaluated in

cultures treated with 10μM and 30μM rifampin, these concentration were selected to identify subtle changes in gene expression. Hepatocytes were able to maintain expression of UGT1A1, however, strain differences were observed in the expression of CYP3A11.

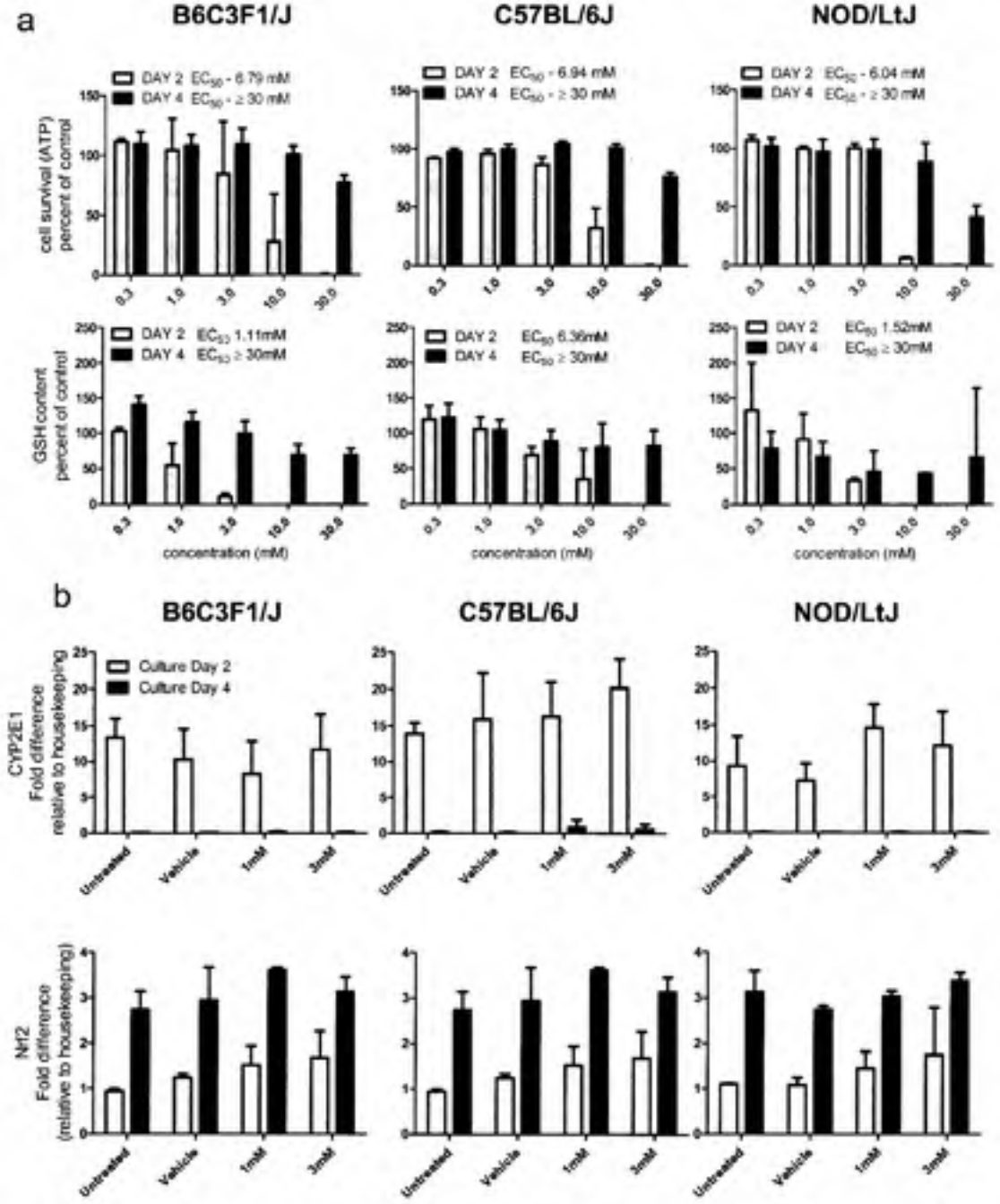


Figure 7: Utility of toxicogenetic model for screening of hepatotoxicity and gene interaction. Cytotoxicity of APAP in hepatocytes derived from 3 inbred mouse strains (B6C3F1/J, C57BL/6J and NOD/LtJ) and two time points, culture Day 2 (grey bars) and Day 4 (black bars). Hepatocytes from male mice (4-6wks of age) were isolated in situ by collagenase perfusion, purified by Percoll gradient centrifugation (final viability ≥ 90%) cultured on BD BiocoatTM 96 well plates in a conventional monolayer for 24 h and 72h, then cultured for a additional 24h in the presence of increasing concentration of APAP (0.3, 1, 3, 10 and 30 mM). (A) Toxicity was assessed by evaluating cellular ATP content and GSH levels. (B) Expression level of key liver specific genes Cyp2E1and Nrf2.

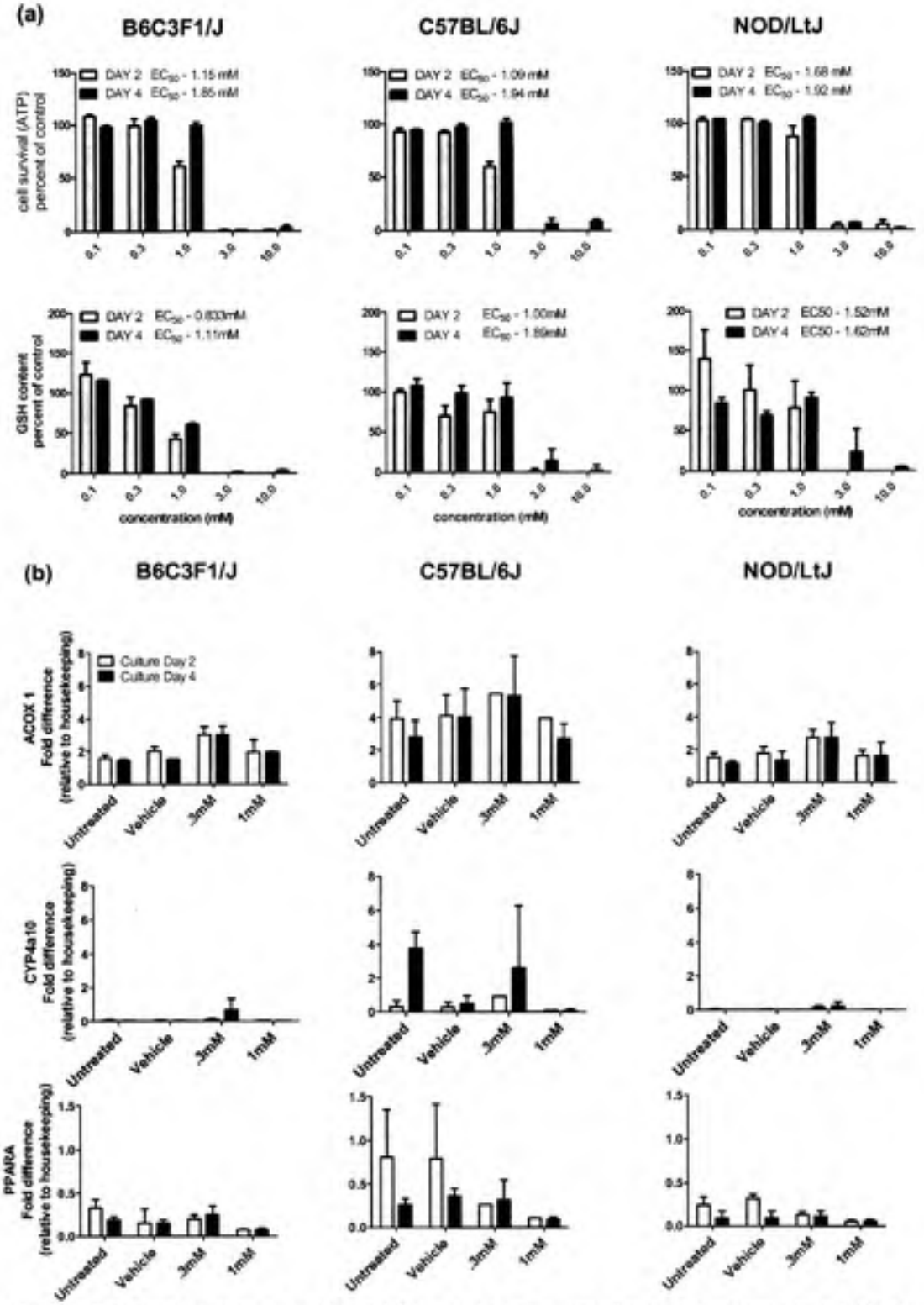


Figure 8: Toxicity of peroxisome perliferator WY-14, 643. Comparison of hepatocyte cytotoxicity in 3 inbred mouse strains (B6C3F1/J, C57BL/6J and NOD/LtJ) and two time points, culture Day 2 (grey bars) and Day 4 (black bars). Hepatocytes from male mice (4-6wks of age) were isolated in situ by (final viability ≥ 90%) and cultured on BD BiocoatTM 96 well plates in a conventional monolayer for 24 h and 72h, then cultured for a additional 24h in the presence of increasing concentrations (0.1, 0.3, 1, 3 and 10mM) of WY-14,643. (A) Toxicity was assessed by evaluating cellular ATP content and GSH levels. (B) Expression level of key liver specific genes Acox1, Cyp4a10 and Ppara.

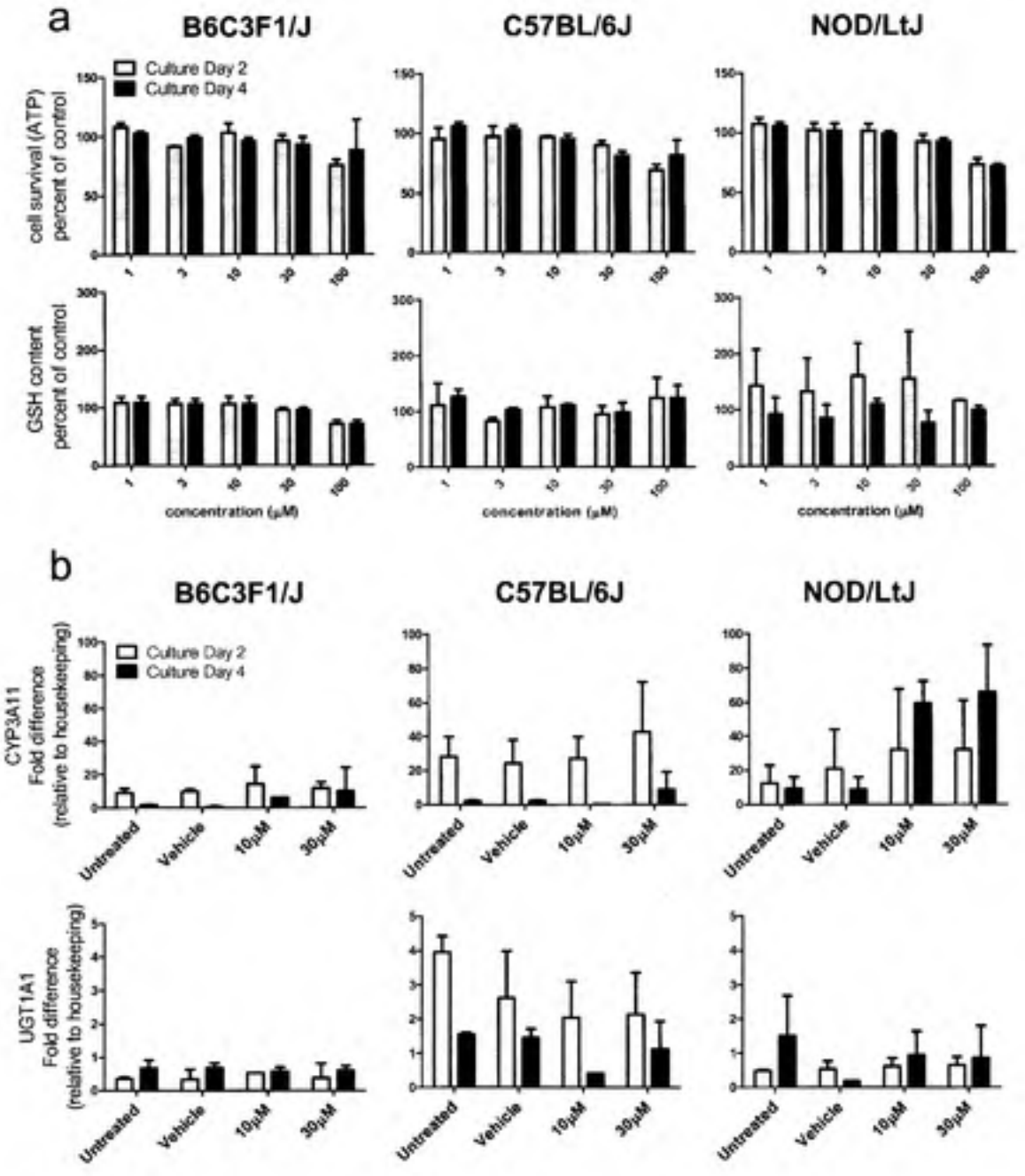


Figure 9: Hepatocyte cytotoxicity in response to treatment with Rifampin. Hepatocytes were isolated, (final viability ≥ 90%) and cultured from in 3 inbred mouse strains (B6C3F1/J, C57BL/6J and NOD/LtJ). Toxicity was measured at two time points, culture Day 2 (grey bars) and Day 4 (black bars). Hepatocytes were treated for 24 h on culture day 1 and 3 in the presence of 1, 3, 10, 30 and 100 μM Rifampin. (A) Toxicity was assessed by evaluating cellular ATP content and GSH levels. (B) Expression of key liver specific genes CYP3a11and UGT1a1.

Chapter 5

Discussion, Limitations, Conclusions and Future Directions

Discussion

Experiments 1 and 2: Assessment of cell function and gene expression

Cell based approaches are well suited for application in hazard identification, prioritization of environmental chemicals and drug discovery (19). Furthermore, *in vitro* toxicity experiments can generate large batteries of information on biological mechanisms of action, including compound potential on metabolic-mediated toxicity (chemicals require metabolic activation) and inter-individual toxicity (response to chemicals based on genotype) (20). Our approach demonstrates the utility of a cultured hepatocyte model developed from a mouse diversity panel. Primary mouse hepatocytes were prepared using standardized isolation and cell culture conditions from a panel of genetically distinct mouse strains. Hepatocytes were isolated with high viability (93%) and good yield (59.3 x 10^6 cells) and results were reproducible upon subsequent isolations, however, there was a failure to maintain the cultured cells in a differentiated state past culture day 3. At this point hepatocytes spread out and formed fibroblast like protrusions.

There are many factors that regulate phenotypic gene expression *in vitro* including, cell-cell communications, cell shape, organization and distribution of the cytoskeleton and extracellular matrix interactions (21-25). Cell-cell contacts help to maintain liver-specific

function and normal gene expression through adhesion dependent signal transduction pathways mediated by the Rho-family of small GTPases (22). Morphological assessment of the cultures at day 3 revealed a significant loss in cell shape and monolayer confluence. Cell morphology has a significant effect on hepatocyte phenotype. Cultured cells that have dedifferentiated and become fibroblastic like exhibit a loss of hepatic phenotype marked by the inability to maintain expression of several transcription factors, an increase in the expression of proto-oncogene's and a decrease in cell and membrane polarity (21).

Our results on basal level function of hepatocytes isolated from 15 strains are comparable. Cells remained viable and functional as indexed by lactate/pyruvate production, urea synthesis and low levels of LDH release. Our findings on expression of key liver specific genes suggest that functionality is maintained in cultured hepatocytes up to day 3, represented by mRNA levels of ALB, CPS-1 and HNF4α. Conversely, the expression of key genes relating to metabolism was not maintained as expected, represented by mRNA levels of CYP1A2, CYP3A11, CYP4A10 and GSTA2. There were some strain differences in the expression of UGT1A1 compared to whole liver samples. We subsequently tested for reproducibility of viability and functionality in three strains, B6C3F1/J, C57BL/6J and NOD/LtJ (n=3). Our results suggest reproducibility is achievable among biological replicates.

Monolayer confluency influences the expression and the inductive capability of several P450's including CYP3A, CYP1A2 and CYP2B6, and furthermore, expression of P450s has been shown to decrease with the loss of confluence (26). These observations are consistent with our findings demonstrating an inability of the cultured cells to maintain expression of genes relating to phase I and II metabolism out to culture day 3.

Liver-enriched transcription factors, including HNF4α, are involved in the regulation of a number of hepatic genes, including P450s (27). The cultured hepatocytes from our study were able to maintain the expression of HNF4α out to culture day 3 compared to whole liver samples. However, these cells were not able to maintain CYP expression out to culture day 3. Although, a number of the studies investigating HNF4α signaling on hepatic gene expression have been performed *in vitro*, these studies may not be representative of the *in vivo* situation. HNF4α may only represent a single factor in a complex regulatory network that mediates the expression of many genes (27;28).

Experiment 3: Hepatotoxicity and gene interaction

In our experiments, hepatocytes were prepared from three strains (B6C3F1/J, C57BL/6J and NOD/LtJ), cultured and treated with four reference compounds to investigate strain-specific responses to treatment and determine whether cultures of hepatocytes can produce data indicative of *in vivo* responses to hepato-toxicants. Prior to treatment with reference compounds, hepatocytes were allowed a 24 h and 72 h adaptation period to the culture environment. This was done to evaluate the effects of culture day on response to treatment. Hepatocytes were treated with increasing concentrations of acetaminophen, phenobarbital, WY-14,643 and rifampin, cell viability and function was measured by the Cell Titer-Glo Cell Viability assay and GSH-Glo (Promega) assay respectively. Strain specific responses were observed in the hepatocytes treated with acetaminophen. NOD/LtJ hepatocytes appeared to be the most sensitive to the cytotoxic action of acetaminophen at culture day 4, following 24 h of treatment, as determined by intracellular ATP content. GSH levels were not found to be significantly different when compared across strains for 30mM acetaminophen treatment at day 4.

Hepatic intracellular glutathione is well known for its pivotal role in the metabolism of acetaminophen. Evaluating glutathione content in the cultured hepatocytes from the three strains revealed notable differences in the rate of GSH depletion in response to treatment with acetaminophen. The variation observed between strains with respect to the rate of GSH depletion is markedly different. Additional studies need to be conducted in more complex culture systems to further investigate the relationship between genotype and sensitivity to acetaminophen.

Furthermore, our results indicate a significant reduction in the cytotoxicity of acetaminophen when tested against primary hepatocytes cultured out to day 3, which suggest a significant change in the metabolic capacity of the cultured hepatocytes. Acetaminophen toxicity is a metabolism dependent pathway via bioactivation by CYP2E1. CYP2E1 is the primary enzyme involved in the formation of the reactive metabolite NAPQI, therefore the observed decrease in sensitivity to acetaminophen can be attributed to the failure of the hepatocytes to maintain expression and activity of CYP2E1. Our findings are consistent with previous studies which found metabolic capacity, transport functions and other enzyme activities change during culturing and identify time as a significant variable (29;30). In primary hepatocyte culture the expression and activity of transport proteins change over the course of culture and the down regulation of genes encoding proteins responsible for drug uptake may contribute to the increase of resistance against acetaminophen toxicity found in hepatocytes cultured out to day 3 (29;31). Additionally, a number of acetaminophen induced cellular alterations may contribute to the outcome of acetaminophen toxicity including oxidative stress, disruption of calcium homeostasis, altered mitochondrial function, protein nitrosylation, Kupffer cell activation, DNA damage and apoptosis among others (31;32).

Interestingly, our results lack a direct correlation with the *in vivo* study conducted in our laboratory, which showed B6C3F1/J as the most sensitive strain to acetaminophen treatment and NOD/LtJ as one of the most resistant strains to acetaminophen treatment. The results from our *in vitro* experiment illustrate the contrary, hepatocytes from NOD/LtJ mice were observed to be the most sensitive to acetaminophen treatment at day 4, while hepatocytes from B6C3F1/J mice were the most resistant at culture day 4. This relationship between toxicity to hepatocytes *in vitro* and drug-induced liver injury *in vivo* remains poorly defined and highlights an important uncertainty factor that warrants exploration (19).

Cytotoxicity was further examined in cultured hepatocytes treated with increasing concentrations of a peroxisome proliferating agent, WY-14,643. Model peroxisome proliferators, such as clofibrate, have been shown to induce peroxisomes in isolated hepatocytes (33-35). Strain-specific responses were observed in hepatocytes treated at culture day 1 with 1mM WY-14,643. B6C3F1/J and C57BL/6J hepatocytes appeared to be more sensitive to treatment with 1mM WY-14,643 at culture day 2 compared to NOD/LtJ hepatocytes, as indexed by intracellular ATP content. Surprisingly, culture day, a significant variable in response to treatment with acetaminophen, appeared to have little effect on response to treatment with WY-14.643.

Interestingly, the expression of CYP4A10 was not detectable in the presence or absence of WY-14,643 treatment in hepatocytes derived from B6C3F1/J and NOD/LtJ. The inductive capability of CYP4A11, the human P450 4A gene, is largely influenced by monolayer confluency. A study conducted by Johnson et al. found that sub-confluent cultures altered the expression of CYP4A11 in human hepatocytes cultured in the presence or absence of elevated PPARα expression or treatment with the peroxisome proliferator WY-14,643

(36). Restoring and maintaining hepatocyte physiology is important for *in vitro* cell culture studies. Restoration of the native architecture and the natural orientation for linked enzymes, transporters and receptors in primary cultures is essential for the simulation of hepatic disposition of drugs *in vivo*. Studies have demonstrated that cell shape, cell density, contact with extracellular matrix and cell polarity all play key roles in the induction of P450 gene expression by model compounds (21). The loss of cell-cell contact, important for the maintenance of liver-specific function and normal gene expression, could help to explain the poor inductive power of CYP4A10 in the cultured hepatocytes. Furthermore, the exposure period may have been an insufficient amount of time to observe induction of CYP4A10 in the cultured cells. Although, the reason for this lack of elevated expression PPARα and CYP4A10 mRNAs in the cultured hepatocytes is unclear, we observed an increase in ACOX1 expression with treatment which is consistent with the observations that mice are sensitive to peroxisome proliferation (37;38).

Treatment with increasing concentrations of rifampin yielded comparable results between strains, as determined by intracellular ATP content and GSH levels. At the highest concentration, 100μM, 30% cell killing was achieved at culture day 2 and 4, following 24 h of treatment, for the three strains. We selected this compound as our negative control. The lack of observed strain-specific responses to rifampin can be attributed to the poor inductive capability of CYP3A11 in response to treatment with rifampin. Inductive response to inducers is significantly different, both quantitatively and qualitatively, among animal species (39). Rifampin is a CYP3A4 specific-inducer, which is found in humans and a poor inducer of CYP3A11, which is found in mice. Several studies have been published on the observed species differences in CYP induction and have identified structural differences in

the nuclear receptor PXR as the molecular basis responsible for the observed species differences in CYP3A induction (40).

Our data supports notion that cultures of hepatocytes derived from a mouse diversity panel can potentially be utilized as a tool to conduct population-based toxicity studies *in vitro*. Our results, however, suggest that a more detailed characterization of baseline hepatic function is necessary. This exercise has also demonstrated the importance of optimizing cell isolation and culture conditions in order to construct population-based *in vitro* models that accurately predict *in vivo* responses to a wide range of hepato-toxicants. Additional studies are needed to fully characterize this relationship.

Limitations

Numerous studies have found that conventional culture systems lack the capacity to maintain long-term cultures. In addition, hepatocytes cultured in a traditional monolayer dedifferentiate and rapidly lose hepatic-specific functions (41). The utility of conventional culture systems with a rigid collagen substratum is also limited by the rapid decline in expression and activity levels of most liver-specific programs (14;42). Many culture systems have been developed to allow for better retention of hepatocyte cytoarchitecture and to foster better retention of the *in vivo* gene expression profiles of liver. Models include co-culture with other liver-derived or non-liver cell types, exposing hepatocytes to a gel substratum, culture in collagen gel sandwiches, and culture in spheroids and in 3D bioreactors (14;42;43). Although, *in vitro* models of hepatocellular toxicity are widely used for investigating cellular response to exposure, these models are limited by the instability of the hepatocyte phenotype and the lack of similarities in responses to toxicants between

cultured hepatocytes and whole organ. A limitation to our culture system is the lack of other liver derived cells. Harrill et al. (2009a) demonstrated that liver sensitivity to acetaminophen *in vivo* is largely influenced by variation in immune response. These findings are consistent with the numerous studies that have identified inflammatory mediators, released by non parenchymal cells, to be important factors in progression of liver injury (44-46). It is known that chemicals which induce peroxisomes in rodent liver increase DNA synthesis and produce profound changes in the liver, including hepatomegaly. Increase in DNA synthesis caused by peroxisome proliferators is largely regulated by the recruitment and stimulation of Kupffer cells, which are a major source of mitogens such as tumor necrosis factor α (47;48). An observed limitation to treating purified hepatocyte cultures with peroxisome proliferators is the lack of other liver derived cells. This observation is supported by work carried out by Parzefall et al. The group conducted a study using purified rat hepatocytes and demonstrated that peroxisome proliferators do not influence DNA synthesis in isolated hepatocytes and found that cytokine production by cultured Kupffer cells can increase DNA synthesis in primary cultures (33).

The majority of *in vitro* studies in the literature are difficult to benchmark against *in vivo*, as temporal changes in gene expression and protein activity is typically compared to values 24 hr after plating. Furthermore, biochemical activities of cultured hepatocytes are difficult to compare to *in vivo* due to differences in dose and exposure, both of which are complex to interpret. Correspondence of in vitro to *in vivo* has been assessed in a few systems using RT-PCR and protein expression levels, showing, for example, the patterns of gene expression maintained by inclusion of DMSO in the culture medium are closer to *in vivo* for many genes, including key regulator of liver metabolism genes, HNF4α (49).

Conclusions

In summary, our current work supports the utility of cultured hepatocytes prepared from a panel of inbred mouse strains as an *in vitro* toxicogenetic model for hepato-toxicity. Primary hepatocytes can be successfully isolated and cultured from a diverse panel of inbred mouse strains using standardized isolation and culture conditions. Hepatocytes were viable and functional as indexed by lactate, pyruvate and urea production. Reproducibility of viability and functionality can be achieved within a given strain over subsequent isolations. Expression of key liver specific genes is maintained in cultured hepatocytes up to day 3, represented by mRNA levels of ALB, CPS-1 and HNF4α. Gene expression of metabolism enzymes in cultured hepatocytes was not maintained as expected, as evidenced by mRNA levels of CYP1A2, CYP3A11, CYP4A10 and GSTA2. Cells were metabolically active and responsive to treatment with acetaminophen at culture day 2, as indexed by cell killing, GSH depletion and expression of key liver specific genes. Furthermore, hepatocytes were responsive to treatment with peroxisome proliferator WY-14,643, evidenced by the upregulation of ACOX1 at culture day 2 and 4.

It is essential to understand the biological mechanisms mediated by underlying genetic determinants and delineate the factors contributing to inter-individual responses to toxicity. This design could provide insight to the genetic factors contributing to inter-individual susceptibility in humans by identifying the unique genotype-specific toxicant-induced changes in metabolism, toxicity and gene expression. Furthermore, this model can operate as a medium to high-throughput screening tool to test a large number of compounds

against densely genotyped backgrounds as well as to improve the predictive power of *in vitro* screening tools.

Future Directions

Scaling up to more complex model culture systems, including co-cultures, Matrigel™ overlay or collagen gel sandwiches and three-dimensional cultures, will help retain hepatic-specific phenotype. Conventional culture systems are limited by the short-term survival and rapid dedifferentiation of hepatocytes in culture. Culturing primary hepatocytes with other liver-derived cell types will play an important role in modulating hepatocyte behavior with regard to viability and functionality. Heterotypic cell-cell interactions are needed to elicit responses similar to what is observed *in vivo*, for compounds that recruit and stimulate Kupffer cells. The extracellular matrix also plays an important role in the long-term survival of hepatocytes in a differentiated state, and furthermore, it can play a significant role in the formation and retention of bile canaliculi. It will be important to incorporate a combination of these approaches in future experiments to optimize the performance of the culture system. In our laboratory experiments are currently being conducted to investigate the effects of culturing primary hepatocytes in a three-dimensional perfused bioreactor system. These studies will help to establish applications that are helpful in maintaining the survival of long-term cultures.

To further characterize the performance of the cultures derived from distinct genotypes it will be important to identify the relationship between enzyme activity and expression. Studies have shown that mRNA levels do not always correlate quantitatively with enzymatic activity. Additionally, this study lacks correlation between multistrain *in*

vivo/*in vitro* response to treatment with acetaminophen. Conducting a microarray analysis comparing the gene expression profiles of whole liver to cultured cells for selected strains may help to identify factors contributing to observed phenotypic differences *in vivo* and *in vitro*.

Chapter 6

Practicum Report

My practicum experience took place at CellzDirect/Invitrogen Corporation (a part of Life Technologies) for 150 hours from August 1, 2008 to October 18, 2009 under the direction of Dr. Stephen Ferguson. During the first phase of my practicum experience I assisted in identifying whether standardized cell isolation and culture conditions are sufficient for the near-physiological maintenance of hepatocytes isolated from different mouse strains. Two studies were conducted; in the first study hepatocytes from 15 mouse lines (n=1) were isolated and cultured out to day 7 and the second study hepatocytes from 3 strains (n=3) were isolated and cultured out to day 7.

Hepatocytes were prepared from male mice 4-6 weeks of age, the isolations were carried out over the course of five weeks, each week hepatocytes from three genetically distinct strains were prepared and cultured. Primary hepatocytes were isolated using a two-step collagenase perfusion followed by Percoll centrifugation. Viable hepatocytes, as determined by the Trypan Blue™ exclusion method, were seeded at a density of 0.3×10^6 million cells per mL. Cell culture media was prepared by supplementing William's E Medium. On the day prior to isolation, I prepared 24-well culture plates by handcoating them with Purecol collagen type 1 and allowed them to dry under UV light for 4 hours. To each well of the culture plate, 0.5mL of the hepatocytes suspension was added to yield a final

cell density of 0.15 x 10^6 million cells per well. In addition to preparing the culture plates, I was responsible for taking photomicrographs of the cell cultures on days 1, 3, 5 and 7. Phase contrast microscopy was utilized to assess morphological changes and staining with calcein AM and ethidium-homodimer 1 was simultaneously employed to qualitatively determine the number of live and dead cells in each well. Furthermore, I was responsible for sample collection over the course of the experiment, which included media and cell harvest. Media was harvested on a daily basis, culture media was removed from two wells from a culture plate and placed in a fresh eppendorf tube and spun at 14,000 rpms for 3 min. and the supernatant was stored at -20°C. The cultured cells were harvested in 200µl of TRIzol reagent or PBS at time points day 1, 3, 5 and 7. The cells were stored at -80°C following the addition of TRIzol or PBS. The second study was carried to assess whether the reproducibility of viability and functionality could be achieved over subsequent isolations. Three mouse strains (n=3) were selected for this study and the experimental design of this study followed that of the first, as described above. My responsibilities for the second study included taking photomicrographs of the cultures and collecting media and harvesting cell cultures.

These studies investigated the feasibility of isolating and culturing hepatocytes from genetically distinct mouse strains using standardized cell isolation and culture conditions. To determine cell functionality the following biochemical markers were evaluated: lactate and pyruvate production, urea synthesis and LDH release. RT-PCR analysis was carried out for key liver specific genes to identify any significant changes in gene expression.

The second phase of my practicum research was devoted to characterizing the viability and functionality of cryopreserved hepatocytes derived from a panel of inbred

mouse strains. Fifteen lots of cryopreserved hepatocytes, representing the 15 strains selected for the first study, were thawed and cultured, cell viability was determined by the Trypan Blue™ exclusion method. Photomicrographs were taken on a daily basis to evaluate cell attachment and morphology. Of the 15 lots thawed and plated only 4 passed the post-cryopreservation plating characterization analysis. Results from this study demonstrate the difficulty of using cryopreserved mouse hepatocytes derived from a panel of inbred mouse strains. For the second phase of my practicum experience I was primarily responsible for managing all of the data collected for the cryopreservation experiments. In addition to data management I was in charge of preparing and delivering a presentation summarizing the study's results.

The third phase of my practicum experience centered focus on improving the culture conditions for primary mouse hepatocytes. Perfusion buffers and cell culture media were supplemented with 2mM glycine. Three mouse strains (n=3) were selected and the experimental design for this study mirrored that of the first study. A with the first phase experiments I was responsible for taking photomicrographs and harvesting the cell cultures at time points day 1, 3, 5 and 7. Additionally, I was responsible for collecting media samples on a daily basis. The results from this study were inconclusive. No marked differences were noted in functionality as indexed by lactate and pyruvate production, urea synthesis and LDH release compared to data collected from the second study.

Mice are widely used in toxicological studies as a model organism and considered to be helpful in addressing the potential drug-induced toxicities and exploring mechanisms of injury. Furthermore, mouse models aid in the identification of unique genetic determinants, which might infer inter-individual differences in susceptibility. The utilization of a

genetically diverse mouse population may be useful as an animal model to understand and predict rare adverse drug events in humans.

Currents approaches to toxicity screening do not address population wide diversity and fail to capture the complexity of human toxicity. The present testing paradigm requires the use of intact animals dosed at high levels not relevant to human exposure. These studies are typically conducted against a single genotype. *In vitro* models are widely used applications in toxicological research and have been identified as suitable alternatives to *in vivo* testing. The work completed in this study addresses some of the limitations to toxicity screening to date by establishing an *in vitro* model for hepatotoxicity that takes into account population wide diversity. This model could be used a novel tool to evaluate differences in response to treatment against multiple genotypes. Additionally, this high throughput approach will allow for the assessment of multiple treatments and endpoints in hepatocytes isolated and cultured from one animal. Although, this approach requires the need for species extrapolation to conduct a risk assessment analysis, it provides researchers with the ability to conduct repeat testing against distinct genotypes. These studies can have a significant impact on the development of new pharmaceuticals and the prioritization of environmental chemicals.

Chapter 7

Global Health Focus: Globalization and Genomics

What is genetics and how does it relate to the human condition? Medical conditions are often caused by mutations or inherited single nucleotide polymorphisms (SNPs), in one or more of an individual's genes. These conditions are identified as genetic disorders. Alteration of a specific gene, as a result of a SNP or mutation, can adversely affect the transcription and activity of proteins encoded by that gene. Protein malformations can affect the cell's ability to function properly, causing problems for whole tissues or organs. Utilizing information generated from whole-genome sequencing presents researchers and clinicians with the unique opportunity to detect-disease related genetic variants and tailor therapies based on genotype.

Soon genetic testing will become an integral part of diagnosing, treating, predicting and preventing common diseases, and furthermore, help to reshape more traditional approaches to health promotion. As the world approaches the post-genomic era health care practitioners will incorporate the use of genetic information to address early childhood, middle-age and older-age medical concerns. Medical practitioners will soon be able to determine the risk profiles for a wide-spectrum of diseases and implement appropriate methods of intervention. Scientists are developing new computer-based methods to aid in the

interpretation of the wealth of genetic information and determine how it relates to the human condition. The price of sequencing an individual's genome and designing individualized therapies based on genetic background may create additional barriers to healthcare for disadvantaged groups.

Many factors contribute to the health disparities present in developed and developing nations. Along with factors such as poverty, access to health care, behavior and environmental factors, scientists are now beginning to accept genetics as a risk factor for health related disparities (56). The complex etiology of health disparities is dependent on interactions between said factors. The well known analogy, "genetics loads the gun, but the environment pulls the trigger," is consistent with what has been published linking genetics and the environment to health disparities. An individual can inherit certain risk factors (i.e. polymorphisms) for a disease but the risk of developing the disease is dependent on exposure to the environmental trigger. Little genetic variation exists between individuals belonging to different socio-economic groups. However, these individuals may be exposed to different levels of carcinogens or other toxicants in their living and working environments. Moreover, certain disadvantaged ethnic groups may have a higher frequency of disease related genes making them more susceptible to factors within their environment.

There are approximately 10,000 environmental chemicals which individuals may be exposed to that are currently in commerce. The vast majority of these chemicals have gone through limited or no toxicity testing (10). The current approaches to toxicity testing are typically conducted against a single genotype and fail to factor in the influence of genetic diversity in response to exposure. As a result current methods to evaluate toxicity fail to capture the complexity of human toxicity with regard to genetic variance. As the world

becomes more connected the utilization of such applications (chemicals) in an environmental setting will pose a risk to consumers worldwide, including individuals of the most susceptible populations. To address such a complex problem as health disparities, interdisciplinary research teams composed of social, environmental and genetic scientists should be established to develop new ways to approach health in low-income and minority communities around the world.

As a result of the Human Genome Project and sequencing of the genomes of model organisms, scientists are using these tools to aid in the discovery of genetic variations in environmental response genes that are responsible for such wide differences in inter-individual variability in toxicity. The National Institute of Environmental Health Sciences has launched a project designed to identify genetic variations within populations and indentify unique genetic determinants of inter-individual susceptibility to environmental agents (56). It is believed that a nation's disease burden can potentially be reduced through better environmental practices, this is especially true for individuals living in low-income and minority communities.

Sequencing of the Human Genome

Completion of the Human Genome Project (HGP) in 2003, coordinated by the U. S. National Institutes of Health (NIH) and the Department of Energy (DOE), has provided researchers with the tools to identify specific points of variation in human DNA that underlie common diseases and effects of environmental exposures. Whole-genome sequencing has played an important role in reducing the time needed to detect and identify unique genetic determinants influencing disease susceptibility (53). The cost of sequencing genomes has dropped significantly; however, the cost of sequencing an individual's genome for medical

purposes remains high. As a result of the HGP, investigators have identified over 1,800 disease genes and engineered 350-biotechnology based products that are currently in clinical trials (53).

The genomes of individuals are distinct, thus generating diversity. The genetic diversity that exists in mankind can be evaluated by comparing the genomes of different individuals. The most common form of genetic variation is the SNP, which accounts for less than 15% of diversity observed among populations originating from different continents; however, the amount of genetic diversity present in a population (as defined by shared culture, geography, physical appearance and gene pool) is significant (54-55). Furthermore, sequence variance affecting gene splicing and regulation plays and important role in influencing disease susceptibility. Therefore, sequencing of the human genome has created a growing market for panels of DNA from individuals originating from many different populations throughout the world and these panels have become widely used applications in biomedical research (51).

As with many technological revolutions in our globalized world, the developed world tends to control and quickly capitalize on goods before the developing world has access to them. Such is the case for population-based research. Furthermore, due to the potential benefits of conducting population-based studies, researchers seek to use genetic resources (samples containing human DNA) of isolated populations to unravel the mysteries behind human diversity, design individualized drug therapies, and find genetic causes of inter-individual variability in toxicity. Remote populations, located in many different regions across the world, are ideal participants for genetic research because there is little outside genetic influence. This provides researchers with the ability to track diseases over many generations. Researchers have made many important discoveries and developed important

biomedical technologies through their efforts, but in some cases their methods of retrieval and utilization of natural and biological material of remote populations has been viewed as unethical and unjust. Many researchers have been accused of biocolonialism—a term that has been used to define the unethical and unjust mining and control of knowledge and biological property for profit—and further exacerbated the culture of mistrust between underserved peoples and the scientific community. In addition to implementing new laws to protect the rights of remote populations, scientists can continue to use alternative models to capture the complexity of human diversity in order to identify disease-related genes.

The HGP has revolutionized the field of biotechnology worldwide and has sparked the development of novel methodologies designed to characterize the genomes of model organisms widely used in biomedical research. Mice are widely used model organisms in biomedical and toxicological research. Additionally, these models are important for understanding the basics of complex disorders by identifying conserved regions on chromosomes where disease traits are located. These models present researchers with the tools to manipulate one or several genes to identify their functions in relation to disease. Current methods of manipulation that are carried out include controlled breeding, producing transgenic and knockout mutant mice, in addition to creating congenic strains. Moreover, mouse models may help to unravel gene-environment interactions and give detailed insights into many aspects of the human condition as well as basic human biology, including the identification of molecular pathways in which a mutated protein acts (58).

The quest to sequence the mouse genome was launched shortly after the completion of the HGP. The mouse genome sequence, from the C57BL/6J strain, reveals about 30,000 genes, a similar number of genes have been identified in the human genome and close

homology exists between many mouse and human gene sequences (57). Therefore, the utility of such models can play a powerful role in identifying genetic causes of human susceptibility.

Appendix A

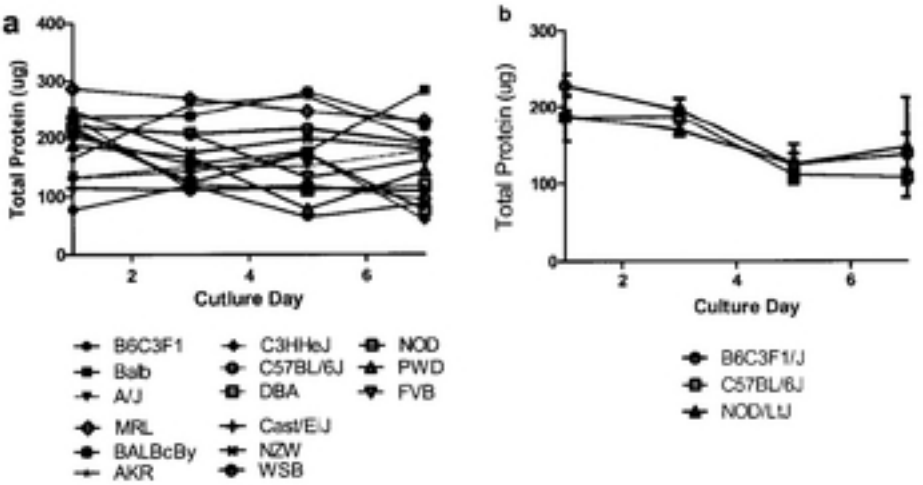
Table 1: Summary of Liver Parenchymal Cell Yields and Viability

Table 1. Summary of strain lineage, liver parenchymal cell yield and viability after isolation. Results are shown for experiment 1 (hepatocytes from 15 inbred lines were isolated and cultured out to day 7 to assess viability and functionality), experiment 2 (hepatocytes from 3 inbred lines were isolated and cultured out to day 7 to assess reproducibility of functionality) and experiment 3 (hepatocytes from 3 inbred lines were treated with 4 reference compounds to evaluate cytoxicity).

Lineage of Strains	Strain	Study	N	[a]Final Yield (millions)	[a]Final Viability (percent)
Castle's Derived	A/J	Expt. 1	2	35 ± 18.03	86.5 ± 6.364
	AKR/J	Expt. 1	1	46	96
	BALBc/J	Expt. 1	1	33.5	86
	BALBc/ByJ	Expt. 1	1	59.5	94
	C3H/HeJ	Expt. 1	1	49	88
	DBA/2J	Expt. 1	1	57	97
	NZW/LacJ	Expt. 1	1	76	88
C57 Derived	B6C3F1/J	Expt. 1	1	75.5	94
		Expt. 2	3	66 ± 12.51	94.3 ± 1.54
		Expt. 3	3	57.6 ± 20.6	91.3 ± 6.4
	C57BL/6J	Expt. 1	1	51	92
		Expt. 2	3	56 ± 3.5	93.67 ± 2.1
		Expt. 3	3	37 ± 1	92.3 ± 4
(+++ mixed)	MRL/MpJ	Expt. 1	1	82	93
Swiss Derived	FVB/NJ	Expt. 1	1	61.5	98
	NOD/LtJ	Expt. 1	1	64	93
		Expt. 2	3	77.7 ± 18.1	95.3 ± 1.2
		Expt. 3	3	42.8 ± 38.5	90.7 ± 1.5
Wild Derived	CAST/EiJ	Expt. 1	1	63	95
	PWD/PhJ	Expt. 1	1	87	94
	WSB/EiJ	Expt. 1	1	60	94

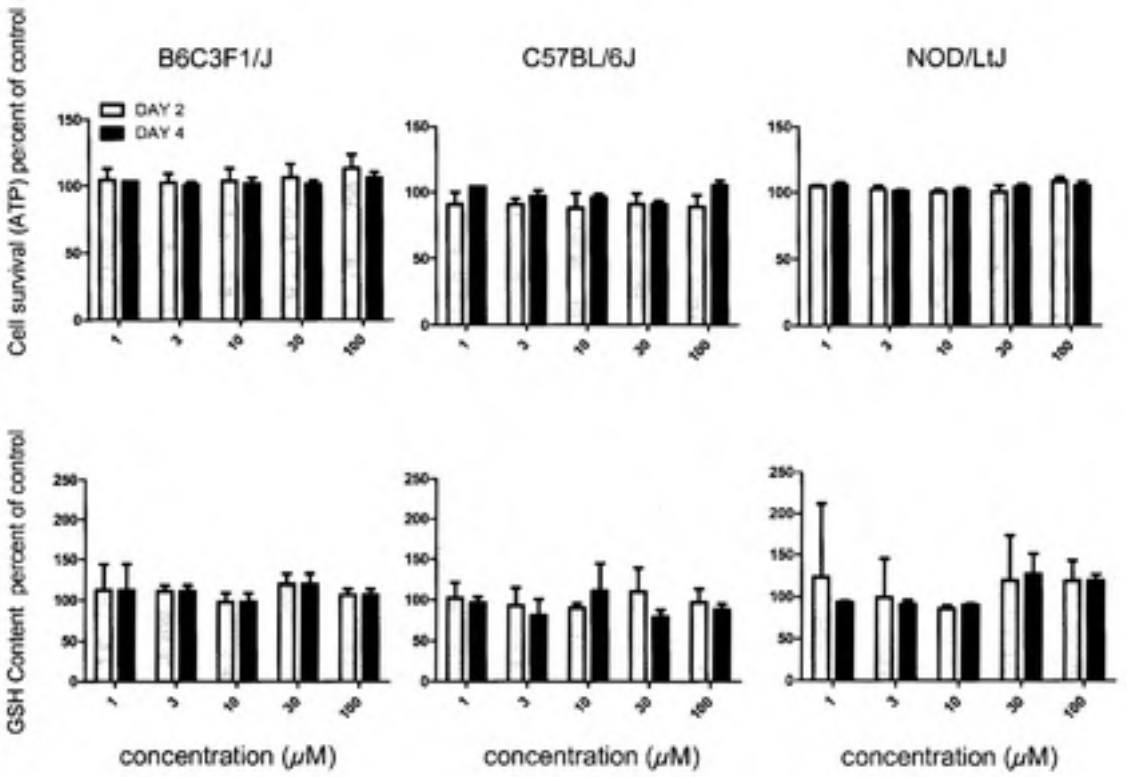
[a]Values represented are Mean ± STDEV

Figure 9: Quantification of Total Protein



Supplementary Figure 1: Total protien content (Pierce BCA kit) evaluated in (A) 15 inbred mouse strains and (B) 3 inbred mouse strains. Colors correspond to the day of hepatocyte preparation (calendar date) and each symbol represents one of the 15 strains (A).

Figure 10: Cytotoxicity of Phenobarbital



Supplementary Figure 2: Comparison of hepatocyte cytotoxicity of Phenobarbital in 3 inbred mouse strains (B6C3F1/J, C57BL/6J and NOD/LtJ) and two time points, culture Day 2 (grey bars) and Day 4 (black bars). Hepatocytes from male mice (4-6wks of age) were isolated by collagenase perfusion, purified by Percoll gradient centrifugation (final viability ≥ 90%) cultured on BD Biocoat™ 96 well plates in a conventional monolayer for 24 h and 72h, then cultured for a additional 24h in the presence of Phenobarbital (1,3,10,30,100 μM). Toxicity was assessed by evaluating cellular ATP content and GSH levels.

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