# Evaluation of strategies to improve in vitro mutagenicity assessment: Alternative sources of S9 exogenous metabolic activation and the development of an in vitro assay based on MutaMouse primary hepatocytes

by

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## Abstract

*In vitro* genetic toxicity tests using cultured bacterial or mammalian cells provide a cost- and time-effective alternative to animal tests. Unfortunately, existing *in vitro* assays are not always reliable. This is in part due to the limited metabolic capacity of the cells used, which is often critical to accurately assess chemical genotoxicity. This limited metabolic capacity necessitates the use of exogenous sources of mammalian metabolic enzymes that can simulate *in vivo* mammalian metabolic activation reactions. In response to this, and other limitations, alongside the worldwide trend to reduce animal testing, there is an acute need to consider various strategies to improve *in vitro* mutagenicity assessment. This thesis first examined the utility of exogenous metabolic activation systems based on human hepatic S9, relative to conventional induced rat liver S9, for routine genetic toxicity assessment. This was accomplished by critically evaluating existing literature, as well as new experimental data. The results revealed the limitations of human liver S9 for assessment of chemical mutagenicity. More specifically, the analyses concluded that, due to the increased risk of false negative results, human liver S9 should not be used as a replacement for induced rat liver S9. To address the limitations of conventional mammalian cell genetic toxicity assays that require exogenous hepatic S9, the thesis next evaluated the utility of an *in vitro* mutagenicity assay based on metabolically-competent primary hepatocytes (PHs) derived from the transgenic MutaMouse. Cultured MutaMouse PHs were thoroughly characterized, and found to temporarily retain the phenotypic attributes of hepatocytes *in vivo*; they express hepatocyte-specific proteins, exhibit the karyotype of typical hepatocytes, and maintain metabolic activity for at least

the first 24 hours after isolation. Preliminary validation of the *in vitro* MutaMouse PH gene mutation assay, using a panel of thirteen mutagenic and non-mutagenic chemicals, demonstrated excellent sensitivity and specificity. Moreover, inclusion of substances requiring a diverse array of metabolic activation pathways revealed comprehensive metabolic competence. Finally, the thesis further investigated the applicability domain of the *in vitro* MutaMouse PH assay by challenging the assay with selected azo compounds. Comparison of these results with those obtained using the *in vivo* MutaMouse TGR (transgenic rodent) assay revealed that MutaMouse PHs can carry out some forms of reductive metabolism. Overall, this thesis demonstrated that a gene mutation assay based on MutaMouse PHs holds great promise for routine assessments of chemical mutagenicity.

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## List of Abbreviations

| | |
|---|---|
| **1,8-DNP** | 1,8-dinitropyrene |
| **2-AA** | 2-aminoanthracine |
| **2-AAF** | 2-acetylaminofluorene |
| **2-AF** | 2-aminofluorene |
| **3-MC** | 3-methylcholanthrene |
| **3-NBA** | 3-nitrobenzanthrone |
| **3Rs** | Reduction, refinement, and replacement |
| **6-AC** | 6-aminochrysene |
| **ATCC** | American Type Culture Collection |
| **AA** | Aromatic amine |
| **ADH** | Alcohol dehydrogenase |
| **AFB1** | Aflatoxin $B_1$ |
| **AhR** | Aryl hydrocarbon receptor |
| **ALDH** | Aldehyde dehydrogenase |
| **ANOVA** | Analysis of variance |
| **ARNT** | AhR nuclear translocator |
| **AROD** | Alkoxyresorufin-*O*-deethylase |
| **BaP** | Benzo[*a*]pyrene |
| **BDI** | Benzenediazonium ion |
| **Bl** | Bladder |
| **BM** | Bone marrow |
| **BMD** | Benchmark dose |

| | |
|---|---|
| **BMDL** | Benchmark dose 90% lower confidence limit |
| **BMDU** | Benchmark dose 90% upper confidence limit |
| **BMR** | Benchmark Response |
| **BPDE** | BaP-diol-epoxide |
| **BraCVAM** | Brazilian Centre for the Validation of Alternative Methods |
| **BROD** | Benzoxyresorufin-*O*-deethylase |
| **BSA** | Bovine serum albumin |
| **CA** | Chromosomal aberration |
| **CAR** | Constitutive androstane receptor |
| **CEPA** | Canadian Environmental Protection Act |
| **CHL** | Chinese hamster lung |
| **CHO** | Chinese hamster ovary |
| **CMP** | Chemicals Management Plan |
| **Co** | Colon |
| **CYP** | Cytochrome P450 |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **DEN** | Diethylnitrosamine |
| **DHS** | Donor horse serum |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DMN** | Dimethylnitrosamine |
| **DMSO** | Dimethyl sulphoxide |
| **E** | Equivocal |
| **ECACC** | European Collection of Cell Cultures |

| | |
|---|---|
| **ECHA** | European Chemicals Agency |
| **EFSA** | European Food Safety Authority |
| **EGF** | Epithelial growth factor |
| **EGTA** | Ethylene glycol tetraacetic acid |
| **EM** | Extensive metabolizer |
| **EMEM** | Eagle's minimum essential medium |
| **ENU** | *N*-nitroso-*N*-nitrosourea |
| **EROD** | Ethoxyresorufin-*O*-deethylase |
| **EU** | European Union |
| **EURL-ECVAM** | European Union Reference Laboratory for Alternatives to Animal Testing |
| **FBS** | Fetal bovine serum |
| **FISH** | Fluorescent *in situ* hybridization |
| **FMN** | Flavin mononucleotide |
| **FSC** | Forward scatter |
| **GPI** | Glycophosphatidylinositol |
| **GS** | Glandular stomach |
| **GST** | Glutathione-*S*-transferase |
| **GTTC** | Genetic Toxicology Technical Committee |
| **HBSS** | Hank's balanced salt solution |
| **HCA** | Heterocyclic amine |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| ***Hprt*** | X-linked hypoxanthine-guanine phosphoribosyltransferase |

| | |
|---|---|
| **HRP** | Horseradish peroxidase |
| **IARC** | International Agency for Research on Cancer |
| **ICATM** | International Cooperation on Alternative Methods |
| **ICH** | International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use |
| **ICR 191** | 6-Chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride |
| **ILSI/HESI** | International Life Sciences Institute/Health and Environmental Sciences Institute |
| **IM** | Intermediate metabolizer |
| **IPCS** | International Programme on Chemical Safety |
| **IQ** | 2-amino-3-methylimidazo[4,5-*f*]quinolone |
| **IVGT** | *In Vitro* Genetic Toxicity |
| **JaCVAM** | Japanese Centre for the Validation of Alternative Methods |
| **LC-MS/MS** | Liquid chromatography with tandem mass spectrometry |
| **LOEL** | Lowest observed effect level |
| **Lv** | Liver |
| **MAD** | Mutual Acceptance of Data |
| **MF** | Mutant frequency |
| **MLA** | Mouse lymphoma Tk gene mutation assay |
| **MN** | Micronucleus |
| **MMR** | Mismatch repair |
| **MOA** | Mode of sction |

| | |
|---|---|
| **MRM** | Multiple reaction monitoring |
| **MROD** | Methoxy-*O*-deethylase |
| **MSH2** | MutS protein homolog 2 |
| **MSH3** | MutS protein homolog 3 |
| **MSH6** | MutS protein homolog 6 |
| **NA** | Not applicable |
| **NAM** | New Approach Methodology |
| **NAT** | *N*-acetyltransferase |
| **NGS** | Next generation sequencing |
| **NICEATM** | U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods |
| **NQO1** | NADPH dehydrogenase, quinone 1 |
| **NS** | Not significant |
| **NTP** | National Toxicology Program |
| **OECD** | Organization for Economic Cooperation and Development |
| **PAH** | Polycyclic aromatic hydrocarbon |
| **PAPS** | 3'-phosphoadenosine-5'-phosphosulfate |
| **PBS** | Phosphate-buffered saline |
| **PBST** | PBS with 2 mL/L Tween 20 |
| **PCB** | Polychlorinated biphenyls |
| **PCPA** | Pest Control Products Act |
| **pfu** | Plaque-forming unit |
| **P-Gal** | Phenyl-β-D-galactopyranoside |

| | |
|---|---|
| **PH** | Primary hepatocyte |
| **PHH** | Primary human hepatocyte |
| **PhIP** | 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine |
| **PM** | Poor metabolizer |
| **PO** | Oral administration |
| **PROD** | Pentoxyresorufin-*O*-deethylase |
| **PXR** | Pregnane X receptor |
| **QC** | Quality control |
| **REACH** | European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals |
| **RNase** | Ribonuclease |
| **RICC** | Relative increase in cell count |
| **RINC** | Relative increase in nuclear count |
| **RPD** | Relative population doubling relative increase in cell count |
| **RXR** | Retinoid X receptor |
| **SCCS** | Scientific Committee on Consumer Safety |
| **SCE** | Sister chromatid exchange |
| **SD** | Standard deviation |
| **SDS** | Sodium dodecyl sulphate |
| **SE** | Standard error |
| **SFM** | Serum-Free Medium |
| **SI** | Small intestine |
| **SNP** | Single nucleotide polymorphism |

| | |
|---|---|
| **SPSF** | Standard Project Submission Form |
| **SSC** | Side scatter |
| **Subcu** | Subcutaneous administration |
| **TBHQ** | *tertiary* butylhydroquinone |
| **TCAG** | The Centre for Applied Genomics |
| **TCDD** | 2,3,7,8-tetrachlorodibenzodioxin |
| **TG** | Test guideline |
| **TGR** | Transgenic rodent |
| ***Tk*** | Thymidine kinase |
| **Trp-P2** | 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole |
| **UDS** | Unscheduled DNA synthesis |
| **UGT** | UDP-glucuronosyltransferase |
| **UM** | Ultrarapid metabolizers |
| **USFDA** | United States Food and Drug Administration |
| **US FIFRA** | United States Federal Insecticide, Fungicide, and Rodenticide Act |
| **WNT** | Working Group of National Coordinators of the Test Guidelines Committee |
| ***xprt*** | Xanthine-guanine phosphoribosyl transferase |
| **XRE** | Xenobiotic response elements |

## Statement of Contributions

***Chapter 2:*** The Utility of Metabolic Activation Mixtures Containing Human Hepatic Post-Mitochondrial Supernatant (S9) for *In Vitro* Genetic Toxicity Assessment

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| Literature survey and data analysis……... | Julie A. Cox<br>Paul A. White |
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# CHAPTER ONE

# Chapter 1: General Introduction

## 1.1 *In Vitro* Genetic Toxicity Testing for Regulatory Evaluations of Chemical Safety

### 1.1.1 Requirements for Genetic Toxicity Testing

Genetic damage is an important endpoint in chemical safety assessments due to its empirical and mechanistic links to debilitating diseases (e.g., cancer, heritable genetic disorders, somatic cell mutation disorders) [Hanahan and Weinberg, 2011; Campbell et al., 2015; Erickson, 2014; Stenson et al., 2003]. For example, exposure to environmental mutagens has been correlated with increased incidence of cancer in humans [Alexandrov et al., 2016; Wu et al., 2015]. Genotoxic events, such as mutations and chromosomal damage, are routinely assessed using a variety of *in vitro* and *in vivo* tests. Events commonly examined include nucleotide substitutions, small insertions and deletions (i.e., indels), whole chromosome loss or gain (i.e., aneuploidy), and aberrations resulting from chromosome breakage and rejoining. Genetic damage resulting in mutations and/or chromosomal aberrations can arise endogenously (e.g., effects of reactive oxygen species, DNA repair and replication errors), as well as via exposure to exogenous agents such as ionizing radiation and xenobiotic chemicals [Jackson and Loeb, 2001; Irigaray and Belpomme, 2010].

### 1.1.2 Current Genetic Toxicity Assessment Test Battery

The International Programme on Chemical Safety (IPCS) recommends that any testing strategy designed to assess genotoxicity should yield information on the ability of a test agent to cause gene mutations, structural chromosomal aberrations and aneuploidy [FAO and WHO, 2009]. As such, the Organization for Economic Cooperation and Development (OECD) has approved and published several standardized test guidelines (TGs) for both *in vitro* and *in vivo* genetic toxicity assays; these TGs are accepted by all OECD member countries.

*In vivo* genetic toxicity tests with OECD TGs include cytogenetic tests, such as the *in vivo* erythrocyte micronucleus (MN) test (TG 474) and the bone marrow chromosomal aberration (CA) test (TG 475), as well as gene mutation and DNA damage tests such as the transgenic rodent (TGR) gene mutation assay (TG 488), and the *in vivo* mammalian alkaline comet assay (TG 489) [OECD, 2016d; OECD, 2016b; OECD, 2013; OECD, 2016g]. These tests are frequently applied in test batteries in order to assess multiple complementary endpoints. The test batteries employed by different jurisdictions generally include both *in vitro* and *in vivo* tests. Importantly, the *in vitro* genetic toxicity assays are inherently far less expensive, time-consuming, and resource-intensive than the *in vivo* assays. Due to these advantages, *in vitro* tests form the cornerstone of routine chemical screening [Zeiger, 2010]. Although the tests specifically required for new substance safety evaluations vary by jurisdiction, *in vitro* components of test batteries generally require assessment of both gene mutations and chromosomal damage (Table 1-I).

**Table 1-I:** *In vitro* genotoxicity tests required under Canadian regulations, as well as regulatory requirements and/or recommendations in other jurisdictions worldwide.

| Legislation/Guideline | *In vitro* requirement | Reference |
|---|---|---|
| CEPA[a] | *In vitro* gene mutation test<br>*AND/OR*[b]<br>*In vitro* chromosomal damage test | [Minister of Justice, 2018] |
| PCPA[c] | Bacterial reverse mutation assay<br>*AND*<br>*In vitro* mammalian cell genotoxicity assay<br>*AND/OR*<br>*In vitro* chromosomal damage assay[d] | [Health Canada, 2013] |
| US FIFRA[e] | *In vitro* MLA[t]<br>*OR BOTH*<br>*In vitro* mammalian cell mutagenicity test<br>*AND*<br>*In vitro* chromosomal damage assay | [Jaeger, 1984; 40CFR158 (U.S. Code of Federal Regulations), 2019] |
| USFDA[g] Redbook | Bacterial gene mutation test<br>*AND*<br>*In vitro* mammalian cell chromosomal damage test<br>*OR*<br>*In vitro* MLA | [USFDA, 2007] |
| ICH[h] | Bacterial gene mutation test<br>*AND*<br>*In vitro* mammalian cell chromosomal damage test[i]<br>*OR*<br>*In vitro* MLA[i] | [ICH, 2011] |
| SCCS[j] | Bacterial reverse mutation assay<br>*AND*<br>*In vitro* MN[k] test | [SCCS, 2018] |
| EFSA[l] | Bacterial reverse mutation assay<br>*AND*<br>*In vitro* MN test | [EFSA Scientific Committee, 2011] |
| REACH[m] | Bacterial gene mutation test<br>*OR BOTH*[b]<br>*In vitro* mammalian cell chromosomal damage test<br>*AND*<br>*In vitro* mammalian cell mutagenicity test | [ECHA, 2017] |
| Chemical Substance Control Law (Japan) | Bacterial gene mutation test<br>*AND*<br>*In vitro* mammalian cell chromosomal damage test<br>*OR*<br>*In vitro* MLA | [METI, 2009] |

| | | |
|---|---|---|
| European Commission (Plant Protection Active Substances) | Bacterial reverse mutation assay<br>*AND*<br>*In vitro* mammalian cell chromosomal aberration test<br>*AND*<br>*In vitro* mammalian cell mutagenicity test | [European Commission, 2013] |

[a] CEPA, Canadian Environmental Protection Act
[b] Conditionally required based on production volume
[c] PCPA, Pest Control Products Act
[d] Only required if not addressed by *in vitro* mammalian cell genotoxicity assay
[e] US FIFRA, United States Federal Insecticide, Fungicide, and Rodenticide Act
[f] MLA, mouse lymphoma *Tk* gene mutation assay
[g] USFDA, United States Food and Drug Administration
[h] ICH, International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use
[i] *In vitro* mammalian genotoxicity tests not required if an *in vivo* assessment of genotoxicity in two different tissues is performed
[j] SCCS, Scientific Committee on Consumer Safety
[k] MN, micronucleus
[l] EFSA, European Food Safety Authority
[m] REACH, European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals

Both bacterial and mammalian cell *in vitro* assays have been developed to detect treatment-induced mutations. The bacterial reverse mutation test remains the most commonly used *in vitro* test (TG 471) (Table 1-II). The test employs a series of *Salmonella typhimurium* and/or *Escherichia coli* mutants that carry specific mutations in the histidine or tryptophan biosynthesis genes (i.e., histidine or tryptophan auxotrophs), and assesses the frequency of reversion to wild-type (i.e., prototophic growth). The *S. typhimurium* and *E. coli* strains employed, which are engineered to confer an ability to detect base-pair substitutions or frameshifts, also carry mutations and/or plasmids that enhance sensitivity for the detection of chemical mutagens [Mortelmans and Zeiger, 2000; OECD, 1997]. Despite the test's reliance on bacteria (i.e., bacterial metabolism and genome structure), Ames test results are routinely used for human health safety assessments (i.e., identification of mutagenic hazard). Gene mutation assays using mammalian cell lines have been established in an effort to develop *in vitro* assays with increased relevance to humans (Table 1-II). These include the *Hprt*/*xprt* mutation test (TG 476) and the thymidine kinase (*Tk*) gene mutation assay (TG 490) [OECD, 2016e; OECD, 2016c]. The *Hprt*/*xprt* and *Tk* assays are frequently performed using L5178Y mouse lymphoma, human TK6, Chinese Hamster ovary (CHO), Chinese Hamster lung (CHL), and Chinese Hamster V79 cell lines. These standardized assays are accepted internationally; moreover, the bacterial reverse mutation assay and/or a mammalian gene mutation assay (i.e., either the *Hprt*/*xprt* or the *Tk* assay) are required by regulatory agencies world-wide (Table 1-I).

**Table 1-II:** *In vitro* bioassays routinely used for genetic toxicity screening prior to regulatory evaluations of new and existing chemicals.

| Test | Endpoint | Commonly used cell lines | OECD[a] test guideline number |
|---|---|---|---|
| Bacterial reverse mutation test | Gene mutation | *Salmonella typhimurium* or *Escherichia coli* strains | 471 |
| *Tk* locus test | Gene mutation | L5178Y, TK6 | 490 |
| *Hprt*/*xprt* test | Gene mutation | CHO[b], CHL[c], V79, L5178Y, TK6, AS52 (for *xprt* only) | 476 |
| CA[d] test | Chromosome damage, nondisjunction | CHO, CHL, V79, TK6, primary human lymphocytes | 473 |
| MN[e] assay | Chromosome damage, nondisjunction | CHO, CHL, V79, L5178Y, TK6, primary mammalian lymphocytes<br>Less well-validated: HT29, Caco-2, HepaRG, HepG2, A549, and primary Syrian Hamster Embryo cells | 487 |

[a] OECD, Organization for Economic Co-operation and Development
[b] CHO, Chinese hamster ovary
[c] CHL, Chinese hamster lung
[d] CA, chromosomal aberration
[e] MN, micronucleus

In addition to the gene mutation assays described above, *in vitro* tests to assess chromosomal damage have also been developed (Table 1-II). *In vitro* CA assays (TG 473) employed to score damage frequency, including chromosome breaks, dicentrics, rings, and whole chromosome losses or gains (i.e., aneuploidy), generally involve microscopic analysis of mammalian cells (e.g., Chinese Hamster fibroblasts) following exposure to a chemical of interest and subsequent metaphase arrest [Hsu et al., 1977; OECD, 2016a]. The *in vitro* MN assay (i.e., TG 487) also assesses exposure-induced chromosomal damage. MNi are small nuclear envelope-bound bodies that form following chromosome breakage or loss and subsequent mitosis [Fenech, 2000]. The *in vitro* MN assay has rapidly gained popularity, due, in part, to its amenability to automated scoring via flow cytometry or automated slide-scanning, its applicability to multiple cell types, and the potential to detect both clastogens and aneugens [Kirsch-Volders et al., 2011; Avlasevich et al., 2006; Varga et al., 2004; OECD, 2016f]. Both the CA and MN assays are required by numerous regulatory agencies for the detection of chromosomal damage (Table 1-I).

### 1.1.3 *In Vitro* Assays – The Path Forward

Recent efforts to conform to changes in the regulatory landscape worldwide have shifted emphasis towards a greater, sometimes exclusive, reliance on *in vitro* tests as tools for human health hazard assessment and regulatory decision-making. These efforts include the 7$^{th}$ Amendment to the European Union (EU) Cosmetics Directive, which prohibits animal-tested cosmetics and cosmetic ingredients, as well as the *Toxicity Testing in the 21$^{st}$ Century* paradigm, which envisions an increased reliance on *in vitro*

techniques [Adler et al., 2011; European Commission, 2009; Krewski et al., 2010]. Overall, problems related to assay execution, reliability and performance (see Section 1.2 below), when combined with the global movement towards *in vitro* toxicity assessment tools, underscores the acute need for improved tools for *in vitro* mutagenicity assessment.

The need for sound and reliable *in vitro* assays has led to the establishment of several international initiatives committed to the development and promotion of alternative *in vitro* test methods. These initiatives include the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the Japanese Centre for the Validation of Alternative Methods (JaCVAM), the Brazilian Centre for the Validation of Alternative Methods (BraCVAM), and several others [Barroso et al., 2016]. Although Canada does not have a national initiative aimed at establishing and validating alternative test methods, the Health Canada's Environmental Health Science and Research Bureau is a founding signatory of the International Cooperation on Alternative Methods (ICATM) that strives to facilitate international cooperation regarding the development of alternative methods [Barroso et al., 2016]. Moreover, Health Canada and Environment and Climate Change Canada have committed to integrating New Approach Methodologies (NAMs), including *in silico*, *in chemico*, and *in vitro* assays, into traditional risk assessment [CMP Science Committee, 2016]. The importance of NAMs for regulatory decision-making was formally recognized at a European Chemicals Agency (ECHA) scientific workshop in April 2016 [ECHA, 2016]. These initiatives and organizations provide a foundation for the development, validation, and deployment of alternative *in vitro* methods.

## 1.2 Drawbacks of *In Vitro* Tools Currently Employed for Genetic Toxicity Assessment

### 1.2.1 Issues Related to Assay Performance

Despite the advantages of in vitro assays in general, and the demonstrated utility of the aforementioned assays, the current battery of *in vitro* genetic toxicity assays has come under fire for having a relatively low predictive capacity with respect to the identification of *in vivo* mutagens and/or carcinogens. A 2005 study by Kirkland et al. sought to evaluate the performance of a conventional *in vitro* genetic toxicity test battery, including the Ames test, the mouse lymphoma *Tk* gene mutation assay (MLA), and *in vitro* MN or CA assays, by measuring the sensitivity (i.e., ability to correctly detect mutagens/carcinogens) and specificity (i.e., ability to correctly detect non-mutagens/non-carcinogens). The study found that although the sensitivity of mammalian cell assays is fairly high, the specificity is often unacceptably low [Kirkland et al., 2005]. This low specificity may lead to the generation of false or misleading positive results that can, in turn, lead to unnecessary *in vivo* follow-up for genotoxic or carcinogenic effects [Kirkland et al., 2007].

### 1.2.2 Issues Related to Metabolic Deficiency

Many chemical mutagens cannot react with DNA directly; rather their metabolites, such as unstable electrophiles, are DNA-reactive [Miller, 1970]. In the field of genetic toxicology, “metabolic activation” refers to the metabolic conversion (i.e., enzymatic catalysis) of otherwise inert substances into DNA-reactive metabolites [H. R. Glatt, 2000]. Importantly, most of the cell types used in the aforementioned *in vitro*

genetic toxicity assays cannot carry out the metabolic reactions that occur in mammals *in vivo* (Table 1-II). For example, *S. typhimurium* and *E. coli*, the bacteria used in bacterial reverse mutation tests, are not capable of carrying out major mammalian drug metabolizing processes, thus limiting their ability to detect genotoxicants that require metabolic conversion to DNA-reactive metabolites. Additionally, the mammalian cells commonly used for genotoxicity assessment typically have diminished endogenous metabolic capacity; therefore, they are also unable to effectively catalyse the metabolic reactions required to generate DNA-reactive compounds [Pelkonen et al., 2013]. The limited metabolic capacity of these test systems necessitates the use of exogenous sources of mammalian metabolic enzymes that can simulate the *in vivo* catalysis that converts parent compounds to DNA-reactive metabolites.

Catalyzed reactions related to xenobiotic metabolism, including reactions pertaining to the aforementioned metabolic activation, are divided into two main categories: Phase I and Phase II. Phase I reactions involve the modification and/or addition of functional groups (e.g., oxidation, reduction, and hydrolysis reactions), whereas Phase II reactions are characterized by conjugation of metabolites to chemical groups (e.g., glutathione, sulphate, glucuronide). Important Phase I reactions, in particular those involved in metabolic activation, are catalyzed by isozymes belonging to the cytochrome P450 (CYP) superfamily [Rendic and Guengerich, 2012]. In general, Phase II reactions utilize the products of Phase I reactions as substrates for conjugation reactions. Phase II metabolic enzymes include N-acetyltransferases (NATs), sulfotransferases (SULTs), glutathione-S-transferases (GSTs), and UDP-glucuronosyltransferases (UGTs) [Rendic and Guengerich, 2012]. Both Phase I and

Phase II reactions are crucial for the metabolic activation of many classes of chemical mutagens.

Importantly, CYP expression can be modified by cellular exposure to specific xenobiotics. Induction of CYP expression is mediated by increased gene transcription, protein stabilization, or RNA stabilization. For example, the expression of genes encoding the CYP1 family of isozymes is regulated via the aryl hydrocarbon receptor (AhR), which, once bound to polycyclic ligands such as polycyclic aromatic hydrocarbons (PAHs), is transported from the cytoplasm into the nucleus via the AhR nuclear translocator (ARNT) [Waxman, 1999]. The ligand-bound AhR-ARNT complex can then bind to xenobiotic response elements (XREs) thereby upregulating CYP1 family gene expression. Certain orphan nuclear receptors, including the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR), also regulate CYP gene transcription. Induction of CYP2A, CYP2B, and CYP2Cis mediated by CAR, and induction of CYP3A is mediated mainly by PXR. In the presence of inducers, CAR and PXR dissociate from co-repressors and recruit co-activators and a dimerization partner, such as the retinoid X receptor (RXR), bind to DNA response elements of promoter regions of target CYP genes, and upregulate transcription [Waxman, 1999]. Interestingly, induction of some CYP isozymes, such as CYP2E1, appears to be facilitated by the stabilization of protein and mRNA levels. In the presence of low doses of ethanol-type inducers, CYP2E1 protein synthesis remains constant, while degradation is reduced [Gonzalez, 2007]. The induction and/or stabilization of metabolic enzymes has a critical impact on the detoxification and metabolic activation of xenobiotic chemicals.

Metabolic activation has been extensively studied for several chemical classes of mutagens and carcinogens, including PAHs, nitrosamines, aromatic amines (AAs), heterocyclic amines (HCAs), nitroarenes, fungal metabolites, and azo compounds (Table 1-III). Many mutagens are activated solely through CYP-mediated oxidation reactions, including PAHs, nitrosamines, and some fungal metabolites. Figure 1 illustrates the Phase I-mediated metabolic activation of the prototypical PAH benzo[*a*]pyrene (BaP) [Jeffrey, 1985; Bauer et al., 1995; Kim et al., 1998]. A variety of mutagens require both Phase I oxidation reactions and Phase II conjugation reactions for metabolic activation. These include AAs and HCAs; Figure 2 illustrates the metabolic activation of a prototypical HCA 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [Schut and Snyderwine, 1999; Cai et al., 2016]. Some mutagens require more complex metabolic activation, including Phase I oxidation and/or reduction reactions, as well as Phase II reactions (e.g., nitroarenes and azo compounds). Figure 3 illustrates the complex metabolic activation of the nitroarene 3-nitrobenzanthrone (3-NBA) [IARC, 2014]. Ideally, *in vitro* genetic toxicity assays should be capable of detecting mutagens requiring diverse modes of metabolic activation.

**Table 1-III:** Metabolic requirements of different classes of chemical genotoxicants

| Chemical class | Example | Metabolic Requirements | References |
|---|---|---|---|
| Polycyclic aromatic hydrocarbons | Benzo[*a*]pyrene (BaP) | CYP1A1<br>CYP1A2<br>CYP3A<br>Epoxide hydrolase | [Jeffrey, 1985; Bauer et al., 1995; Kim et al., 1998] |
| Aromatic amines | 2-Acetylaminofluorene (2AAF) | CYP1A1<br>CYP1A2<br>SULT<br>NAT | [Heflich and Neft, 1994] |
| Heterocyclic amines | 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) | CYP1A1<br>CYP1A2<br>SULT<br>NAT<br>UGT | [Schut and Snyderwine, 1999; Cai et al., 2016] |
| Nitroarenes | 1,8-Dinitropyrene (1,8-DNP) and 3-Nitrobenzanthrone (3-NBA) | CYP1A1<br>CYP1A2<br>NQO1<br>NAT<br>SULT | [IARC, 2014; Arlt et al., 2003; Arlt et al., 2005] |
| Nitrosamines | Dimethylnitrosamine (DMN) | CYP2E1 | [Chowdhury et al., 2012; Yamazaki et al., 1992; Hoffmann and Hecht, 1985] |
| Fungal metabolites | Aflatoxin $B_1$ (AFB1) | CYP1A2<br>CYP3A | [Gallagher et al., 1994] |
| Azo compounds | Direct Black 38 | Bacterial azoreductase<br>NQO1<br>NAT<br>SULT | [Manning et al., 1985; Martin et al., 1982; Møller and Wallin, 2000] |

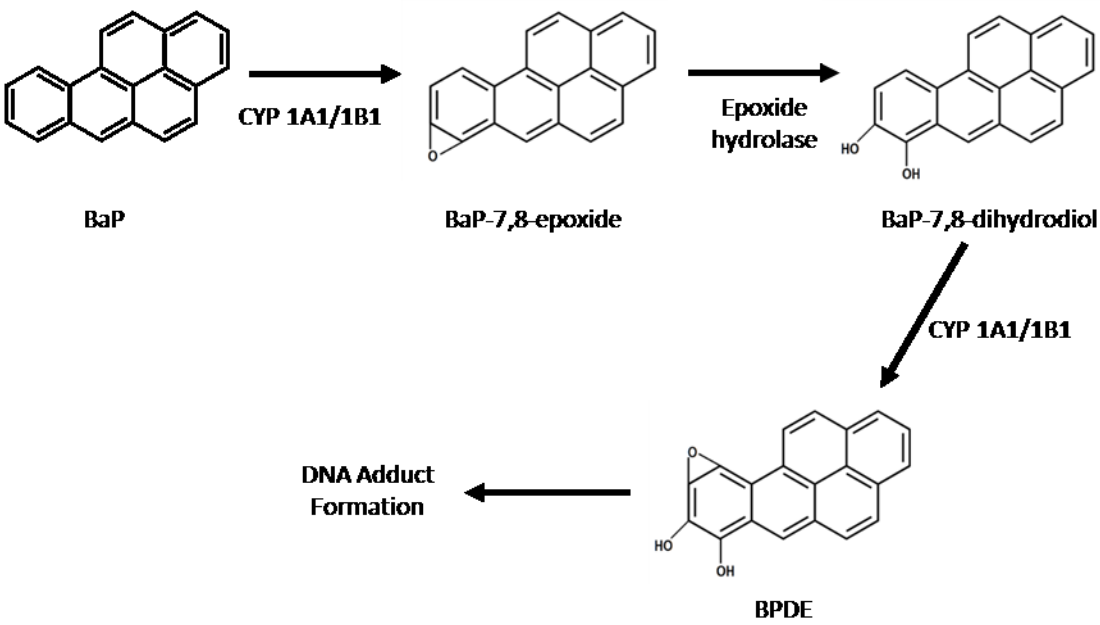


**Figure 1-1:** Metabolic activation of benzo[*a*]pyrene (BaP), illustrating the Phase I enzymes involved and the DNA-reactive metabolite BaP-7,8-dihydrodiol-9,10-epoxide (BPDE). Stereochemical features are not indicated.

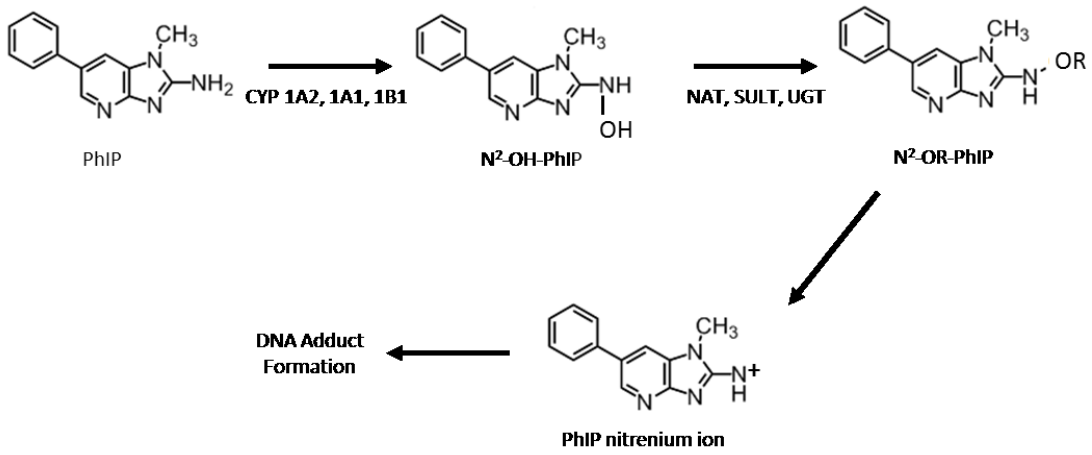


**Figure 1-2:** Metabolic activation of 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) demonstrating the Phase I and Phase II enzymes involved, and the formation of the DNA-reactive nitrenium ion. Stereochemical features are not indicated.

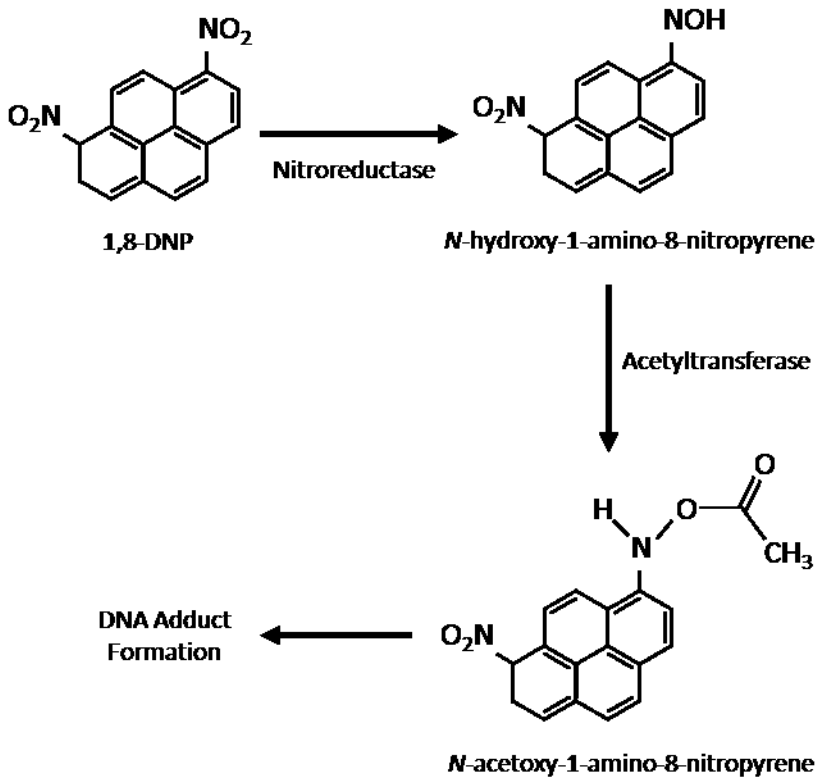


**Figure 1-3:** Metabolic activation of 1,8-dinitropyrene (1,8-DNP), demonstrating the role of enzymes capable of nitroreductase activity (i.e., NQO1). Stereochemical features are not indicated.

As noted earlier, both bacterial and mammalian cells commonly used for genetic toxicity assessment lack endogenous mammalian metabolic capacity. More specifically, in order to simulate *in vivo* mammalian xenobiotic activation and detoxification, *in vitro* assays commonly employ mammalian metabolic enzyme preparations; these preparations are routinely added to bacterial and mammalian cell cultures [Ku et al., 2007]. The most popular source of exogenous metabolic activation enzymes is, by far, S9 (i.e., post-mitochondrial supernatant) prepared from the livers of male Sprague-Dawley rats treated with enzyme-inducing agents such as Aroclor 1254, or a combination of ß-naphthoflavone and phenobarbital [OECD, 1997]. Aroclor 1254, a commonly used inducing agent, is a commercial mixture of polychlorinated biphenyls (PCBs) [Ku et al., 2007] that includes dioxin-like PCBs (i.e., coplanar), which are AhR agonists, as well as other PCBs that are CAR and PXR agonists. Phenobarbital and β-naphthoflavone are ligands of CAR and AhR, respectively, thus they are often used together to stimulate the production of hepatic CYPs prior to preparation of S9 from Sprague-Dawley rat liver. Induced rat liver S9 is a key component of the standardized "S9 metabolic activation mixture", alongside an NADPH-generating system. S9 metabolic activation mixtures are a required component of both bacterial and mammalian genetic toxicity assays.

Although induced rat liver S9 is routinely used in *in vitro* genotoxicity assays, its use carries several drawbacks. Firstly, Aroclor 1254-induced rat liver S9 favours CYP-mediated Phase I oxidation reactions over other types of reactions, including reduction reactions and Phase II conjugation reactions [H. R. Glatt et al., 1981]. The components necessary for reductive metabolism or Phase II conjugation, such as flavin mononucleotide (FMN) for reductive reactions, and 3'-phosphoadenosine-5'-

phosphosulfate (PAPS) for sulfotransferase enzymes, are not generally included for typical regulatory assessments [To et al., 1982; Prival and Mitchell, 1982]. This overrepresentation of CYP-catalysed reactions, and underrepresentation of other metabolic enzymes and processes, may hamper the activation of mutagens requiring both Phase I and Phase II metabolism, and/or mutagens requiring reductive metabolism (Table 1-III; Figures 2 and 3). Secondly, induced rat liver S9 is known to be cytotoxic to cultured mammalian cells, thus limiting exposure durations [Kugler et al., 1987; Madle, 1981]. Finally, with respect identification and assessment of human health hazards, the human relevance of a homogenized liver preparation from a chemically induced rodent has been called into question [Ku et al., 2007]. These drawbacks have, in part, led to the development of new approaches for *in vitro* genotoxicity assessment, such as the use of metabolically competent cells.

### 1.2.3 Issues Related to Genome Structure, Genotype, and Genetic Stability

Additional weaknesses of cell lines commonly employed for *in vitro* genotoxicity assessment relate to genome structure, genotype, and/or genetic stability. For example, bacterial reverse mutation tests employ *S. typhimurium* or *E. coli* as test organisms; the genomes of these organisms are fundamentally different from mammalian cells, thus raising concerns about the relevance of the bacterial reverse mutation test as a tool for human risk assessment. More specifically, bacterial genomes typically carry one circular chromosome plus plasmids, thus they cannot be used to examine chemically-induced chromosomal aberrations. Additionally, the immortalized cells often used for mammalian cell genetic toxicity assessments typically have aberrant karyotypes that include

numerous deletions, translocations and duplications. Consequently, some commonly used cell lines, such as mouse lymphoma L5178Y $Tk^{+/-}$ cells, show characteristics that are similar to oncogenically-transformed tumour cells, including impaired p53 function [Storer et al., 1997]. Functional p53 protein plays a crucial role in DNA repair [Honma and Hayashi, 2011], and cell lines with non-functional p53 may display an inflated mutant frequency (MF) with respect to normal tissue *in vivo*. This could lead to an increase in false positive predictions for mutagenic and carcinogenic activity *in vivo*. Addressing these weaknesses by using cells that are genetically stable and karyotypically normal may improve in the reliability and performance of *in vitro* genetic toxicity tests.

### 1.2.4 Issues Related to Scoring Strategies

Conventional assays for the enumeration of chemically-induced mutations and chromosomal damage often involve time-consuming scoring tasks. For example, enumeration of CAs (TG 473) requires laborious microscopic observation of cells arrested in metaphase [OECD, 2016a]. This requirement has been alleviated by the transition to the use of the MN assay (TG 487), whereby evidence of chromosomal damage can be rapidly scored by flow cytometry [OECD, 2016d]. With respect to scoring chemically-induced mutations, the commonly employed assays necessitate laborious clone isolation and enumeration. For example, the *Hprt/xprt* gene mutation assays require a minimum of 7 to 9 days to allow expression of the mutant phenotype, during which, the cells must be regularly sub-cultured to maintain exponential growth. Following this expression period, cells must be re-plated at an appropriate density, with and without selective medium, and allowed to proliferate for approximately 7 to 12 days to permit

colony formation [OECD, 2016e]. Similarly, the *Tk*-based gene mutation assays require a phenotypic expression period of 2 to 4 days, followed by a period of 10 to 14 days to allow for the formation of mutant colonies [OECD, 2016c]. In order to improve the alignment of *in vitro* mutagenicity assessment tools with the principles laid out in *Toxicity Testing in the 21st Century* (e.g., more rapid screening, increased reliance on *in vitro* tools), emphasis should be placed on developing *in vitro* tests that employ rapid and efficient procedures for endpoint scoring.

## 1.3 Strategies to Overcome Outlined Drawbacks and Deficiencies

### 1.3.1 Alternate S9 Sources

Although Aroclor 1254- or phenobarbital/β-naphthoflavone-induced rodent liver S9 is most commonly used as an exogenous *in vitro* source of mammalian metabolic enzymes, a variety of other metabolic enzyme inducing agents can be used in the preparation of S9 fractions. Other AhR ligands, including 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD), have been utilized as CYP inducers [Tang and Friedman, 1977]. Additionally, phenobarbital and β-naphthoflavone can be used separately to induce specific metabolic enzymes, e.g., CYP2A, CYP2B, CYP2C, and CYP3A by phenobarbital, and CYP1A1, CYP1A2, and CYP1B1 by β-naphthoflavone [Waxman, 1999]. Ethanol or pyrazole is also occasionally used as a CYP2E1 inducer; ethanol and pyrazole-induced S9 fractions are known to be effective in the activation of *N*-nitrosamines [Burke et al., 1994; Mori et al., 2002]. Overall, the use of alternative inducers to Aroclor 1254 or phenobarbital/β-naphthoflavone is often reserved for

applications wherein specific metabolic enzymes are known or suspected to be involved in the activation of a particular chemical or class of chemicals.

S9 fractions are most often prepared from the liver, since this organ is associated with the activation and detoxification of xenobiotics via CYP-catalysed reactions; however, S9 can be prepared from virtually any tissue. Extrahepatic tissues such as the lung, kidney, and gastrointestinal tract are also known to be metabolically competent for the production of mutagenic metabolites, and they have been used as sources of S9 [Bartsch et al., 1980; Hutton and Hackney, 1975; Mori et al., 1987; Frandsen and Alexander, 2000]. Such extra-hepatic S9 fractions can be valuable for comparative studies examining the roles of different tissues in the metabolic activation or detoxification of chemicals of interest.

Hepatic S9 from a variety of mammalian species has also been explored. Although the molecular biology of xenobiotic-metabolizing enzymes is widely conserved across mammals, there exist noteworthy species-specific differences [Guengerich, 1997]; these discrepancies can lead to significant differences in *in vitro* genetic toxicity assay results [Hengstler et al., 1999]. For example, compounds such as nitrosamines and aflatoxins are metabolized in humans by CYP2A6 (Table 1-III); however, the substrate specificities of the CYP2 family enzymes are known to vary widely among different mammalian species [Guengerich, 1997]. More specifically, human CYP2A6 has drastically different substrate specificity in comparison with other mammalian CYP2A enzymes, such as rodent CYP2A enzymes, which have steroid hydroxylation activities [Honkakoski and Negishi, 1997; Crespi et al., 1990]. The CYP3A subfamily of enzymes also demonstrates considerable interspecies variability. For example, rifampicin is not an

inducer of rat CYP3A enzymes, but it is a potent inducer of human CYP3A4, which is known to be involved in the activation of PAHs and AFB1 (Table 1-III) [Lu and Li, 2001]. The known species-specific differences in S9 metabolic profiles can influence the type of S9 chosen for the assessment of specific agents. For example, knowledge about the metabolic activation of azo compounds, which requires azo bond reduction and release of aromatic amines, has led to a preference for hamster S9, and a markedly different activation mixture. More specifically, mutagenicity assessment of azo compounds commonly employs hepatic S9 from uninduced hamsters, in an activation mixture containing FMN to facilitate reductive metabolism. The approach has led to the development of a modified version of the Ames test sometimes referred to as the Prival assay [Prival and Mitchell, 1982; Prival et al., 1984; Prival et al., 1988]. Although species-specific attributes of different S9 types can be exploited to better assess certain test agents (e.g., the Prival method); they may also limit the reliability of inter-species results extrapolation.

The aforementioned characteristics of exogenous metabolic activation mixtures from induced rat hepatic S9, as well as the well-studied interspecies variability with respect to metabolic enzyme substrate specificity and inducibility, have stimulated interest in the use of human liver S9. Human liver S9 has become easier to acquire commercially in recent years, and has been discussed as a more relevant alternative for the identification of mutagens and assessment of human health hazards, relative to hepatic S9 from chemical-induced rodents [Ku et al., 2007]. Few investigations have evaluated the utility of human liver S9 for routine genetic toxicity testing; however, there is some evidence suggesting that human liver S9 may more effectively activate certain

mutagens, such as AAs, and less effectively activate others mutagens, such as PAHs, in comparison with induced rat liver S9 [Beaune et al., 1985]. Although the concept of using human liver S9 for genetic toxicity testing appears promising, it requires thorough examination before being implemented in any regulatory framework.

### 1.3.2 Use of Cells with Improved Metabolic Competence

Numerous tools have been used to create metabolically competent cells, thus improving the relevance and concordance of *in vitro* assessments relative to *in vivo* testing. These include isolation of metabolically-competent cells lines such as HepaRG, which was derived from a tumour in a patient who suffered from hepatocellular carcinoma and hepatitis C [Gripon et al., 2002]. The HepaRG cell line has been shown to be a useful tool for toxicological and pharmacological assessments. For example, HepaRG cells have been used in the *in vitro* MN and comet assays [Hégarat et al., 2014]. Alternatively, some researchers have transfected existing cell lines with specific Phase I and/or II enzymes. These include V79-derived cell lines genetically engineered to express human CYP1A2, CYP2E1, SULT 1A1 and/or NAT 2, as well as the MCL-5 cell line, a human B-lymphoblastoid line that expresses transfected cDNAs for CYP1A2, CYP2A6, CYP2E1, CYP3A4, and microsomal epoxide hydrolase [H. Glatt et al., 2005; Chevereau et al., 2017; Crespi et al., 1991]. Although these cell lines show promise for toxicological applications, they are not designed to permit efficient and convenient scoring of exposure-induced mutations.

Since hepatocytes carry out most liver-related functions, including the detoxification and metabolic activation of xenobiotic chemicals, primary hepatocytes

(PHs) are an attractive, metabolically competent cell type for *in vitro* genetic toxicity assessment [Ulrich et al., 1995; Gao et al., 2008; Ishibashi et al., 2009]. Many strides have been made towards *ex vivo* culturing of metabolically competent, proliferative PHs. For example, two-stage collagenase perfusion methods have been developed to isolate high yields of healthy PHs from rodents for *in vitro* culturing [Klaunig et al., 1981]. Growth media containing selected supplements, such as insulin, as well as collagen- or matrigel-coated culture surfaces, have been employed to maintain the morphology and metabolic activity of PHs in culture [Kreamer et al., 1986; Laishes and Williams, 1976]. Importantly, since hepatocytes in a healthy liver typically proliferate at a very low rate, and proliferation is essential for fixation of genetic damage into mutations (i.e., via replicative processes), techniques must be employed to confirm that the rate of cell turnover is sufficient for the detection of mutations. Fortunately, PH proliferation *in vitro* can be achieved with the addition of epithelial growth factor (EGF) [Block et al., 1996; Muller-Tegethoff et al., 1997].

Cultured PHs have been used to assess *in vitro* genotoxic endpoints that require replication, including mutation formation and cytogenetic damage (e.g., MN formation) [Eckl and Raffelsberger, 1997; Muller-Tegethoff et al., 1997; Chen et al., 2010]. More elaborate *in vitro* models, such as co-cultures of human PHs and stromal cells (i.e., the HepatoPac system), have also been developed to maximize metabolic competence and cellular viability; however, these techniques are not well suited for convenient scoring of genetic damage [Chan et al., 2013; Khetani and Bhatia, 2008]. Cellular proliferation *in vitro*, together with maintained metabolic competence, makes cultured PHs promising tools for *in vitro* genetic toxicity testing.

### 1.3.3 Innovations that Increase Throughput and Performance

The shift towards increased reliance on *in vitro* methods, coupled with the aforementioned scoring difficulties associated with several currently advocated genetic toxicity assays, has led to the development of improved scoring approaches. For example, as noted earlier, a flow cytometry-based methodology for MN scoring (i.e., the MicroFlow™ assay), which was developed by Litron Laboratories (Rochester, NY), has substantially increased the throughput of the *in vitro* MN assay [Bryce et al., 2008; OECD, 2016f]. Similarly, the CometChip® assay has greatly increased the throughput and ease of the *in vitro* comet assay, a test designed to quantify DNA damage including abasic sites, crosslinks, and strand breaks [Wood et al., 2010; Ge et al., 2014]. The *in vitro PIG-A* assay in human B-lymphoblastoid cell lines, which is analogous to the *in vivo* rodent erythrocyte *Pig-a* gene mutation assay, measures the frequency of mutations at the X-linked *PIG-A* gene encoding an enzyme critical to the formation of glycophosphatidylinositol (GPI) anchor proteins. *Pig-a* mutation frequency, or more precisely the *Pig-a* mutant phenotype frequency, is measured by flow cytometric assessment of the frequency of cells that are unable to bind a fluorescent ligand [Rees et al., 2017]. *In vitro* gene mutation assays utilizing cells derived from TGRs have also been developed; they take advantage of the convenient mutation-scoring system inherent to TGR systems, as described in more detail in Section 1.3.4 [White et al., 2019]. These developments allow for high(er) throughput sample processing relative to conventional *in vitro* scoring strategies, thus supporting the *Toxicity Testing in the 21st Century* paradigm.

Researchers have also sought to develop assay variants that directly address the low specificity associated with some *in vitro* assays. Fowler and colleagues demonstrated that the rate of false or misleading positive results associated with the *in vitro* mammalian MN assay can be reduced by employing p53-competent human-derived and/or primary cells, and by using appropriate cytotoxicity measurement techniques (e.g., relative population doubling (RPD) or relative increase in cell count (RICC)) [Fowler, Smith et al., 2012a; Fowler, Smith et al., 2012b]. These recommendations suggested by Fowler et al. are designed to improve the reliability of existing, conventional *in vitro* mammalian cell genetic toxicity assays.

### 1.3.4 Use of Cells with Transgenic Vectors

In order to address issues that hamper efficient endpoint scoring for existing *in vitro* gene mutation assays, several *in vitro* assays have been developed that employ cells derived from TGRs. The *in vivo* TGR somatic and germ cell mutation assay (TG 488) was approved by the OECD in July 2011; the TG was later revised in 2013 [OECD, 2013]. TGRs used for *in vivo* chemical safety assessments include the MutaMouse, the Big Blue® Mouse and Rat, the pUR288 *lacZ* Plasmid Mouse, and the *gpt* Delta Mouse and Rat. The *in vivo* TGR mutagenicity assessment systems rely on rodents whose genomes carry multiple copies of chromosomally-integrated shuttle vectors or plasmids containing mutation target genes. Once recovered from genomic DNA, plasmid or phage shuttle vectors can be used to reliably score mutations in a transfected bacterial host [Lambert et al., 2005]. *In vivo* TGR mutation assays allow for chemical-induced mutations to be detected in any tissue of the organism; therefore, in principle, cell lines

derived from any TGR tissue will carry the mutation target-containing shuttle vectors, and can be used for mutagenicity assessment *in vitro*.

More than 20 TGR-based *in vitro* assays have been developed for mutagenicity assessment [White et al., 2019]. These include both cells derived from the TGR models themselves, and existing cell lines that have been transfected with shuttle vectors carrying mutation targets. Examples of the former include the immortalized FE1 cell line derived from MutaMouse lung tissue [White et al., 2003; Berndt-Weis et al., 2009; Maertens et al., 2017; Hanna, 2018], fibroblasts from the Big Blue® Mouse and Rat [Besaratinia et al., 2018], embryonic fibroblasts from the *lacZ* Plasmid Mouse [Mahabir et al., 2009], and the immortalized GDL1 cell line derived from *gpt* Delta Mouse lung fibroblasts [Takeiri et al., 2006]. Rat2 embryonic fibroblasts are an example of a cell line that has been transfected with a TGR shuttle vector. In the case of Rat2 cells, they have been transfected with λ-LIZ, the same shuttle vector that is carried by the Big Blue® Mouse and Rat [Erexson et al., 1998]. Due to their inherent transgenic mutation targets, and the well-established techniques for mutation scoring, *in vitro* assays utilizing cells derived from these systems present promising alternatives to existing, conventional *in vitro* gene mutation assays.

### 1.3.5 Combination of Primary Cell, Metabolic Competence, and TGR Scoring

Recently, mutagenicity assays using PHs derived from both the MutaMouse and the pUR288 *lacZ* Plasmid Mouse have been developed [Chen et al., 2010; Zwart et al., 2012]. These novel assays take advantage of the metabolic competence and karyotypic stability of PHs, and the reliable MF scoring system of TGR models. PHs from both of

these TGR models have been shown to be capable of reliably detecting increases in MF following exposure to known mutagens requiring metabolic activation [Chen et al., 2010; Zwart et al., 2012; Luijten et al., 2016]. More specifically, MutaMouse PHs have been shown to be capable of detecting mutagenicity following exposure to BaP, PhIP, 3-nitrobenzanthrone (3-NBA), and cigarette smoke condensate, whereas pUR288 *lacZ* Plasmid Mouse hepatocytes have yielded positive results following exposure to BaP, cyclophosphamide, etoposide, bleomycin, *N*-ethyl-*N*-nitrosourea (ENU), and mitomycin C in the absence of any exogenous source of mammalian metabolic enzymes [Chen et al., 2010; Zwart et al., 2012; Luijten et al., 2016]. Additionally, examinations of metabolic capacity have demonstrated that PHs from the pUR288 *lacZ* Plasmid Mouse express BaP-inducible CYP1A1/1A2 activity, as measured by ethoxyresorufin-*O*-deethylase (EROD), as well as UGT and GST activities [Zwart et al., 2012; Luijten et al., 2016]. These examples indicate that PHs from TGR systems can be used as the basis for novel mammalian gene mutation assays that would constitute alternatives to existing mammalian cell *in vitro* mutagenicity tests. However, before any novel test can be used, thorough validation is required to establish the assay's suitability for routine use, as well as define its reliability, performance and limitations.

## 1.4 Validation of New (Geno)toxicity Tests

Prior to being adopted for routine use in regulatory decision-making, any toxicity assay must be extensively validated. The aforementioned OECD TG program offers standardized toxicology test protocols that are accepted by the OECD member countries. Indeed, the OECD TG program operates under the Mutual Acceptance of Data (MAD)

principle that aims to eliminate conflicting or redundant testing requirements, thus lowering trade barriers related to human and environmental safety issues [OECD, 2005]. In order to ensure test reliability for safety assessments, the OECD has established recommended criteria for the acceptance of new assay protocols and establishment of new TGs. These criteria are inspired by the "Solna Principles" developed at an OECD workshop in Solna, Sweden; they apply to both *in vitro* and *in vivo* tests [OECD, 1996]. The recommended criteria include the following [OECD, 2005]:

1. *Test definition*, encompassing both the rationale behind the test method, and the relationship between the test endpoint and the biological effect of interest, as well as the proposed test's limitations;
2. *Intra-laboratory repeatability and reproducibility*, as assessed by evaluating the variability of results between different operators and different dates within the same laboratory;
3. *Inter-laboratory transferability*, as assessed by the ability of a test procedure to be accurately and reliably performed in at least one other independent laboratory;
4. *Inter-laboratory reproducibility*, which typically requires a formal validation study including multiple competent laboratories and blind testing of both positive and negative test agents;
5. *Predictive capacity*, as measured by the sensitivity and specificity of the assay with respect to existing toxicity data in relevant species;
6. *Applicability domain*, which is defined following thorough validation, elucidates the chemical classes, mechanisms of action, and ranges of responses that the test method can reliably assess;

7. *Performance standards*, comprising the essential test method components, minimum list of reference chemicals, and accuracy and reliability values.

The OECD suggests a workflow for assay validation beginning with informal pre-validation prior to formal inter-laboratory validation [OECD, 2005]. The pre-validation steps should begin with a thorough optimization and characterization of all aspects of the proposed test and its protocol. This should be followed by an initial assessment of inter-laboratory transferability. If, at any point, the assay fails to demonstrate acceptable performance, it may be further refined, or, failing that, rejected. Once the novel method has proven to be robust, reliable, and transferable, it can continue on to formal inter-laboratory validation.

Following completion of the validation process described above, a Standard Project Submission Form (SPSF) for a novel TG, along with supporting data, is submitted to the Working Group of National Coordinators of the Test Guidelines Committee (WNT). Subsequently it undergoes rigorous critical review by various committees to ensure that the proposed TG is both relevant and reliable [OECD, 2005; Gourmelon and Delrue, 2016]. More specifically, the committees ensure that data obtained using the proposed TG should be adequately predictive of an endpoint of interest. The data obtained should also be of equivalent or greater value for risk assessment than data obtained using existing methods. The TG should be amenable to standardization, thus requirements for specialized equipment or materials should be minimized. The TG should also be cost- and resource-effective, i.e., not prohibitively expensive and does not require large quantities of unusual reagents. Finally, scientific, ethical, and/or economic justification for the use of the proposed TG, relative to existing

TGs, should be provided. Particular emphasis should be placed on respecting the 3 Rs of animal welfare, i.e., replacement, reduction, and refinement [OECD, 2005; Russell and Burch, 1959]. Once sanctioned by the OECD, and accepted by regulatory authorities under the MAD principle, any results obtained using the TG can be utilized for regulatory decisions in any OECD member country.

## 1.5 Thesis Objectives

### 1.5.1 Thesis Objectives

This project seeks to address the fundamental drawbacks of *in vitro* assays routinely employed to assess genetic toxicity, with emphasis placed on metabolic considerations, endpoint scoring, and assay performance, via the development and preliminary validation of an alternative *in vitro* mammalian cell mutagenicity assay based on PHs from the MutaMouse. More specifically, the project encompasses 3 overarching objectives:

1. A critical examination of the utility of exogenous metabolic activation systems based on human hepatic S9 for routine genetic toxicity assessment (i.e., as an alternative to induced rat liver S9) (**Chapter 2**).
2. The structural, genetic, and enzymatic characterization of cultured MutaMouse PHs (**Chapter 3**).
3. Evaluation of the performance of an *in vitro* genetic toxicity assessment system based on PHs from the MutaMouse (**Chapter 4**). This performance evaluation includes investigations regarding the applicability domain of the *in vitro* MutaMouse PH gene mutation assay, including critical examinations of responses

to chemicals requiring complex metabolic activation (i.e., azo compounds). (**Chapter 5**).

### 1.5.2 Research Chapter Overviews

**Chapter 2 – "The Utility of Metabolic Activation Mixtures Containing Human Hepatic Post-Mitochondrial Supernatant (S9) for *In Vitro* Genetic Toxicity Assessment"**

*Objective:*

Assess the utility of human liver S9 for *in vitro* genotoxicity assessment (i.e., relative to induced rat liver S9), and formulate recommendations regarding its routine use in chemical safety assessments.

*Tasks:*

- Quantitatively review all available dose-response data pertaining to the use of human liver S9 for *in vitro* genetic toxicity assessment.
- Compare metabolic enzyme specific activity profiles of human liver S9 and induced rat liver S9.
- Employ literature review and experimentation to compare *in vitro* genetic toxicity results based on human liver S9 with those based on assays employing induced rat liver S9. To account for the paucity of published information, experimentation will be used to generate additional *in vitro* genetic toxicity data.

## Chapter 3 – "The development and pre-validation of an *in vitro* mutagenicity assay based on MutaMouse primary hepatocytes. Part I: Isolation, structural, genetic and biochemical characterization"

*Objective:*

Thoroughly characterize cultured MutaMouse PHs with respect to biochemical, structural, and genetic attributes. The characterised cell system will constitute the foundation for development and establishment of a novel *in vitro* gene mutation assay.

*Tasks:*

- Optimise PH isolation and culture protocols to yield metabolically competent, proliferative cultures of MutaMouse PHs.
- Characterise cultured MutaMouse PHs on the basis of morphology and expression of hepatocyte-specific markers.
- Evaluate the karyotype of cultured MutaMouse PHs.
- Determine the *in vitro* proliferation rate of MutaMouse PHs.
- Via the use of gene expression and metabolic enzyme activity analyses, assess the basal and induced metabolic capacity of cultured MutaMouse PHs.

**Chapter 4 – "The development and pre-validation of an *in vitro* mutagenicity assay based on MutaMouse primary hepatocytes, Part II: Assay performance for the identification of mutagenic chemicals"**

*Objective:*

Examine the ability of an *in vitro* gene mutation assay based on MutaMouse PHs to correctly evaluate a series of carefully selected test chemicals.

*Tasks:*

- Evaluate assay performance with respect to ability to correctly determine the mutagenic hazard of 9 known mutagens, including direct-acting mutagens (i.e., *N*-ethyl-*N*-nitrosourea [ENU] and 6-Chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride [ICR 191]), and mutagens requiring diverse enzymes for metabolic production of DNA-reactive metabolites (i.e., BaP, AFB1, 2-acetlyaminofluorene [2-AAF], PhIP, 1,8-DNP, 3-nitrobenzanthrone [3-NBA], and dimethylnitrosamine [DMN]).
- Evaluate the ability of the assay to correctly classify 4 known non-mutagens, including 2 true negatives (i.e., ampicillin trihydrate and D-mannitol) and 2 misleading positives (i.e., *tertiary* butylhydroquinone [TBHQ] and eugenol).

**Chapter 5 – "The mutagenic activity of select azo compounds in MutaMouse target tissues *in vivo* and primary hepatocytes *in vitro*"**

*Objective:*

To determine the utility of MutaMouse PHs for *in vitro* mutagenicity assessment of chemicals that undergo complex metabolism and activation. More specifically, compare the *in vitro* and *in vivo* mutagenicity results of selected azo compounds.

*Tasks:*

- Assess the *in vivo* mutagenicity of selected azo compounds (i.e., Direct Black 38, Sudan I, and Para Red) in relevant tissues of the MutaMouse (i.e., the bone marrow, glandular stomach, small intestine, colon, liver, and bladder) following repeat-dose oral exposure.
- Assess the mutagenicity of the selected azo compounds in the MutaMouse PH gene mutation assay, and compare these results with those obtained using the *in vivo* assay.
- Comparative interpretation of *in vivo* and *in vitro* results to define the applicability domain and utility of the *in vitro* MutaMouse PH gene mutation assay for the assessment of chemical mutagenicity.

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# CHAPTER TWO

# Chapter 2: The Utility of Metabolic Activation Mixtures Containing Human Hepatic Post-Mitochondrial Supernatant (S9) for *In Vitro* Genetic Toxicity Assessment

## 2.1 Preamble: Authors, Affiliations, and Style

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## 2.2 Abstract

*In vitro* genotoxicity assessment routinely employs an exogenous metabolic activation mixture to simulate mammalian metabolism. Activation mixtures commonly contain post-mitochondrial liver supernatant (i.e., S9) from chemically induced Sprague Dawley rats. Although Organization for Economic Cooperation and Development (OECD) test guidelines permit the use of other S9 preparations, assessments rarely employ human-derived S9. The objective of this study is to review and evaluate the use of human-derived S9 for *in vitro* genetic toxicity assessment. All available published genotoxicity assessments employing human S9 were compiled for analysis. To facilitate comparative

analyses, additional matched Ames data using induced rat liver S9 were obtained for certain highly cited chemicals. Historical human and induced rat S9 quality control reports from Moltox were obtained and mined for enzyme activity and mutagenic potency data. Additional *in vitro* micronucleus data were experimentally generated using human and induced rat S9. The metabolic activity of induced rat S9 was found to be higher than human S9, and linked to high mutagenic potency results. This study revealed that human S9 often yields significantly lower Salmonella mutagenic potency values, especially for polycyclic aromatic hydrocarbons, aflatoxin B1, and heterocyclic amines (~3-350-fold). Conversely, assessment with human S9 activation yields higher potency for aromatic amines (~2-50-fold). Outliers with extremely high mutagenic potency results were observed in the human S9 data. Similar trends were observed in experimentally generated mammalian micronucleus cell assays, however human S9 elicited potent cytotoxicity L5178Y, CHO, and TK6 cell lines. Due to the potential for reduced sensitivity and the absence of a link between enzyme activity levels and mutagenic potency, human liver S9 is not recommended for use alone in *in vitro* genotoxicity screening assays; however, human S9 may be extremely useful in follow-up tests, especially in the case of chemicals with species-specific metabolic differences, such as aromatic amines.

## 2.3 Introduction

The noteworthy correlation between mutagenic and carcinogenic hazard has resulted in an understandable regulatory preoccupation with accurate detection of genetic toxicity and concomitant regulatory actions to protect human health. The routine

screening of chemicals for genotoxic and/or mutagenic activity was first proposed more than half a century ago [1, 2], and genotoxic events, such as gene mutations and chromosomal damage, are routinely assessed using a variety of *in vitro* and *in vivo* tests. The convenience and affordability of *in vitro* test systems for routine screening resulted in *in vitro* assays forming the cornerstone of most regulatory systems to assess genetic toxicity [2]. More recently, with efforts to conform to changes in the regulatory landscape worldwide (e.g., 7th Amendment to the European Union (EU) Cosmetics Directive, Tox21, ToxCast, etc.), emphasis has shifted away from *in vivo* testing towards a greater, sometimes exclusive, emphasis on *in vitro* testing and predictive tools for human health risk assessment and regulatory decision-making [3]. The current *in vitro* battery of genetic toxicity assays employed by most regulatory agencies comprises a bacterial reverse mutation assay (Ames test), an *in vitro* mammalian cell gene mutation assay such as the mouse lymphoma assay (MLA) or the X-linked hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) assay, and an *in vitro* mammalian cell chromosome damage assay such as the micronucleus (MN) or chromosome aberration (CA) test, and formerly, the sister chromatid exchange (SCE) assay [2].

Metabolic activation is a crucial consideration in genetic toxicity testing. Due to the metabolic deficiencies of *S. typhimurium* and other microorganisms, mammalian metabolic enzyme preparations, such as post-mitochondrial or 9000 x g tissue homogenate supernatants, generally dubbed S9, can be added to *in vitro* cultures to simulate *in vivo* xenobiotic activation and detoxification [4]. Rodent liver S9 is similarly used in *in vitro* mammalian cell genetic toxicity assays. *In vitro* mammalian cell genetic toxicity assays frequently utilize immortalized and/or transformed cell lines that are often

metabolically deficient. Like the aforementioned prokaryotes, these cell lines are incapable of activating many promutagens in the absence of an exogenous metabolic enzyme preparation such as rodent liver S9.

S9 can be prepared from a variety of tissues; however, it is most often prepared from the liver, since this organ is associated with detoxification of xenobiotics via cytochrome P450 (CYP) catalysed reactions. Liver S9 from a variety of mammalian species has been used in genotoxicity assays, and there exist noteworthy species-, strain-, and sex-specific differences [5]. These discrepancies can lead to significantly different responses in genotoxicity assays when using hepatic preparations from different sources [6].

The most popular source of exogenous metabolic enzymes for *in vitro* genetic toxicity testing, by far, is S9 prepared from the livers of male Sprague-Dawley rats treated with enzyme-inducing agents such as Aroclor 1254 or a combination of ß-naphthoflavone and phenobarbital [7]. Aroclor 1254, a commonly used inducing agent, is a commercial mixture of polychlorinated biphenyls (PCBs) [4] that includes dioxin-like coplanar PCBs that are aryl hydrocarbon receptor (AhR) agonists, thus inducers of CYP1A1, CYP1A2, and CYP1B1, as well as non-coplanar PCBs that are constitutive androstane receptor (CAR) and pregnane X receptor (PXR) agonists, thus inducers of members of the CYP2 and CYP3 families of enzymes. Alternative commercial PCB mixtures, such as Kaneclor 400, also exist. Other AhR ligands, such as 3-methylcholanthrene (3-MC) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD), have also been utilized as enzyme inducers in genetic toxicology [8]. Phenobarbital and β-naphthoflavone, which are ligands of CAR and AhR, respectively, are also often used to

stimulate the production of CYPs in rodents such as the Sprague-Dawley rat prior to S9 preparation [9]. The use of the phenobarbital/β-naphthoflavone induction system permits avoidance of Aroclor 1254, which is a restricted agent in many countries.

Despite the convention to employ induced hepatic S9 from the rat, alternative activation systems are recommended for specific types of agents. For example, hepatic S9 from uninduced hamsters is frequently used for mutagenicity assessment of azo-compounds in a modified Ames test. The technique, which is sometimes referred to as the Prival assay, has been shown to be highly effective for the detection of mutagenic azo dyes [10-12].

Although Aroclor 1254-induced rat S9 has become the standard for metabolic activation mixtures employed with *in vitro* genotoxicity assays, several concerns have arisen with respect to its routine use for genotoxicity assessment. The most serious concerns relate to the relevance of using a homogenized liver preparation from a chemically induced rodent to identify substances that may pose genotoxic risks to humans. In addition, the use of activation mixtures containing rat liver homogenate with cultured mammalian cell lines is hampered by marked cytotoxicity [13, 14]. In comparison with hepatic S9 from chemical-induced rodents, human liver S9 could be considered more relevant to the identification and assessment of human health hazards.

There are some obvious caveats associated with the use of human liver S9 for *in vitro* genetic toxicity assessment. For example, deviation from a system that employs controlled experimental animals could introduce uncontrolled variability across lots of human-derived S9. Humans, as a species, are extremely variable with respect to metabolic capacity, and factors such as age and genotype are known to affect the ability

to metabolize xenobiotics [15]. With respect to genotypic variability (i.e., genetic polymorphisms), fast and slow metabolizer phenotypes have been described for numerous enzymes, such as those in the CYP1, CYP2, and CYP3 families, as well as *N*-acetyltransferases (NATs) and glutathione-*S*-transferases (GSTs). Many of the known polymorphisms have been associated with certain ethnic groups [16, 17], and the differences are known to markedly impact the toxicity of xenobiotic substances and the efficacy of certain drugs.

In addition to variability associated with human polymorphisms, humans are also potentially exposed to a wide range of agents (e.g., foods, therapeutic products) that can modify the expression levels of hepatic enzymes. As such, the activity and expression of metabolic enzymes involved in the production of DNA-reactive substances can be expected to vary markedly from one individual to another. For example, tobacco smoke contains AhR agonists that induce CYP1A1, CYP1A2, and CYP1B1 expression, and many commonly consumed therapeutic agents are also known to significantly alter enzyme activity levels. The combination of genetic polymorphisms and exposures to substances that modify enzymatic activity levels results in selected sub-populations displaying elevated sensitivities to certain genotoxic agents [18-20].

The International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) project committee on the Relevance and Follow-up of Positive Results in *In Vitro* Genetic Toxicity (IVGT) Testing, the predecessor of the Genetic Toxicology Technical Committee (GTTC), was tasked with investigating the causes of the low specificity associated with many *in vitro* genetic toxicity assessment tools (i.e., high frequency of false positives for animal carcinogenicity) [21]. Part of this

international exercise involved evaluating options for improving the performance of existing test systems. For example, the project committee considered ways to standardise the source and handling of mammalian cell lines employed for genotoxicity assessment [22]. In addition, the committee was charged with evaluating the utility of exogenous activation systems containing human liver S9 in comparison with the conventional systems containing induced rat liver S9. The objective of this study is to review the available scientific literature pertaining to the use of human S9 for genetic toxicity assessment, and via the use of meta-analysis of published mutagenicity test results, as well as the generation of new experimental data, assess the utility of human S9, relative to induced rat liver S9, for *in vitro* genetic toxicity assessments. Since few studies employed human S9 for mammalian *in vitro* genotoxicity assessment, the new experimental data largely pertain to assessments in mammalian cells.

## 2.4 Materials and Methods

### 2.4.1 Literature survey

Using the PubMed and Scopus databases, the scientific literature was rigorously searched to obtain all publications pertaining to the use of human liver S9 for genetic toxicity assessment (updated in December 2013). The search employed various combinations of the following search terms: human, S9, liver, hepatic, mutation, exogenous, metabolism, metabolic enzymes, post-mitochondrial supernatant, clastogen, strand break, DNA, micronucleus, *Hprt*, Ames, salmonella, mutagen, carcinogen, and risk assessment. The overwhelming majority of published research on the topic pertains to the use of human S9 with the Salmonella reverse mutation assay; mutagenic potency

data were collected from published studies and compiled in an Excel Workbook. In cases where the Salmonella mutagenicity results were only presented in figures, image analysis of digitised images was employed to regenerate the concentration-response data (e.g., GetData Graph Digitizer 2.26).

After the initial search for human S9 data, matching rat liver S9 results were collected for all chemicals wherein more than ten mutagenic potency results, or observations, were collected for *S. typhimurium* (i.e., Ames test) strains employed in combination with human S9. Ten or more human S9 observations were found for 2-aminoanthracene (2-AA) in TA100, 2-aminofluorene (2-AF) in both TA100 and TA98, 3-MC in TA100, 2-acetylaminofluorene (2-AAF) in both TA100 and TA98, aflatoxin B1 (AFB1) in both TA100 and TA98, benzo[*a*]pyrene (BaP) in TA100, 2-amino-3-methylimidazo[4,5-*f*]quinolone (IQ) in TA98, 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) in TA98, and dimethylnitrosamine (DMN) in TA1535. For comparative purposes, approximately 20 Aroclor-induced rat hepatic S9 observations for each of these chemical-strain combinations were collected from the literature and the National Toxicology Program (NTP) database [23]. The majority of the collected rat data was generated using S9 from Sprague-Dawley rats.

To critically examine the enzymatic profiles of Aroclor 1254-induced Sprague-Dawley rat liver S9; and moreover, to compare the profiles to those of human S9 preparations, we obtained historical quality control (QC) reports from Molecular Toxicology (Moltox) Inc. (Boone, NC). The pooled human S9 QC reports contain information about human donors, including age, race, sex, cause of death, medical conditions, substance use, and cold ischemia time. QC reports for both human-derived

and Aroclor 1254-induced rat-derived liver S9 contain lot-specific alkoxyresorufin-*O*-deethylase (AROD) specific activity values (i.e., ng substrate per min per mg microsomal protein): more specifically ethoxyresorufin-*O*-deethylase (EROD), benzoxyresorufin-*O*-deethylase (BROD), methoxy-*O*-deethylase (MROD), and pentoxyresorufin-*O*-deethylase (PROD) specific activities. Additionally, these reports contained the results of Ames tests conducted using the specified S9 lot. The human S9 reports utilized BaP and 2-AA as positive controls, whereas Aroclor 1254-induced rat S9 reports utilized 6-aminochrysene (6-AC) and 2-AA.

### 2.4.2 Human liver S9 usage survey

A survey of 24 members of the Mammalian Mutagenicity Society, a special interest group associated with the Japanese Environmental Mutagenesis Society, was performed. This survey comprised 16 questions regarding the members' use of human liver S9 in genotoxicity testing *in vitro* (Supplementary Material).

### 2.4.3 Test chemicals

All chemicals and reagents were purchased from Sigma/Aldrich Dorset UK, unless otherwise stated.

### 2.4.4 Metabolic activation

Sprague Dawley rat liver S9 was obtained from the hepatic microsomal fraction of Aroclor 1254-treated and untreated rats and provided by Molecular Toxicology Inc. (Boone, NC, USA). Human S9 was obtained from Molecular Toxicology Inc. and BD

Biosciences (Oxford, UK). S9 was stored at -70°C or below until use. On the day of use, S9 mix was prepared by the addition of culture medium or buffers containing cofactors for NADPH generation to the S9 fraction. For Ames testing, the final S9 mix contained 4 mM NADP, 25 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl and 10% (v/v) S9 mix [24]. For human S9 experiments using mammalian cells, the S9 mix yielded final culture concentrations of 6 mM NADP and 24 mM isocitrate [25]. A final S9 concentration of 50 μL/plate was used in the Ames assay and 2% to 10% v/v was used for all mammalian cell tests.

### 2.4.5 Ames test

Standard plate incorporation assays were performed using *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 and *Escherichia coli* strain WP2uvrA(pKM101) according to published methods [26-28]. All test compounds were dissolved in dimethyl sulphoxide (DMSO). In all tests, there were three plates for the control and each concentration of test compound. Plates were scored using a Perceptive Instruments Sorcerer image analyser.

### 2.4.6 Cell lines

All cell lines were confirmed to be mycoplasma free. Mouse lymphoma L5178Y cells, clone 3.7.2C, were obtained from Dr. J. Cole, (MRC Cell Mutation Unit, University of Sussex, Brighton, UK). TK6 human lymphoblast cells and Chinese Hamster Ovary (CHO) cells were obtained from the European Collection of Cell Cultures (ECACC). L5178Y and TK6 cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK)

supplemented with 10% heat inactivated donor horse serum (DHS), 2 mM L-glutamine, 2 mM sodium pyruvate, 1% Pluronic F68 (L5178Y cells only), 200 IU/mL penicillin, and 200 μg/mL streptomycin. CHO cells were cultured in McCoy's medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 2 mM sodium pyruvate. Cells were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

### 2.4.7 Treatment with test chemicals

All test compounds were dissolved in DMSO. Treatment exposure time was 3 hours. 1 x $10^7$ L5178Y cells or 2 x $10^7$ TK6 cells were suspended in 10 mL RPMI containing 2.5% DHS. CHO cells were seeded at 5 x $10^4$ in McCoys medium containing 10% FBS and incubated overnight before treatment in McCoys medium containing 2.5% FBS. The serum concentration is reduced during treatment to limit protein binding of extra-cellular metabolites. For all test agents, solvent or test chemical solution was added to the cell cultures in duplicate at 1% v/v. Following treatment, CHO cells were washed once and received 3 mL fresh McCoys medium containing 10% FBS. TK6 and L5178Y cells were centrifuged, washed once and re-suspended in 10 mL RPMI containing 10% DHS at final cell concentrations of 4 x $10^5$/mL or 2 x $10^5$/mL, respectively. Cultures were incubated for approximately 24 hours prior to preparation of microscope slides [29].

As recommended in the Organization for Economic Cooperation and Development (OECD) test guideline 487, cytotoxicity was measured by Relative Population Doubling (RPD) as indicated below [30].

$$RPD = \frac{Number\ of\ population\ doublings\ in\ treated\ cultures}{Number\ of\ population\ doublings\ in\ control\ cultures} \times 100$$

where,

$$Population\ doubling = \frac{\log(\frac{Post\ treatment\ cell\ number}{Initial\ cell\ number})}{log2}$$

### 2.4.8 Preparation and scoring of slides for micronuclei enumeration

CHO cells were trypsinised and counted using a haemocytometer. TK6 and L5178Y were also counted using a Coulter™ Counter. Microscope slides were prepared by centrifuging 2 x $10^4$ cells in a Cytospin 3 (Shandon) centrifuge (200 x g for 8 minutes) and fixed with methanol. Slides were stained with acridine orange and scored at 200x magnification using a Zeiss Axioplan microscope. All identified micronuclei were confirmed by eye to be separate and within the cytoplasm, to have an intact cytoplasmic membrane, and to be less than one third of the diameter of the main nucleus. Where possible, a total of at least 1000 cells per duplicate culture were scored.

### 2.4.9 Statistical analysis

All data analyses were carried out using SAS v.9.2 for Windows. Salmonella mutagenic potency values (i.e., the slope of the initial linear portion of the concentration-response function) for experimentally-generated data (i.e., 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole [Trp-P2] and PhIP), and published concentration-response data obtained from the literature, were determined using ordinary least-squares linear regression. Comparisons of mean enzyme activity or mutagenic potency values across data groups (e.g., S9 source) were investigated using least-squares analysis of variance (ANOVA). Post-hoc comparison of means across treatment groups employed Duncan's Multiple Range Test. In most cases, the values were $\log_{10}$ transformed to equalise the variance across the groups and meet the assumptions of least squares ANOVA.

Homogeneity of variance was evaluated using the Bartlett test, and the distribution of the residuals evaluated using the Kolmogorov-Smirnov test and visual inspection of a normal probability plot. For all observations obtained from Ames test data, results were deemed positive if a dose response was apparent, and the number of induced revertants reached at least two-fold the number of spontaneous. Contingency table analysis was used to examine the association between mutagenicity test outcome (i.e., positive or negative) and S9 source (i.e., rat or human). The statistical significance of the association was evaluated using the Pearson chi squared statistic. A Chi-square test with Yates-like modification was used to compare the induced frequency of micronucleated cells with matched negative controls, thereby evaluating statistical significance.

## 2.5 Results

### 2.5.1 Data collection

An extensive review of the scientific literature revealed 53 publications that employed human S9 preparations for *in vitro* mutagenicity assessment. The vast majority of these publications, 45 in total, describe the use of human S9 for Salmonella mutagenicity assessment, with only 7 publications describing the use of human S9 for genotoxicity assessment in mammalian cells (i.e., MLA, *Hprt*, SCE, CA, MN, and *in vitro* Comet) (see Supplementary Tables 2-I and 2-II). One publication described the use of human S9 in the bacterial *umu* assay. As described in the Materials and Methods section, an additional literature search was performed to collect approximately 20 matched Aroclor-induced rat liver S9 Ames test results for all chemicals wherein more

than ten observations were found that used human liver S9 with a particular strain of *S. typhimurium*. This search yielded an additional 54 publications and 16 NTP reports.

Many of the publications found during the literature survey described the use of liver-derived S9 from other species, in addition to human. Wherever possible, mutagenic potency values associated with all S9 sources mentioned in the publications were collected from Salmonella mutagenicity studies or calculated from reported concentration-response data. In total, 1340 mutagenic potency values were obtained from the literature across 108 compounds tested in 9 Ames test strains. An additional 10 Ames test results, comprising 6 human S9 and 4 rat S9 observations, were generated experimentally for PhIP and Trp-P2. Of the 1350 total observations, 678 utilized human S9, 312 utilized Aroclor 1254-induced rat S9, 106 utilized rat S9 induced by a combination of phenobarbital and β-naphthoflavone, 30 utilized rat S9 treated with other inducers (e.g., 3-MC or TCDD), 84 utilized uninduced rat S9, 40 utilized uninduced hamster S9, 18 utilized induced hamster S9, 29 utilized uninduced mouse S9, 6 utilized induced mouse S9, 17 utilized uninduced pig S9, 12 utilized uninduced monkey S9, 11 utilized uninduced rabbit S9, 5 utilized uninduced dog S9, and 2 utilized uninduced guinea pig S9.

### 2.5.2 Enzymatic activity profiles

Comparisons across lots of Moltox hepatic S9 was accomplished via the analysis of AROD activity values. Certain ethers of the fluorescent chemical resorufin are known to be metabolized to their parent compound by specific CYPs [31, 32], and CYP activity

is commonly assessed as EROD activity for CYP1A1 and CYP1A2, BROD for CYP2B1 and CYP3A, MROD for CYP1A2, and PROD for CYP2B1 and CYP2B2.

Moltox provided historical QC results for 100 lots of Aroclor 1254-induced rat liver S9 and 19 lots of human liver S9. The rat liver S9 QC results contain enzymatic activity measured as EROD, BROD, MROD, and PROD. An analysis of AROD activity data from the QC results of Moltox S9 lots demonstrates the dramatic upregulation of enzymatic activity associated with Aroclor 1254 treatment (Table 2-I). In comparison, typical AROD specific activities (mean ± SE) for phenobarbital/β-naphthoflavone-induced rat liver S9 from Moltox are 5883.2 ± 520.8, 6525.2 ± 792.1, 833.2 ± 112.8, and 1649.0 ± 175.9 pmoles/min/mg protein for EROD, BROD, MROD, and PROD, respectively (N=16). Typical AROD specific activities (mean ± SE) for uninduced hamster liver S9 from Moltox, which is frequently used for the assessment of *N*-nitroso compounds and azo compounds, are 78.4 ± 21.4, 180.1 ± 36.8 , 198.8 ± 52.8, and 12.8 ± 3.1 pmoles/min/mg protein for EROD, BROD, MROD, and PROD, respectively (N=6).

**Table 2-I:** Comparison of mean AROD activity levels in uninduced and Aroclor 1254-induced rat liver S9 and human liver S9 preparations.

| Assay | Mean activity (SEM[a]) in pmoles/min/mg protein | | |
|---|---|---|---|
| | Uninduced rat liver S9 (N = 100) | Aroclor 1254-induced rat liver S9 (N = 100) | Human liver S9 (N = 19) |
| EROD | 55.2 (2.8) | 6580.9 (338.2) | 94.6 (42.1) |
| BROD | 80.0 (3.9) | 3098.1 (160.6) | 40.1 (17.3) |
| MROD | 17.4 (0.8) | 1856.1 (96.4) | 101.6 (33.9) |
| PROD | 28.9 (1.2) | 790.1 (36.9) | ND[b] |

[a]SEM, standard error of the mean
[b]ND, No Data

The Moltox QC sheets for human liver S9 contain enzymatic activity values expressed as EROD, BROD, and MROD. Not surprisingly, the data for human S9 lots indicate that the levels of EROD, BROD and MROD are far lower in comparison to the Aroclor-induced rat liver S9 values summarised in Table 2-I. Figure 2-1 illustrates mean AROD activity levels and their distributions for human liver S9 and Aroclor 1254-induced rat liver S9. The data show that mean levels of EROD, BROD and MROD activity are on average 55-, 52- and 16-fold greater in Aroclor 1254-induced rat liver S9 compared with human liver S9, respectively. The induced rat liver S9 activity values follow a normal distribution. Conversely, although the majority of human S9 lots varied very little, the data indicate that the distributions are positively skewed with outliers showing enzymatic activities that are 22.0-, 19.3- and 10.9-fold above the geometric mean for EROD, BROD and MROD, respectively.

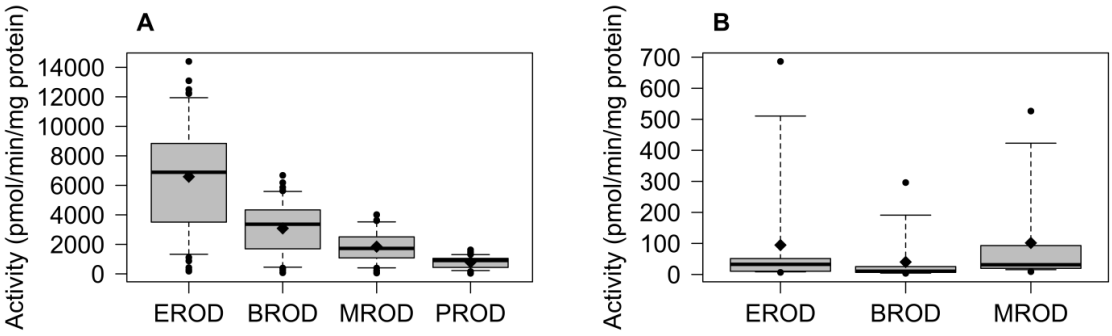



**Figure 2-1:** Distribution of cytochrome P450 (CYP) enzyme activity levels in Moltox lots of Aroclor 1254-induced rat liver S9 (A) and Moltox lots of human liver S9 (B). The enzyme activity levels were measured as EROD, BROD, MROD, and PROD activities. Solid lines represent medians, diamonds represent means, box edges represent first and third quartiles, whiskers represent 5th and 95th percentiles, and circles represent outliers.

### 2.5.3 Ames mutagenic activity comparison

Chemical data wherein more than ten potency observations based on human liver S9 in a particular Ames strain were found in the literature underwent a comparative analysis with induced rat liver results. Analysis of published mutagenic potency data indicates that metabolic activation by human liver S9 generally leads to lower values in comparison with those observed with induced rat liver S9 (Table 2-II, Figure 2-2). For example, the mean mutagenic potencies for the PAHs BaP and 3-MC were approximately 4- and 8-fold higher in TA100 when tested using Aroclor 1254-induced rat liver S9, respectively (Figures 2-2A and 2-2B). The potencies of AFB1 in TA100 and TA98 are approximately 5- and 40-fold greater, respectively, when tested in the presence of Aroclor 1254-induced rat liver S9 (Figures 2-2C and 2-2D). In the presence of Aroclor 1254-induced rat liver S9, IQ yields TA98 potency values that are on average approximately 350-fold greater than those observed in the presence of human liver S9 (Figure 2-2K). Similarly, the TA98 potency of HCAs Trp-P2 and PhIP are approximately 200- and 8-fold greater, respectively, when tested in the presence of rat liver S9 treated with any inducer (Figures 2-2L and 2-2M). It is interesting to note the considerable variability of the mutagenic potency data for both IQ and Trp-P2 when tested in the presence of human liver S9.

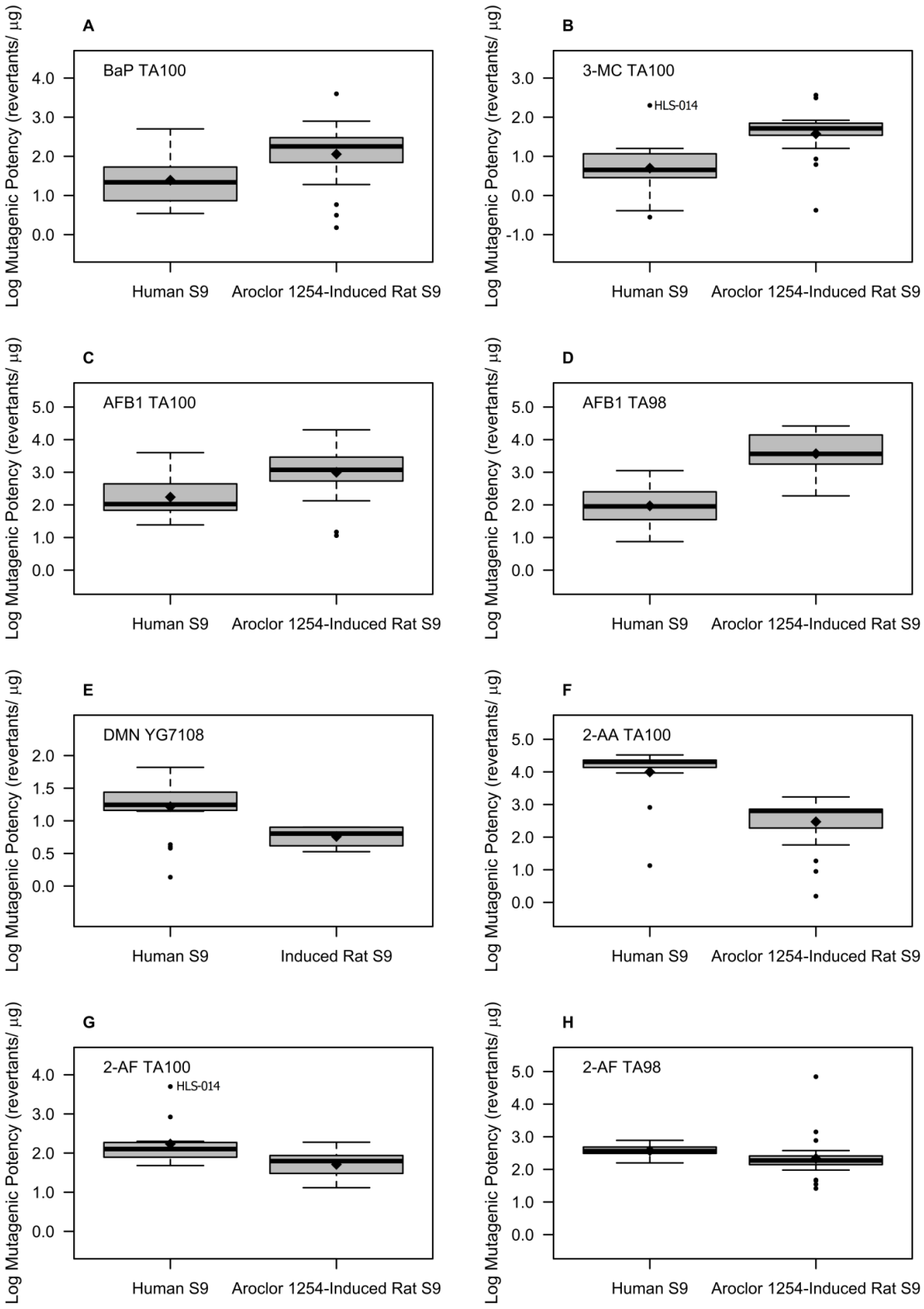
**Table 2-II:** Summary of analysis of variance (ANOVA) results comparing Ames test mutagenic potency data obtained using induced rat liver S9 and human liver S9

| Compound | Strain | N | Geometric Mean Mutagenic Potency (revertants/µg) | | Fold Difference (Induced Rat S9/ Human S9) | F Ratio (p Value) |
|---|---|---|---|---|---|---|
| | | | Induced Rat S9 | Human S9 | | |
| BaP | TA100 | 45 | 66.3 [a] | 18.0 | 3.7 | 9.6 (p=0.003) |
| 3-MC | TA100 | 35 | 42.3 [a] | 5.6 | 7.5 | 16.1 (p=0.0003) |
| AFB1 | TA100 | 51 | 688.8 [a] | 148.3 | 4.6 | 14.3 (p=0.0004) |
| AFB1 | TA98 | 41 | 3233.0 [a] | 77.5 | 41.7 | 71.6 (p<0.0001) |
| DMN | YG7108 | 27 | 5.5 [b] | 12.7 | 0.4 | 4.7 (p=0.04) |
| 2-AA | TA100 | 38 | 161.0 [a] | 6957.4 | 0.02 | 32.2 (p<0.0001) |
| 2-AF | TA100 | 37 | 47.7 [a] | 151.2 | 0.3 | 13.0 (p=0.001) |
| 2-AF | TA98 | 42 | 184.2 [a] | 368.0 | 0.5 | 2.2 (NS [c]) |
| 2-AAF | TA100 | 29 | 8.6 [a] | 34.3 | 0.2 | 6.3 (p=0.02) |
| 2-AAF | TA98 | 59 | 13.4 [a] | 85.3 | 0.2 | 37.8 (p<0.0001) |
| IQ | TA98 | 51 | 24069.3 [a] | 68.1 | 353.4 | 19.8 (p<0.0001) |
| Trp-P2 | TA98 | 16 | 4821.2 [b] | 23.8 | 202.3 | 6.4 (p=0.02) |
| PhIP | TA98 | 23 | 220.3 [b] | 27.5 | 8.0 | 5.9 (p=0.2) |

[a]Geometric mean derived from mutagenic potency values generated using Aroclor 1254-induced rat liver S9 only

[b]Geometric mean derived from mutagenic potency values generated using induced rat liver S9 prepared following treatment with any inducing agent

[c]NS, not significant at $p<0.05$


A
BaP TA100
B
3-MC TA100
HLS-014
C
AFB1 TA100
D
AFB1 TA98
E
DMN YG7108
F
2-AA TA100
G
2-AF TA100
HLS-014
H
2-AF TA98
Log Mutagenic Potency (revertants/ µg)
Human S9
Aroclor 1254-Induced Rat S9
Induced Rat S9

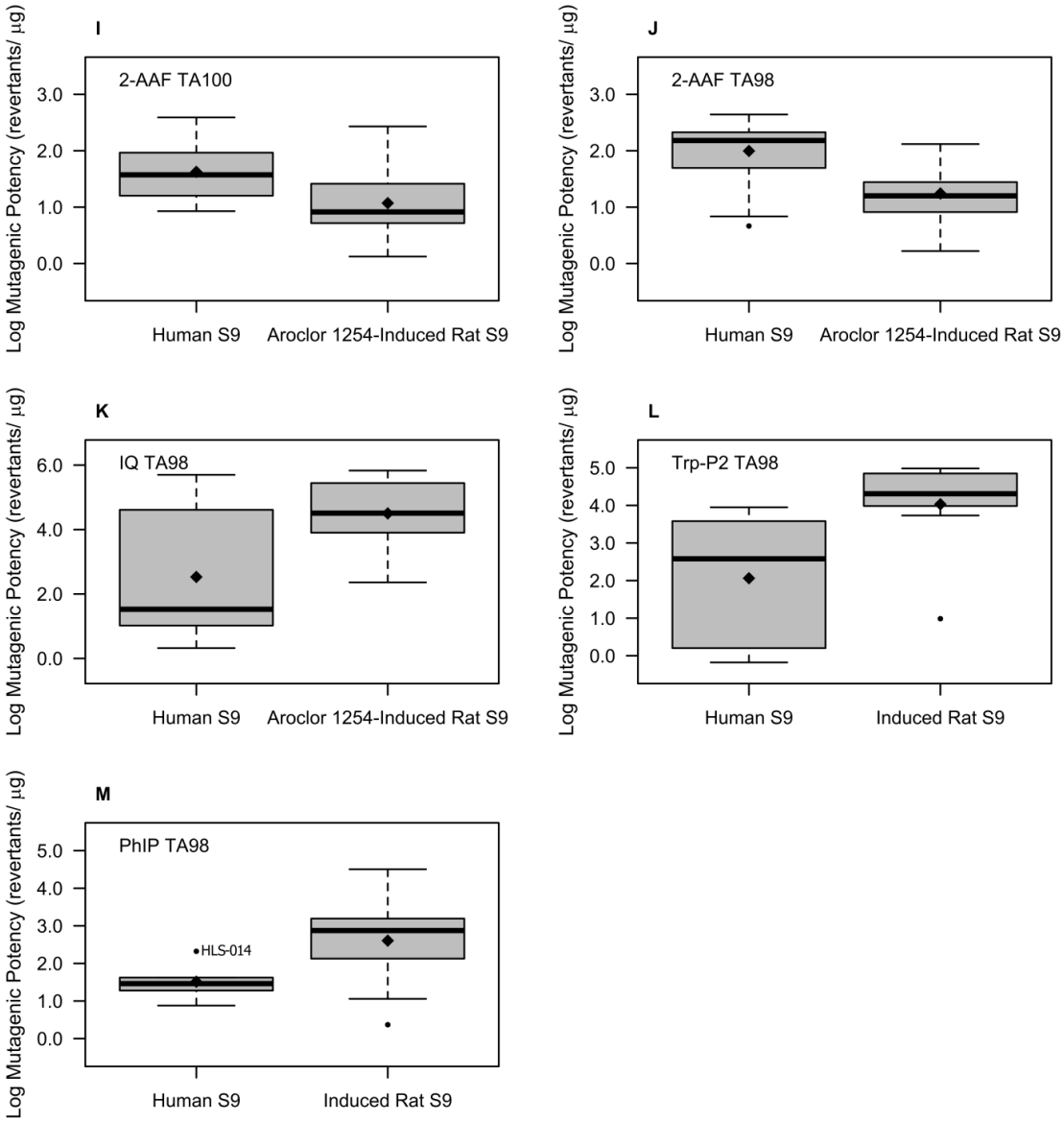



**Figure 2-2:** Distribution of published Salmonella mutagenic potency values generated using human liver S9 and induced rat liver S9.The data comprise chemicals and Ames test strains for which an abundance of data was available in the literature. The analysis includes BaP with TA100 (A), 3-MC with TA100 (B), AFB1 with TA100 (C), AFB1 with TA98 (D), DMN with YG7108 (E), 2-AA with TA100 (F), 2-AF with TA100 (G), 2-AF with TA98 (H), 2-AAF with TA100 (I), 2-AAF with TA98 (J), IQ with TA98 (K), Trp-P2 with TA98 (L), and PhIP with TA98 (M). All analyses compare human liver S9 with Aroclor 1254-induced rat liver S9, except for DMN with YG7108 (E), Trp-P2 with TA98 (L), and PhIP with TA98 (M), which examined responses associated with induced rat liver S9 prepared using any inducer. Solid lines represent medians, diamonds represent means, box edges represent first and third quartiles, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles, and circles represent outliers.

Unlike the mutagenic compounds discussed above, AAs such as 2-AA, 2-AF and 2-AAF, as well as the nitrosamine DMN, often yield mutagenic potency values generated in the presence of Aroclor 1254-induced rat liver S9 that are significantly lower than those observed with human liver S9. The mean mutagenic potency of 2-AA is approximately 40-fold higher when tested on TA100 with human liver S9, in comparison with Aroclor 1254-induced rat liver S9 results (Figure 2-2F). The mutagenic potency of 2-AF is on average 3-fold higher when tested on TA100 with human liver S9, in comparison with Aroclor 1254-induced rat liver S9 results (Figures 2-2G). For TA98, 2-AF mutagenic potency differences between induced rat and human S9 were not significant (Figure 2-2H). The average mutagenic potencies of 2-AAF on TA100 and TA98 are approximately 4- and 6-fold greater, respectively, in the presence of human liver S9 compared to Aroclor 1254-induced rat liver S9 (Figures 2-2I and 2-2J). Finally, the nitrosamine DMN also showed slightly higher mutagenic potency in the presence of human liver S9 in comparison with that observed in the presence of induced rat liver S9. The average mutagenic potency of DMN on strain YG7108 is approximately 2-fold higher in the presence of human liver S9 in comparison with hepatic S9 from rats treated with any inducer (Figure 2-2E).

With respect to the distribution of human S9 potency values for the aforementioned chemicals, it is interesting to note that the published values include several exceptional outliers and data points that impart a marked positive skew (i.e., exceptionally high human S9 results). These outliers relate to human liver S9 sources/lots employed in several published studies (see Figure 2-2). One lot of human liver S9 in particular that was used in several studies by Hakura et al. (i.e., lot HLS-14) yielded

extremely high mutagenic potency values relative to the human liver S9 average [33-35]. The authors of those studies noted that this lot of human liver S9 (Figure 2-2B, 2-2G, and 2-2M) showed exceptional enzymatic activity (e.g., EROD and CYP3A4), and was obtained from an individual taking medication for asthma.

The survey of the literature also revealed a limited amount of mutagenic potency data for other compounds and mixtures. Cigarette smoke condensate, for example, was found to yield TA1538 mutagenic potency with induced rat liver S9 that is 200-fold higher than human liver S9 ($p=0.002$, $N=8$). Similarly, 4-aminobiphenyl, a carcinogenic precursor to azo compounds, showed a 6-fold higher TA1538 mutagenic potency in the presence of induced rat liver S9 compared with human liver S9 ($p=0.02$, $N=9$). In addition, 5 observations of TA98 mutagenic potency for coal tar extract in the presence of human liver S9 had a mean mutagenic potency of 1.7 revertants/µg; less than 10% of the potency level observed with Aroclor 1254-induced rat liver S9 (i.e., 19.5 revertants/µg). Conversely, 1-nitropyrene results showed a significantly higher mutagenic potency (2.5-fold) in the presence of human liver S9 in comparison with induced rat liver S9 ($p=0.02$, $N=5$).

The relationship between enzyme activity and mutagenic potency was examined using AROD activity data and Ames test results obtained from Moltox QC sheets (Figure 2-3). EROD activity is positively correlated with 2-AA mutagenic potency assessed in the presence of Aroclor 1254-induced rat S9. The 2-AA mutagenic potency appears to plateau when EROD activity is very high (Figure 2-3A). Despite the variations in the mutagenic potency of BaP across S9 lots, potency is also positively related to EROD activity (Figure 2-3B). In contrast, the QC sheet data indicate that there is no consistent

empirical relationship between the mutagenic activity of positive controls and the EROD activity of human liver S9 lots. More specifically, as shown in Figure 2-3C and 2-3D, 2-AA and 6-AC mutagenic potency data are highly variable, and there is no significant relationship between mutagenic potency and human S9 EROD activity. The trends were similar for BROD, MROD, and PROD (data not shown).

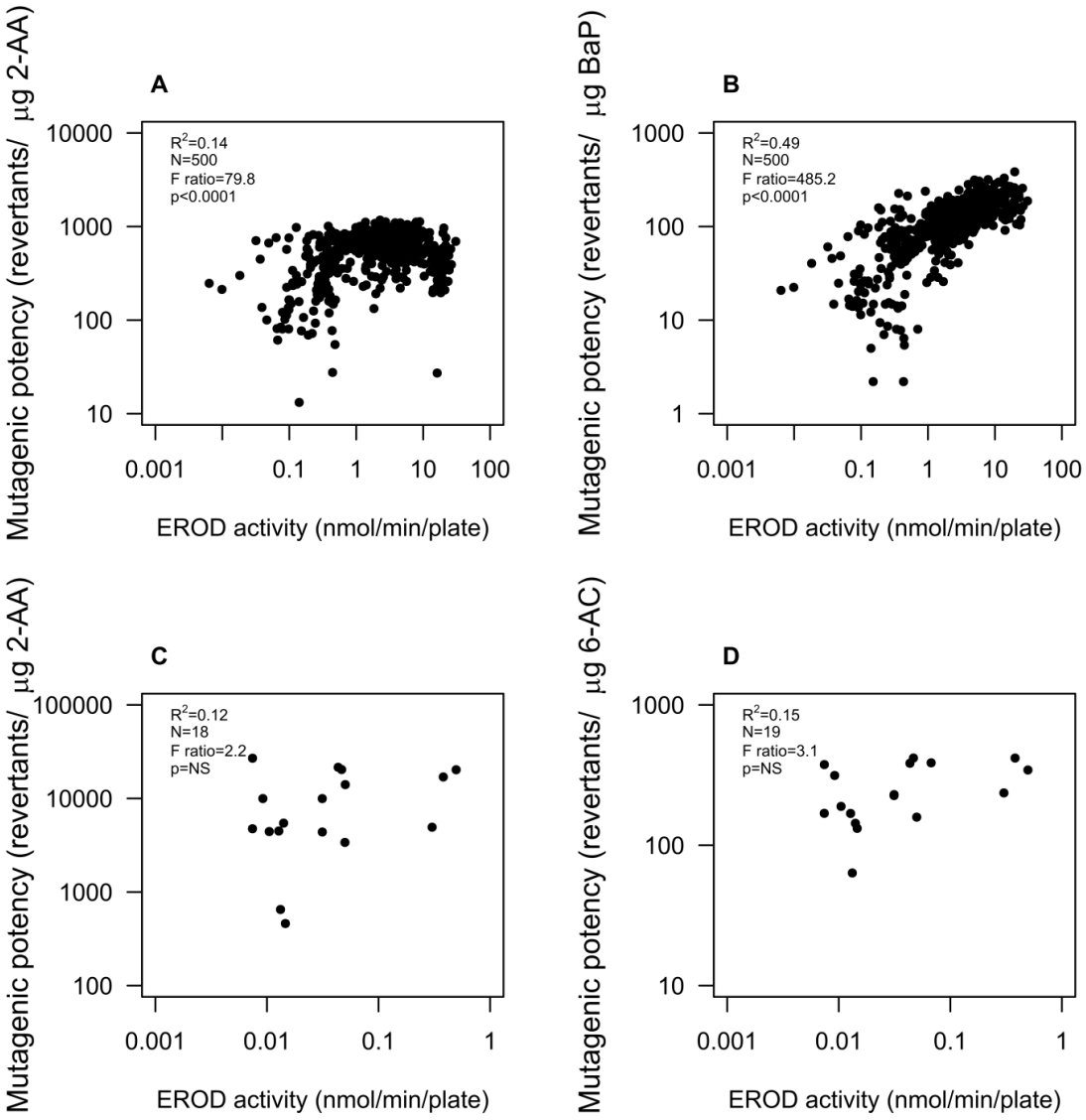



**Figure 2-3:** Relationships between the mutagenic potency of positive controls and hepatic S9 EROD activity. Mutagenic potency values obtained for 2-AA (A) and BaP (B) in the presence of Aroclor 1254-induced rat liver S9, and 2-AA (C) and 6-AC (D) in the presence of human liver S9 were plotted against EROD activity in nmol/min/plate. NS, not significant.

In addition to comparative assessments of mutagenic potency, we also investigated the influence of S9 source (i.e., human liver versus Aroclor 1254-induced rat liver) on test outcome (i.e., positive or negative). In other words, we used published results to assess the ability of a test system employing human liver S9 to successfully identify a known mutagen. Table 2-III summarises the frequency of positive and negative outcomes for 11 mutagens. For the compounds and bacterial strains examined, the collected data show stark differences in test outcomes for 2-AAF and BaP on TA100. In both cases, more than 40% of the total responses obtained in the presence of human liver S9 failed to meet the criteria for a positive test outcome determination. In addition to the compounds listed in Table 2-III, a limited amount of data were also available for 1,7-phenanthroline on TA100 (N=3), 2,4-diaminotoluene on TA100 (N=3), 3-MC on TA98 (N=11), benzo[*a*]anthracene on TA100 (N=5), chrysene on TA100 (N=5), dibenz[*a*,*c*]anthracene on TA100 (N=3), dibenz[*a*,*h*]anthracene on TA100 (N=5), 4,4'-methylene-bis-(2-chlorobenzeneamine) on TA100 (N=4), methyl-*tert*-butylether on TA102 (N=2), *o*-phenylenediamine on TA98 (N=2), *o*-tolidine on TA100 (N=3), and Trp-P-2 on TA100 (N=3). In all cases the reported responses were consistently negative in the presence of human liver S9 and consistently positive in the presence of induced rat liver S9. The only compound found for which all reported responses were positive in the presence of human liver S9 and negative in the presence of induced rat liver S9 was 1-naphthylamine on TA100 (N=3).

**Table 2-III:** Frequency of positive Salmonella mutagenicity responses for selected mutagens assessed with Aroclor 1254-induced rat liver S9 and human liver S9

| Compound | Strain | Frequency of Positive Calls | | Chi-Squared | P-value |
|---|---|---|---|---|---|
| | | Aroclor 1254-Induced Rat Liver S9 (N) | Human Liver S9 (N) | | |
| BaP | TA100 | 100% (27) | 55% (33) | 16.4 | <0.0001 |
| 3-MC | TA100 | 86% (21) | 65% (26) | 2.52 | NS [a] |
| AFB1 | TA100 | 100% (19) | 100% (32) | NA | NA [b] |
| AFB1 | TA98 | 100% (21) | 100% (20) | NA | NA |
| 2-AA | TA100 | 100% (22) | 100% (16) | NA | NA |
| 2-AF | TA100 | 100% (25) | 100% (12) | NA | NA |
| 2-AF | TA98 | 100% (26) | 100% (17) | NA | NA |
| 2-AAF | TA100 | 100% (21) | 50% (16) | 13.4 | <0.0004 |
| 2-AAF | TA98 | 100% (22) | 100% (37) | NA | NA |
| IQ | TA98 | 100% (19) | 100% (23) | NA | NA |
| PhIP | TA98 | 100% (14) | 63% (8) | 6.1 | <0.02 |

[a]Not significant at $p<0.05$
[b]Not applicable

The use of human liver S9 from an individual instead of pooled human liver S9 was also examined. Our survey of the literature, in addition to data supplied by Moltox, indicates that rat liver S9 is almost always prepared using pooled liver homogenates prepared using organs from numerous animals. Moltox confirmed that although S9 lots have been prepared using organs from as few as 6 animals and as many as 40-45 animals, lots of Aroclor-induced rat liver S9 prepared for retail sale are generally prepared from 25-40 livers (Moltox Inc., personal communication). Human liver S9 produced by Moltox for retail sale generally includes pooled liver homogenate from five individuals. In contrast, published studies have employed human liver S9 preparations from single donors, as well as lots prepared using livers from several donors. Although the published data are very limited, we compared mutagenicity results obtained with human liver S9 from a single donor with responses obtained using pooled lots of human liver S9 prepared with organs from several donors. Analysis of the available published data failed to reveal any significant difference between Ames mutagenic potency results obtained using pooled human liver S9 versus results obtained using S9 prepared from individual donor organs.

### 2.5.4 Effect of S9 concentration

Although data were limited, it was possible to analyse the correlation between mutagenic potency and the concentration of human S9 in mg protein/plate for AFB1, DMN, diethylnitrosamine (DEN), 2-AAF, and IQ. There is a significant positive correlation between potency and S9 concentration for 2-AAF tested on TA98 (N=36, F

ratio=19.2, $p<0.0001$) (data not shown); however, significant relationships were not found for any of the other chemicals examined (data not shown).

### 2.5.5 Mammalian cell data

A publication by Johnson et al., detailing an analysis that evaluated the utility of human liver S9 for the *in vitro* mammalian SCE and CA assays, recommended optimization of an isocitrate dehydrogenase-mediated NADPH generating system and also noted that, to achieve positive results, higher concentrations of S9 and promutagens are required for human liver S9 in comparison with induced rat liver S9 [25]. For example, a concentration of 1.5% induced rat liver S9 was sufficient to yield a positive response for 20 μM BaP, whereas a concentration of 10% human liver S9 was necessary to elicit a positive response from 300 μM BaP. Cytotoxicity to mammalian cell lines and reduced sensitivity are known drawbacks associated with the use of human liver S9 (BioReliance, Covance Inc., personal communications).

Due to the paucity of data in the published literature, additional data were experimentally generated to examine the utility of human liver S9 for *in vitro* genetic toxicity assessment with mammalian cell assays. Cytotoxicity to mammalian cell lines was observed for both human and induced rat liver S9. A lot of human liver S9 from Moltox (lot 2637) induced substantial cytotoxicity, measured as RPD, in three tested cell lines, L5178Y mouse lymphoma, CHO and human TK6, in the absence of promutagen exposure. Severe toxicity was apparent with Moltox lot 2637 at a concentration of 2% in L5178Y cells and 4% for CHO and human TK6 cells (data not shown). Human liver S9 obtained from BD (lot 73024) appeared to be bacterially contaminated, but demonstrated

little to no cytotoxicity in the three cell lines up to the maximum tested concentration of 10% (data not shown).

MN induction by BaP and 2-AAF was assessed in L5178Y mouse lymphoma cells exposed in the presence of an S9 mix containing either Aroclor 1254-induced rat liver S9 or human liver S9 (Figure 2-4). Unlike Aroclor 1254-induced rat liver S9, the human liver S9 from BD lot 73024 did not lead to a significant induction in micronuclei following exposure to BaP at either of the tested S9 concentrations (Figure 2-4A). Conversely, human liver S9 from BD lot 73024 yielded a higher frequency of MN in response to 2-AAF exposure in comparison with Aroclor 1254-induced rat S9 (Figure 2-4B). The 2-AAF results also showed that induction of MN is enhanced when the cells are exposed in the presence of an increased concentration of human liver S9 (i.e., 10% versus 2%). It is important to note that higher concentrations of human S9 also led to higher cytotoxicity. A 10% concentration of BD human liver S9 lot 73024 yielded a reduction in survival, as measured by RPD, that exceeds the OECD test guideline [30], and the cells may have suffered significant cell cycle delay leading to an underestimation of genotoxicity. MN induction could not be assessed with Moltox human S9 lot 2906, for either BaP or 2-AAF, due to severe cytotoxicity (Figure 2-4C and 2-4D). In all cases, human liver S9 was more cytotoxic than rat liver S9.

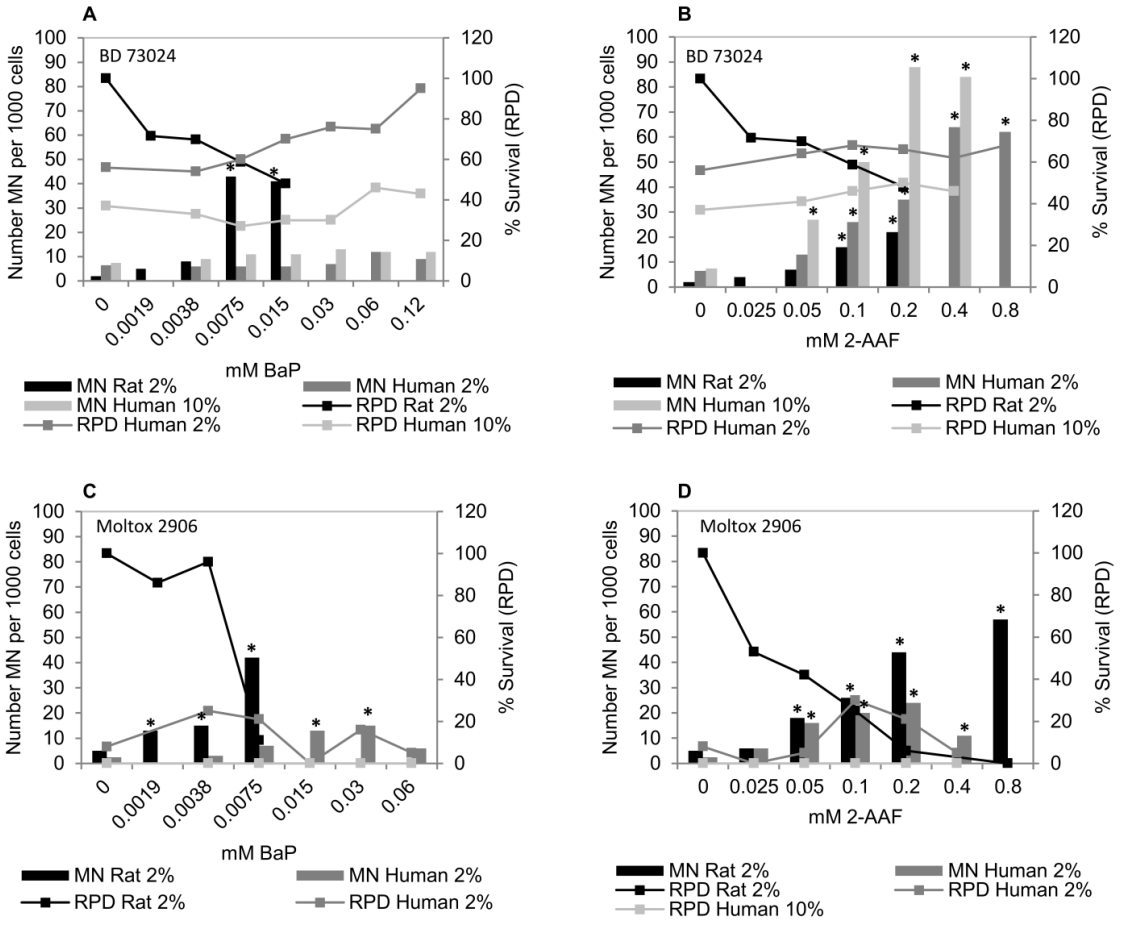



**Figure 2-4:** Micronucleus induction in L5178Y cells following exposures to BaP and 2-AAF in the presence of 2% Aroclor 1254-induced rat liver S9 or human liver S9 at concentrations of 2% and 10%. Human liver S9 was obtained from BD and Moltox, and used to assess induced MN frequency following exposures to BaP (A and C, respectively) and 2-AAF (B and D, respectively). * Significant increase above control ($p<0.05$).

### 2.5.6 Human liver S9 usage survey

A survey of 24 members of the Mammalian Mutagenicity Society, a special interest group associated with the Japanese Environmental Mutagenesis Society, was conducted to gather information related to the use of human liver for test article screening. Most of the researchers surveyed had not used human liver S9, and the reasons given included (1) not needing to evaluate the genotoxicity of human-specific metabolites (40.9% of respondents), (2) using synthesized metabolites to assess genotoxicity (18.2% of respondents), (3) poor reproducibility relative to rodent S9 (12.5% of respondents), (4) lack of information regarding utility of human liver S9 (12.5% of respondents), (5) low enzyme activity (8.3% of respondents), and (6) protocols that restrict the use of human material (4.5% of respondents). The general opinion of those that have not used human liver S9 was that there is poor reproducibility between lots, lower sensitivity relative to induced rat liver S9; and moreover, that the effectiveness of human-derived S9 for test article screening is relatively unknown.

Only 29.2% of the individuals surveyed had used human liver S9 in an *in vitro* genotoxicity test. Of those who had used human liver S9, 85.7% only used S9 pooled from several (pooled) donors. The survey respondents revealed that human liver S9 was used for (1) measuring metabolite concentrations for an *in vitro* cytogenetic assay (85.7% of respondents), (2) measuring metabolite concentrations for an Ames test (57.1% of respondents), (3) genotoxicity assessment via the Ames test (42.9% of respondents), (4) measuring metabolite concentrations for an MLA (42.9% of respondents), (5) genotoxicity assessment via and *in vitro* cytogenetic assay (28.6% of respondents), and (6) genotoxicity assessment via the MLA (14.3% of respondents). In addition,

respondents indicated that human liver S9 was used to (1) add to the weight of evidence given that previous tests using induced rat liver S9 were positive (42.9% of respondents), (2) to catalyse the formation of a human metabolite that cannot be generated using rat liver S9 (28.6% of respondents), (3) to assess human-specific metabolites (14.3% of respondents), and (4) to compare results with human S9 to results obtained with bacteria expressing human CYPs (14.3% of respondents). Drawbacks to using human liver S9 were noted and these included (1) difficulty selecting concentrations for positive controls (57.1% of respondents), (2) differences between lots of human liver S9 with respect to enzyme activity and test results (28.6% of respondents), (3) requirement to design new quality control protocols (14.3% of respondents), and (4) difficulty obtaining human liver S9 in a timely fashion (14.3% of respondents). Of the respondents that had used human liver S9, 14.3% reported obtaining a negative result with human S9 and a positive result with rodent S9. Interestingly, 14.3% of respondents reported instances where Ames tests for a particular compound were negative with both human and rat liver S9, but a CA test for the same compound yielded a positive result with human liver S9 and a negative result with rat liver S9. Of those who had used human liver S9, 85.7% stated that they would work with it again. Overall, 50% of respondents indicated that human liver S9 should be used in cases where the synthesis of the metabolite is difficult by any other means or the metabolite is human specific. Similarly, 50% of respondents stated that, as part of a weight of evidence approach, human liver S9 should be used to follow up tests conducted using rat liver S9.

## 2.6 Discussion

Post-mitochondrial supernatants, more commonly known as S9, derived from mammalian liver are extensively used as sources of exogenous metabolic enzymes for *in vitro* genotoxicity assessment. Rat liver S9 obtained from chemically-induced animals is the most popular source of exogenous metabolic enzymes for routine *in vitro* testing. However, its relevance for detecting human mutagenic hazards has been called into question [36]. Indeed, S9 derived from human liver has become increasingly available in recent years, and at first glance, appears to offer the promise of an exogenous source of metabolic enzymes with improved relevance to humans. Through comparisons with the traditionally used induced-rat hepatic S9, this study sought to critically evaluate the utility of human liver S9 for identification of chemically-induced mutagenic hazards. The study is the first attempt to collect, collate, and analyse mutagenic potency data from the published literature to assess the performance of human liver S9 in relation to induced rat liver S9 that has been routinely used for the past 40 years to identify and assess mutagenic hazards.

The most notable characteristic that immediately differentiates human liver S9 from the more conventionally used Aroclor 1254-induced rat liver S9 is its relatively low, variable, and highly skewed enzymatic activity, as measured by AROD activity, and the concomitant highly variable mutagenic potency values. Conversely, relative to human liver S9, Aroclor 1254-induced rat liver S9 has extremely high, normally-distributed enzymatic activity measured by AROD activity, and generally yields less variable mutagenic potency values. Some variability in Ames mutagenic potencies may be due to inter-laboratory inconsistencies and differences between protocols (i.e., plate

incorporation and pre-incubation); however, these sources of variability likely played a smaller role than inter-species enzyme activity differences.

The aforementioned variability in the enzymatic profiles of human liver S9 samples, relative to Aroclor-induced rat liver S9, would certainly be expected to affect the results of *in vitro* mutagenicity assessments. Indeed the data presented here, which included both published data and new experimental data, indicate that mutagenicity responses elicited in the presence of human liver S9 are often far lower than those observed with induced rat liver S9. This was observed for a variety of known mutagens that require different CYP isozymes. More specifically, the collected data revealed that the Salmonella mutagenic potency of PAHs, AFB1, and HCAs, which are converted to DNA reactive metabolites by CYP1A1/1A2, CYP1A2/3A, CYP1A1/1A2 and conjugation enzymes, respectively, are on average, substantially reduced when tested with human liver S9, relative to Aroclor-induced rat liver S9. The significant empirical relationships between mutagenic potency and the AROD activity levels of induced rat liver S9 supports the notion that the activity of CYP1, CYP2, and CYP3 family isozymes alter the influence of different lots of induced rat liver S9 on mutagenic potency. In contrast, the data obtained from the Moltox QC sheets indicated that the ability of human liver S9 to convert chemical mutagens to DNA-reactive products is not empirically dependant on AROD levels. Unlike the mutagenic potency data for the aforementioned mutagens, AA results (i.e., 2-AF, 2-AAF and 2-AA) for both Salmonella mutagenicity and micronucleus induction in L5178Y mouse lymphoma cells indicate that responses obtained using human liver S9 can exceed those of induced rat liver S9.

A handful of investigations into the utility of human liver S9 can be found in the scientific literature. One of the first studies examining human liver S9 in the Ames test found that it was generally less active than phenobarbital-induced rat liver S9 for 20 known carcinogens, except for 2-AA, 2-AAF, 4-aminobiphenyl, 6-AC, and AFB1, all of which produced qualitatively similar results regardless of the S9 used [37]. This early work only examined a human liver S9 preparation from one individual; however, a later study by Beaune et al. investigating 10 different human liver S9 samples yielded additional evidence that human liver S9 is more active towards certain classes of carcinogens. Beaune et al. found that the AAs 2-AA and 2-AF are far more mutagenic when activated by human liver S9 than by uninduced or Aroclor-induced rat liver S9. In contrast, human liver S9 was found to be far less effective for the PAH 3-MC than Aroclor-induced rat liver S9 [38]. Similarly, in the large-scale analysis of published Salmonella mutagenic potency data presented herein, it was observed that human liver S9 yielded higher mutagenic potencies for AAs, and lower mutagenic potencies for PAHs.

The lower mutagenicity of AAs in the presence of Aroclor-induced rat liver S9, relative to human liver S9, appears to be due to interspecies differences in hepatic levels and functions of CYP1A1 and CYP1A2. Rat hepatic CYP1A1 is responsible for *C*-oxidation, leading to the ring-hydroxylation and subsequent detoxification of AAs, and rat hepatic CYP1A2 is responsible for *N*-oxidation, leading to activation of AAs to DNA-reactive metabolites. Humans have extremely low levels of hepatic CYP1A1, thus AAs preferentially undergo *N*-oxidation through CYP1A2 in the liver, leading to AA activation and mutagenic activity [39, 40]. Induction by chemicals such as Aroclor 1254 dramatically elevates the activity of rat hepatic CYP1A1, thus the production of reactive

AA metabolites is decreased relative to humans and uninduced rats [38]. The inverse correlation of increased CYP activity and lower AA mutagenicity has been observed in several studies [38, 41, 42]. Indeed, the analyses presented here suggest that the mutagenic activity of aromatic amines can decline when Aroclor-induced rat liver S9 EROD activity exceeds 1 nmole per min per mg protein (Figure 2-3A).

Unlike aromatic amines, PAHs appear to be much less mutagenic with human liver S9 than with induced rat liver S9. A high frequency of negative results was obtained for both BaP and 3MC in the presence of human liver S9, but not rat liver S9 (Table 2-III). Frequent negatives for PAHs tested with human liver S9 are likely the result of the aforementioned low expression of CYP1A1, a key enzyme in PAH metabolism and activation, in human liver relative to rat liver. Unlike the human liver, human lungs have relatively high levels of CYP1A1, thus PAH exposure has been shown to increase the risk of lung cancer [43]. Additionally, human liver S9 does not benefit from the approximately 120-fold boost in EROD (i.e., CYP1A1 and CYP1A2) activity afforded to rat liver S9 by Aroclor induction.

In comparison to bacterial mutagenesis studies, relatively few published studies have used human liver S9 for *in vitro* mammalian cell genetic toxicity assays [25, 44-48]. Johnson et al. conducted a comprehensive investigation regarding the use of human and Aroclor 1254- or phenobarbital/β-naphthoflavone-induced rat liver S9 to assess the clastogenicity of BaP, AFB1, DMN, DEN, and 2-AAF, compounds that require different CYP isozymes for activation. More specifically, genotoxic activity was assessed using the SCE and CA induction assays in CHO cells, and with the exception of BaP, the results showed that human liver S9 was capable of converting all tested chemicals to

mutagenic metabolites that induce CAs. Additionally, it was observed that a stronger CA response for 2-AAF was obtained using human liver S9, relative to induced rat liver S9, echoing the results obtained in bacterial tests, and the results obtained herein. It is important to note that the Johnson et al. study utilized an isocitrate dehydrogenase-based NADPH generation system, observing that it is an essential component of an optimized S9 cofactor mix; and moreover, increased the S9 concentration to maximize the observed mutagenic response [25]. This cofactor mix is an alternative to the cofactor mix concentrations initially described by Ames et al. and Maron and Ames, which all bacterial mutagenesis assays analysed in this study utilised, with very few exceptions [24, 26].

The current study is the first to assess the utility of human liver S9 for the *in vitro* micronucleus assay. Similar to the aforementioned bacterial and mammalian cell test results, the results obtained indicate that human liver S9 was not effective at converting BaP to a clastogenic metabolite, but it was more effective than Aroclor 1254-induced rat liver S9 at converting 2-AAF to a metabolite that induces micronucleus formation (Table 2-II, Figure 2-2A, 2-2I and 2-2J). It was observed that higher concentrations of human liver S9 (i.e., 10% instead of 2%) yielded higher MN frequencies in L1587Y cells treated with 2-AAF; a similar observation was made by Johnson et al. [25]. It is important to note that increasing S9 concentrations can be problematic since higher S9 concentrations can lead to increased extra-cellular protein binding of active metabolites, thus lowering genotoxicity.

Significant cytotoxicity was observed with Moltox human liver S9 lot 2637 tested using three commonly used cell lines; CHO and TK6 cells were less sensitive to human

liver S9-induced cytotoxicity than L5178Y cells. However, this severe cytotoxicity was not seen for all tested lots, and BD lot 73024 exhibited far less cytotoxicity in the three cell lines examined. These limited data suggest that mammalian cell cytotoxicity will limit the utility of human liver S9 for routine assessment of *in vitro* genetic toxicity. The cytotoxicity of human liver S9 may be attributable to NADP-induced lipid peroxidation and the production of reactive oxygen species [49]. However, this contention does not explain why the same level of cytotoxicity is not observed with induced rat liver S9. It is possible that the longer cold ischemia time for human subjects relative to experimental rodents (i.e., chilling time in the absence of blood supply) results in differential production of reactive oxygen species in respective S9 fractions. If so, the NADPH-generating cofactor mixtures may need to be further optimized to permit effective use of human liver S9 in mammalian cell assays. Inter-species variability may also be playing a role, and further investigations would be required to delineate the processes underlying the cytotoxicity of human liver S9.

Medications, alcohol use, smoking status, and diet can also influence metabolic enzyme activity, and thus, modulate the ability of S9 preparations to convert test compounds to DNA-reactive products. Drug-induced inhibition and induction of human metabolic enzymes is extremely well documented [50]. For example, phenobarbital, a commonly-prescribed antiepileptic drug, is a potent inducer of several CYPs via CAR/PXR agonism [51]. Ethanol is an effective CYP2E1 inducer; thus, chronic alcoholism can lead to an increased risk of *in vivo* mutagenesis through the CYP2E1-mediated metabolism of promutagens such as nitrosamines [52, 53]. Smoking and occupational exposures to combustion by-products can upregulate CYP1A1, CYP1A2,

and CYP1B1 activity through AhR agonism [54-56], and lastly, some common foods, such as cruciferous vegetables, charred meat, grapefruit, and garlic, contain compounds that can significantly increase or decrease the activity of selected metabolic enzymes [57-60].

The potential effects of therapeutic products were apparent in our review and analysis. Hakura et al. noted large inter-individual variations between human liver S9 preparations, and described one S9 sample in particular, dubbed HLS-014, that elicited Salmonella mutagenicity responses for 13 known mutagenic carcinogens that were equal to or greater than responses elicited in the presence of phenobarbital/β-naphthoflavone-induced rat liver S9. Additional analyses indicated that the level of total CYP protein in the HLS-014 S9 sample was much greater in comparison with all other S9 lots examined; moreover, the level of CYP3A4 activity in HLS-014 was exceptionally high. The unusually high activity of HLS-014 was thought to be linked to the human donor's asthma medication, which may act as a receptor agonist [33-35]. Not surprisingly, our meta-analysis of published Salmonella mutagenicity data, which includes data from Hakura et al., revealed high variability in human S9 enzymatic activities (e.g., AROD activity levels), as well as mutagenicity responses to 3-MC, 2-AF, and PhIP that include noteworthy outliers.

The human liver S9 lots prepared by Moltox, which were used in the reviewed studies and our mammalian MN induction assessments, include both male and female organ donors, of a wide range of ages. Although there is insufficient data to assess the influence of age or sex on the ability of human liver S9 lots to activate promutagens, the literature indicates that the activity of xenobiotic metabolizing enzyme genes and enzyme

activity levels can vary with sex and age. For example, a transcriptional profiling study of 112 female and 112 male human livers revealed that the expression of 40 xenobiotic metabolism-related genes is significantly influenced by sex [61]. Enzyme activity levels gradually increase during the first year of life [62], although age-related variability in adulthood is considerably more subtle. Nevertheless, CYP1A2, CYP2D6, and CYP2E1 activities in adulthood decrease slightly with age [15].

Genotypic variations in the genes that encode xenobiotic metabolizing enzymes (i.e., Phase I and II) include numerous single nucleotide polymorphisms (SNPs) that are over- or under-represented in certain ethnic groups. For example, several members of the CYP2 family, such as CYP2D6, are highly polymorphic in humans [63], with poor (PM), intermediate (IM), extensive (EM), and ultrarapid (UM) metabolizers present to different extents in different ethnic groups. For example, the CYP2D6 PM phenotype is far more prevalent among Caucasians than Asians, and the UM phenotype is predominant among Middle Eastern populations [64], with more rapid metabolism being associated with an increased risk of adverse effects attributable to increased production of DNA-reactive metabolites. *N*-acetyltransferase 2 (NAT2), a phase II enzyme vital to the activation and detoxification of arylamines, is another example of a metabolic enzyme that is highly polymorphic in humans, with fast, slow, and intermediate acetylator phenotypes being differentially prevalent in different ethnic groups [65]. The slow aceylator phenotype, which is less common in people of East Asian descent, has been linked to an increased overall risk of bladder cancer [66]. Unfortunately, commercially available human liver S9, which is predominantly prepared from Caucasian organ donors, cannot effectively reflect human metabolic diversity. Thus, the average pooled human liver S9 preparation

is unlikely to be able to capture the full spectrum of activities in human xenobiotic metabolizing enzymes.

The aforementioned variability in human xenobiotic metabolism is well documented, and known to be linked to genetic polymorphisms, environmental factors (e.g., occupation, tobacco smoking, diet, therapeutic product use, etc.), age, and sex. Thus, with respect to an individual's ability to convert chemical mutagens into DNA-reactive metabolites, it is reasonable to expect a great deal of variation in human sensitivity to mutagenic effects, and this variability suggests that human liver S9 prepared from individual organs cannot be reliably employed to identify mutagenic hazards. Indeed, data collected and presented herein indicate that use of human liver S9 can contribute to an inability to detect known mutagenic carcinogens such as BaP, 3-MC, and PhIP (Table 2-III). It has been suggested that high-activity human liver S9 samples, such as the aforementioned HLS-014, and S9 with average activity, such as pooled S9, should each be used for effective identification of human mutagenic hazards [67]. However, the results presented herein reveal that even pooled human liver S9 can yield negative responses for a known mutagenic carcinogen. On the other hand, there is evidence to suggest that some positive genotoxicity results obtained using rat liver S9 may have limited relevance to humans due to the production of rat-specific metabolites that are not produced in humans. Interestingly, a study that analysed metabolite profiles of 16 drugs generated using Aroclor-induced rat liver S9 and pooled human liver S9 showed that metabolites generated by human liver S9 were generally also generated by induced rat liver S9. However, rat liver S9 generally produced substantially elevated levels of metabolites and consumed far more of the parent compound [68].

The aforementioned survey of Japanese laboratories routinely conducting genetic toxicity assessments revealed that some researchers employ Aroclor-induced rat liver S9 for compound screening, with targeted follow-ups employing human-derived S9, where appropriate. Although this survey was small, it provides an indication of the general opinions regarding the use of human liver S9 for genetic toxicity assessment. The survey also revealed that researchers recognise several drawbacks related to the use of human liver S9; notably, lower sensitivity and lot-to-lot variability. Indeed, the variability of human liver S9, and the concomitant risk of false negatives, suggests that chemically-induced rat liver S9 is effective and pragmatic, albeit conservative, for identifying chemical mutagens. Targeted follow-up studies with human-derived S9 can be used to provide data regarding interspecies differences in the metabolic activation of a given chemical, as well as to investigate the relevance of a genotoxicity result for human hazard identification.

In conclusion, despite the potential for increased human relevance, S9 preparations from human livers should not be solely used for *in vitro* genotoxicity assessment. Rat liver S9 is more likely to correctly identify mutagens, and limit the likelihood of undesirable false negative results. Additionally, the ability of human liver S9 to generate DNA-reactive metabolites does not appear to be dependent on AROD levels, and the enzymatic attributes of human liver S9 that contribute to its ability to convert chemical test articles to DNA-reactive metabolites remain unknown. Nevertheless, human liver S9 may be very useful for confirmatory assays that follow those employing induced rat liver S9. This is especially true for classes of chemicals

where species-specific differences in metabolism are known or suspected (e.g., aromatic amines).

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# CHAPTER THREE

# Chapter 3: The development and pre-validation of an *in vitro* mutagenicity assay based on MutaMouse primary hepatocytes. Part I: Isolation, structural, genetic and biochemical characterization

## 3.1 Preamble: Authors, Affiliations, and Style

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## 3.2 Abstract

In order to develop an improved *in vitro* mammalian cell gene mutation assay, it is imperative to address the known deficiencies associated with existing assays. Primary hepatocytes isolated from the MutaMouse are ideal for an *in vitro* gene mutation assay due to their metabolic competence, their "normal" karyotype (i.e., neither transformed nor immortalized), and the presence of the MutaMouse transgene for rapid and reliable mutation scoring. The cells were extensively characterized to confirm their utility. Freshly isolated cells were found to have a hepatocyte-like morphology, predominantly

consisting of binucleated cells. These cells maintain hepatocyte-specific markers for up to 3 days in culture. Analyses revealed a normal murine hepatocyte karyotype with a modal ploidy number of 4n. Fluorescence *in situ* hybridization (FISH) analysis confirmed the presence of the lambda shuttle vector on chromosome 3. The doubling time was determined to be 22.5 ± 3.3 h. Gene expression and specific enzymatic activity of key Phase I and Phase II metabolic enzymes were maintained for at least 8 and 24 h in culture, respectively. Exposure to β-naphthoflavone led to approximately 900- and 9-fold increases in *Cyp1a1* and *Cyp1a2* gene expression, respectively, and approximately 2-fold induction in cytochrome P450 (CYP) 1A1/1A2 specific activity. Exposure to phenobarbital resulted in an approximately 2-fold increase in CYP2B6 enzyme specific activity. Following this characterization, it is evident that MutaMouse primary hepatocytes have considerable promise for *in vitro* mutagenicity assessment. The performance of these cells in an *in vitro* gene mutation assay is assessed in Part II.

## 3.3 Introduction

*In vitro* genetic toxicity tests are critical components of the toxicity assessment batteries typically employed for chemical safety evaluations and subsequent regulatory decisions [Kirkland et al., 2007]. *In vitro* genetic toxicity assays currently used for regulatory purposes include both bacterial assays (e.g., the *Salmonella* reverse mutation test), as well as mammalian cell assays (e.g., the *Hprt* and *xprt* gene mutation tests, the *in vitro* micronucleus assay, the Mouse Lymphoma Assay (MLA), and the TK6 gene mutation assay). The current battery of *in vitro* genotoxicity assays has a lengthy history; indeed they have been prominent tools for protecting human health [Zeiger, 2010]. In

addition, attendant changes to the regulatory landscape, such as the 7th Amendment to the European Union (EU) Cosmetics Directive, are stimulating increased reliance on *in vitro* tools that augment or even replace *in vivo* tests for routine chemical screening [Adler et al., 2011; European Commission, 2009; Tice et al., 2013]. This shift away from *in vivo* models towards predictive *in vitro* tools, combined with the issues posed by specific mammalian *in vitro* tests, as discussed below, highlights the acute need to develop and adopt improved and/or alternative *in vitro* methods.

Although the aforementioned mammalian cell genotoxicity assays are highly sensitive, and have been well validated for routine use in regulatory assessments (i.e., Organization for Economic Cooperation and Development [OECD] Test Guidelines 476, 487 and 490) [OECD, 2016b; OECD, 2016d; OECD, 2016c], they present noteworthy drawbacks. First, none of the cell lines employed for these assays, such as the L5178Y, TK6, Chinese hamster ovary (CHO), Chinese hamster lung (CHL), and V79 cell lines, are metabolically competent, necessitating the use of exogenous activation mixtures containing, for example, Aroclor-1254-induced or phenobarbital/β-naphthoflavone-induced rodent liver S9 [Johnson et al., 1996; Cox et al., 2016]. Unfortunately, the use of rodent liver S9 is problematic due to its cytotoxicity, the over-representation of Phase I cytochromes P450 (CYPs), lack of Phase II enzyme activity, and poor penetration of exogenously formed metabolites into the cell [Madle et al., 1986; Cox et al., 2016; Ku et al., 2007; Glatt et al., 1981; Kirkland et al., 1989]. Second, immortalized cells often used for genotoxicity assessment typically have aberrant and/or unstable karyotypes that include numerous deletions, duplications and translocations. Consequently, many commonly used cell lines, such as mouse lymphoma L5178Y $Tk^{+/-}$ cells, show

characteristics that are similar to oncogenically-transformed tumour cells, including impaired p53 function [Storer et al., 1997]. Genomic instability may also lead to genomic drift and subsequent differences in characteristics of the cell stocks used for routine genetic toxicity assessment [Lorge et al., 2016]. It is anticipated that an *in vitro* assay that addresses these shortcomings could deliver more reliable and relevant results in comparison to existing *in vitro* genotoxicity assays.

Transgenic rodent (TGR) systems, such as the MutaMouse, have been shown to yield consistent and reliable results for detection of chemical mutagens and mutagenic carcinogens [Lambert et al., 2005; OECD, 2013]. *In vitro* assays utilising cells from TGR systems have previously been developed in an effort to complement the *in vivo* TGR assays. For example, a spontaneously immortalized cell line derived from the lung of the MutaMouse has been successfully employed in an *in vitro* gene mutation assay [White et al., 2003]. This cell line, named FE1, exhibits significant benzo[*a*]pyrene (BaP)-inducible *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* gene expression; however, the addition of induced rat liver S9 is required to detect mutations induced by chemicals that are activated by other metabolic enzymes [White et al., 2003; Arlt et al., 2008; Berndt-Weis et al., 2009]. The FE1 *in vitro lacZ* gene mutation assay is currently undergoing validation according the multi-step process advocated by the OECD [OECD, 2005].

More recently, mutagenicity assays using primary hepatocytes derived from both the MutaMouse and the pUR288 *lacZ* plasmid mouse have been developed [Chen et al., 2010; Zwart et al., 2012]. Primary hepatocytes carry several advantages over immortalized cell lines, including endogenous metabolic competence and karyotypic stability. Indeed, MutaMouse primary hepatocytes results have shown concentration-

dependent increases in mutant frequency (MF) in response to BaP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and 3-nitrobenzanthrone (3-NBA), and a significant increase in MF following exposure to cigarette smoke condensate (CSC) [Chen et al., 2010]. Both fresh and cryopreserved primary hepatocytes from the pUR288 *lacZ* plasmid mouse have been shown to proliferate in culture; moreover, they have BaP-inducible CYP1A1/1A2 activity as measured by ethoxyresorufin-*O*-deethylase (EROD), functional p53, and yielded results for 16 known mutagens and non-mutagens that indicate excellent sensitivity and specificity [Zwart et al., 2012; Luijten et al., 2016]. Collectively, these studies demonstrate the potential utility of primary hepatocytes from TGR systems as alternatives to existing mammalian cell *in vitro* mutagenicity tests.

Although the aforementioned study by Chen et al. [2010] showed that primary hepatocytes from the MutaMouse can be used to detect known mutagens, the cells and their various attributes have not been well characterized. The present study, which is Part I in a two-part series, constitutes the next step in the development and establishment of an *in vitro* assay based on primary hepatocytes from the MutaMouse. More specifically, the work characterises MutaMouse primary hepatocytes on the basis of their morphology, proliferative capacity, expression of markers indicative of cell type, karyotype, and metabolic capacity. Part II examines the performance of an *in vitro* gene mutation assay based on MutaMouse primary hepatocytes (i.e., the ability to effectively assess mutagenic hazard).

## 3.4 Materials and Methods

### 3.4.1 Materials and reagents

Dulbecco's modified Eagle's medium (DMEM), William's E medium, phosphate-buffered saline (PBS), foetal bovine serum (FBS), epithelial growth factor (EGF), penicillin-streptomycin reagent, Hank's balanced salt solution (HBSS), proteinase K, trypan blue, colcemid, and SYTOX® green nucleic acid stain were obtained from Life Technologies Inc. (Burlington, Ontario). Corning® Biocoat™ type I collagen-coated culture dishes and coverslips, and Matrigel®-coated culture dishes were obtained from VWR International (Mississauga, Ontario). CIzyme™ collagenase HA and BP protease were obtained from VitaCyte LLP (Indianapolis, Indiana). American Type Culture Collection (ATCC) Eagle's minimum essential medium (EMEM), DMEM, and F-12K medium were obtained from Cedarlane (Burlington, Ontario). VectaShield hardset mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Burlington, Ontario). Dexamethasone, human insulin, dimethylsulphoxide (DMSO), Percoll®, bovine serum albumin (BSA), resorufin ethyl ether, resorufin sodium salt, fluorescamine, ribonuclease (RNase) A, and IGEPAL CA-630 were obtained from Sigma-Aldrich Canada Co. (Oakville, Ontario). Bacteriophage lambda cl857 DNA was obtained from Roche Diagnostics (Laval, Quebec).

### 3.4.2 Isolation and culture of primary hepatocytes

The TGR MutaMouse (i.e., BALB/c x DBA2, mouse strain 40.6), carrying the bacteriophage lambda shuttle vector containing the bacterial *lacZ* target gene [Gossen et al., 1989], was bred and maintained locally under conditions approved by the Health Canada Ottawa Animal Care Committee. Hepatocytes were isolated from MutaMouse

liver according to the two-step collagenase technique proposed by Seglen [1976] with the addition of a Percoll® isodensity purification step [Kreamer et al., 1986]. This study was restricted to female MutaMouse specimens that were not required for colony maintenance breeding purposes, thus primary hepatocytes were isolated from mice that ranged in age from 8 to 18 weeks. Primary hepatocytes were isolated from anesthetized mice following a retrograde perfusion using a blanching solution (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 1 mM ethylene glycol tetraacetic acid [EGTA], 100 U/mL penicillin-streptomycin in HBSS) and a collagenase-containing solution (2000 U/mL collagenase HA and 250 U/mL BP protease in DMEM), as previously described [Klaunig et al., 1981; Chen et al., 2010]. The isolated cells were counted via hemocytometer using trypan blue exclusion. Successful perfusions yielded populations of hepatocytes that showed at least 80% viability. The cells were then plated onto collagen-coated culture dishes using Attachment Medium (20 U/L human insulin, $4x10^{-6}$ mg/mL dexamethasone, 10% FBS, and 100 U/mL penicillin-streptomycin in DMEM), and incubated at 37°C and 5% $CO_2$. Two hours (t = 2 h) following plating, the Attachment Medium was replaced with Serum-Free Medium (SFM; 10 mM HEPES, 2 mM L-glutamine, 10 mM pyruvate, 0.35 mM L-proline, 20 U/L human insulin, $4x10^{-6}$ mg/mL dexamethasone, 0.01 μg/mL EGF, and 100 U/mL penicillin-streptomycin in Williams Medium E), and the plates incubated at 37°C and 5% $CO_2$.

### 3.4.3 Culture of other cell lines

C2C12 mouse muscle myoblasts, RAW 264.7 mouse macrophages, A549 human lung carcinoma cells, and HepG2 human hepatocellular carcinoma cells were acquired

from the ATCC through Cedarlane (Burlington, Ontario). C2C12 and RAW 264.7 cells were cultured in DMEM, A549 cells were cultured in F-12K medium, and HepG2 cells were cultured in EMEM. All media were supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. All incubations were carried out at 37°C and 5% $CO_2$.

### 3.4.4 Time-lapse imaging

Time-lapse videos of the primary hepatocytes in culture were captured using the JuLi Live Cell Movie Analyser (NanoEnTek, Seoul, South Korea). Images were captured at 10x magnification at 10 min intervals, beginning 2 h following plating, for 120 h.

### 3.4.5 Immunocytochemistry

Freshly isolated hepatocytes suspended in Attachment Medium, or cultured positive control cells (Supplementary Table 3-I) suspended in their optimal medium, were plated onto collagen-coated glass coverslips (hepatocytes) or sterilized uncoated glass coverslips (positive control cell lines) at $2.5x10^5$ cells/well in 6-well culture dishes, and incubated at 37°C and 5% $CO_2$. After the hepatocytes had incubated for 2 h, the Attachment Medium was replaced with SFM, and the cells returned to the incubator for 24 or 72 h. Following 24 or 72 h incubation, the cells were rinsed with PBS, then fixed and permeabilized (if appropriate) in 4% paraformaldehyde and 0.1% Triton X-100 in PBS for 15 min at room temperature. The cells were then washed with PBS and incubated with 1% BSA in PBS with 2 mL/L Tween 20 (PBST) for 30 min to block non-specific antibody binding. The cells were incubated with the primary antibody of interest in 1% BSA in PBST for 1 h at room temperature or overnight at 4°C. Following washing,

the cells were incubated with the secondary antibody for 1 h at room temperature in the dark. If necessary, this process was repeated for a second cell marker with a second set of primary and secondary antibodies. The antibodies used in these analyses are listed in Supplementary Table 3-I. Antibodies against albumin and cytokeratin 18 were used to stain hepatocytes [Wells et al., 1997; Alpini et al., 1994]. The presence of hepatic bile duct cells, fibroblasts, stellate cells, and Küpffer cells was determined using antibodies against cytokeratin 19, vimentin, desmin, and F4/80, respectively [Van Eyken et al., 1987; Yokoi et al., 1984; Kruglov et al., 2002; Li et al., 2014]. The coverslips were mounted on glass slides using VectaShield hardset mounting medium containing DAPI and sealed with clear nail polish. Slides were imaged using a TCS SP8 confocal laser scanning microscope from Leica Microsystems (Concord, Ontario).

### 3.4.6 Measurement of nuclear abundance

Relative nuclear abundance was measured to examine hepatocyte proliferation. Relative nuclear abundance was quantified by flow cytometry as described previously with some modifications [Nüsse et al., 1994; Avlasevich et al., 2006; Bryce et al., 2007]. Briefly, cultured hepatocytes were lysed through the addition of Lysis Buffer I (0.584 mg/mL NaCl, 1 mg/mL sodium citrate, 0.5 µL/mL IGEPAL, 0.7 U/mL RNase A, and 0.5 µM SYTOX® green nucleic acid stain) directly to the plates following removal of SFM and washing with PBS. Following incubation for 1 h in the dark at room temperature with gentle rocking, Lysis Buffer II (85.6 mg/mL sucrose, 15 mg/mL citric acid, and 0.5 µM SYTOX® green nucleic acid stain) was added to the plates, and the plates were incubated for an additional 30 min in the dark at room temperature with gentle rocking. To

normalize nuclei counts, 150 µL of a suspension of 6 µm polystyrene microspheres was added to each sample of lysate. The microspheres are labelled with a fluorescent dye with excitation/emission maxima of 488/515 nm (Cell Sorting Set-up Beads for Blue Lasers, Life Technologies, Burlington, Ontario). Each microsphere-lysate sample was diluted 1:10 prior to flow cytometric analysis. Data were acquired using a BD Biosciences FACScalibur flow cytometer (BD Biosciences, Mississauga, Ontario) equipped with a 488 nm laser. Instrumentation settings and data acquisition were facilitated using CellQuest Pro software (BD Biosciences). Data analysis was performed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Turku, Finland). SYTOX® green and bead fluorescence emission were captured in the FL1 channel (530/30 band-pass filter). Events were scored as nuclei following the application of key criteria (i.e., within a side scatter (SSC) vs. forward scatter (FSC) region, within a region that excludes doublets, and within a FSC vs FL1 region) (Supplementary Figure 3-1).

Nuclei counts were normalized to number of haploid genomes and presented relative to bead counts according to the following equation:

$$\frac{(population_{2N} \times 2) + (population_{4N} \times 4) + (population_{8N} \times 8)}{population_{beads}},$$

wherein $population_{2N}$ represents the number of events in the 2n population, $population_{4N}$ represents the number of events in the 4n population, $population_{8N}$ represents the number of events in the 8n population, $population_{beads}$ represents the number of events in the bead population. These data were generated following the acquisition of at least 15,000 events, wherein events comprise both nuclei and beads.

The doubling time was calculated using the following equation:

$$\frac{\ln(2)}{a},$$

wherein *a* represents the slope of the linear portion of the relationship between the natural logarithm of the nuclei counts versus time. The doubling time was presented as the mean of 5 biological replicates (i.e., primary hepatocytes isolated from 5 different mice).

### 3.4.7 Karyotype analysis and fluorescent *in situ* hybridization (FISH)

Primary hepatocytes were seeded in 100 mm petri dishes at $1.2x10^6$ cells per dish. Two days post-isolation at approximately 70% confluence, cultured hepatocytes were treated with 50 ng/mL colcemid in SFM for 1 h. Following colcemid treatment, the dishes were incubated with 1 mL of a 0.05% Trypsin-EDTA solution at 37°C and 5% $CO_2$. Trypsinization was stopped after 5 min with the addition of Attachment Medium, and the cells were gently collected in 15 mL tubes. The tubes were centrifuged for 10 min at 220 x *g*. The cell pellet was gently resuspended in 75 mM KCl. After 15 min, 6 to 8 drops of fixative (3:1 methanol to acetic acid) were added to each tube and the tubes immediately centrifuged for 10 min at 1000 rpm. The pellet was resuspended in 75 mM KCl once more and 6 to 8 drops of cold fixative were added to each tube. The tubes were agitated to mix and then filled with cold fixative. Tubes were stored at -20°C overnight.

The G-to-FISH karyotype analysis was performed by The Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, ON). The fixed cells were mounted on slides and digested with pancreatin for 35 s prior to Giemsa staining. A probe for the transgene was prepared from bacteriophage lambda cI857 DNA (Roche Diagnostics, Laval, Quebec) and labelled with SpectrumOrange. A control probe corresponding to the 3Hv locus on mouse chromosome 3 was prepared and labelled with

SpectrumGreen. The probe mixture consisted of 1 part lambda SpectrumOrange probe, 1 part mouse 3Hv locus SpectrumGreen probe, 2 parts mouse Cot-1 DNA, and 7 parts hybridization buffer (50% deionized formamide and 10% dextran sulphate in 2x saline-sodium citrate buffer, pH 7). The probe mixture was denatured at 75°C for 5 min, and incubated at 37°C for 30 min to re-anneal repetitive sequences to mouse Cot-1 DNA. The slides were denatured at 65°C for 20 s before the denatured probe mixture was applied to the slides. The slides were hybridized overnight at 37°C in a lightproof, humidified oven. The chromosomes were counterstained with DAPI. As is the standard at TCAG, 20 metaphases were analysed in order to allow for the detection of clonal chromosomal abnormalities with lower level mosaicism [Hook, 1977].

### 3.4.8 Ethoxyresorufin-*O*-deethylase (EROD) activity assay

EROD is a measure of CYP1A1 and CYP1A2 specific activity. Primary hepatocytes were suspended in 15 mL tubes at $1.2x10^6$ cells per 10 mL of Attachment Medium (i.e., for the 0 h timepoint) or seeded in 100 mm petri dishes at $1.2x10^6$ cells per dish (i.e., for the 2, 8, 24, and 48 h collection timepoints). The suspended hepatocytes were immediately centrifuged at 50 x *g* for 3 min, rinsed with PBS, frozen on dry ice and transferred to a -80°C freezer. Two h post seeding, the medium for the plated hepatocytes was changed to SFM or SFM containing 33 μM β-naphthoflavone. At 2, 8, 24, and 48 h post seeding, dishes of cultured hepatocytes were rinsed with PBS, frozen on dry ice and transferred to a -80°C freezer. The EROD specific activity of primary MutaMouse hepatocytes was then measured using a modification of a method described previously [Kennedy et al., 1995; Kennedy and Jones, 1994]. Resorufin was measured with

excitation/emission wavelengths of 530/590 in nm and total protein was measured with excitation/emission wavelengths of 400/460 in nm using a SpectraMax Gemini EM Microplate Reader (Molecular Devices, San Jose, CA). Fluorescence values were converted to quantities of resorufin and total protein by comparison with simultaneously measured standard curves. EROD specific activity was measured for 3 biological replicates.

### 3.4.9 Metabolite analysis by LC-MS/MS

CYP2B, CYP3A, UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT) specific activities were measured by liquid chromatography with tandem mass spectrometry (LC-MS/MS) quantification of testosterone and 7-hydroxycoumarin metabolites. Briefly, primary hepatocytes were suspended in 15 mL tubes at $1.2x10^6$ cells per 10 mL of Attachment Medium or seeded in 100 mm petri dishes at $1.2x10^6$ cells per dish. The suspended hepatocytes were immediately treated with 100 μM testosterone or 200 μM 7-hydroxycoumarin in SFM and incubated at 37°C with gentle shaking for two h. The suspended hepatocytes were then centrifuged at 50 x *g* for 3 min. Two h post seeding, the medium for the plated hepatocytes was changed to plain SFM, SFM containing 33 μM β-naphthoflavone, or SFM containing 100 μM phenobarbital. At 2, 8, 24, and 48 h post seeding, hepatocytes were treated with 100 μM testosterone or 200 μM 7-hydroxycoumarin in SFM and incubated at 37°C and 5% $CO_2$ for 2 h. Subsequently, the supernatant was removed and precipitated with two volumes of ice-cold acetonitrile, shaken vigorously for 10 min, and centrifuged at 5000 x *g* for 10 min to remove all particles. The particle-free supernatant samples were analysed for testosterone and 7-

hydroxycoumarin metabolites, specifically, 6β-hydroxytestosterone, 16β-hydroxytestosterone, 7-hydroxycoumarin glucuronide, and 7-hydroxycoumarin sulfate at Charles Rivers Laboratories Inc. (Cambridge, UK). Testosterone, 16β-hydroxytestosterone, 6β-hydroxytestosterone, and 7-hydroxycoumarin were measured using a Xevo tandem quadrupole mass spectrometer (TQ-MS) (Waters UK, Elstree, United Kingdom). 7-Hydroxycoumarin sulphate and 7-hydroxycoumarin glucuronide were measured using a Xevo TQ-S (Waters UK, Elstree, United Kingdom). Instrument parameters, multiple reaction monitoring (MRM) parameters, and chromatographic conditions are provided in Supplementary Tables 3-II, 3-III, 3-IV, and 3-V.

### 3.4.10 Gene expression

Primary hepatocytes were suspended in 15 mL tubes at $1.2x10^6$ cells per 10 mL of Attachment Medium or seeded in 100 mm petri dishes at $1.2x10^6$ cells per dish. RNA was isolated from cells 0, 2, 8, 24, and 48 h post-isolation using Qiagen RNeasy kits (Toronto, Ontario), with three biological replicates, according to manufacturer's instructions. RNA quality was assessed by the Agilent RNA ScreenTape Assay (Mississauga, Ontario) using the Agilent 2200 Tapestation System (Mississauga, Ontario) and all samples achieved $RIN^e$ quality scores of at least 8.5. cDNA was synthesized using Qiagen $RT^2$ First Strand kit (Toronto, Ontario) according to manufacturer's instructions. cDNA was prepared and applied to Qiagen Mouse Drug Metabolism $RT^2$ profiler PCR arrays (catalog #PAMM-002Z) (Toronto, Ontario) (Supplementary Table 3-VI). The $C_t$ values were determined using a BioRad CFX96 real-time PCR thermal cycler (Mississauga, Ontario). A $C_t$ cut-off of 35 was applied.

### 3.4.11 Statistical analyses

Statistical analyses were performed using RStudio version 1.0.136 (RStudio, Boston, MA, USA) software. Values are expressed as means ± standard error (SE). Comparisons between multiple conditions were performed with ANOVA, followed by Tukey's Honest Significance Test. Real-time qPCR data was normalized to the housekeeping gene, β-2 microglobulin, and analysed using the Livak method with significance calculated using the Student's T-test [Livak and Schmittgen, 2001]. The significance of the slope of the nuclear proliferation data was assessed using least-squares linear regression. The threshold for statistical significance was defined as $p \leq 0.05$.

## 3.5 Results

The isolated cells are frequently binucleated (Figure 3-1). Binucleated hepatocytes were visually enumerated in micrographs of 5 cultures and the proportion of binucleated cells was determined to be 78.1% ± 1.9% (data not shown). They present clear cytoplasms and cluster in small islands. Primary hepatocytes grown on collagen-coated plates appear to maintain a cuboidal morphology for roughly the first 24 h, before developing a branched, spindle-shaped appearance. This apparent de-differentiation of the *in vitro* hepatocytes has been confirmed via time-lapse imaging (Supplementary Video 3-1).

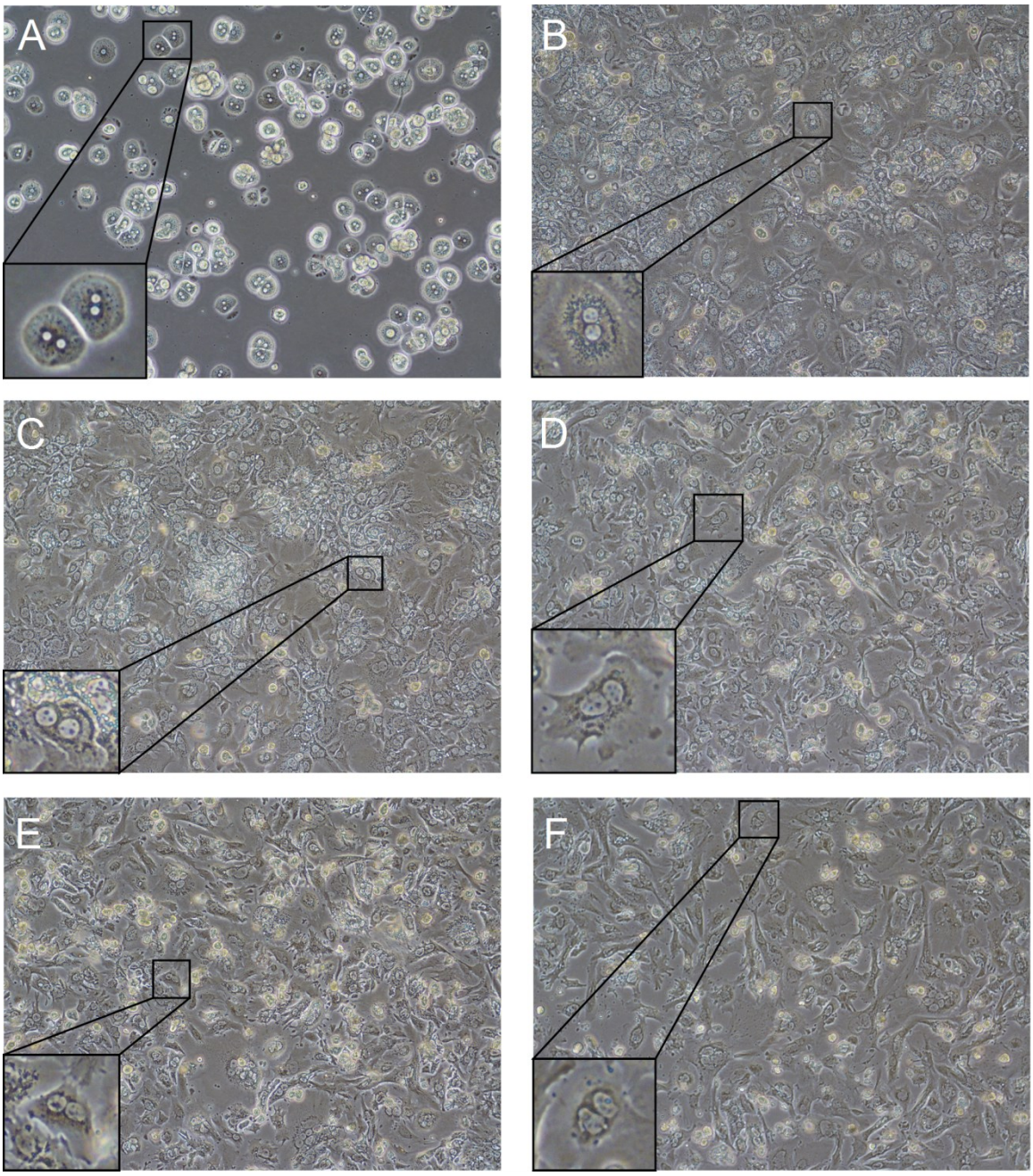


**Figure 3-1:** Time series of MutaMouse primary hepatocytes cultured on collagen-coated petri dishes. Images acquired 2 (panel A), 24 (panel B), 48 (panel C), 72 (panel D), 96 (panel E), and 120 (panel F) h post-isolation (100X magnification). Inset images display 300x magnified images of binucleated hepatocytes

Immunocytochemical analyses of cell-specific markers permit the distinction between hepatic cell types. The results show that virtually all primary MutaMouse hepatocytes express albumin and cytokeratin 18, two hepatocyte-specific markers, for at least 72 h in culture (Figure 3-2, panels A and B). The immunocytochemical analyses did not yield any evidence for the presence of hepatic bile duct cells, stellate cells, or Küpffer cells (data not shown). Vimentin staining, which was used to detect fibroblasts in the primary hepatocyte cultures, was sometimes observed as early as 1 day following hepatocyte isolation (Figure 3-2, panel C), with staining increasing over time (Figure 3-2, panel D). Roughly 10 to 20% of hepatocytes appear to express vimentin on day 1, and roughly 50 to 70% of hepatocytes appear to express vimentin on day 3.

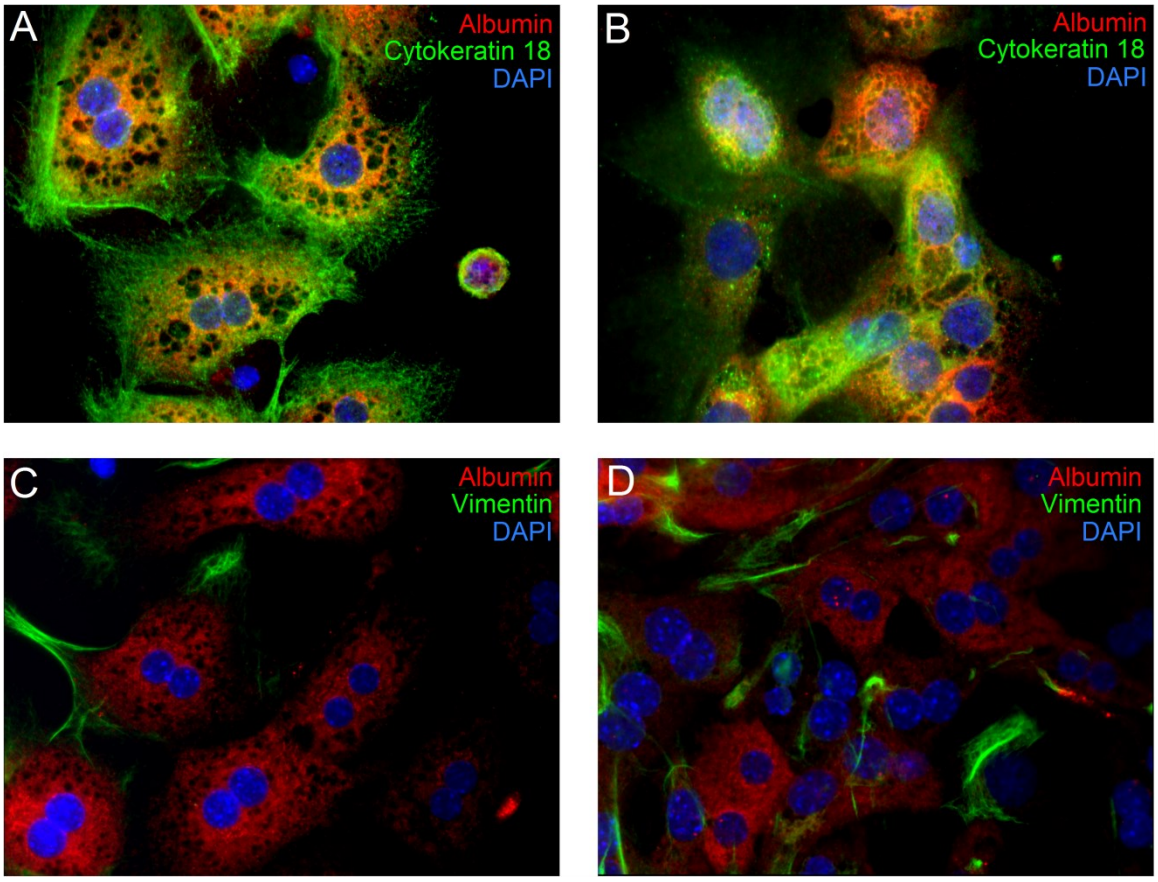


**Figure 3-2:** Representative immunofluorescent images of freshly isolated MutaMouse primary hepatocytes in culture for 24 (panels A and C) and 72 (panels B and D) h post-isolation. Hepatocyte-specific markers albumin (all panels) and cytokeratin 18 (panels A and B only) are shown in orange/red and green, respectively. The co-expression of cytokeratin (green) and albumin (red) in panels A and B caused labelled albumin to appear orange; in contrast labelled albumin appears red in the lower panels. Marker of fibroblasts, vimentin (panels C and D only), is shown in green. Fixed cells were treated with primary antibodies, labelled with secondary antibodies, and counterstained with the nuclear stain DAPI (blue) (600x magnification).

The karyotype of the cultured primary MutaMouse hepatocytes was assessed using a G-to-FISH analysis. The karyotype analysis revealed a modal chromosomal number of 80 (Figure 3-3 and Table 3-I). Out of the 20 metaphases analyzed, 1 is 2n, 16 are 4n, 1 is 5n, and 2 are 8n (Table 3-I). Aneuploidy was evident in many of the metaphases analyzed. However, it should be noted that some instances of perceived aneuploidy may have been due to the technical artefact of metaphases overspreading. Five of the metaphases analysed were found to have chromosomal aberrations, mainly chromosomal breakages, including terminal deletions. FISH using bacteriophage λGT10 DNA labelled with SpectrumOrange, confirmed the presence of the *lacZ* transgene on chromosome 3 in all metaphases examined (Figure 3-3 and Table 3-I).

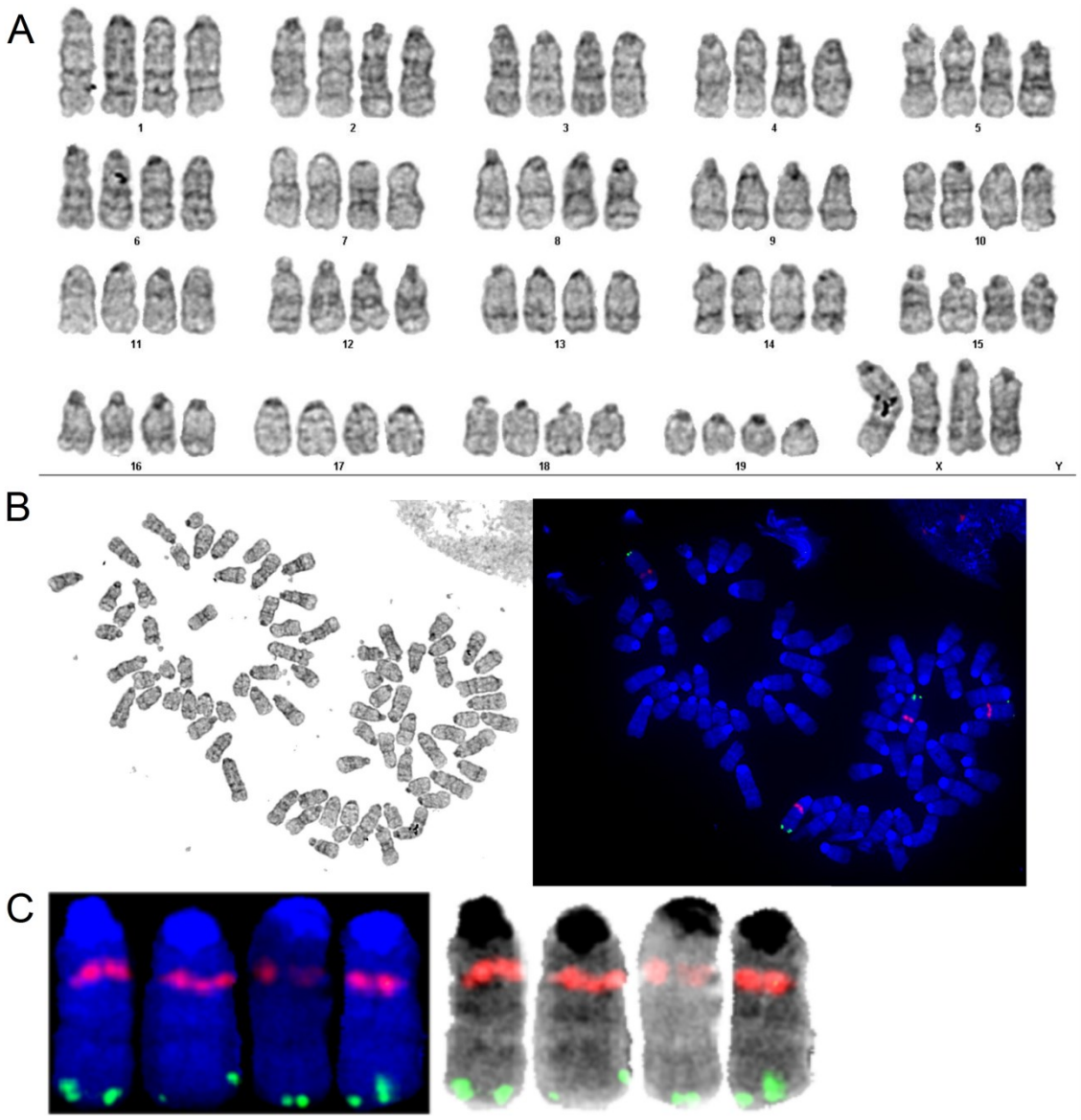


**Figure 3-3:** Representative karyotype and fluorescent *in situ* hybridization (FISH) results for cultured MutaMouse primary hepatocytes. Panel A shows a representative G-banding karyotype of a 4n MutaMouse primary hepatocyte. Panel B shows the metaphase spread as seen in the original brightfield (left) and fluorescent (right) micrographs. Panel C shows representative FISH result on chromosome 3 as seen in the fluorescent micrograph (left), and with reverse-DAPI banding (right), indicating λGT10 DNA (pink/orange) and the control H4 locus (green). The chromosomes were stained with DAPI, λGT10 DNA was labeled with SpectrumOrange, and a positive control probe consisting of mouse DNA from the chromosome 3 H4 locus was labeled with SpectrumGreen.

**Table 3-I:** G-to-FISH karyotype summary.

| Number of cells | Chromosome count (ploidy) | FISH Detection of Bacteriophage λ DNA on chromosome 3 (number of signals detected) |
|---|---|---|
| 1 | 160 (8n)[a] | Yes (8) |
| 1 | ~153 (8n)[a] | Yes (8) |
| 1 | 99 (5n) | Yes (6) |
| 10 | 80 (4n) | Yes (4) |
| 2 | 79 (4n) | Yes (4) |
| 1 | 78 (4n) | Yes (4) |
| 2 | 73 (4n) | Yes (3) |
| 1 | 70 (4n) | Yes (3) |
| 1 | 40 (2n) | Yes (2) |

[a]Unclear whether these were octoploid cells or two proximate tetraploid nuclei

The proliferation of MutaMouse primary hepatocytes in culture was quantified using relative counts of nuclei measured by flow cytometry. Three discrete populations of nuclei were observed using this approach; they are presumed to represent the polyploid states observed via karyotypic analyses (i.e., 2n, 4n, and 8n) (Supplementary Figure 3-1). The three populations were normalized to their respective assumed ploidy number, pooled, and the nuclei/bead ratio was determined each day for 5 consecutive days following isolation (Figure 3-4 A). The calculated doubling time was 22.5 ± 3.3 h (n=5). Hepatocyte proliferation was visually confirmed using time-lapse microscopy (Figure 3-4 B and Supplementary Video 3-1). Visual evaluation estimates the cell confluence to be ~30% at the start of culture, peaking at ~90% at 72 hours, and falling to ~80% at 96 h, and 120 h.

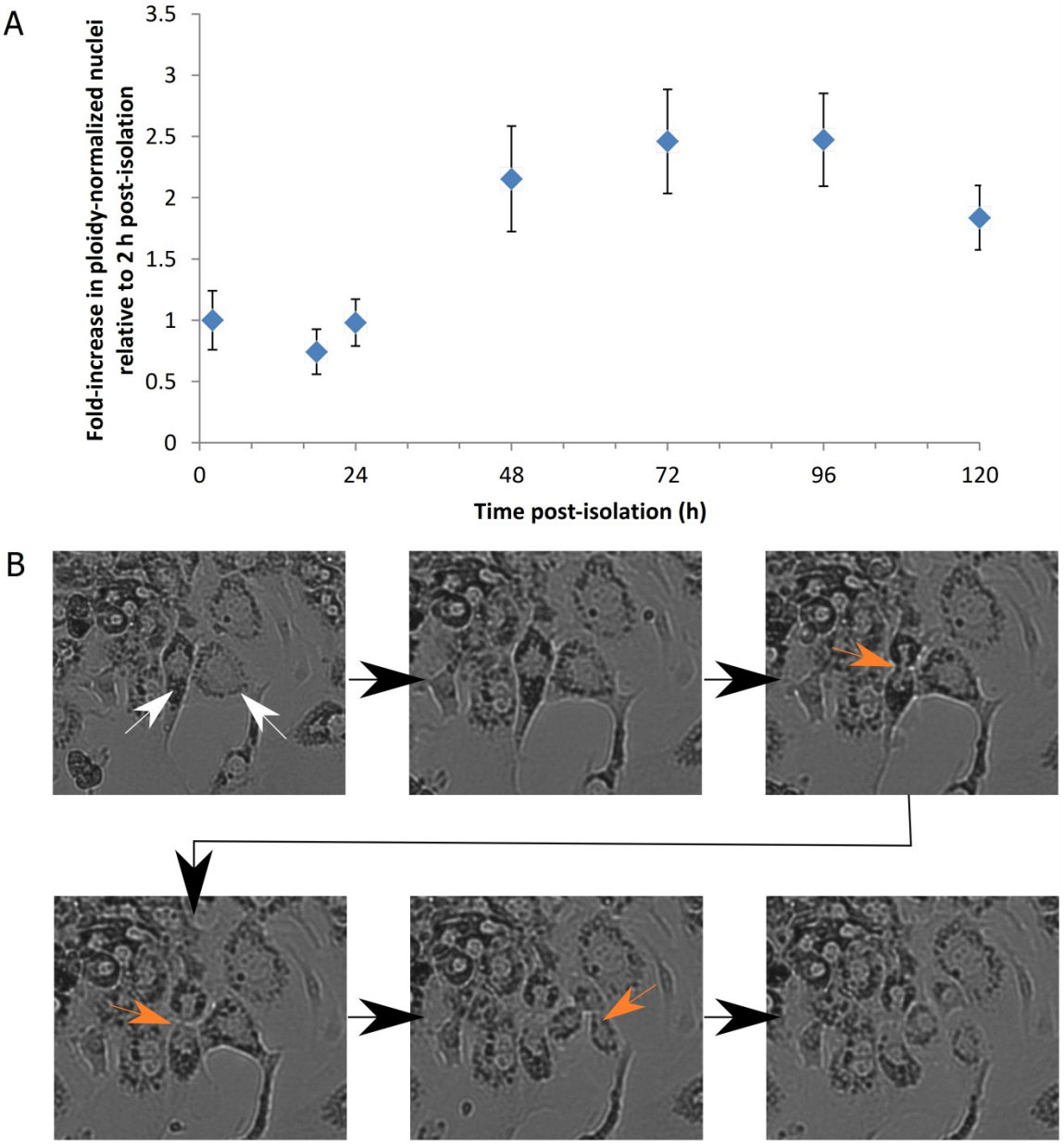


**Figure 3-4:** The proliferation of MutaMouse primary hepatocytes in culture. Panel A shows the temporal changes in the abundance of ploidy-normalised MutaMouse hepatocyte nuclei. The mean fold-increase in nuclei to bead ratio is presented (± SE; n=5), relative to 2 h post-isolation. Regression analysis revealed $r^2$ = 0.52, $p < 0.005$ for the linear portion of the relationship (i.e., 18 h to 48 h), yielding a doubling time estimate of 22.5 ± 3.3 h. Sytox Green-stained hepatocyte nuclei were mixed with a standardized volume of fluorescent beads, and temporal changes in abundance assessed using flow cytometry. Panel B is a representative sequence of both mononucleate and binucleate primary hepatocytes dividing in culture. The images (250x magnification), which were acquired 36 h post-isolation at 10 min intervals, show two cells (white arrows) undergoing division (orange arrows).

The Phase I metabolic enzyme capacity of MutaMouse primary hepatocytes was assessed using gene expression and specific enzyme activity analyses. The results show that the gene expression of many genes encoding Phase I enzymes, including genes encoding a variety of CYPs, cytochrome $b_5$ reductase, various alcohol dehydrogenases (ADHs), and epoxide hydrolase 2, as measured by real-time qPCR, were stable until the 2 h post-isolation time point; however, they begin to decline sharply 8 h post-isolation (Figure 3-5 A and Supplementary Table 3-VII). The expression of certain Phase I enzymes, such as *Cyp1a1*, encoding CYP1A1, *Nqo1*, encoding NADPH dehydrogenase, quinone 1 (NQO1), *Aldh1a1*, encoding aldehyde dehydrogenase (ALDH) 1A1, and *Ephx1*, encoding epoxide hydrolase 1, did not follow this trend. *Cyp1a1* exhibits ~6.4- and ~16.7-fold increases in gene expression 2 and 8 h post-isolation, respectively, followed by a return to the baseline expression level (Figure 3-5 B). Similarly, *Nqo1* showed ~7.2- and ~5.7-fold increases in gene expression 8 and 24 h post-isolation, respectively, followed by a return to the original expression level (Figure 3-5 B). *Aldh1a1* exhibited a ~2.7-fold increase in relative gene expression level 24 h post-isolation before returning to the level observed in freshly isolated hepatocytes (Figure 3-5 C). The gene expression analysis of *Ephx1* reveals ~3.0- and ~3.8-fold increases in relative expression 24 and 48 h post-isolation, respectively (Figure 3-5 C). Interestingly, the specific catalytic activities of CYP1A1/1A2, CYP2B, and CYP3A, measured by EROD activity, testosterone 16β-hydroxylation, and testosterone 6β-hydroxylation, respectively, remain fairly stable through the first 24 h post-isolation, followed by significant reductions in specific enzyme activity at 48 h post-isolation (Figure 3-5 D-F).

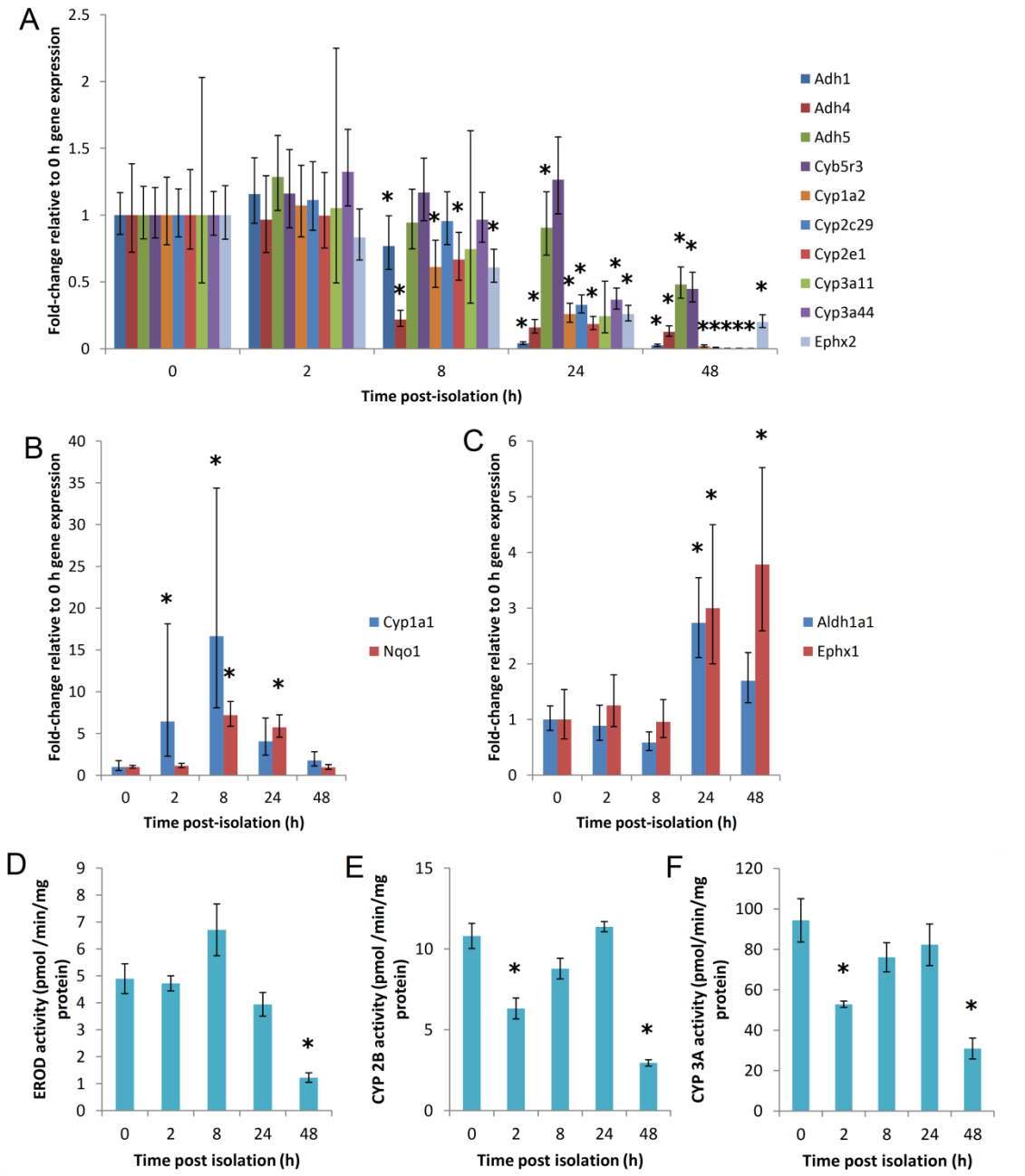


**Figure 3-5 :** Temporal trends in gene expression (panels A-C) and specific enzyme activity (panels D-F) for key Phase I metabolic enzymes. Fold-change gene expression changes of *Adh1*, *Adh4*, *Adh5*, *Cyb5r3*, *Cyp1a2*, *Cyp2c29*, *Cyp2e1*, *Cyp3a11*, *Cyp3a44*, and *Ephx2* (panel A), as well as *Cyp1a1* and *Nqo1* (panel B), and *Aldh1a1* and *Ephx1* (panel C), were quantified using a RT$^2$ Profiler PCR array. The specific activity of CYP1A1/1A2 were quantified by EROD (panel D). The specific activities of CYP2B (panel E) and CYP3A (panel F) were measured as testosterone 16β-hydroxylation and testosterone 6β-hydroxylation, respectively. *, significantly different from expression or specific activity at 0 h ($p \leq 0.05$). Mean fold changes ± SE (n = 3) presented (i.e., relative to expression or specific activity 0 h post-isolation). The genes encode the following metabolic enzymes: *Adh1*, alcohol dehydrogenase 1; *Adh4*, alcohol dehydrogenase 4; *Adh5*, alcohol dehydrogenase 5; *Cyb5r3*, cytochrome $b_5$ reductase 3; *Cyp1a2*, CYP1A2; *Cyp2c29*, CYP2C29; *Cyp2e1*, CYP2E1; *Cyp3a11*, CYP3A11, *Cyp3a44*, CYP3A44; *Ephx2*, epoxide hydrolase 2; *Cyp1a1*, CYP1A1; *Nqo1*, NAD(P)H dehydrogenase, quinone 1; *Aldh1a1*, aldehyde dehydrogenase 1A1; *Ephx1*, epoxide hydrolase 1.

In addition to temporal changes in metabolic activity, the induction of gene expression and enzyme activity following exposure to aryl hydrocarbon receptor (AhR) and constitutive androstane receptor (CAR) agonists (i.e., β-naphthoflavone and phenobarbital) was investigated. Induced metabolic enzyme gene expression and specific activity was assessed 24 h post-isolation. Exposure to β-naphthoflavone elicited significantly enhanced expression of the genes encoding CYP1A1 and CYP1A2 approximately 900- and 9-fold, respectively (Figure 3-6 A and B). CYP1A1/1A2 specific enzyme activity (i.e., EROD activity) is also significantly increased (~2-fold) following β-naphthoflavone treatment (Figure 3-6 C). Exposure to phenobarbital yields a significant induction (~2-fold) in CYP2B specific enzyme activity, as measured by testosterone 16β-hydroxylation (Figure 3-6 D).

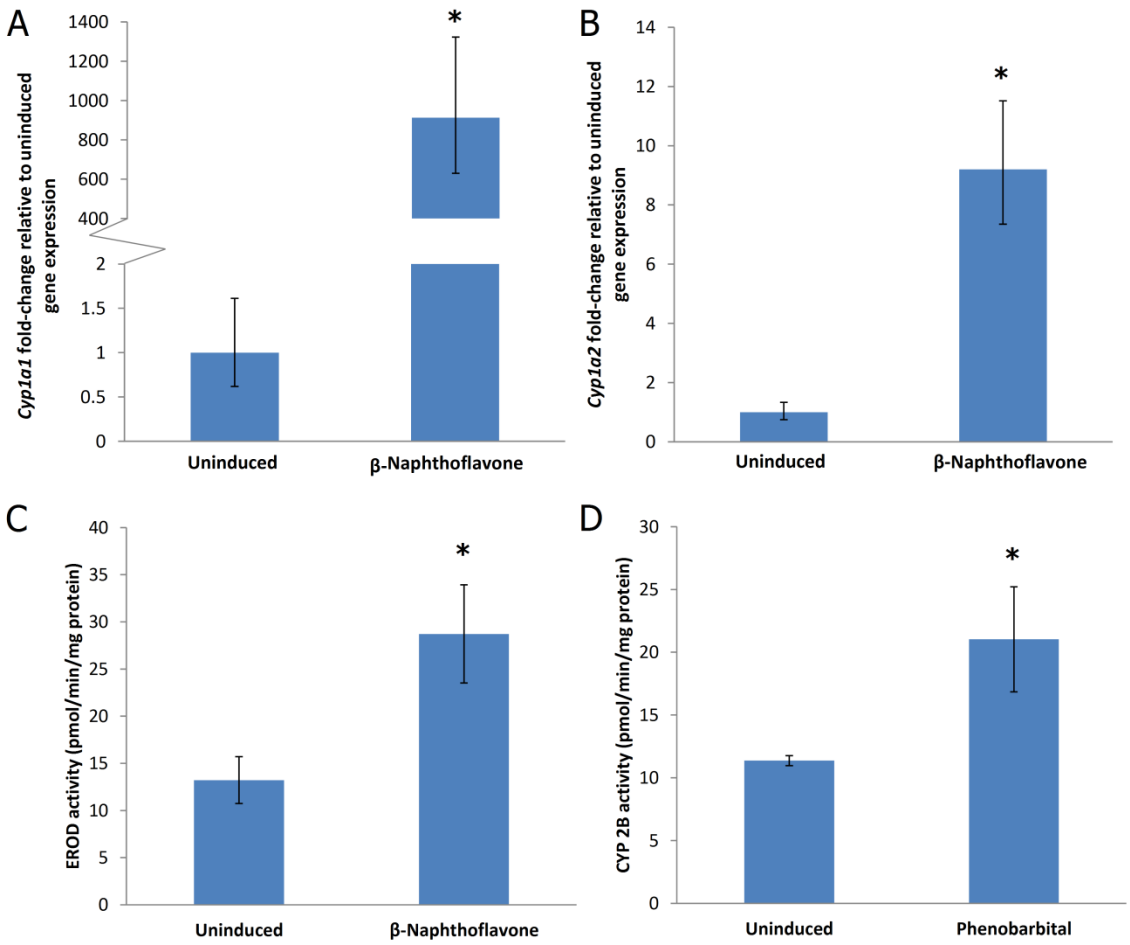


**Figure 3-6 :** The expression and specific activities of key Phase I metabolic enzymes following 22 h induction with the Ah receptor agonist β-naphthoflavone or the CAR agonist phenobarbital 24 h post-isolation. β-naphthoflavone-induced changes in gene expression was observed for *Cyp1a1* (panel A) and *Cyp1a2* (panel B). The β-naphthoflavone-induced increase in the specific enzymatic activity of CYP1A1/1A2 is shown in panel C. Phenobarbital-induced increase in the specific enzymatic activity of CYP2B is shown in panel D. Data presented as mean ± SE for triplicate samples, except panel C where n=7. *, significantly different from control at $p \leq 0.05$.

Gene expression and activity of Phase II metabolic enzymes were also assessed. Similar to the Phase I enzymes, the gene expression of several Phase II enzyme genes began to decline 8 h post-isolation (Figure 3-7 A and Supplementary Table 3-VII). *Chst*, encoding carbohydrate SULT 1, *Gstm5*, encoding glutathione-S-transferase (GST) μ5, *Gstp5*, encoding glutathione-*S*-transferase (GST) π5, *Mgst3*, encoding microsomal GST 3, *Nat2*, encoding NAT 2, and *Gsta1*, encoding GST α1, were exceptions to this rule (Figure 3-7 B and C). *Chst* and *Nat2* showed ~6.2- and ~3.6-fold increases in gene expression 24 h post-isolation, respectively, followed by a return to original levels 48 h post-isolation (Figure 3-7 B). Similarly, the gene expression of *Mgst3* showed a ~1.5-fold increase 8 h post-isolation prior to returning to the baseline level (Figure 3-7 B). On the other hand, *Gstm5* and *Gstp1* showed ~2.2- and ~8.7-fold increases in gene expression 8 h post-isolation, respectively, and do not return to original gene expression levels over the course of 48 h (Figure 3-7 B). Interestingly, *Gsta1* gene expression exhibits several thousand-fold increase over the matched 0 h control 8 and 24 h post-isolation. At 48 h post-isolation, the relative gene expression level of *Gsta1* decreases; however, it is still more than 400-fold higher than the 0 h control (Figure 3-7 C). Contrary to the gene expression results, SULT specific enzyme activity, measured as 7-hydroxycoumarin sulfation, showed no significant change over time relative to the specific enzymatic activity of freshly isolated cells (Figure 3-7 D) and UGT specific enzyme activity, measured as 7-hydroxycoumarin glucuronidation, exhibited a gradual increase over time (Figure 3-7 E).

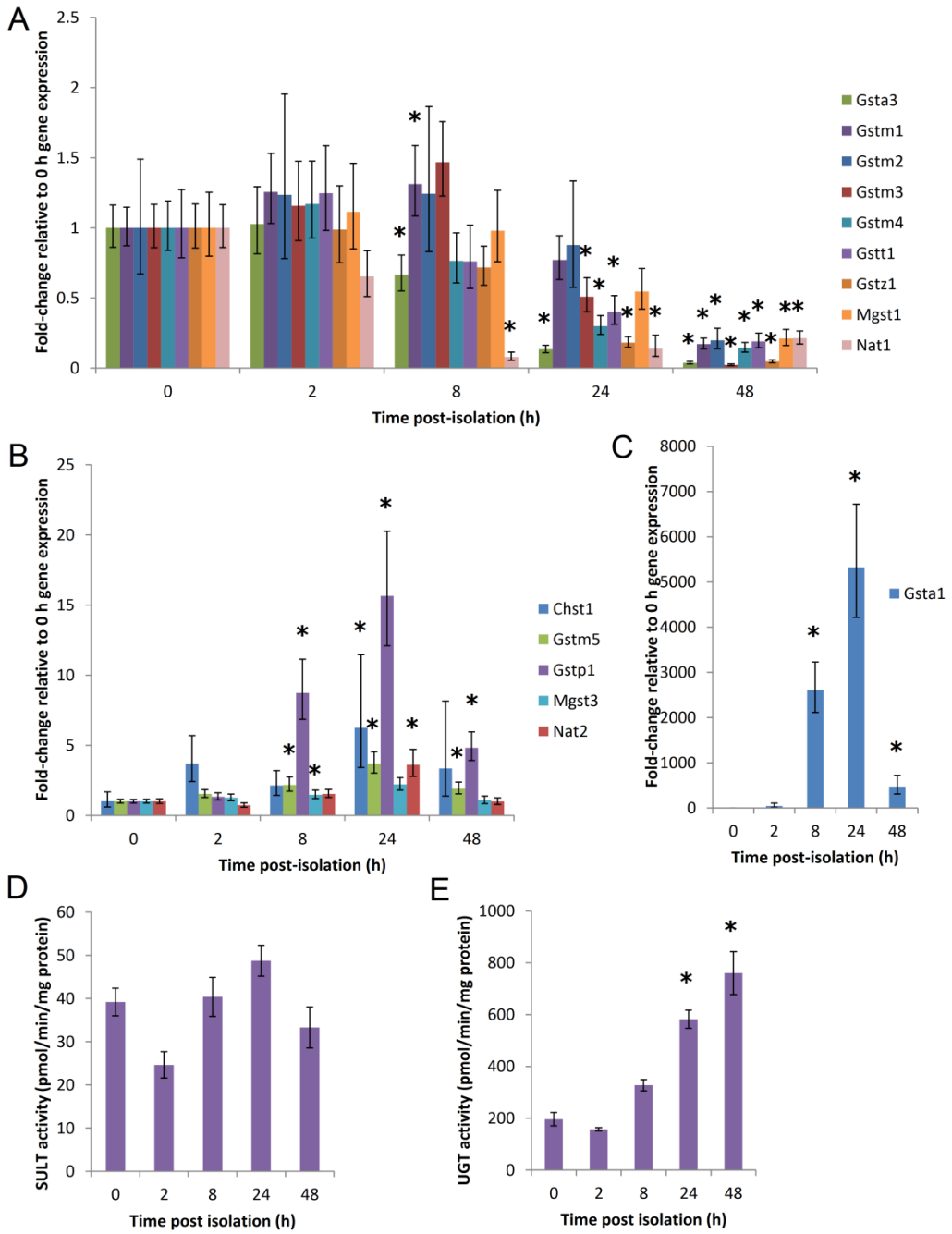


**Figure 3-7 :** Gene expression (Panels A, B, and C) and specific enzyme activity (Panels D and E) of key Phase II metabolic enzymes. Fold-changes in the gene expression of *Gsta3*, *Gstm1*, *Gstm2*, *Gstm3*, *Gstm4*, *Gstt1*, *Gstz1*, *Mgst1*, and *Nat1* (Panel A), as well as *Chst1*, *Gstm5*, *Gstp1*, Mgst3, *and Nat2* (Panel B), and *Gsta1* (Panel C) were quantified using an RT$^2$ Profiler PCR array. The specific activities of SULT (Panel D) and UGT (Panel E) were measured by 7-hydroxycoumarin sulfation and 7-hydroxycoumarin glucuronidation, respectively. *, significantly different from expression or specific activity at 0 h ($p \leq 0.05$). Mean fold changes ± SE (n = 3) presented (i.e., relative to expression or specific activity 0 h post-isolation). The genes encode the following metabolic enzymes: *Gsta3*, GST α1; *Gstm1*, GST μ1; *Gstm2*, GST μ2; *Gstm3*, GST μ3; *Gstm4*, GST μ5; *Gstt1*, GST θ1; *Gstz*, GST ζ1; *Mgst1*, microsomal GST 1; *Nat1*, NAT 1, *N*-acetyl transferase 1; *Chst1*, carbohydrate SULT 1; *Gstm5*, GST μ5; *Gstp1*, GST π1; *Mgst3*, microsomal GST 3; *Nat2*, NAT 2; *Gsta1*, GST α1.

## 3.6 Discussion

The use of metabolically competent and karyotypically stable primary hepatocytes circumvents many of the disadvantages posed by currently employed *in vitro* mammalian cell genetic toxicity assays. Moreover, the use of primary hepatocytes from a TGR developed for *in vivo* scoring of induced somatic and germ cell mutations (i.e., OECD Test guideline 488) [OECD, 2013] would permit adoption of a well-validated transgene mutation scoring system. Nevertheless, routine use of TGR primary hepatocytes is not without its challenges. Hepatocytes must be isolated, they must be capable of replicating in culture, and they must retain and maintain the metabolic capacity required for effective generation of DNA-reactive metabolites. This study constitutes a thorough characterization of MutaMouse primary hepatocytes, including detailed information about their structural, biochemical, and karyotypic characteristics, and thus their potential to be used as the cornerstone of an *in vitro* mammalian cell gene mutation assay.

The protocol used to isolate MutaMouse primary hepatocytes resulted in robust cultures of essentially pure hepatocytes. The isolated cells are frequently binucleated, a known characteristic of mammalian hepatocytes (Figure 3-1) [Gerlyng et al., 1993]. MutaMouse primary hepatocytes in culture tend to closely associate with other hepatocytes in small clusters, which has been shown to enhance hepatocyte function [Dohda et al., 2003]. Rodent hepatocytes *in vivo* are spherical [Klaunig et al., 1981; Arterburn et al., 1995]. The isolated cells maintain a cuboidal, *in vivo*-like appearance for the first 24 h in culture, followed by a temporal shift towards a flatter, spindle-shaped, elongated morphology typically associated with fibroblasts (Figure 3-1 and

Supplementary Video 3-1). This shift, known as de-differentiation, has been well described *in vitro* [Elaut et al., 2006; Rowe et al., 2010]. The de-differentiation process involves a loss of phenotype, including hepatocyte-specific functions, such as a gradual decline of metabolic activity, and results in the cultured hepatocytes more closely resembling generic, proliferative cells. A hallmark of the de-differentiation process is the expression of vimentin [Godoy et al., 2009]. Indeed, vimentin, a well-known fibroblast marker, is increasingly expressed in cultured MutaMouse hepatocytes (e.g., 3 days post-isolation, Figure 3-3 D). Vimentin staining in the culture is only observed in cells that are also expressing albumin, thus suggesting that these are hepatocytes undergoing de-differentiation, rather than another cell type (e.g., fibroblasts). Moreover, the results show that the cultures are not contaminated by other liver cell types. Thus, the results obtained collectively indicate that the MutaMouse primary hepatocyte cultures are initially composed of healthy hepatocytes, and the cultures are not overtaken by a rapidly proliferating cell type (e.g., fibroblasts) over time in culture.

As noted, the karyotype analysis revealed extensive polyploidy and instances of aneuploidy, with attendant flow cytometric analyses confirming three distinct populations of nuclei (Table 3-I and Supplementary Figure 3-1). Both the karyotype and flow cytometry results revealed that the 4n population is the most abundant. As mentioned, binucleation and polyploidization are common in hepatocytes [Guidotti et al., 2003; Duncan et al., 2010], and aneuploidy is also a known characteristic of mammalian hepatocytes both *in vivo* and *in vitro*. The latter has been postulated to be a mechanism for adaptation to stress; however, aneuploidy has been observed in normal, adult murine hepatocytes *in vivo* at a frequency of ~60% in the absence of any toxic insult [Duncan et

al., 2012; Duncan et al., 2010]. The karyotype analysis also confirmed the presence of the MutaMouse λGT10-*lacZ* shuttle vector. Bacteriophage λGT10 DNA was identified using FISH, confirming the presence of the *lacZ* transgene on chromosome 3, as previously shown *in vivo* [Blakey et al., 1995]. The presence of the *lacZ* transgene confirms that the isolated primary hepatocytes can be used to score induced mutations in much the same way as is currently done with the MutaMouse FE1 cell line (i.e., via the phenyl-β-D-galactopyranoside [P-Gal] positive selection assay) [White et al., 2003; Hoorn et al., 1993]. Indeed, Part II documents the use of MutaMouse primary hepatocytes to reliably detect chemical mutagens, including those that require Phase I and/or Phase II metabolic capacity.

Since cell division is required for mutagenesis, it is necessary to demonstrate that the isolated hepatocytes can proliferate in culture. This is challenging since genomic and/or nuclear division in hepatocytes is often divorced from cytokinesis [Duncan et al., 2010; Guidotti et al., 2003]. In other words, the hepatocyte genome replicates, but there is not necessarily an increase in cell number. The role of hepatocyte binucleation remains unclear, but it appears to be linked to polyploidization [Guidotti et al., 2003]. Indeed, the hepatocytes observed herein are frequently multinucleated; moreover, flow cytometric analysis of isolated nuclei revealed three distinct populations of nuclei with increasing DNA content (Supplementary Figure 3-1). The karyotype analyses, discussed above, indicate that the recorded flow cytometric events are indeed indicative of 2n, 4n and 8n nuclei. Cellular proliferation was observed using time-lapse imaging, and the results showed unusual cytokinetic events that illustrate the complex kinetics of hepatocyte proliferation, such as a single hepatocyte generating 3 daughter cells (Figure 3-4 B and

Supplementary Video 3-1). Indeed, genomic and/or nuclear divisions without cytokinesis complicate measurement of growth rate, and temporal change in the abundance of ploidy-corrected nuclei was used to assess proliferation rate [Guidotti et al., 2003; Gerlyng et al., 1993]. The analyses of this data (Figure 3-4 A) revealed a mean doubling time of 22.5 ± 3.3 h, similar to the doubling time of MutaMouse FE1 lung cells [White et al., 2003]. The OECD test guideline for the *in vitro* mammalian chromosomal aberration test suggests a sampling time of at least 1.5 doubling times [OECD, 2016a]. Given an adequate sampling time that allows for the variability around the MutaMouse primary hepatocyte doubling time (e.g., 72 h), this doubling time should permit fixation of transgene mutations. Importantly, the flow cytometry-based method presented here, and utilized to measure primary hepatocyte proliferation, can easily be repurposed to assess chemically-induced cytotoxicity using an adaptation of the "relative increase in cell count" (RICC) metric (i.e., relative increase in nuclear counts, or RINC) [Nüsse et al., 1994; Bryce et al., 2007; Avlasevich et al., 2006; OECD, 2016c].

Key metabolic enzymes that are involved in the activation of prototypical classes of bioactivated chemical mutagens have been detected in cultured MutaMouse primary hepatocytes (Table 3-II). Polycyclic aromatic hydrocarbons (PAHs) are activated to an electrophilic metabolite through a series of Phase I reactions, involving CYP1A1, CYP1A2, and CYP3A, as well as epoxide hydrolase [Bauer et al., 1995; Kim et al., 1998]. Like PAHs, the metabolic activation of mutagenic mycotoxins, including aflatoxins, is mediated by CYPs, in particular, CYP1A2 and CYP3A [Gallagher et al., 1994]. Nitrosamines, such as dimethylnitrosamine and diethylnitrosamine, are also activated by Phase I metabolism, specifically CYP 2E1 [Chowdhury et al., 2012;

Yamazaki et al., 1992]. Mutagenic phosphoramides, such as cyclophosphamide, require CYP2B and CYP3A for activation [Xie et al., 2003]. Azoxyglycosides, such as plant-derived cycasin, require the Phase I enzymes, CYP2E1, ADH, and ALDH, for conversion to a mutagenic metabolite [McMahon et al., 1991; Sohn et al., 2001]. The Phase I metabolic enzyme NQO1 has been postulated to have both azoreductase and nitroreductase activity and has been implicated in the activation of azo compounds and nitro-PAHs [Arlt et al., 2005; Møller and Wallin, 2000; Huang et al., 1979]. Aromatic amines (AAs), including 2-acetylaminofluorene, and heterocyclic amines (HCAs), such as PhIP, generally require both Phase I (e.g., CYP1A1 and CYP1A2) and Phase II (e.g., SULT, NAT, and/or UGT) enzymatic reactions to generate DNA-reactive nitrenium or carbenium ions [Schut and Snyderwine, 1999; Heflich and Neft, 1994; Cai et al., 2016]**.** Although GSTs are well-known for their detoxification and antioxidant functions, they have also been implicated in the mutagenic activation of halogenated hydrocarbons, such as 1,2-dibromo-3-chloropropane [Miller et al., 1986; van Bladeren et al., 1980]. The metabolic enzymes required in each of the examples above are present in MutaMouse primary hepatocytes, as measured by either enzyme gene expression or specific enzyme catalytic activity, thus illustrating their utility in an *in vitro* gene mutation assay.

**Table 3-II:** Specific catalytic activity and gene expression of metabolic enzymes in MutaMouse primary hepatocytes. Selected genes are known to be involved in the activation of prototypical classes of bioactivated chemical mutagens.

| Metabolic Enzyme | Chemical classes requiring enzyme for activation | Presence in MutaMouse primary hepatocytes | |
|---|---|---|---|
| | | Specific activity | Expression |
| *Phase I* | | | |
| ADH | Azoxyglycoside | ND[a] | Yes |
| ALDH | Azoxyglycoside | ND | Yes |
| CYP1A1 | PAH[b], AA[c], HCA[d] | Yes | Yes |
| CYP1A2 | PAH, Mycotoxin, AA, HCA | Yes | Yes |
| CYP2B[e] | Phosphoramide | Yes | ND |
| CYP2E1 | Nitrosamine, azoxyglycoside | ND | Yes |
| CYP3A[f] | PAH, Mycotoxin, Phosphoramide | Yes | Yes |
| Epoxide hydrolase | PAH | NA | Yes |
| NQO1 | Azo compound, Nitro-PAH | ND | Yes |
| *Phase II* | | | |
| SULT | AA, HCA | Yes | Yes |
| NAT | AA, HCA | ND | Yes |
| UGT | HCA | Yes | ND |
| GST | Halogenated hydrocarbon | ND | Yes |

[a] ND, not determined
[b] PAH, polycyclic aromatic hydrocarbon
[c] AA, aromatic amine
[d] HCA, heterocyclic amine
[e] Murine CYP2B9, CYP2B10, CYP2B13, CYP2B19, and CYP2B23 are closely related to human CYP2B6 [Nelson et al., 2004]
[f] Murine CYP3A11, CYP3A13, CYP3A16, CYP3A25, CYP3A41, CYP3A44, and CYP3A57 are closely related to human CYP3A4 [Nelson et al., 2004]

The temporal changes in enzyme gene expression profiles and specific activity levels were examined to further elucidate the utility of these cells in a gene mutation assay. This investigation demonstrated that the expression of many major metabolic enzyme genes in cultured MutaMouse primary hepatocytes is maintained for up to 8 h in culture, and the specific catalytic activity of major metabolic enzymes is maintained for at least 24 h (Figures 3-5 A, D-F and Figure 3-7 A). The temporal discrepancy between expression and activity is likely due to differences between mRNA and protein turnover and stability [Yang et al., 2008]. These results support findings by Mathijs, et al., [2009] wherein the transcriptional changes in sandwich-cultured murine hepatocytes were examined by microarray following 0, 42, and 90 h of cultivation. That study found that Phase I metabolic enzyme expression generally declined over time, and that Phase II gene expression either declined or showed no significant change [Mathijs et al., 2009]. The maintenance of basal metabolic enzyme gene expression was generally prolonged in the Mathijs et al. study, relative to the study presented here, as has been observed in sandwich cultures versus monolayer cultures [Tuschl and Mueller, 2006]. Collectively, the results presented herein indicate that MutaMouse primary hepatocytes are most metabolically active during the first 24 h of culture; therefore, the first 24 h of culture are the ideal timeframe for exposure to test chemicals that require metabolic bioactivation.

Although the specific activity and gene expression of many of the metabolic enzymes assessed in this study declined over time, there were some exceptions. Some enzymes, including *Cyp1a1*, *Nqo1*, *Aldh1a1*, *Chst1*, *Mgst3*, and *Nat2*, exhibit an initial increase in gene expression, followed by a return to the levels observed in freshly isolated hepatocytes (Figure 3-5 B-C and Figure 3-7 B). A similar trend is seen for *Ephx1*, *Gstm5*

and *Gstp1*, although the relative expression of these genes remains elevated 48 h post-isolation (Figure 3-5 C and Figure 3-7 B). These expression patterns potentially indicate a delayed "recovery" of expression of these genes following an initial dampening of expression in freshly isolated hepatocytes, as has been seen for CYP1A1 in primary rat hepatocytes [Tuschl and Mueller, 2006]. Interestingly, the relative gene expression of *Gsta1* increases dramatically to a 5000 fold-increase over freshly isolated hepatocytes 24 h post-isolation. Upregulation of *Gsta1* has been linked to murine hepatocellular injury, and its increased expression over time in MutaMouse primary hepatocytes is likely due to trauma caused to the hepatic architecture during the cell isolation process [Liu et al., 2014]. Mathijs et al [2009] similarly noted upregulation for *Cyp1a1*, several GSTs, including *Gstm1* and *Gstm5*, and several NATs, including *Nat2*, over time. Similarly, UGT specific activity increases over time. UGT is known to have increased activity in response to membrane perturbants and preferentially metabolizes hydrophobic molecules [Bock, 1977], and UGT has also been implicated in the regulation of endogenous lipids, thus affecting proliferation and differentiation [Dates et al., 2015; Radominska-Pandya et al., 1999]. Since hepatocyte isolation is an extremely disruptive process that unavoidably yields both healthy and damaged cells, harvest-induced cellular stress may be leading to a sustained induction of UGT specific activity. Importantly, despite any potential cellular disruption during the isolation process, MutaMouse primary hepatocytes maintain metabolic competence for at least the first 24 h of culture and exhibit expression patterns typical of murine hepatocyte cultures.

Other than the use of induced rodent liver S9, there are currently few metabolically competent options for use in *in vitro* gene mutation assays. The HepaRG

cell line is sometimes used in genetic toxicity assays where metabolic competence is desired due to its consistently high metabolic activity [Aninat et al., 2006; Lambert et al., 2009]. Interestingly, comparisons of MutaMouse primary hepatocytes and HepaRG cells indicates that MutaMouse hepatocytes have ~4 to ~10-fold higher specific activity for CYP1A1/1A2, SULT, and UGT, approximately equivalent specific activity for SULT, and one tenth the specific activity for UGT [Kratochwil et al., 2017; Jossé et al., 2012]. Overall, the metabolic competence of MutaMouse primary hepatocytes is similar to, or exceeds, HepaRG cells. This comprehensive metabolic profile, coupled with the presence of the MutaMouse transgene, make these cells ideally suited for *in vitro* assessment of chemically-induced mutations.

Primary human hepatocytes (PHHs) are the gold standard with respect to *in vitro* metabolic activity; thus, it useful to comparatively scrutinise the activity of the cells discussed herein. EROD specific activity values for fresh PHHs are between 0.14 and 0.96 pmol/min/mg protein, 24 hours post-seeding [Alexandre et al., 2002; Truisi et al., 2015]; values for cryopreserved PHH cultures vary between 1.68 and 6.73 pmol/min/mg protein 96 hours post-thawing [Roymans et al., 2005]. PHH CYP3A4 specific activity levels, measured by testosterone 6β-hydroxylation, are between 26.6 and 67.4 pmol/min/mg protein 96 hours post-thawing [Roymans et al., 2005]; one study noted 55.0 pmol/min/mg protein for freshly cultured cells [Lübberstedt et al., 2011]. Herzog et al. [2016] noted that UGT specific activity levels in freshly isolated PHH from two donors were 104.6 and 251.8 pmol/min/mg protein, respectively, as measured by hydroxycoumarin glucuronidation 48 hours post-seeding [Herzog et al., 2016]. Thus, the

values recorded for MutaMouse primary hepatocytes (Figure 3-5 D and F and Figure 3-7 E) are comparable to, or exceed, those recorded for PHHs.

Metabolic enzyme inducibility is indicative of the ability of primary hepatocytes to respond to xenobiotic insults. *Cyp1a1* and *Cyp1a2* gene expression is strongly induced following 22 h treatment with β-naphthoflavone (i.e., ~900- and ~9-fold, respectively). The increase in CYP1A1/1A2 specific catalytic enzyme activity (i.e., ~2-fold) is relatively modest, but the fold-increase is similar to what has been observed in BaP-induced pUR288 *lacZ* plasmid mouse hepatocytes (i.e., ~5-fold) [Zwart et al., 2012]. The EROD specific activity observed in β-naphthoflavone-induced primary MutaMouse hepatocytes is ~100-fold higher than that observed in β-naphthoflavone-induced rat primary hepatocytes [Lnenikova et al., 2018]. In addition, the modest ~2-fold induction in CYP1A1/1A2 and CYP2B specific activities observed in MutaMouse primary hepatocytes following exposures to β-naphthoflavone and phenobarbital, respectively, is similarly observed in HepaRG cells [Wang et al., 2015]. These results demonstrate that the inducibility of MutaMouse primary hepatocytes is similar to, or exceeds, that which is seen in other hepatocyte cultures.

By using a TGR mutation scoring system that is already internationally accepted and validated, and combining it with the metabolic competence and genetic stability of a normal primary hepatocyte, several of the problems plaguing current mammalian cell mutagenicity assays can be overcome. Indeed, utilization of hepatocytes from TGR models for *in vitro* mutagenicity assessment has already shown considerable promise [Chen et al., 2010; Zwart et al., 2012; Luijten et al., 2016]. However, those earlier works did not present a thorough analysis of the genetic, structural, and metabolic

characteristics that must be considered to objectively evaluate the potential utility of an assay based on primary hepatocytes. The results presented herein indicate that primary hepatocytes can readily be harvested from the MutaMouse; they are structurally and karyotypically normal and they proliferate in culture. The isolated hepatocytes are metabolically active for 24 h after isolation, and the observed activity is suitable for bioactivation of numerous known mutagens. Proliferation occurring after 24 h can permit the genetic damage to become fixed.

Routine use of primary hepatocytes from TGRs will require quality assurance criteria to ensure that the cells are functioning according to accepted standards. Cryopreservation of TGR primary hepatocytes has previously been described [Luijten et al., 2016], and could be employed to aid the distribution of MutaMouse primary hepatocytes. Although specification of precise quality assurance criteria will be necessary, it is not possible to precisely specify criteria at this time. Nevertheless, it is possible to provide some guidance with respect to the minimum acceptable level of metabolic activity. For example, based on levels recorded in this and other studies [Zwart et al., 2012; Luijten et al., 2016], it could be stated that TGR primary hepatocytes must have a baseline EROD specific activity level of at least 3 pmol/min/mg protein.

In conclusion, this work presents a thorough characterization of MutaMouse primary hepatocytes, in particular the cytological features that reflect their potential to be used for routine genetic toxicity assessments of new and legacy chemicals. Both this study and Part II focus on the use of MutaMouse primary hepatocytes in gene mutation assays; however, these cells could also be used *in vitro* for the assessment of chromosomal damage (e.g., micronucleus induction). The next step in the evaluation of

their utility for routine chemical screening involves structured testing of selected mutagens and non-mutagens, as recommended by the European Center for the Validation of Alternative Methods (EURL-ECVAM) [Kirkland et al., 2008]. Indeed, Part II presents mutagenicity assessments of 9 known mutagens, 2 known non-mutagens, and 2 compounds reported to elicit false positives *in vitro* and the results therein indicate high sensitivity and specificity. Part II also further discusses the criteria set out by the OECD Test Guideline program for the validation of novel toxicological test procedures and the additional criteria that this assay must meet prior to regulatory acceptance. It is anticipated that the attributes of MutaMouse primary hepatocytes, and their utility for chemical screening, will provide a foundation for their adoption as the cornerstone of a robust (i.e., sensitive and specific) *in vitro* mammalian cell mutagenicity assay that effectively complements existing *in vitro* tests (e.g., bacterial reverse mutation), and permits robust prioritization for follow-up *in vivo* testing.

## 3.7 References


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# CHAPTER FOUR

# Chapter 4: The development and pre-validation of an *in vitro* mutagenicity assay based on MutaMouse primary hepatocytes, Part II: Assay performance for the identification of mutagenic chemicals

## 4.1 Preamble: Authors, Affiliations, and Style

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Style: This chapter was prepared according to the style guide for *Environmental and Molecular Mutagenesis*

## 4.2 Abstract

As demonstrated in Part I, cultured MutaMouse primary hepatocytes (PHs) are suitable cells for use in an *in vitro* gene mutation assay due to their metabolic competence, their "normal" phenotype, and the presence of the MutaMouse transgene for reliable mutation scoring. The performance of these cells in an *in vitro* gene mutation assay is evaluated in the present study, Part II. A panel of thirteen mutagenic and non-mutagenic compounds was selected to investigate the performance of the MutaMouse PH *in vitro* gene mutation assay. The nine mutagens represent a range of classes of chemicals and include mutagens

that are both direct-acting and requiring metabolic activation. All the mutagens tested, except for ICR 191, elicited significant, concentration-dependent increases in mutant frequency (MF) ranging from 2.6- to 14.4-fold over the control. None of the four non-mutagens, including two misleading, or "false", positives (i.e., *tertiary* butylhydroquinone [TBHQ] and eugenol), yielded any significant increases in MF. The benchmark dose (BMD) covariate approach facilitated ranking of the positive chemicals from most (i.e., 3-nitrobenzanthrone [3-NBA], benzo[*a*]pyrene [BaP], and aflatoxin $B_1$ [AFB1]) to least (i.e., *N*-ethyl-*N*-nitrosourea [ENU]) potent. Overall, the results of this preliminary validation study suggest that this assay may serve as a complimentary tool alongside the standard genotoxicity test battery. This study, alongside Part I, illustrates the promise of MutaMouse PHs for use in an *in vitro* gene mutation assay, particularly for chemicals requiring metabolic activation.

## 4.3 Introduction

Regulatory evaluations of new and existing substances always require genetic toxicity assessment, and this generally includes *in vitro* assessments of mutagenic activity. Although bacterial mutagenicity testing (e.g., Salmonella reverse mutation test) is most commonly used for *in vitro* mutagenicity assessment, tiered testing regimes employed in different jurisdictions require or accept *in vitro* mutagenicity assessments in cultured mammalian cells. For example, the United States Federal Insecticide, Fungicide, and Rodenticide Act (US FIFRA) Pesticide Assessment Guidelines require that *in vitro* mammalian cell mutagenicity tests are performed [Jaeger, 1984]. Similarly, the Canadian Environmental Protection Act (CEPA) requires an *in vitro* test for gene mutation, with

and without metabolic activation, for certain substances not on the Domestic Substances List (DSL) [Minister of Justice, 2018]. The United States Food and Drug Administration (USFDA) Redbook, the European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), and the International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1), and Japan's Chemical Substance Control Law all require, under certain conditions, an *in vitro* assessment of either chromosomal damage or mutagenesis [USFDA, 2007; ICH, 2011; ECHA, 2017; METI, 2009].

The mammalian cell genotoxicity assays that are currently used for regulatory assessments have all been extensively validated. Indeed, assays routinely used for regulatory evaluations and decision-making, such as the *Tk* gene mutation assay, have internationally-accepted Organization for Economic Cooperation and Development (OECD) test guidelines (TG). However, there are only two OECD TGs for *in vitro* mutagenicity assessment in cultured mammalian cells, the aforementioned *Tk* locus gene mutation test (i.e., TG 490), and the *Hprt*/*xprt* mutation test (i.e., TG 476) [OECD, 2016a; OECD, 2016c]. Although these assays have a long history of successful use for chemical safety assessments, they have several shortcomings; some of which are associated with the inherent traits of the cell lines used (e.g., L5178Y, TK6, Chinese hamster ovary, Chinese hamster lung, and Chinese Hamster V79 cell lines). These include lack of metabolic competence [OECD, 2016a; OECD, 2016c], aberrant karyotypes [Storer et al., 1997], and/or genomic instability [Lorge et al., 2016]. Moreover, these assays require laborious, time-consuming isolation and enumeration of

mutant clones [OECD, 2016c; OECD, 2016a]. To provide alternatives, immortalized cell lines derived from transgenic rodents such as the MutaMouse have been used to develop *in vitro* mammalian cell mutagenicity assays. One example is the *in vitro* mutagenicity assessment assay conducted in MutaMouse FE1 lung epithelial cells. This assay shows considerable promise for routine *in vitro* assessment of mutagenicity, and the assay is now partially validated [Maertens et al., 2017; Hanna, 2018]. Although FE1 cells have some endogenous metabolic capacity (e.g., cytochrome P450 [CYP] 1A1), and can convert some genotoxic agents into reactive metabolites (e.g., benzo[*a*]pyrene [BaP]), the cells do not have a full complement of Phase I and II metabolic enzymes [White et al., 2003; Maertens et al., 2017; Hanna, 2018]. In contrast, primary hepatocytes (PHs) from transgenic rodents, including the MutaMouse and the pUR288 *lacZ* Plasmid Mouse, have a more extensive complement of Phase I and II enzymes; as such, they are excellent candidates for the development of an *in vitro* mammalian cell mutagenicity assay [G. Chen et al., 2010; Zwart et al., 2012; Luijten et al., 2016].

Although in vitro mutagenicity tests based on cells (e.g., PHs) from transgenic rodents such as the MutaMouse and *lacZ* Plasmid Mouse show considerable promise, assays used for regulatory purposes must be validated to ensure adequate performance and reliability. The OECD TG program specifies criteria for the validation of novel toxicological test procedures. More specifically, to assess the performance and reliability of a novel test, the OECD requires the generation of information regarding test definition, intra-laboratory variability, inter-laboratory transferability, inter-laboratory reproducibility, predictive capacity, applicability domain, and performance standards [OECD, 2005]. In Part I of this two-part series, we characterized MutaMouse PHs;

demonstrating that they proliferate in culture, are karyotypically stable, carry the *lacZ* transgene vector for enumeration of chemically-induced mutations, and express a comprehensive complement of Phase I and II metabolic enzymes (e.g., CYP1A1, CYP1A2, CYP2B, CYP2E1, CYP3A, sulfotransferases (SULTs), UDP-glucuronosyl transferases (UGTs), glutathione-*S*-transferases (GSTs), *N*-acetyltransferases, etc.) [Cox et al., 2018].

This study (i.e., Part II) aims to elucidate the predictive capacity and applicability domain of the assay. The predictive capacity is the ability of an assay to accurately predict the intended endpoint (e.g., gene mutation), whereas the applicability domain refers to the range of chemicals that can be reliably assessed [OECD, 2005]. Since MutaMouse PHs are metabolically most active in the first 24 h of culture, and this is followed by a period of proliferation (doubling time of 22.5 ± 3.3 h), the assay protocol includes a 6 h exposure to the chemical of interest within the first 24 h, followed by a sampling time of 72 h [Cox et al., 2018]. The chemicals selected for the performance evaluation include known mutagens, known non-mutagens, and compounds that have been reported to elicit a misleading positive *in vitro* that is not manifested *in vivo* [Kirkland et al., 2008; Kirkland et al., 2016]. These chemicals (i.e., *t*-butylhydroquinone and eugenol), which are not DNA-reactive, can indirectly elicit genotoxicity *in vitro*. The positive *in vitro* results are likely artifacts due to strain of cells used, cytotoxicity, or perturbations to the cell culture conditions [Fowler, Smith et al., 2012a; Fowler, Smith et al., 2012b]. Most of the chemicals investigated, which cover a range of chemical classes, have been suggested by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) for the validation of novel *in vitro* genotoxicity assays

[Kirkland et al., 2008; Kirkland et al., 2016]. More specifically, in an effort to characterize and evaluate assay performance and applicability domain, we have examined a mutagenic nitrosourea, an acridine mutagen, a polycyclic aromatic hydrocarbon (PAH), an aromatic amine (AA), a heterocyclic amine (HA), a mycotoxin, two nitroarenes, and a nitrosamine, each of which has a unique mode of action and unique metabolic requirements. The test set of chemicals included 4 non-mutagens, 2 of which yield spurious positives in *in vitro* assays, as described above. Collectively, these assessments constitute an initial characterization of the performance and applicability domain (i.e., pre-validation) of the *in vitro* mutagenicity assay in MutaMouse PHs.

## 4.4 Materials and Methods

### 4.4.1 Materials and Reagents

The CAS numbers and sources of all test chemicals are presented in Table 4-I. Dulbecco's modified Eagle's medium (DMEM), William's E medium, phosphate-buffered saline (PBS), foetal bovine serum (FBS), epithelial growth factor (EGF), penicillin-streptomycin reagent, Hank's balanced salt solution (HBSS), proteinase K, trypan blue, and SYTOX® green were obtained from Life Technologies Inc. (Burlington, Ontario). Corning® Biocoat™ type I collagen-coated culture dishes. CIzyme™ collagenase HA and BP protease were obtained from VitaCyte LLP (Indianapolis, Indiana). Dexamethasone, human insulin, dimethylsulphoxide (DMSO), Percoll®, bovine serum albumin (BSA), and IGEPAL CA-630 were obtained from Sigma-Aldrich Canada Co. (Oakville, Ontario). Phenyl-β-D-galactopyranoside (P-Gal) was obtained from MJS

BioLynx (Brockville, Ontario). TransPak Packaging Extract was obtained from Agilent Technologies Canada (Mississauga, Ontario).

### 4.4.2 Isolation and Culture of PHs

Female MutaMouse specimens were bred and maintained locally under conditions approved by the Health Canada Ottawa Animal Care Committee. Fresh MutaMouse PHs were isolated as specified in the companion manuscript [Cox et al., 2018]. Briefly, cells were obtained using a two-step collagenase technique with the addition of a Percoll® isodensity purification step [Seglen, 1976; Kreamer et al., 1986]. The cells were plated at a density of $1.2x10^6$ cells/dish onto 100 mm collagen-coated culture dishes using Attachment Medium (20 U/L human insulin, $4x10^{-6}$ mg/mL dexamethasone, 10% FBS, and 100 U/mL penicillin-streptomycin in DMEM), and incubated at 37°C and 5% $CO_2$. Two hours (t = 2 h) following plating, the Attachment Medium was replaced with Serum-Free Medium (SFM; 10 mM HEPES, 2 mM L-glutamine, 10 mM pyruvate, 0.35 mM L-proline, 20 U/L human insulin, $4x10^{-6}$ mg/mL dexamethasone, 0.01 µg/mL EGF, and 100 U/mL penicillin-streptomycin in Williams Medium E), and the plates were incubated at 37°C and 5% $CO_2$.

**Table 4-I:** Sources of chemicals used for *in vitro* exposures of MutaMouse PHs

| Chemical | CAS number | Source |
|---|---|---|
| ENU[a] | 759-73-9 | Sigma-Aldrich (Oakville, Ontario) |
| ICR 191[b] | 17070-45-0 | Sigma-Aldrich (Oakville, Ontario) |
| BaP[c] | 50-32-8 | Moltox (Boone, North Carolina) |
| AFB1[d] | 1162-65-8 | Sigma-Aldrich (Oakville, Ontario) |
| 2-AAF[e] | 53-96-3 | Sigma-Aldrich (Oakville, Ontario) |
| PhIP[f] | 105650-23-5 | Moltox (Boone, North Carolina) |
| 1,8-DNP[g] | 42397-65-9 | Courtesy of Dr. I. Lambert (Carleton University) |
| 3-NBA[h] | 17117-34-9 | Courtesy of Dr. V. Arlt (King's College London) |
| DMN[i] | 62-75-9 | Sigma-Aldrich (Oakville, Ontario) |
| Ampicillin trihydrate | 7177-48-2 | Sigma-Aldrich (Oakville, Ontario) |
| D-Mannitol | 69-65-8 | Sigma-Aldrich (Oakville, Ontario) |
| TBHQ[j] | 1948-33-0 | Sigma-Aldrich (Oakville, Ontario) |
| Eugenol | 97-53-0 | Sigma-Aldrich (Oakville, Ontario) |

[a] ENU, *N*-ethyl-*N*-nitrosourea
[b] ICR 191, 6-Chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride
[c] BaP, Benzo[*a*]pyrene
[d] AFB1, Aflatoxin $B_1$
[e] 2-AAF, 2-Acetylaminofluorene
[f] PhIP, 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
[g] 1,8-DNP, 1,8-Dinitropyrene
[h] 3-NBA, 3-Nitrobenzanthrone
[i] DMN, Dimethylnitrosamine
[j] TBHQ, *tertiary* butylhydroquinone

### 4.4.3 Chemical Exposure and DNA Isolation

MutaMouse PHs were exposed to test chemicals as described by Chen et al., [2010], with some modifications. Briefly, stock solutions of the chemicals described in Table 4-I were prepared in DMSO. After 18 hours of culture, MutaMouse PHs were exposed to the chemicals of interest in SFM with 1% DMSO for 6 hours at 37°C and 5% $CO_2$. Three biological replicates (i.e., separate experiments using primary hepatocytes from three different donor mice) were used for each test chemical. Following exposure, the medium was replaced with fresh SFM and the hepatocytes were incubated for a further 72 hours prior to lysis and DNA isolation.

Following the 72 hour sampling period, the SFM was replaced with lysis buffer (10 mM Tris pH 7.6, 10 mM ethylenediaminetetraacetic acid [EDTA], 150 mM sodium chloride, 1% sodium dodecyl sulphate [SDS], and 1 mg/mL proteinase K. The DNA was isolated by phenol chloroform extraction as previously described with an additional chloroform step [Gingerich et al., 2014]. DNA was precipitated with ethanol, spooled onto a sealed Pasteur pipette, washed with 70% ethanol, dried, dissolved in $TE^{-4}$ buffer (10 mM Tris pH 7.6 and 0.1 mM EDTA), and stored at 4°C.

### 4.4.4 Mutant Frequency (MF) Determination

The frequency of *lacZ* mutants was determined using the P-Gal positive selection method as previously described [Gingerich et al., 2014; Lambert et al., 2005; Vijg and Douglas, 1996; G. Chen et al., 2010]. Briefly, TransPak was used to retrieve and package bacteriophage λgt10*lacZ* vectors from MutaMouse PH DNA. *E. coli* cells (*E. coli* C lacZ -, galE -, recA -, Kanr, pAA119) [Gossen et al., 1992] were allowed to adsorb the phage

particles; cells were plated with P-Gal selective medium and incubated overnight at 37°C. Plaques were scored manually, and MF was calculated as the ratio of mutant plaque-forming units (pfu) to total pfu determined from non-selective plates (i.e., without P-Gal).

### 4.4.5 Cytotoxicity Determination

Cytotoxicity was measured using the relative increase in nuclear counts (RINC) metric. RINC was quantified by flow cytometry as described previously with some modifications [Nüsse et al., 1994; Avlasevich et al., 2006; Bryce et al., 2007; Cox et al., 2018]. Briefly, cultured hepatocytes were lysed using Lysis Buffer I (0.584 mg/mL NaCl, 1 mg/mL sodium citrate, 0.5 µL/mL IGEPAL, 0.7 U/mL RNase A, and 0.5 µM SYTOX® green nucleic acid stain). Following a 1 hour incubation, Lysis Buffer II (85.6 mg/mL sucrose, 15 mg/mL citric acid, and 0.5 µM SYTOX® green nucleic acid stain) was added to the plates. To normalize nuclei counts, 150 µL of a suspension of 6 µm fluorescently labelled polystyrene microspheres was added to each sample of lysate. Ploidy was normalized as described previously [Cox et al., 2018]. The microspheres have excitation/emission maxima of 488/515 nm (Cell Sorting Set-up Beads for Blue Lasers, Life Technologies, Burlington, Ontario). Each microsphere-lysate sample was diluted 1:10 prior to flow cytometric analysis. Data were acquired using a BD Biosciences FACScalibur flow cytometer (BD Biosciences, Mississauga, Ontario) equipped with a 488 nm laser. Instrumentation settings and data acquisition were facilitated using CellQuest Pro software (BD Biosciences). Data analysis was performed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Turku, Finland). SYTOX® green and bead fluorescence emission were captured in the FL1 channel (530/30 band-

pass filter). Events were scored as nuclei following the application of key criteria (i.e., within a side scatter (SSC) vs. forward scatter (FSC) region, within a region that excludes doublets, and within a FSC vs FL1 region).

RINC values were calculated using a modification of the relative increase in cellular counts (RICC) formula [OECD, 2016b]:

$$RINC = \frac{Increase\ in\ relative\ number\ of\ nuclei\ in\ treated\ cultures\ (final-initial)}{Increase\ in\ relative\ number\ of\ nuclei\ in\ control\ cultures\ (final-initial)},$$

wherein the initial count was obtained at the beginning of the exposure period and the final count was obtained 72 hours following the end of the exposure period.

In accordance with the OECD test guidelines for the *in vitro* mammalian cell gene mutation assays using the *Hprt*, *xprt*, and thymidine kinase genes, any positive responses elicited from concentrations with RINC values lower than 0.2 were interpreted with caution [OECD, 2016a; OECD, 2016c].

### 4.4.6 Statistical Analyses

The *lacZ* mutant frequency data were analyzed in using RStudio version 1.0.136 (RStudio, Boston, MA, USA) software using the glm function. The quasi-Poisson distribution family was used to account for over-dispersion, and the offset was designated as the natural log of total pfu [Haynes, 1989]. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment (i.e., Chi-squared test), and custom contrasts statements were employed to evaluate the statistical significance of responses at selected doses or concentrations [Arlt et al., 2008]. The resulting p-values were corrected for multiple comparisons using the Bonferroni method. P-values were considered to be significant if they were lower than 0.05. Results for a

given chemical were deemed positive if a significant response was obtained for the Chi-squared test for overall treatment effect and at least 1 concentration yielded a significant MF increase above the concurrent vehicle control. A negative result was called if neither of these conditions were met. A chemical was deemed equivocal if only 1 of these conditions was met.

### 4.4.7 Benchmark Dose (BMD) Modeling

BMD analysis was performed on all positive *lacZ* MF data using PROAST version 65.5 in R. The analysis employed chemical as a covariate. Both exponential and Hill nested model families were fit to the data. A benchmark response (BMR) of 100% (i.e., a 2-fold MF increase over control) was selected, as it has been previously used for the assessment of *lacZ* MF data; it lies within the range of observed results, thus allowing for optimal resolution of confidence intervals [Wills et al., 2016; Long et al., 2018].

## 4.5 Results

The vehicle control data (Figure 4-1) show a statistically normal distribution with a mean MF of $11.0 \times 10^{-5}$ (SEM = $0.80 \times 10^{-5}$, N = 32), and $5^{th}$ and $95^{th}$ percentiles of $5.3 \times 10^{-5}$ and $18.7 \times 10^{-5}$, respectively. These data were compiled from all available experiments (N = 32) performed over the course of 4 years.

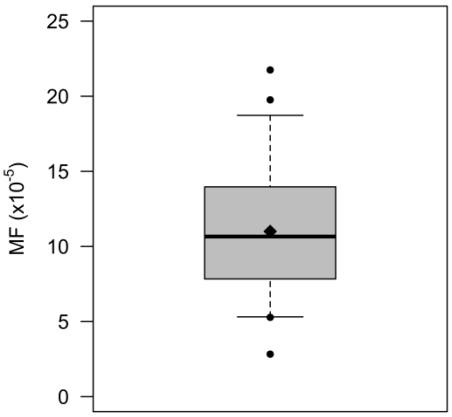


**Figure 4-1:** Spontaneous *lacZ* mutant frequency (MF) for MutaMouse primary hepatocytes (PHs). The solid line represents the median (10.7 x $10^{-5}$), the diamond represents the mean (11.0 x $10^{-5}$), the box limits represent the 25$^{th}$ and 75$^{th}$ percentiles (7.8 x $10^{-5}$ and 14.0 x $10^{-5}$, respectively), the whiskers represent the 5$^{th}$ and 95$^{th}$ percentiles (5.3 x $10^{-5}$ and 18.7 x $10^{-5}$, respectively), and the solid circles represent outliers that exist beyond the 5$^{th}$ and 95$^{th}$ percentiles. N = 32. Standard error of the mean (SEM) = 0.80 x $10^{-5}$.

The sensitivity of MutaMouse PHs in an *in vitro* gene mutation assay was investigated following exposure to nine well-characterized mutagens. For all chemicals tested, the MF values of treated cells were compared to concurrently run vehicle controls to determine significance. Of the mutagens assessed, all but ICR 191 yielded a significant MF increase over control for at least one concentration, and, more importantly, a significant overall treatment effect (Figure 4-2 B). The three top concentrations of ENU elicited significant MF increases over control with a maximum response of 4.2-fold at 1000 µg/mL (Figure 4-2 A). A significant MF increase was observed in MutaMouse PHs exposed to BaP at the four highest concentrations tested, culminating in a maximum fold-increase of approximately 11-fold at 10 µg/mL (Figure 4-2 C). AFB1 yielded a significant MF increase at 0.5 µg/mL with a 3.6-fold MF increase, which falls to a significant 2.5-fold MF increase at 1 µg/mL (Figure 4-2 D). This trend is accompanied by increased cytotoxicity as the RINC falls from 0.6 to 0.3. 2-AAF yielded a significant response at the top two concentrations tested with a 2.6-fold MF increase above control at 5 µg/mL (Figure 4-2 E). PhIP yielded significant MF increases at the top three concentrations tested with a 3.7-fold increase at 10 µg/mL (Figure 4-2 F). 1,8-DNP yielded a significant MF increase of 3.6-fold at 10 µg/mL (Figure 4-2 G). 3-NBA elicited a significant MF fold-increase of 8.2 at 0.5 µg/mL, this was accompanied by a sharp increase in cytotoxicity (Figure 2 H). DMN showed significant increases in MF at the top two concentrations tested, with a maximum fold-increase of 14.4-fold at 200 µg/mL (Figure 4-2 I).

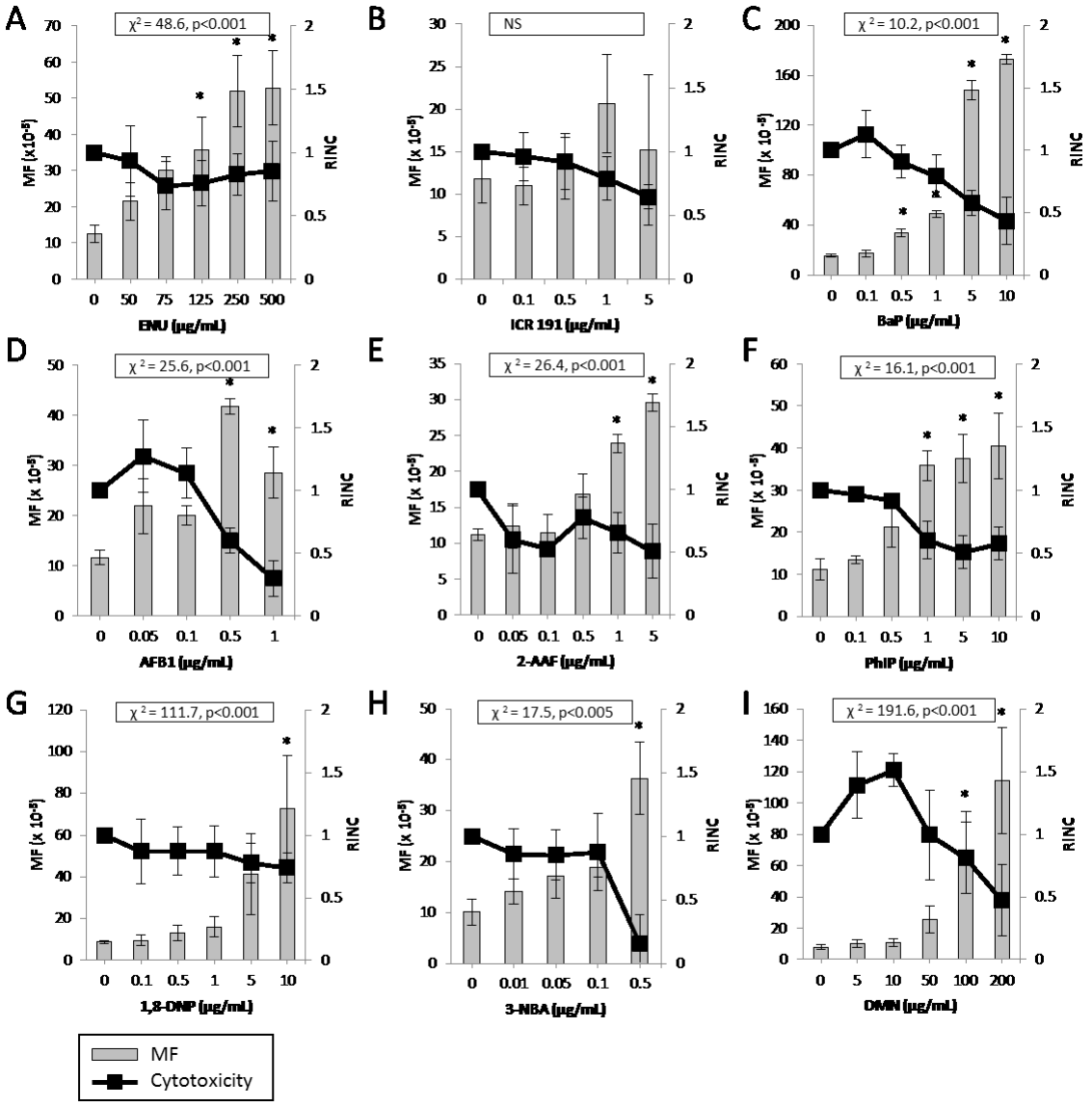


**Figure 4-2:** Induced *lacZ* transgene mutant frequency (MF) in MutaMouse primary hepatocytes (PHs) exposed to known mutagens. MutaMouse PHs were exposed to ENU (A), ICR 191 (B), BaP (C), AFB1 (D), 2-AAF (E), PhIP (F), 1,8-DNP (G), 3-NBA (H), and DMN (I). Grey bars represent MF ± Standard error of the mean (SEM) and black squares represent relative increases in nuclear counts (RINC) ± SEM, a measure of cytotoxicity. Asterisks indicate MF values that are significantly elevated relative to control ($p<0.01$). Inset boxes show statistical results for the overall concentration-response relationship. N = 3 for all observations, except for 0.05 µg/mL AFB1, wherein N = 2 for MF data. NS, not significant.

The MutaMouse PH gene mutation assay did not yield any significant MF increases for any of the non-mutagenic chemicals tested, including the aforementioned misleading positives (Figures 4-3 and 4-4). The results obtained for eugenol demonstrate a significant treatment effect, despite the absence of a significant response at any concentration tested (Figure 4-4 B). Eugenol could not be tested at higher concentrations due to cytotoxicity.

To rank the potencies of all chemicals that elicited a positive response in the MutaMouse PH gene mutation assay, the confidence intervals of the $BMD_{100}$ values were plotted in order of decreasing potency (i.e., from lowest to highest $BMD_{100}$) (Figure 4-5). There was little to no difference between the exponential and Hill models. The ranking of chemicals from most to least potent was: 3-NBA, BaP, AFB1, 1,8-DNP, 2-AAF, PhIP, DMN, followed by ENU. The $BMD_{100}$, BMDL, and BMDU values for each chemical are presented in Supplementary Table 4-I.

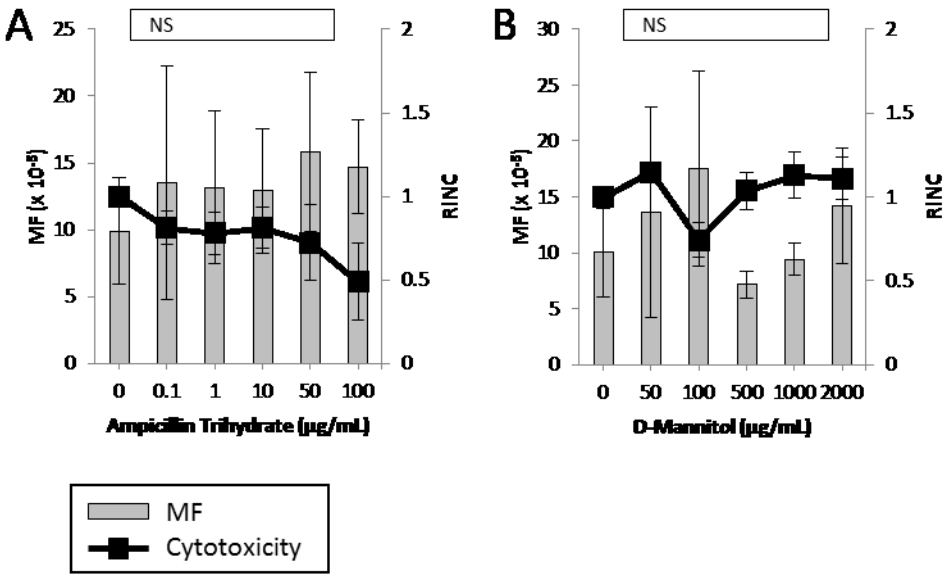


**Figure 4-3:** Induced *lacZ* transgene mutant frequency (MF) in MutaMouse primary hepatocytes (PHs) exposed to non-DNA-reactive chemicals (i.e., known non-mutagens). MutaMouse PHs were exposed to ampicillin trihydrate (A) and D-mannitol (B). Grey bars represent MF ± Standard error of the mean (SEM) and black squares represent relative increases in nuclear counts (RINC) ± SEM, a measure of cytotoxicity. Inset boxes show statistical results for the overall concentration-response relationship. N = 3 for all observations. NS, not significant.

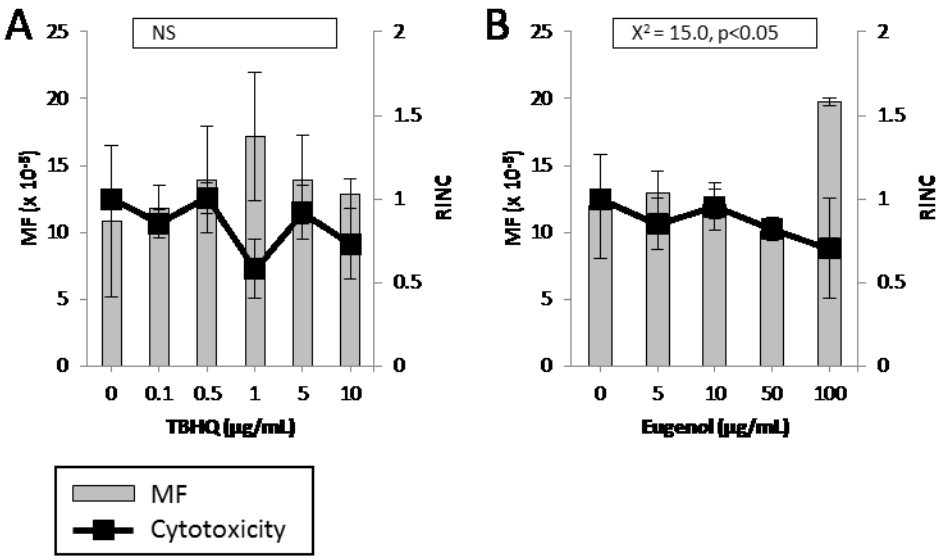


**Figure 4-4:** Induced *lacZ* transgene mutant frequency (MF) in MutaMouse primary hepatocytes (PHs) exposed to non-DNA reactive chemicals that have been shown to elicit positive results in other *in vitro* genotoxicity assays (i.e., misleading positives). MutaMouse PHs were exposed to TBHQ (A) and Eugenol (B). Grey bars represent MF ± standard error of the mean (SEM) and black squares represent relative increases in nuclear counts (RINC) ± SEM, a measure of cytotoxicity. Inset boxes show statistical results for the overall concentration-response relationship. N = 3 for all observations. NS, not significant.

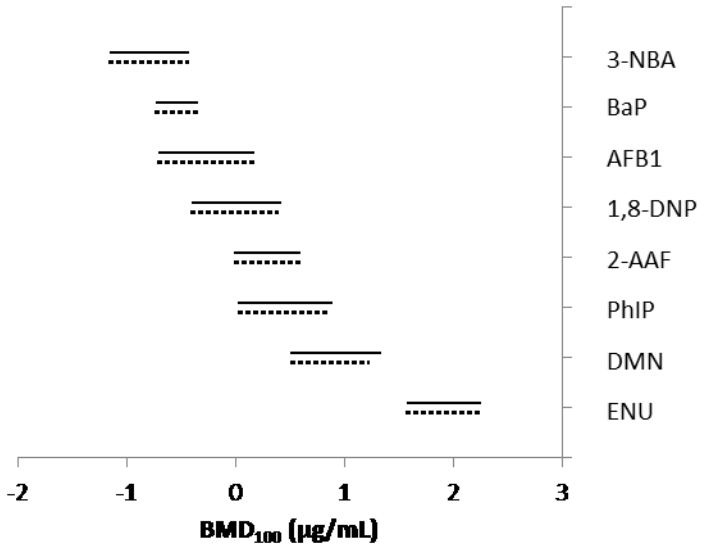


**Figure 4-5:** Benchmark dose (BMD) values (i.e., $BMD_{100}$) with two-sided 90% confidence intervals generated using BMD covariate analysis of MutaMouse primary hepatocyte (PH) mutant frequency (MF) dose-response data. BMD analysis was only conducted for agents shown to elicit significant positives responses. Solid lines represent the fitted exponential model, whereas dotted lines represent the Hill model.

## 4.6 Discussion

This study aimed for an initial characterization of the performance and applicability domain of the *in vitro* gene mutation assay in MutaMouse PHs. Along with Part I, which extensively characterized cultured MutaMouse PHs, this study represents a first step towards the validation and acceptance of this test method. In this preliminary validation study, initial conclusions regarding the predictive capacity and applicability domain were obtained by challenging the assay with a set of mutagenic and non-mutagenic chemicals.

The OECD assay validation guidelines require the careful examination of historical negative controls, thus throughout the development of the MutaMouse PH assay, all spontaneous MF values have been recorded and compiled. A total of 32 spontaneous MF values were compiled from separate experiments performed over four years (Figure 4-1). The mean spontaneous MF value observed for MutaMouse PHs (i.e., $11.0 \times 10^{-5} \pm 0.80 \times 10^{-5}$) was comparable to that observed for PHs from the pUR288 lacZ plasmid mouse, which have a mean spontaneous MF of $9.1 \times 10^{-5}$, and a 95th percentile of $12.0 \times 10^{-5}$, [Luijten et al., 2016]. The spontaneous MF observed for MutaMouse PHs is also similar to what is observed in MutaMouse liver *in vivo* (i.e., $6.31 \times 10^{-5} \pm 1.3 \times 10^{-5}$) [Lemieux et al., 2011]. Previous work in MutaMouse PHs, using a different isolation and culture protocol, elicited a mean spontaneous MF value of $14.2 \times 10^{-5} \pm 1.7 \times 10^{-5}$ across 11 biological replicates [G. Chen et al., 2010]. The FE1 cell line, derived from MutaMouse lung epithelium has a higher mean spontaneous MF of $51 \pm 0.9 \times 10^{-5}$ (N = 460) [Maertens et al., 2017]. With a mean spontaneous MF value of 11.0, a 95th percentile of 18.7, and a conventional $\alpha$ level of 0.05 for the p-value, the

MutaMouse PH gene mutation assay can reliably detect significant increases in MF that are ~1.7-fold higher than the vehicle control [Hayashi et al., 2011].

In Part I, it was shown that cultured MutaMouse PHs encompass a virtually full complement of Phase I and Phase II metabolic enzymes. In order to assess the functional utility of the PHs' complement of metabolic enzymes, in comparison to conventional gene mutation assays, this study examined chemicals with a range of metabolic activation pathways and modes of action (Supplementary Table 4-II). Table 4-II compares the responses of the chemicals assessed in the current study to their responses in the pUR288 lacZ plasmid mouse PH gene mutation assay, the *in vitro* MutaMouse FE1 gene mutation assay, the MLA, and the *Hprt* test. To our knowledge, no gene mutation assay using an immortalized or transformed cell line can cover this range of chemicals without the addition of an exogenous source of mammalian metabolic enzymes (i.e., rodent liver S9).

**Table 4-II:** Comparisons of chemical responses across a range of *in vitro* gene mutation assays. Citations for the indicated responses are provided in the text.

| Chemical | MutaMouse PHs | pUR288 *lacZ* Plasmid Mouse PHs | MutaMouse FE1 | MLA | *Hprt* |
|---|---|---|---|---|---|
| *True positive* | | | | | |
| ENU | Positive | Positive | Positive (-S9) | Positive (-S9) | Positive (-S9) |
| ICR 191 | Negative | NT | Positive (-S9) | Positive (-S9) | Positive (-S9) |
| BaP | Positive | Positive | Positive (±S9) | Positive (+S9) | Positive (+S9) |
| AFB1 | Positive | NT[a] | Positive (±S9) | Positive (+S9) | Positive (+S9) |
| 2-AAF | Positive | NT | Positive (±S9) | Positive (±S9) | Positive (+S9) |
| PhIP | Positive | NT | Positive (+S9) | Positive (+S9)[b] | Positive (+S9) |
| 1,8-DNP | Positive | NT | NT | Positive (-S9) | Pos/Neg[c] (±S9) |
| 3-NBA | Positive | NT | Positive (±S9) | Positive[d] | Positive[d] |
| DMN | Positive | NT | Negative (+S9) | Positive (+S9) | Positive (+S9) |
| *True negative* | | | | | |
| Ampicillin trihydrate | Negative | NT | Negative (-S9) | Negative (±S9) | NT |
| D-Mannitol | Negative | Negative | Negative (-S9) | Negative (±S9) | NT |
| *Misleading positive* | | | | | |
| TBHQ | Negative | NT | Negative (±S9) | NT | NT |
| Eugenol | Negative | NT | Negative (±S9) | Positive (-S9) | NT |

[a] NT, not tested
[b] Human TK6 assay
[c] Both positive and negative responses have been observed
[d] Mutagenicity was assessed at *Tk* and *Hprt* loci in the human lymphoblast cell lines, h1A1v2 and MCL-5, that have been transfected with CYP1A1, and CYP1A2, CYP2A6, CYP2E1, and CYP3A4, respectively

As a nitrosourea and a strong alkylating agent, ENU is a direct-acting agent and, thus, does not require metabolic activation in order to become DNA-reactive [Doak et al., 2007]. It is known to cause transitions and transversions following O2- and O4-alkylation of thymine residues, and O6-alkylation of guanine residues, respectively [Douglas et al., 1995]. It yields a positive response in conventional gene mutation assays, such as the MLA and *Hprt* test, without the addition of induced rodent S9 [Nishi et al., 1984; T. Chen et al., 2002; Doak et al., 2007; Kirkland et al., 2008; Kirkland et al., 2016]. In the present study, ENU induced a concentration-dependent increase in MF culminating in a 4.2-fold increase over control (Figure 4-2 A). The magnitude of the observed response is comparable to what is seen in pUR288 lacZ plasmid mouse PHs and MutaMouse FE1 cells (i.e., ~4- and ~6-fold increases, respectively) [Luijten et al., 2016; White et al., 2003].

ICR 191 is an acridine half-mustard that, like ENU, interacts with DNA without metabolic conversion to a DNA reactive metabolite [Ferguson and Denny, 1990]. Unlike ENU, ICR 191 is known to intercalate within the DNA molecule and cause +1 frameshifts in regions with consecutive guanine residues [Taft et al., 1994]. Like ENU, ICR 191 is positive in conventional gene mutation assays, as well as the MutaMouse FE1 gene mutation assay, without the addition of S9 [Nishi et al., 1984; Mitchell et al., 1997; White et al., 2003; Doerr et al., 1989]. Interestingly, ICR 191 did not yield a significant increase in MF in MutaMouse PHs, nor was a significant treatment effect detected (Figure 4-2 B). It should be noted that the variability at the two highest concentrations was relatively high compared to the other chemicals tested. Uninduced murine liver S9 has been shown to dramatically reduce the mutagenicity of ICR 191 in the Ames test (i.e.,

Salmonella strain TA1537), whereas untreated murine lung S9 had little effect on mutagenicity [De Flora et al., 1982]. This suggests that mouse liver metabolic enzymes are better able to detoxify ICR 191 than mouse lung metabolic enzymes, which may account for the discrepancy between the MutaMouse PH response and the result observed for Mouse FE1 pulmonary cells [White et al., 2003] (Table 4-II). The high metabolic competency of MutaMouse PHs may be reducing the mutagenicity of ICR 191 in this *in vitro* system.

BaP is one of several highly mutagenic polycyclic aromatic hydrocarbons (PAH) that are by-products of incomplete combustion processes. BaP is considered a prototypical PAH that is known to form DNA adducts following Phase I metabolic activation involving CYP1A1, CYP1A2, and CYP3A, as well as epoxide hydrolase [Jeffrey, 1985; Bauer et al., 1995; Kim et al., 1998]. These enzymatic reactions convert BaP to a DNA-reactive metabolite, such as BaP-diol-epoxide (BPDE), to form adducts with guanine and adenine residues [Jeffrey, 1985]. BaP is typically a strong positive in the MLA and *Hprt* tests with the addition of S9 [Mitchell et al., 1997; Oberly et al., 1990; Bradley et al., 1981; Kirkland et al., 2008; Kirkland et al., 2016]. BaP induced a 11-fold concentration-dependent MF increase over control in MutaMouse PHs, which is comparable to what is observed in pUR288 lacZ plasmid mouse PHs (i.e., 9.8-fold), but less than what is observed in MutaMouse FE1 cells (i.e., ~25-fold) [Zwart et al., 2012; White et al., 2003] (Figure 4-2 C).

AFB1 is a mutagenic mycotoxin, food contaminant, and known human carcinogen that is metabolized to AFB1-8,9-epoxide, a DNA-reactive metabolite, that is capable of forming adducts with guanine residues [Gallagher et al., 1994; Essigmann et

al., 1977]. This activation is carried out by Phase I CYPs that are present in MutaMouse PH cultures, in particular, CYP1A2 and CYP3A. Like BaP, AFB1 is generally a strong positive in the MLA and *Hprt* assay with S9 [Bradley et al., 1981; Preisler et al., 2000; Kirkland et al., 2008; Kirkland et al., 2016]. AFB1 induced a 3.6-fold concentration-dependent increase over control in MutaMouse PHs, which is similar to the ~3-fold increase seen in MutaMouse FE1 cells [Hanna, 2018] (Figure 4-2 D).

2-AAF and PhIP are both mutagenic aromatic amines that undergo similar metabolic activation pathways to become DNA-reactive. 2-AAF is a prototypical aromatic amine in mutagenesis studies [Heflich and Neft, 1994]. The heterocyclic amine, PhIP, is a by-product of the Maillard, or browning, reaction that occurs during the cooking of meat [Jägerstad et al., 1983]. Aromatic amines, such as 2-AAF and PhIP, require both Phase I enzymes, such as CYP1A1 and CYP1A2, as well as Phase II enzymes, such as, SULT, NAT, and/or UGT to generate DNA-reactive nitrenium or carbenium ions [Schut and Snyderwine, 1999; Heflich and Neft, 1994; Cai et al., 2016]. Both 2-AAF and PhIP are positive in the MLA and *Hprt* tests with the addition of S9 [Mitchell et al., 1997; Oberly et al., 1990; Morgenthaler and Holzhäuser, 1995; Kirkland et al., 2008; Kirkland et al., 2016]. 2-AAF and PhIP induced 2.6- and 3.7-fold increases in MF above control in MutaMouse PHs (Figure 4-2 E and F). Once again, these values are comparable to the ~1.7- and ~5.5-fold increases observed in MutaMouse FE1 cells following exposure to 2-AAF and PhIP, respectively [White et al., 2003; Hanna, 2018].

Nitroarenes, such as 1,8-DNP and 3-NBA, are mutagenic compounds found in both diesel and gasoline engine exhaust [IARC, 2014]. The metabolic activation pathway of 1,8-DNP is presumed to require both nitroreduction and acetyltransferase metabolic

activity to yield the DNA-reactive metabolite, *N*-acetoxy-1-amino-8-nitropyrene, that is capable of forming guanine adducts [IARC, 2014]. Similarly, the proposed metabolic activation pathway for 3-NBA involves nitroreductase activity, as well as *N*-acetyltransferase and/or sulfotransferase catalytic activity to yield a highly reactive nitrenium ion that can form guanine or adenine adducts [IARC, 2014; Arlt et al., 2003; Arlt et al., 2005]. This pathway may also involve CYP1A1, CYP2A6, CYP2B6, or CYP3A4 oxidation, following reduction to 3-aminobenzanthrone. The nitroreduction required for the activation of nitroarenes sets these chemicals apart from the other compounds examined in this study. Interestingly, in the companion manuscript, NADPH dehydrogenase, quinone 1 (NQO1), a cytosolic enzyme capable of nitroreduction, was shown to be expressed in cultured MutaMouse PHs. 1,8-DNP is positive in the MLA without the addition of S9, but its response in the *Hprt* test depends on the cell line in which it is tested [Edgar, 1985]. When tested in the *Hprt* assay in CHO cells, it elicited a strong mutagenic response; however, in HepG2 cells, 1,8-DNP yielded a negative response [Edgar and Brooker, 1985; Eddy et al., 1986]. 3-NBA has yielded positive results at *Tk* and *Hprt* loci in the human lymphoblast cell lines, MCL-5 and h1A1v2, which have been transfected with human CYPs [Phousongphouang et al., 2000; Arlt et al., 2008]. Both 1,8-DNP and 3-NBA yielded positive results in the MutaMouse PH gene mutation assay, with induced fold-changes in MF of 8.2 and 3.6, respectively (Figure 4-2 G and H). Of the two, only 3-NBA has been assessed in MutaMouse FE1 cells, yielding a ~5-fold increase in MF above solvent control [Arlt et al., 2008].

Mutagenic nitrosamines, like DMN, are by-products of some industrial processes and water treatment. They are also found in cigarette smoke and in some foods, such as

cured meat and beer. DMN is activated by Phase I enzymes, including CYP2E1, to methyldiazohydroxide, followed by potential formation of a diazonium ion that methylates nucleic acids, particularly guanine residues [Chowdhury et al., 2012; Yamazaki et al., 1992; Hoffmann and Hecht, 1985]. DMN has yielded positive results in both the MLA and *Hprt* assays with the addition of S9 [Mitchell et al., 1997; Bradley et al., 1981; Kirkland et al., 2016; Kirkland et al., 2008]. DMN induced a 14.4-fold increase in MF above control in MutaMouse PHs (Figure 4-2 I), but, interestingly, did not elicit a significant positive response in MutaMouse FE1 cells in the presence or absence of S9 [Hanna, 2018].

This study included four non-mutagenic chemicals to offer some insight into the specificity of the MutaMouse PH gene mutation assay. D-Mannitol and ampicillin trihydrate are known to be non-mutagenic, and are consistently negative in gene mutation assays [Mitchell et al., 1997; Maertens et al., 2017; Kirkland et al., 2016; Kirkland et al., 2008]. These chemicals both yielded negative results in the MutaMouse PH gene mutation assay (Figure 4-3 A and B). TBHQ, one of the misleading positive chemicals assessed in this study, has not been tested in either of the more conventional gene mutation tests (i.e., MLA or *Hprt*), but has yielded a negative result in the FE1 assay [Maertens et al., 2017]. Eugenol, another misleading positive chemical has elicited a positive result in the MLA test, but a negative result in the FE1 assay [Mitchell et al., 1997; Maertens et al., 2017]. Both TBHQ and eugenol have tested positive in the *in vitro* chromosome aberration test and positive for micronucleus in p53-deficient hamster cells. They have both also resulted in negative results for the *in vitro* micronucleus assay in p53-functional human cells [NTP, 1986; NTP, 1982; Fowler et al., 2012a; Kirkland et al.,

2008; Kirkland et al., 2016]. TBHQ and eugenol are part of a larger subset of chemicals that are not DNA-reactive, but are thought to disturb cell culture conditions or exert toxicity on cells *in vitro* in a way that leads to an apparent genotoxic response. These chemicals have both elicited negative results *in vivo*; however, this data is rather limited and mainly restricted to hematopoietic tissues (e.g., the bone marrow and blood) [Kirkland et al., 2016]. Eugenol has also elicited negative results in the liver as measured by the unscheduled DNA synthesis (UDS) assay and the *in vivo* MutaMouse assay [Rompelberg, Steenwinkel et al., 1996; Rompelberg, Evertz et al., 1996]. Oxidative stress and cytotoxicity are thought to be the major factors influencing the positive results seen for TBHQ and eugenol, respectively [Kirkland et al., 2008; Kirkland et al., 2016; Fowler et al., 2012a; Fowler et al., 2012b]. Neither TBHQ nor eugenol yielded a significant increase in MF over the solvent control in MutaMouse PHs at any concentration tested (Figure 4-4 A and B). Due to the relatively high RINC obtained at the top concentration of TBHQ, this chemical should be tested at a higher range of concentrations. Eugenol did elicit a significant result for the overall treatment effect (i.e., Chi-squared for overall effect); however, this compound was too cytotoxic to test at higher concentrations (i.e., no DNA could be isolated for MF testing) and the effect appeared to be driven solely by the highest concentration tested. Following the criteria outlined in the Materials and Methods, ampicillin trihydrate, D-mannitol, and TBHQ are negative and eugenol is equivocal in the MutaMouse PH gene mutation assay.

This study employed the BMD approach to quantitatively examine the MutaMouse PH gene mutation assay results and compare the responses of the various chemicals (Figure 4-5). By fitting a model to concentration-response data, BMD analysis

yields a concentration that elicits a specified response. The BMD covariate approach builds on this concept by incorporating a covariate, such as compound, to rigorously compare potencies within an endpoint [Wills et al., 2016]. The use of a covariate refines BMD confidence intervals and improves the precision of BMD values [Wills et al., 2016; Slob and Setzer, 2014]. The results revealed that slope, maximal response, and variance were conserved across all chemicals for both the fitted exponential and Hill models. When comparing the results obtained, no significant distinction in potency can be made between chemicals with overlapping confidence intervals. 3-NBA, BaP, AFB1, and 1,8-DNP appear to be the most potent chemicals in this assay, and it appears that there could be a link between potency and mode of action. As discussed above, 3-NBA, BaP, AFB1, and 1,8-DNP all undergo Phase I metabolic activation, and elicit similar forms of DNA damage (i.e., guanine and adenine adducts). 2-AAF, PhIP, and DMN are less potent than 3-NBA and BaP. 2-AAF and PhIP are both aromatic amines with similar modes of activation involving both Phase I and Phase II metabolism, and genotoxic effects, as described above. DMN has a separate mode of action as it generally exerts its genotoxicity by methylating guanine residues following CYP2E1 metabolism. ENU yielded the lowest BMD100 (i.e., potency), and, like DMN, it is known to be a DNA alkylating agent, albeit a direct-acting one. Although this preliminary study only examines a small subset of mutagenic chemicals, the BMD covariate results indicate that DNA adduct-forming promutagens requiring Phase I metabolic activation are the most potent in this assay, followed by DNA adduct-forming promutagens requiring both Phase I and Phase II metabolic activation, and finally, DNA alkylating agents. The lower potency of ENU, relative to compounds forming bulky adducts, such as BaP, was also

observed in the MutaMouse FE1 and pUR288 lacZ plasmid mouse PH *in vitro* gene mutation assays; however, ENU is more potent than BaP in the MutaMouse gene mutation assay in the liver and bone marrow *in vivo*, and of equivalent potency in the small intestine [Gocke et al., 2009; Long et al., 2018; Hanna, 2018; Luijten et al., 2016]. The BMD covariate analysis presented herein provides further insight into the genotoxic effects of the compounds studied in metabolically competent cells; it also demonstrates the utility of the BMD covariate approach for comparative analysis of *in vitro* dose-response data.

It is important to note that the ability to employ *in vitro* tools to effectively and efficiently assess genotoxicity is a critical component of the evolving paradigm for *Toxicity Testing in the 21st Century* [Krewski et al., 2010]. This paradigm calls for adoption of high(er) throughput screening tools for efficient (geno)toxicity assessment and mode of action (MOA) determination. Moreover, effective adoption of novel *in vitro* tools is consistent with global initiatives aimed at replacing, reducing, refining the use of animals for (geno)toxicity assessment and attendant regulatory decision-making [Adler et al., 2011; European Commission, 2009]. In this regard, *in vitro* mutagenicity assessment using MutaMouse primary hepatocytes is aligned with global initiatives to modernise mutagenicity assessment. The *in vivo* MutaMouse assay can require more than 20 animals per compound, PHs isolated from a single MutaMouse specimen provide enough cells to test one to three chemicals. Nevertheless, it must also be noted that the MutaMouse PH mutagenicity assay described herein is nowhere near as efficient as some recently-developed, high throughput *in vitro* genotoxicity reporter assays. These assays, such as the ToxTracker® assay [Hendriks et al., 2012], the MultiFlow® assay [Bryce et

al., 2016], and the TGx-DDI toxicogenomic biomarker assay [Buick et al., 2015], can rapidly and simultaneously assess multiple cellular responses indicative of DNA damage, thereby efficiently identifying genotoxicants and elucidating MOA. While such assays can indeed be categorized as high throughput, they cannot detect the endpoints that are requisite in the aforementioned legislative frameworks (i.e., CEPA, FIFRA, etc.), i.e., mutations and/or chromosome damage. Thus, in the short- to medium-term it will be necessary to develop and adopt high(er) throughput mutagenicity assays such as that presented herein.

This study, alongside Part I, constitutes an important first step towards the validation of the MutaMouse PH *in vitro* gene mutation assay. The positive responses elicited by BaP, AFB1, 2-AAF, PhIP, DMN, 1,8-DNP, and 3-NBA illustrate that the MutaMouse PHs are capable of converting these mutagenic compounds to their reactive metabolites without the addition of exogenous S9. The results thus far indicate that the applicability domain of the assay encompasses chemicals that require Phase I and/or Phase II metabolism. This is consistent with the companion paper (i.e., Part I) where we demonstrated that MutaMouse PHs maintain maximal metabolic enzyme activity for at least the first 24 hours in culture. Since the exposures in this study take place from hours 18 to 24 post-isolation, we expected that the cells would be capable of activating promutagens during this time; indeed, that is what was observed.

Due to their karyotypically normal phenotype, metabolic capacity, and DNA-repair proficiency, it is not unreasonable to assert that some direct-acting chemicals may not be detected in this assay. The negative result for ICR 191 illustrates a gap in this assay's applicability domain. Testing of a larger set of both positive and negative

chemicals, including more *in vitro* misleading positive chemicals and *in vitro* false positive chemicals (e.g., chemicals that are positive in the Ames test and negative *in vivo*) will further resolve the sensitivity (i.e., ability to correctly identify mutagens) and specificity (i.e., ability to correctly identify non-mutagens) of the assay, and extend the elucidation of the applicability domain. Although positive controls were not included in this preliminary study, it is recommended that BaP and PhIP be included in future studies as positive controls for Phase I and Phase II assessment, respectively, at 10 μg/mL. Future work includes the development of a cryopreservation protocol, as has been developed for pUR288 lacZ Plasmid Mouse PHs, to facilitate the use of these cells for routine screening; moreover, their distribution to laboratories interested in adopting the assay [Luijten et al., 2016]. This study indicates that this system shows great promise as a metabolically competent complement to bacterial mutagenicity tests, particularly for compounds that require metabolic activation.

## 4.7 References


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# CHAPTER FIVE

# Chapter 5: The mutagenic activity of select azo compounds in MutaMouse target tissues *in vivo* and primary hepatocytes *in vitro*

## 5.1 Preamble: Authors, Affiliations, and Style

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Style: This chapter was prepared according to the style guide for *Mutation Research*

## 5.2 Abstract

The mutagenicity of Direct Black 38, Sudan I, and Para Red were evaluated in the *in vivo* MutaMouse assay and the *in vitro* MutaMouse primary hepatocyte (PH) assay. Direct Black 38 is an International Agency for Research on Cancer (IARC) Group 1 carcinogen and a prototypical benzidine-based azo compound that requires azo-reduction to yield a DNA-reactive metabolite. Sudan I and Para Red are structurally related azo compounds that have been detected as illegal contaminants in foods. Sudan I is an *in vivo* mutagen, and both it and Para Red are known to be mutagenic *in vitro.* Sudan I is oxidized by hepatic and/or bladder enzymes to yield a mutagenic metabolite, but little is known about Para Red. In the present study, Direct Black 38 elicited a significant mutagenic response in the bone marrow, glandular stomach, small intestine and colon *in vivo*, and in PHs *in vitro***.** Sudan I elicited a weak positive response in the bone marrow and a marginally

significant treatment effect in the bladder ($p = 0.059$); it did not elicit a significant response in PHs *in vitro*. Para Red elicited a positive response in the colon, as well as in PHs *in vitro*, albeit at a cytotoxic concentration. The findings are well aligned with the known mechanisms of action of Direct Black 38 and Sudan I; they suggest that intestinal azo-reduction plays an important role in the activation of Para Red. The MutaMouse PH results illustrate the ability of this assay to detect chemicals requiring azo-reduction; however, they also demonstrate a gap in applicability domain, as MutaMouse PHs elicit a negative response following exposure to Sudan I. Elucidation of the mechanisms underlying this gap will require further study.

## 5.3 Introduction

Approximately 60-80% of all commercially used colourants are azo compounds [1]. Azo colourants are synthetically manufactured, and used extensively in paints, textiles, personal care products, and inks [2]. There is evidence that some azo compounds may pose a genotoxic and/or carcinogenic hazard; the International Agency for Research on Cancer (IARC) has classified 37 azo compounds with respect to their carcinogenicity. Three compounds, described as dyes metabolized to benzidine via azo-reduction, have been classified as Group 1 carcinogens (known human carcinogens), eleven compounds have been classified as Group 2B carcinogens (possible human carcinogens), and twenty-three compounds have been classified as Group 3 agents (not classifiable as to their carcinogenicity to humans) [3]. The Government of Canada's Chemicals Management Plan (CMP) recently completed an assessment of 358 aromatic azo- and benzidine-based substances [2]. The assessment concluded that, although azo compounds pose a hazard,

and Canadians are exposed through their use in commercial products, the exposure is too low to pose a significant risk. Nevertheless, it also concluded that these substances would be a concern if exposure levels increase.

Numerous cohort and case-control studies conducted over the last 50 years have shown a correlation between exposure to benzidine, and some benzidine-based dyes, and a higher incidence of cancers, especially bladder cancer [4]. These data are further supported by animal carcinogenicity studies, toxicokinetic studies, and *in vitro* mechanistic studies. Thus, effective health hazard/risk evaluation of azo compounds must consider metabolism, the potential for enzymatic cleavage to yield carcinogenic aromatic amines and/or benzidine. Benzidine-based dyes make up only a small subset of azo bond-containing compounds currently used in consumer products. For the most part, there is a paucity of information regarding the carcinogenicity, genotoxicity, and metabolism of azo compounds in general, thus it is imperative to apply tools that involve metabolically competent cells. The need for effective genetic toxicity screening tools is particularly acute, since azo compound data are lacking, and testing is complicated by low solubility and the metabolic limitations of available tools [5]. Despite the low solubility that complicates genetic toxicity screening, it should be noted that human exposures to insoluble azo pigments can occur via direct contact with very common consumer products (e.g., plastics and polymers, paper, surface coatings) [5].

Azo-reduction, rather than oxidation, is thought to be the major route of bioactivation of most azo compounds. It has been estimated that out of the several thousand azo compounds that have been produced, at least 500 can potentially yield carcinogenic aromatic amines through azo-reduction [6]. In mammals, azo-reduction can

occur in either the gut or the liver, and may also be catalysed by bacteria on the skin [6]. Azoreductase activity in the liver is mediated by cytochrome P450 (CYP) isozymes in the microsomal fraction, and NADPH quinone oxidoreductase in the cytosolic fraction [5]. Moreover, it is believed that anaerobic bacterial azoreductase activity is important in the gastrointestinal metabolism of soluble azo dyes. Dye metabolites can subsequently be absorbed via the gut and further processed in the liver. Once released, the aromatic amine can undergo *N*-hydroxylation and *O*-acetylation to eventually yield DNA-reactive nitrenium or carbenium ions [7,8].

The aforementioned complex routes of metabolic bioactivation of azo compounds pose a problem for conventional *in vitro* genotoxicity assays, thus necessitating modifications of standard assay protocols. One notable protocol modification is the so-called Prival variation of the Ames/Salmonella mutagenicity assay. The modification utilizes uninduced hamster liver S9, instead of the more conventional Aroclor 1254-induced rat liver S9, and flavin mononucleotide (FMN) in the co-factor mix [9-11]. FMN serves as a reducing agent to facilitate azo reduction. Unlike induced rat liver S9, uninduced hamster S9 does not preferentially detoxify benzidine and benzidine-based compounds, thus yielding mutagenic metabolites [9]. Conventional mammalian cell genetic toxicity assays typically use cell lines that are not metabolically competent, thus relying on the addition of Aroclor 1254-induced rat liver S9. Aroclor 1254-induced rat liver S9 enables CYP mediated Phase I oxidation reactions, and typically the S9 mix does not include the cofactors necessary for Phase II conjugation reactions (e.g., 3'-phosphoadenosine-5'-phosphosulfate [PAPS] for sulfotransferase enzymes) or reductive metabolism (e.g., flavin mononucleotide [FMN]) [9,12,13]. Additionally, induced rat

liver S9 is known to cause cytotoxicity in cultured mammalian cell lines [14,15]. An assay that incorporates cells that are metabolically competent, such as primary hepatocytes (PHs), would serve as a helpful tool for the assessment of azo compounds, due to their metabolic activation mechanisms requiring both reductive and oxidative metabolism.

Direct Black 38 is a prototypical benzidine-based azo dye (Figure 5-1 A); its metabolism is known to release benzidine. Based on the results of epidemiologic studies in occupational settings, and carcinogenicity studies in experimental animals, Direct Black 38 and similar benzidine-based azo dyes, have been classified by IARC as known human carcinogens (Table 5-I) [16-20]. Direct Black 38 elicits positive responses in the Ames/Salmonella mutagenicity assay *in vitro*, and these responses are substantially more potent in assays modified to include reductive metabolism (Table 5-I). Direct Black also yields positive responses in the unscheduled DNA synthesis (UDS), micronucleus (MN), and comet assays *in vivo* (Table 5-I). Direct Black 38 has not been tested in any *in vitro* genotoxicity assay in mammalian cells, however. Direct Black 38 is thought to be metabolically activated mainly via azo-reduction by anaerobic bacteria in the intestinal tract [20-25].

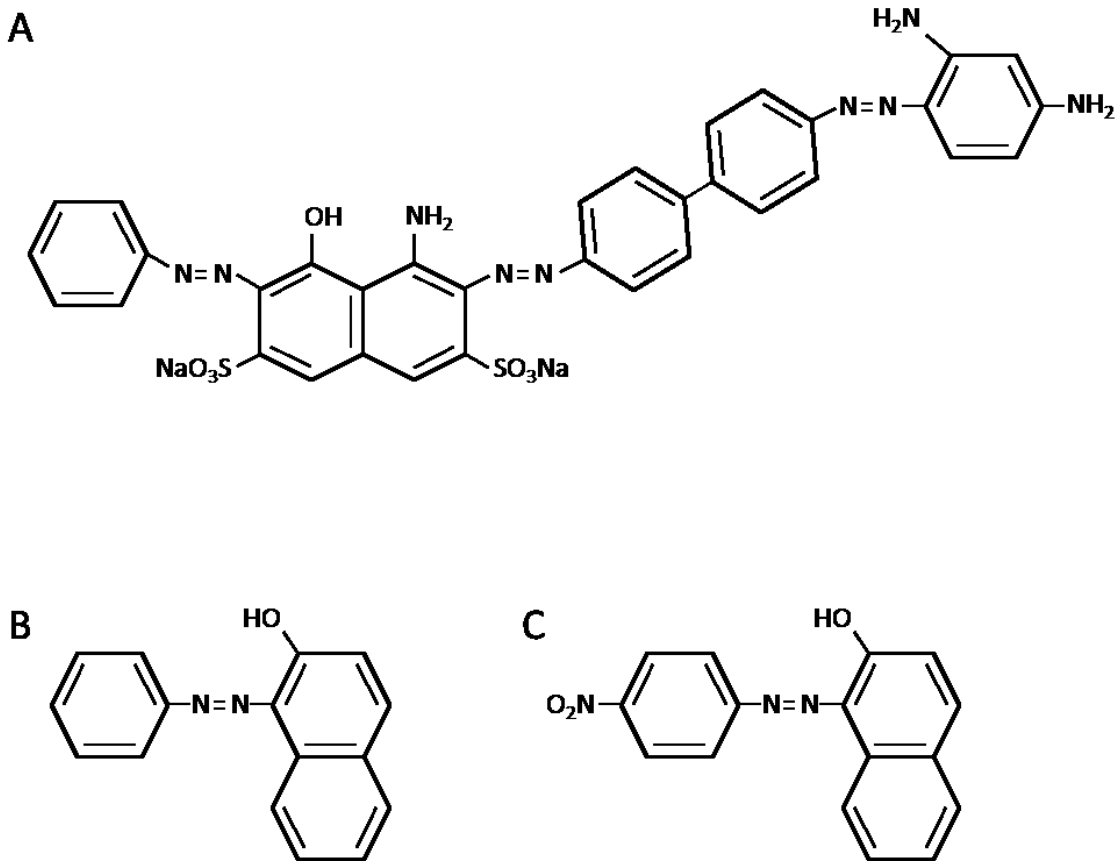


**Figure 5-1:** Structures of Direct Black 38 (A), Sudan I (B), and Para Red (C).

**Table 5-I:** Summary of genotoxicity and carcinogenicity data for Direct Black 38, Sudan I, and Para Red.

| Chemical (CAS) | Genotoxicity profile | | | Carcinogenicity findings | References |
|---|---|---|---|---|---|
| | Ames Test[a] | *In vivo* genotoxicity tests | *In vitro* mammalian cell tests | | |
| Direct Black 38 (1937-37-7) | +ve[b] in TA1538 (+S9) and TA98 (+S9)<br>-ve[c] in TA1538 (-S9), TA98 (-S9), TA100 (±S9), and TA1535 (±S9) | +ve for UDS[d] in rat liver and MN[e] in rat bone marrow<br>+ve for comet in mouse stomach, colon, liver, bladder, brain and bone marrow | NA[f] | IARC Group 1 - Increased risk of human bladder and colorectal cancer<br>Mice (P.O.[g]) – liver and mammary gland tumours<br>Rats (P.O.) – liver, bladder, and colon tumours | [20,91-104] |
| Sudan I (842-07-9) | -ve in TA97 (-S9), TA98 (-S9 & Prival), TA100 (±S9 & Prival), TA1535 (±S9), TA1537 (±S9), TA1538 (±S9)<br>+ve in TA97 (+S9), TA1538 (+ hamster S9)<br>Weakly +ve in TA98 (+S9) in 1 study; –ve in 2 other studies | E[h] or -ve for UDS in rat liver<br>+ve for MN in rat bone marrow<br>Weakly +ve for MN in mouse bone marrow in 1 study and -ve in 1 other | +ve for *Hprt*[i] in AHH-1 and MCL-5 cells<br>+ve for MN in AHH-1, MCL-5, and HepG2 cells<br>E or -ve in MLA[j] (+S9)<br>+ve for SCE[k] in CHO[l] cells (±S9).<br>-ve for CA[m] in CHO cells (±S9)<br>+ve for comet in HepG2 cells | IARC Group 3.<br>Mice (P.O.) – No increase in tumours<br>Mice (Subcu[n]) – liver tumours<br>Mice (bladder implantation) – bladder tumours.<br>Rat (P.O.) – No increase in tumours | [34-36,61,62,65,82,83,105-116] |
| Para Red (6410-10-2) | +ve in TA98 (+S9) and TA1538 (+S9)<br>-ve in TA98 (-S9), TA1535 (±S9), and TA1538 (-S9) | NA | +ve for *Hprt* in AHH-1 cells<br>+ve for MN in AHH-1 and MCL-5 cells | NA | [36,117] |

[a] Standard Ames test (i.e., not Prival method), unless indicated
[b] +ve, positive
[c] -ve, negative
[d] UDS, unscheduled DNA synthesis assay
[e] MN, micronucleus assay
[f] NA, not available
[g] P.O., oral administration
[h] E, equivocal
[i] *Hprt*, hypoxanthine phosphoribosyltransferase forward mutation assay
[j] MLA, mouse lymphoma assay
[k] SCE, sister chromatid exchange assay
[l] CHO, Chinese hamster ovary cells
[m] CA, chromosome aberration
[n] Subcu, subcutaneous administration

Sudan I and Para Red are structurally related, as they are both 1-amino-2-naphthol-based azo compounds (Figure 5-1 B and C). Sudan I is well-studied, in part because it is the simplest in a series of azo compounds that are extensively used world-wide in oils, waxes, printing inks, textiles, and cosmetics [5,26]. In 2003, European authorities reported Sudan I contamination in chilli products [27,28]; this report was followed by numerous additional reports of foodstuffs contaminated by Sudan I and related dyes, including Para Red [29-33]. Sudan I has been designated by IARC as Group 3; the available information did not permit classification of its carcinogenicity to humans (Table 5-I) [34,35]. Sudan I has elicited positive responses in the Ames test, *in vitro* mammalian cell genotoxicity assays (i.e., the *Hprt* gene mutation assay in AHH-1 and MCL-5 cells, the MN assay in AHH-1, MCL-5, and HepG2 cells, the sister chromatid exchange [SCE] assay in CHO cells, and the comet assay in HepG2 cells), as well as several *in vivo* genotoxicity assays (Table 5-I). Para Red has also elicited positive responses in the Ames test, as well as the *in vitro* MN assay and the *Hprt* forward mutation assay (Table 5-I). Unlike Direct Black 38 and many other azo compounds, the main route of Sudan I activation is not via hepatic or bacterial azo-reduction, but rather via hepatic CYPs and/or bladder peroxidases [26]. Very little data is available concerning the metabolism and mutagenic mode of action of Para Red; however, its structural similarity to Sudan I suggests that its activity may be similar [36].

In the present study, the mutagenicity of azo compounds Direct Black 38, Sudan I, and Para Red are assessed *in vivo* in several tissues of the MutaMouse, and *in vitro* in MutaMouse PHs. Although Direct Black 38 and Sudan I are relatively well-studied, neither has been assessed in a transgenic rodent (TGR) assay; Para Red is an under-

studied azo compound. Mutagenicity assessment in several MutaMouse tissues will provide insight into the modes of action of the studied compounds; indeed, information to determine whether the mechanism of action of Para Red is, as predicted, similar to Sudan I (i.e., oxidative metabolic activation), or rather similar to Direct Black 38 (i.e., reductive metabolic activation). These mutagenicity assessments will provide additional insight into the metabolic activation of the selected azo compounds. Preliminary characterization and validation studies have demonstrated that the *in vitro* MutaMouse PH gene mutation assay is capable of detecting mutagens requiring diverse types of metabolic activation [37,38]. This comparison of *in vitro* and *in vivo* methodologies will provide important information regarding the utility of the *in vitro* MutaMouse PH assay to accurately assess compounds that undergo complex metabolism *in vivo*.

## 5.4 Materials and Methods

### 5.4.1 Materials and Reagents

Direct Black 38 (95% dye purity) and Sudan I (95% dye purity) were synthesized by TC Scientific (Edmonton, Alberta). Para Red (95% dye purity) was obtained from Sigma-Aldrich Canada (Oakville, Ontario). The composition of the impurities in the azo compounds is not known. Dulbecco's modified Eagle's medium (DMEM), William's E medium, phosphate-buffered saline (PBS), foetal bovine serum (FBS), epithelial growth factor (EGF), penicillin-streptomycin reagent, Hank's balanced salt solution (HBSS), proteinase K, trypan blue, and Sytox® green were obtained from Life Technologies (Burlington, Ontario). Corning® Biocoat™ type I collagen-coated culture dishes were obtained from VWR International (Mississauga, Ontario). CIzyme™ collagenase HA

(high activity) and BP (*Bacillus polymyxa*) protease were obtained from VitaCyte LLP (Indianapolis, Indiana). Dexamethasone, human insulin, dimethylsulphoxide (DMSO), olive oil, Percoll®, bovine serum albumin (BSA), and IGEPAL CA-630 were obtained from Sigma-Aldrich Canada (Oakville, Ontario). Phenyl-β-D-galactopyranoside (P-Gal) was obtained from MJS BioLynx (Brockville, Ontario). TransPak Packaging Extract was obtained from Agilent Technologies Canada (Mississauga, Ontario).

### 5.4.2 Animal Treatment

All MutaMouse animals used in this study were bred and maintained locally under conditions approved by the Health Canada Animal Care Committee. Adult male MutaMouse animals aged 9 to 10 weeks were housed individually on a 12 h light / 12 h dark cycle, and provided standard rodent chow and water *ad libitum*. The animals were dosed daily via oral gavage for 28 days. Each dose group, including vehicle controls, contained 5 animals. Direct Black 38 was dissolved in water (250, 500, and 1000 mg/kg body weight/day). Sudan I was dissolved in olive oil and Para Red were dissolved in olive oil with 1% DMSO. Sudan I was tested using doses of 100, 200 and 300 mg/kg body weight/day and Para Red was tested using doses of 100, 200, and 400 mg/kg body weight/day. Doses were selected based on preliminary range-finding studies performed for each chemical; however, despite being well-tolerated in the range-finding study, the animals in the 300 mg/kg Sudan I dose group displayed unacceptable toxicity and were euthanized by cervical dislocation before the end of the main-study gavage period. Partway through the study, due to aspiration of the chemical into the lungs, one animal in the Sudan I 100 mg/kg group died shortly following gavage dosing. This reduced the

group size to 4. All remaining animals were euthanized 3 days following the end of the gavage period [39] by cardiac puncture under isoflurane anaesthesia, followed by cervical dislocation and chest cavity opening. Tissues, including the bone marrow, glandular stomach, small intestine, colon, liver, and bladder, were collected, processed, flash frozen in liquid nitrogen, and stored at -80° C according to previously established methods [40].

### 5.4.3 Isolation, culture, and exposure of PHs

MutaMouse PHs were isolated as described previously [37]. Briefly, cells were obtained using a two-step collagenase technique, with the addition of a Percoll® isodensity purification step [41,42]. The cells were plated at a density of $1.2x10^6$ cells/dish onto 100 mm collagen-coated culture dishes using Attachment Medium (20 U/L human insulin, $4x10^{-6}$ mg/mL dexamethasone, 10% FBS, and 100 U/mL penicillin-streptomycin in DMEM), and incubated at 37°C and 5% $CO_2$. Two hours (t = 2 h) following plating, the Attachment Medium was replaced with Serum-Free Medium (SFM; 10 mM HEPES, 2 mM L-glutamine, 10 mM pyruvate, 0.35 mM L-proline, 20 U/L human insulin, $4x10^{-6}$ mg/mL dexamethasone, 0.01 µg/mL EGF, and 100 U/mL penicillin-streptomycin in Williams Medium E), and the plates were incubated at 37°C and 5% $CO_2$.

MutaMouse PHs were exposed to test chemicals as described previously [38]. Briefly, stock solutions of the azo compounds were prepared in DMSO. After 18 hours of culture, PHs were exposed to the Direct Black 38, Sudan I, and Para Red at concentrations of 5, 10, 25, 50, and 100 µg/mL in SFM with 1% DMSO for 6 hours at 37°C and 5% $CO_2$. Three biological replicates (i.e., separate experiments using PHs from

three different donor mice) were used for each test chemical. Following exposure, the medium was replaced with fresh SFM, and the hepatocytes were incubated for a further 72 hours prior to lysis and DNA isolation.

### 5.4.4 Cytotoxicity Measurement

Cytotoxicity was measured using the relative increase in nuclear counts (RINC) metric. The RINC metric is analogous to the widely accepted relative increase in cell counts (RICC) metric [43]. RINC was quantified by flow cytometry using a method described previously [37,38,44-46]. Briefly, cultured hepatocytes were lysed, and fluorescently labelled polystyrene microspheres added to each sample to normalize nuclei counts [37,38]. Each microsphere-lysate sample was diluted 1:10 prior to flow cytometric analysis. Data were acquired using a BD Biosciences FACScalibur flow cytometer (BD Biosciences, Mississauga, Ontario) equipped with a 488 nm laser. Instrumentation settings and data acquisition were facilitated using CellQuest Pro software (BD Biosciences). Data analysis was performed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Turku, Finland). SYTOX® green and bead fluorescence emission were captured in the FL1 channel (530/30 band-pass filter). Events were scored as nuclei following the application of key criteria (i.e., within a side scatter (SSC) vs. forward scatter (FSC) region, within a region that excludes doublets, and within an FSC vs FL1 region). RINC values were calculated as previously described [37,38].

### 5.4.5 DNA Isolation and Mutant Frequency (MF) Analysis

Bone marrow, glandular stomach, small intestine, colon, liver, and MutaMouse PHs were lysed as previously described [38,40]. Bladder tissue was homogenized in lysis buffer (1 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) using a glass Dounce tissue grinder, 1 mg/mL proteinase K and 1% SDS were added to the suspensions, and the homogenate was incubated overnight at 37° C with shaking. DNA for all tissues was isolated by phenol-chloroform extraction as previously described, with an additional chloroform extraction step [38,40]. DNA was precipitated with ethanol, spooled onto a sealed Pasteur pipette, washed with 70% ethanol, dried, dissolved in $TE^{-4}$ buffer (10 mM Tris pH 7.6 and 0.1 mM EDTA), and stored at 4°C.

The frequency of *lacZ* mutants was determined using the P-Gal positive selection method as previously described [40,47-49]. Briefly, TransPak was used to retrieve and package λgt10*lacZ* vectors from MutaMouse genomic DNA. *E. coli* cells (*E. coli* C lacZ -, galE -, recA -, Kanr, pAA119) [50] were allowed to adsorb the phage particles; cells were plated with P-Gal selective medium and incubated overnight at 37°C. Plaques were scored manually, and MF was calculated as the ratio of mutant plaque-forming units (pfu) to total pfu determined from non-selective plates (i.e., without P-Gal). N = 3 for all *in vitro* results, except for Para Red at 25 μg/mL, where N=2 due to cytotoxicity. Although all of the compounds were tested at 5, 10, 25, 50, and 100 μg/mL; however, cytotoxicity was too severe above 10 μg/mL and 25 μg/mL for Direct Black 38 and Para Red, respectively, to obtain sufficient DNA for scoring. N = 5 for all tissues, except for the Sudan I 100 mg/kg dose group, wherein N = 4 due to a premature death (see Section 2.2), and the Direct Black 38 1000 mg/kg glandular stomach sample group, the Para Red 200 mg/kg glandular stomach sample group, the Direct Black 38 control small intestine

sample group, the Direct Black 38 control colon sample group, and the Para Red 200 mg/kg colon sample group, wherein N = 4 due to low DNA yields.

### 5.4.6 Statistical Analyses

The *lacZ* mutant frequency data were analyzed in RStudio version 1.0.136 (RStudio, Boston, MA, USA) using the glm function. The quasi-Poisson distribution family was used to account for over-dispersion, and the offset was designated as the natural log of total pfu. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment (i.e., Chi squared test), and custom contrasts statements were employed to evaluate the statistical significance of responses at selected doses or concentrations [51]. The resulting p-values were corrected for multiple comparisons using the Bonferroni method.

## 5.5 Results

### 5.5.1 Mutagenicity in Various MutaMouse Tissues *In Vivo*

MF for the *lacZ* transgene was evaluated in 6 tissues *in vivo*: bone marrow, glandular stomach, small intestine, colon, liver and bladder. Following Direct Black 38 exposure, a significant treatment effect was observed in the bone marrow ($\chi^2 = 17.1$, $p < 0.001$), glandular stomach ($\chi^2 = 20.2$, $p < 0.005$), small intestine ($\chi^2 = 12.3$, $p < 0.005$), and colon ($\chi^2 = 20.1$, $p < 0.005$), with maximal 5.9-, 5.3-, 3.1-, and 8.5-fold increases in MF over control, respectively (Figure 5-2 A). For all tissues, at least one dose elicited a MF that was significantly elevated over control ($p < 0.05$). Sudan I only elicited a significant treatment effect in bone marrow ($\chi^2 = 36.1$, $p < 0.005$) (Figure 5-2 B). The

7.1-fold increase over control at the 200 mg/kg dose was marginally significant with a p-value of 0.052. A marginally significant treatment effect was observed in the bladder of mice exposed to Sudan I ($\chi^2 = 10.9$, $p = 0.059$), but there was no significant dose-specific increase in MF over control. Para Red exposure induced a significant treatment effect in the colon ($\chi^2 = 8.9$, $p < 0.001$), with a statistically significant 1.4-fold increase above control at the 400 mg/kg dose ($p < 0.05$) (Figure 5-2 C). The *in vivo* results are summarized in Table 5-II.

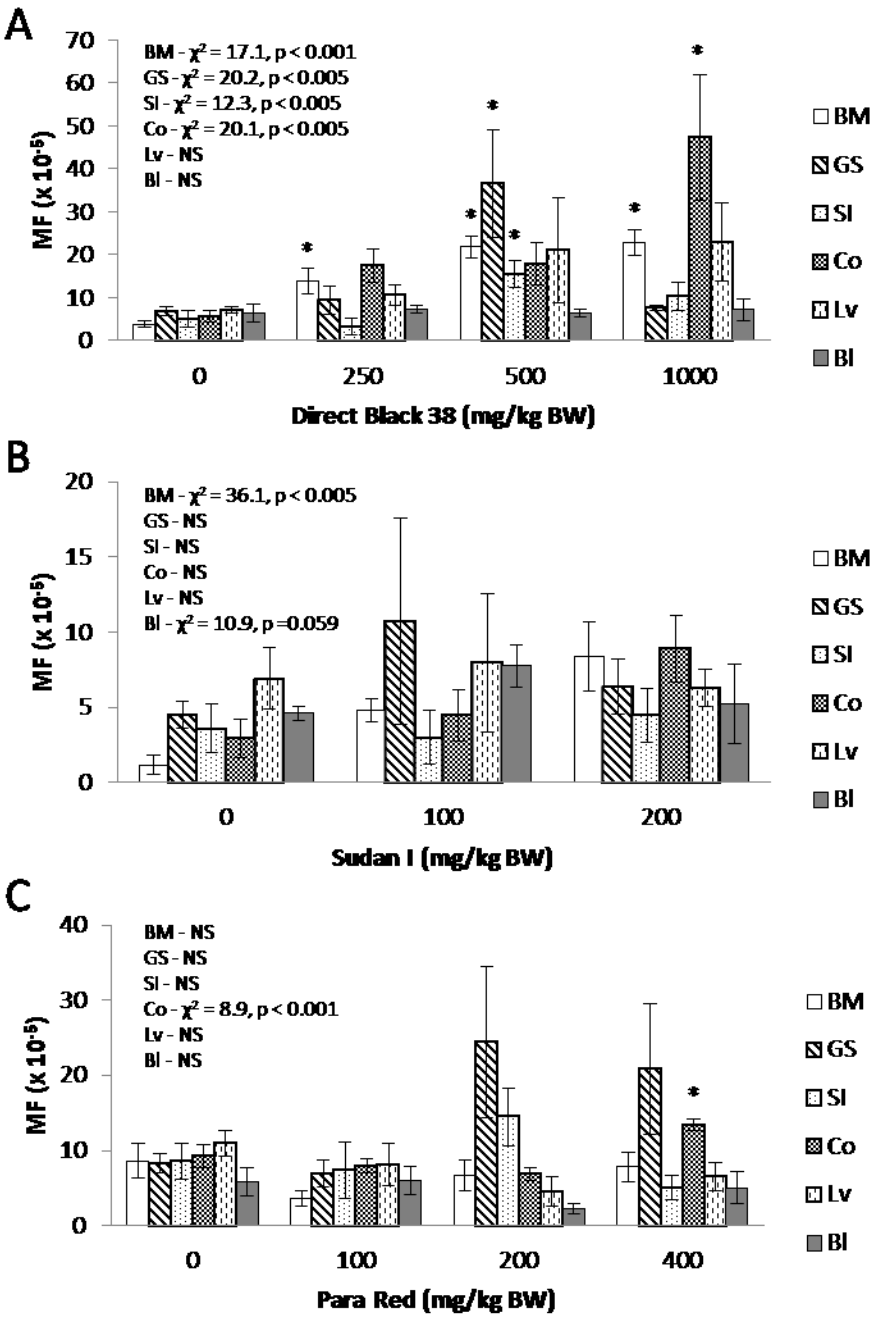


**Figure 5-2:** Induced *lacZ* MF in tissues from MutaMouse specimens exposed to Direct Black 38 (A), Sudan I (B), and Para Red (C). Bars represent average MF ± SEM. Asterisks indicate MF values that are significantly elevated relative to the concurrent control ($p<0.05$). Inset boxes show statistical results for the overall treatment effect. N = 5 for all observations, except in cases noted in the Materials and Methods. NS, not significant; BM, bone marrow; GS, glandular stomach; SI, small intestine; Co, colon; Lv, liver; Bl, bladder.

### 5.5.2 Mutagenicity in MutaMouse Primary Hepatocytes *in vitro*

MF was also evaluated following *in vitro* of exposure of MutaMouse PHs. Direct Black 38 induced a significant treatment effect ($\chi^2 = 4.2$, $p < 0.005$), with a maximal MF increase of 2.2-fold over control at 10 μg/mL ($p < 0.05$) (Figure 5-3 A). Sudan I exposure did not elicit any significant treatment effect (i.e., increase in MF) up to a test concentration that decreased RINC to 0.18 (Figure 5-3 B). Para Red induced a marginally significant ($\chi^2 = 12.6$, $p = 0.055$) treatment effect; a statistically significant MF increase of 5.0-fold over control was observed at 25 μg/mL (Figure 5-3 C). However, this concentration elicited a marked cytotoxicity (i.e., RINC < 0.20). The *in vitro* results are summarized in Table 5-II.

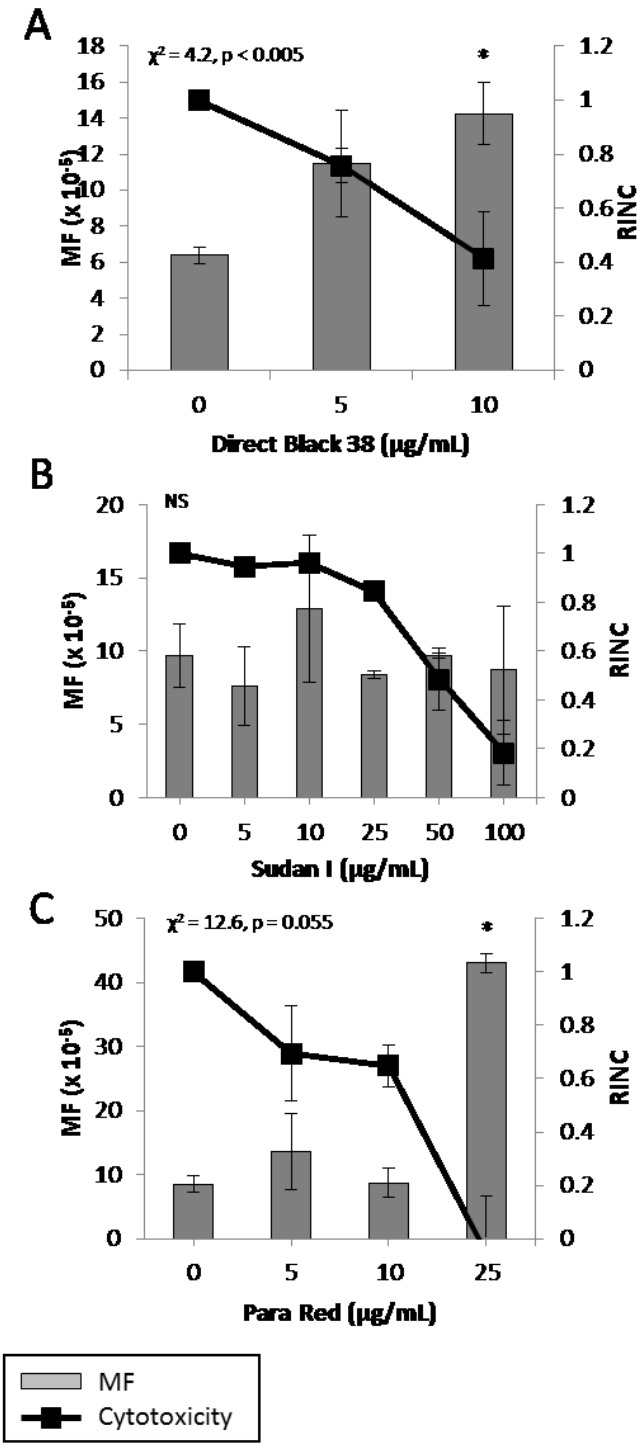


**Figure 5-3:** Induced *lacZ* MF in MutaMouse PHs exposed to Direct Black 38 (A), Sudan I (B), and Para Red (C). Grey bars represent MF ± SEM and black squares show relative increases in nuclear counts (RINC) ± SEM, a measure of cytotoxicity. Asterisks indicate MF values that are significantly elevated relative to the concurrent control ($p<0.05$). Inset boxes show statistical results for the overall treatment effect. N = 3 for all observations, except for 25 µg/mL Para Red, wherein N = 2 due to cytotoxicity. NS, not significant.

**Table 5-II:** Summary of mutagenicity results for Direct Black 38, Sudan I and Para Red - various MutaMouse tissues *in vivo* and MutaMouse primary hepatocytes (PHs) *in vitro*

| | Direct Black 38 | Sudan I | Para Red |
|---|---|---|---|
| *In vivo* | | | |
| Bone marrow | +[a] | +/-[c] | - |
| Glandular stomach | + | - | - |
| Small intestine | + | - | - |
| Colon | + | - | + |
| Liver | -[b] | - | - |
| Bladder | - | +/- | - |
| | | | |
| *In vitro* | | | |
| PHs | + | - | +/- |

[a] +, significant treatment-related effect and at significant increase in MF for at least one dose
[b-] -, no significant treatment-related effect, and no significant increase in MF above control
[c] +/-, not clearly positive or negative, see text for details

## 5.6 Discussion

The present study investigated the mutagenic activity of Direct Black 38, Sudan I, and Para Red, both *in vitro* and *in vivo*. The selected tissues are either known targets of these chemicals (Table 5-I), and/or sites of contact in the gastrointestinal tract. The doses and concentrations tested are much higher than Canadian oral exposure estimates for benzidine-based azo compounds related to Direct Black 38 (e.g., 0.027 μg/kg body weight per day in infants exposed to Acid Red 97-containing textiles) and Sudan I (i.e., up to 8.1 μg/kg body weight per day in children exposed to Sudan I-containing ballpoint pen ink); however, the data generated by the assays in this study are intended for hazard identification and must account for chronic exposures. The responses of the chemicals varied greatly; Direct Black 38 elicited the strongest response in the widest range of tissues, as well as a clear positive response in PHs *in vitro* (Figures 5-2 A and 5-3 A, Table 5-II). Sudan I elicited a positive response in bone marrow, a marginally significant treatment effect in the bladder, and a negative response in PHs *in vitro* (Figure 5-2 B and 5-3 B, Table 5-II). Para Red, the least well-studied of the three chemicals, elicited a positive response in the colon, and a positive response in PHs (Figures 5-2 C and 5-3 C, Table 5-II). The pattern of results obtained offer insight into the mechanisms of action of these compounds. Moreover, the ability to appropriately detect mutagenic activity using an *in vitro* assay based on PHs.

Direct Black 38 is a human and animal carcinogen that targets the bladder, colon, liver, and mammary glands (Table 5-I); as mentioned in the Introduction, the mechanism of action of involves azo bond cleavage and release of benzidine. Azoreductase activity is present in both the liver and the intestine; in the former it is mediated by CYPs and

NADPH quinone oxidoreductase (NQO1), in the latter by intestinal microflora [5,52-54]. Benzidine released following azo-reduction can then be transported to other tissues where further modifications via acetyl- or sulfotransferases result in the formation of electrophilic species (e.g., nitrenium, carbenium) that can readily react with DNA bases (e.g., guanine) to form adducts [24,55]. Interestingly, bacterial azo-reduction of Direct Black 38 has been shown to be much more efficient than hepatic azo-reduction [23,25].

The strongest Direct Black 38 response was manifested in the colon, a known site of bacterial azo-reduction; this was followed by bone marrow, glandular stomach, and small intestine. The bone marrow effect is particularly is interesting since it is distal from the gastrointestinal tract, thus requiring systemic circulation of Direct Black 38 metabolites. Interestingly, the highest dose (i.e., 1000 mg/kg) yielded a lower MF than the 500 mg/kg dose in glandular stomach and small intestine. The drop in MF in these tissues at the highest dose is presumably due to cytotoxicity in these tissues. Despite Direct Black 38 being linked to bladder tumours in humans and rats, and liver tumours in mice and rats, no significant response was observed in either of these tissues. Lack of response in these tissues may be a consequence of low cellular turnover rates (i.e., mitotic index) that dramatically reduce the likelihood of mutation fixation in MutaMouse specimens [56]. Indeed, urothelial cells (i.e., the cells that line the bladder) and hepatocytes *in vivo* have been shown to have extremely slow turnover rates. The turnover rate of mammalian urothelial basal cells is approximately 3 to 6 months, the slowest of any mammalian epithelial cells, and the turnover rate of murine hepatocytes is approximately 6 to 13 months [57-60]. Thus, future studies of compounds such as Direct Black 38 should enumerate DNA adducts in tissues with low cellular turnover, and/or

mutant frequency after far longer sampling times. Indeed, OECD test guideline 488 indicates that longer sampling times (i.e., 28 days instead of 3 days) may be required for tissues with low rate of cellular turnover (e.g., liver, bladder, etc) [39].

A significant response was also observed in PHs exposed to Direct Black 38 *in vitro*. This is not unexpected since, unlike hepatocytes *in vivo*, *ex vivo* MutaMouse PHs proliferate relatively rapidly [37]. Moreover, as noted in a previous study, murine PHs express a full complement of CYPs and NQO1, which can confer azoreductase activity [37]. This positive *in vitro* result echoes what has been observed in experiments using rat PHs. More specifically, Bos et al. [1984] demonstrated that Direct Black 38 incubated with rat PHs resulted in the formation of diacetylbenzidine, a DNA-reactive, reductive metabolite, without the addition of acetyl coenzyme A. The results confirm that, despite difficulty of detecting *in vivo* mutagenic hazard in known tumour sites, MutaMouse PHs can reliably detect a carcinogenic azo compound known to be activated via azo-reduction. Unfortunately, due to a paucity of published information, the ability to detect Direct Black 38 mutagenicity in MutaMouse PHs *in vitro* cannot be compared to results obtained using other mammalian cells (Table 5-II).

Sudan I is hepatocarcinogenic in mice following subcutaneous administration; it produces bladder tumours in albino and (C57 X IF)$F_1$ mice following wax pellet implantation [61-65] (Table 5-I). However, the wax pellet implantation studies are no longer deemed appropriate since the pellet itself can irritate the bladder epithelium [66]. As mentioned in Section 1, Sudan I, unlike Direct Black 38, does not require azo-reduction to become metabolically converted to a DNA-reactive agent. In fact, azo-reduction seems to mainly lead to detoxification of Sudan I with the production of aniline

and 1-amino-2-naphthol [5]. There are two main routes of activation for Sudan I: hepatic oxidation by CYP1A1 and CYP3A, and metabolism by bladder peroxidases [5,26]. Cytochrome P450-mediated oxidation of Sudan I leads to the formation of the benzenediazonium ion (BDI). BDI is formed following metabolism by rat liver microsomes [67,68], and it forms 8-(phenylazo)guanine adducts with calf thymus DNA *in vitro* and rat liver DNA *in vivo* [69,70]. CYP1A1 and CYP3A metabolism also leads to the formation of *C*-hydroxylated detoxification by-products [67,71,72]. Sudan I is an AhR agonist and strongly induces CYP1A1 and NQO1 in rats [73,74]. Peroxidases, including horseradish peroxidase (HRP) and mammalian prostaglandin H synthase, can metabolize both Sudan I and *C*-hydroxylated Sudan I metabolites to DNA-reactive species [75-79]. High levels of peroxidases (i.e., prostaglandin H synthase) are found in the urinary bladder, where CYP activity is known to be very limited [80]. The same DNA adducts observed *in vitro* following HRP-mediated metabolism have been detected in the urinary bladder of Fisher 344 rats following oral administration of Sudan I [81]. Thus, the metabolic processes underlying the mutagenicity and/or hepatocarcinogenicity of Sudan I likely include several enzymatic systems that collectively catalyse the generation of several DNA-reactive species (e.g., BDI, peroxidation products of *C*-hydroxylated Sudan I).

In this study, Sudan I elicited a significant overall treatment effect and marginally significant MF increase in the bone marrow, as well as a marginally significant overall treatment effect in the bladder (Figure 5-2 B). The bone marrow result suggests that Sudan I was metabolically activated and systemically circulated; this result echoes the positive *in vivo* MN assay results observed in both rat and mouse bone marrow [82,83].

Although the observed bladder response is only marginally significant, it is consistent with the aforementioned role of bladder peroxidases in metabolic activation. Moreover, published information regarding Sudan I activation in the liver and bladder, and information regarding Sudan I cancer target tissues (i.e., liver and bladder), support an expectation of liver and bladder effects. The marginal overall treatment effect for bladder, and the accompanying lack of a significant MF increase at any of the tested doses, may be due, as noted for Direct Black 38, to the slow turnover of bladder epithelial cells. Moreover, pattern of results suggests that the experiment was underpowered. In order to improve the ability to detect an effect in the bladder, future work should employ increased numbers of animals per dose group, particularly for the most relevant dose(s) (e.g., 100 mg/kg). Interestingly, no response was observed in the liver. Similar to the bladder results, this may be due to low cell turnover necessitating, as noted for Direct Black 38, a longer sampling time. Moreover, as noted for Direct Black 38, DNA adduct analyses of the liver and bladder could provide important information to elucidate the respective ability of the liver and bladder to activate Sudan I *in vivo*. Although this study has provided evidence of mutagenic activity in the bone marrow and bladder, additional investigations are needed to fully characterise genetic toxicity; moreover, to understand mechanism of action and tissue-specific metabolism.

Sudan I did not elicit a significant increase in MF in MutaMouse PHs exposed *in vitro* (Figure 5-3 B). This lack of response is interesting, considering that Sudan I elicited a positive response in AHH-1 and MCL-5 cells in the *in vitro Hprt* gene mutation assay [36]. Indeed, published information about the metabolism and activation of Sudan I, combined with available information about MutaMouse PH metabolic capacity, suggest

that the substance should be converted into a DNA-reactive metabolite in MutaMouse PHs (e.g., CYP1A1-mediated catalysis to DNA-reactive BDI). Moreover, the rapid proliferative capacity of MutaMouse PHs should lead to fixation of elevated *lacZ* transgene mutations. The discrepancy in response between these *in vitro* systems may be related to the strong CYP1A1 and NQO1 induction capabilities of Sudan I [74]. AHH-1 is an immortal human B lymphoblastoid cell line that inducibly expresses CYP1A1. MCL-5, which is derived from AHH-1, expresses particularly high levels of CYP1A1, and has been transfected with 2 plasmids: one containing 2 copies of CYP3A4 cDNA and 1 copy of CYP2E1, and one containing 1 copy each of CYP1A2, CYP2A6, and microsomal epoxide hydrolase. Although these cell lines show induced fold-change increases in CYP1A1 activity that is similar to MutaMouse PHs, the absolute magnitude of both their basal and induced activity are approximately 10-fold lower than that of MutaMouse PHs [37,84-86]. Thus, failure to elicit a positive response in MutaMouse PHs, when combined with the results for MCL-5 and AHH-1 cells, and the observation of CYP1A1 generation of detoxified *C*-hydroxylation products, suggest that the level of CYP1A1 activity in MutaMouse PHs is preferentially producing detoxified metabolites. Additionally, the induction of NQO1 in MutaMouse PHs, which has azoreductase activity, and is not known to be present in AHH-1 or MCL-5 cells, may also be contributing to the detoxification of Sudan I. Indeed, although convincing evidence has yet to be published, there has been speculation that NQO1 may lead to the reduction and detoxification of Sudan I [74]. The roles of CYP1A1 and NQO1 in Sudan I metabolism and mutagenicity *in vitro* could be confirmed by retesting Sudan I in the presence of CYP1A1 and NQO1 inhibitors, such as α-naphthoflavone and dicumerol, respectively

[87,88]. Furthermore, it may also be useful to perform a follow-up study that assesses the frequency of Sudan I adducts. Such follow-up studies could evaluate hypotheses regarding the inability to elicit mutations in MutaMouse PHs, which, by extension, would shed light on the utility of MutaMouse PHs for identifying the mutagenic hazards of azo compounds.

As mentioned in the Introduction, very little is known about the mechanism of action of Para Red; although, due to its structural similarity, it is presumed to act in a similar fashion to Sudan I. In other words, oxidation mediated by hepatic and bladder enzymes that lead to production of DNA-reactive metabolites. Interestingly, it is known that human intestinal microflora can catalyze azo-reduction of Para Red, yielding 1-amino-2-naphthol and 4-nitroaniline. In contrast, azo-reduction of Sudan I yields 1-amino-2-naphthol and aniline [89]. Unlike aniline, which is not mutagenic in the Ames test, 4-nitroaniline induces mutations in Salmonella TA98 in the presence of induced rat liver S9 [90]. Thus, although there is a paucity of information about the metabolism and mutagenicity of Para Red, there is some evidence to suggest that intestinal azo-reduction may play a more important role in comparison with Sudan I. Indeed, the results obtained show that Para Red can elicit a positive response in the colon *in vivo* (Figure 5-2 C, Table 5-II), which in turn suggests that colonic azoreductase activity is yielding 4-nitroaniline, i.e., a mutagenic metabolite. This is in stark contrast to the mutagenic mechanism of Sudan I, which requires oxidation.

Para Red also yielded a positive result in MutaMouse PHs *in vitro*, albeit only when tested at a highly cytotoxic concentration (i.e., 25 μg/mL) (Figure 5-3 C). Para Red also elicited a positive response in the *Hprt in vitro* gene mutation assay in AHH-1 cells,

with a lowest observable effect level (LOEL) of 7.5 μg/mL in the absence of cytotoxicity [36]. These observations suggest that CYP1A1 mediated catalysis likely plays a role in the mutagenic activation of Para Red. Although Para Red is mutagenic in both systems, the discrepancy with respect to the active concentration is likely related to the different metabolic profiles of MutaMouse PHs and AHH-1 cells [37,84]. Further studies involving select enzyme inhibitors, such as the CYP1A1 inhibitor α-naphthoflavone, would lead to an improved understanding regarding the metabolic activation and mutagenicity of Para Red. Identification and quantification of Para Red-induced DNA adducts would similarly contribute to mode of action determination.

An important goal of the present study was generation of information about the *in vivo* mutagenicity and mechanisms of action of selected azo compounds. None of the tested compounds had previously been analysed in a TGR gene mutation assay, and, using the MutaMouse system, the work examined *in vivo* mutagenic activity in selected tissues. The target tissues identified in the Direct Black 38 experiment support the important role of intestinal microflora in the metabolic activation (i.e., azo reduction) of this potent carcinogen. The effects observed in the bone marrow and bladder, and the absence of effects in the colon, of Sudan I-exposed animals supports the proposed oxidation-mediated activation of this food contaminant; reaffirming that bacterial azo-reduction is not a major route of activation [26]. Importantly, the study investigated the mutagenicity of the common food contaminant Para Red, and found that, despite close structural similarity to Sudan I, it targets different tissues (i.e., the colon). This finding suggests that azo-reduction by anaerobic bacteria in the colon is likely leading to the production of mutagenic 4-nitroaniline; demonstrating that, in comparison with Sudan I,

azo-reduction plays a more important role in the activation of Para Red. Overall, the present study's *in vivo* evaluation of Direct Black 38, Sudan I, and Para Red offers insight into the proposed mechanisms of action of these chemicals, and identifies data gaps that could be filled by, for example, DNA adduct analyses, additional TGR assays with longer sampling times for key tissues such as the liver and bladder, and enzyme inhibition experiments. Additionally, investigating the mutation spectra of these chemicals in their target tissues could elucidate further details regarding their mechanisms of action.

The study was also designed to evaluate the utility of the *in vitro* MutaMouse PH assay to reliably assess the mutagenicity of compounds that undergo complex metabolism *in vivo*. Previously, our group showed that MutaMouse PHs are metabolically competent and capable of detecting mutagens with a variety of metabolic activation requirements (i.e., polycyclic aromatic hydrocarbons, aromatic amines, mycotoxins, nitroarenes, and nitrosamines) [37,38]. The chemicals previously examined require both Phase I oxidation reactions and Phase II conjugation reactions; however, none necessitated reductive metabolism for generation of DNA-reactive metabolites. The current study illustrates the ability of the MutaMouse *in vitro* PH gene mutation assay to detect chemicals requiring azo-reduction (e.g., Direct Black 38 and Para Red). However, it also demonstrates a gap in the applicability domain of this assay with respect to azo compounds that do not require azo-reduction for metabolic activation (i.e., Sudan I). The negative Sudan I response was unexpected in light of the fact that this compound elicits positive responses in other *in vitro* mammalian cell gene mutation assays; it indicates that further studies are warranted regarding the roles of key metabolic enzymes in the detoxification versus

activation of compounds such as Sudan I (i.e., CYP1A1 in MutaMouse PHs) [36]. This apparent limitation and possible lack of sensitivity requires further study. Precise determination of the ability of the MutaMouse PH assay to assess the mutagenicity of azo compounds will require testing of additional azo compounds, including some well-studied compounds with existing *in vivo* and *in vitro* data. Nevertheless, the results presented here indicate that this assay is capable of reductive metabolism, and thus is a useful tool for the assessment of azo compounds.

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# CHAPTER SIX

# Chapter 6: General Conclusions

## 6.1 Summary of Study Outcomes

The overall goal of this thesis was to scrutinise the weaknesses of conventional, *in vitro* mammalian cell mutagenicity assays, and subsequently address these weaknesses via the development of a novel *in vitro* mutagenicity assay based on metabolically-competent primary hepatocytes (PHs) derived from the transgenic MutaMouse. The results of the presented studies elucidate and clarify the limitations of human liver S9, establish and partially validate an *in vitro* MutaMouse PH mutagenicity assay, and enhance current understanding regarding the metabolic activation and mutagenicity of selected azo compounds. The investigation regarding the utility of human liver S9 for routine genetic toxicity assessment concluded that, due to the increased risk of false negative results (i.e., negative response *in vitro* for known *in vivo* mutagens), it should not be used as a replacement for induced rat liver S9. To address the limitations of conventional mammalian cell genetic toxicity assays that require exogenous sources of mammalian metabolic enzymes (i.e., hepatic S9), the thesis subsequently focused on the development of the *in vitro* MutaMouse PH mutagenicity assay. This began with a thorough characterization of cultured MutaMouse PHs, which demonstrated that they temporarily retain the phenotypic attributes of hepatocytes *in vivo*, express hepatocyte-specific proteins, exhibit the karyotype of typical hepatocytes, and maintain metabolic activity for at least the first 24 hours after isolation. Moreover, the observed capacity of PHs to proliferate in culture is sufficient to assess chemically-induced mutations. Preliminary validation of a mutagenicity assessment assay based on MutaMouse PHs,

which takes advantage of the window of optimal metabolic enzyme activity, demonstrated excellent sensitivity and specificity. With respect to the investigated azo compounds, comparisons of the results obtained using the *in vitro* MutaMouse PH mutagenicity assay with those obtained using the *in vivo* MutaMouse TGR assay revealed that MutaMouse PHs can carry out some forms of reductive metabolism. The importance of these findings is summarized below.

**Chapter 2 – "The Utility of Metabolic Activation Mixtures Containing Human Hepatic Post-Mitochondrial Supernatant (S9) for *In Vitro* Genetic Toxicity Assessment"**

*Objective*: Assess the utility of human liver S9 for *in vitro* genotoxicity assessment (i.e., relative to induced rat liver S9), and formulate recommendations regarding its routine use in chemical safety assessments.

*Outcomes and Importance*: This study demonstrates that genetic toxicity assays, such as the Ames test and the *in vitro* MN assay, conducted in the presence of human hepatic S9 generally yield lower mutagenic potency values relative to those obtained using induced rat liver S9. Importantly, some assessments of noteworthy mutagens, including known mutagenic carcinogens, based on human liver S9 yielded negative Ames test results (i.e., false negatives); induced rat liver S9 did not elicit negative results for these chemicals. Interestingly, the use of human liver S9 yields higher mutagenic potencies for certain chemicals relative to induced rat liver S9, in particular, aromatic amines (AAs) and dimethylnitrosamine (DMN). New experimentally-generated data confirms that, in comparison with Aroclor 1254-induced rat liver S9, human liver S9 is far more cytotoxic to the mammalian cells frequently used for *in vitro* mutagenicity assessment (i.e.,

L5178Y mouse lymphoma, CHO and human TK6). With respect to genetic toxicity assessment for routine assessments of chemical safety, the results presented in **Chapter 2,** which indicate a risk of false negative results, do not support the use of human liver S9 as a replacement for induced rodent liver S9.

**Chapter 3 – "The development and pre-validation of an *in vitro* mutagenicity assay based on MutaMouse primary hepatocytes. Part I: Isolation, structural, genetic and biochemical characterization"**

*Objective*: Thoroughly characterize cultured MutaMouse PHs with respect to biochemical, structural, and genetic attributes. The characterised cell system will constitute the foundation for development and establishment of a novel *in vitro* mutagenicity assay.

*Outcomes and Importance*: Through its extensive characterization of cultured MutaMouse PHs, this study demonstrates the suitability of the cells as the foundation of an *in vitro* mutagenicity assay. The results indicated that MutaMouse PHs are structurally and karyotypically normal with respect to *in vivo* phenotype. Since hepatocytes are frequently binucleated and polyploid, measuring proliferation was challenging. For the purpose of this study, the temporal change in the amount of ploidy-corrected nuclei, which was measured using flow cytometry, permitted accurate quantification of genomic doublings. The technique yielded a doubling time of 22.5 ± 3.3 hours. Proliferation was also visually observed using time-lapse imaging (Supplementary Video 3-1). Importantly, the gene expression and enzymatic activity results, which monitored key Phase I and Phase II metabolic enzymes required for activation of known chemical mutagens, were maintained for at least 8 and 24 h in culture, respectively. The gene expression and

activity of cytochromes P450 (CYPs) 1A1/1A2, and CYP2B were observed to be inducible by β-naphthoflavone and phenobarbital, respectively.

**Chapter 4 – "The development and pre-validation of an *in vitro* mutagenicity assay based on MutaMouse primary hepatocytes, Part II: Assay performance for the identification of mutagenic chemicals"**

*Objective*: Examine the ability of an *in vitro* mutagenicity assay based on MutaMouse PHs to correctly evaluate a series of carefully selected test chemicals.

*Outcomes and Importance*: This study shows that the *in vitro* MutaMouse PH mutagenicity assay is capable of detecting mutagens requiring Phase I and/or Phase II metabolism. The assay was found to have excellent sensitivity and specificity. The aforementioned flow cytometry-based proliferation measurement technique was employed to measure cytotoxicity as concentration-related changes in relative increase in nuclear counts (RINC). Additionally, the spontaneous MF was observed to be similar to that of MutaMouse liver tissue *in vivo* (i.e., $11.0 \times 10^{-5} \pm 0.80 \times 10^{-5}$), and to be consistent across experiments and animals (i.e., low variability across PH batches). This study demonstrates that the *in vitro* MutaMouse PH mutagenicity assay can be reliably employed for chemical safety assessments.

**Chapter 5 – "The mutagenic activity of select azo compounds in MutaMouse target tissues *in vivo* and primary hepatocytes *in vitro*"**

*Objective*: To determine the utility of MutaMouse PHs for *in vitro* mutagenicity assessment of chemicals that undergo complex metabolism and activation. More specifically, compare the *in vitro* and *in vivo* mutagenicity results of selected azo compounds.

*Outcomes and Importance*: This study shows that the *in vitro* MutaMouse PH mutagenicity assay is capable of correctly identifying chemical mutagens that require azo-reduction; moreover, the study revealed new mechanistic information regarding the mutagenic activity of selected azo compounds. The *in vivo* results reaffirmed the vital role of azo-reduction for Direct Black 38. The *in vivo* results also supported the oxidation-mediated activation of Sudan I that has been proposed in the literature [Stiborová et al., 2009]. Interestingly, despite its structural similarity to Sudan I, the results obtained, which show that Para Red targets different tissues *in vivo* (i.e., the colon), suggest that intestinal azo-reduction plays an important role in its metabolic activation. The *in vitro* MutaMouse PH mutagenicity assay was capable of detecting the mutagenicity of both Direct Black 38 and Para Red; this important finding suggests that MutaMouse PHs are capable of azo-reduction, an enzymatic reaction that is not facilitated by standard S9 mixes. Sudan I yielded a negative response in this assay, which reveals a potential gap in the applicability domain that requires further investigation.

## 6.2 Overall Fulfillment of Thesis Objectives

The results presented in **Chapters 2 – 5** collectively indicate that the objectives outlined in **Chapter 1** were achieved.

*Objective 1:* A critical examination of the utility of exogenous metabolic activation systems based on human hepatic S9 for routine genetic toxicity assessment (i.e., as an alternative to induced rat liver S9).

*Outcomes:* This objective was fulfilled. As described above, using an evaluation of both the existing literature and new experimental data, **Chapter 2** successfully investigated the

utility of human liver S9 for routine genetic toxicity assessment. This examination concluded with the recommendation that, for routine genetic toxicity testing, human liver S9 not be used as a replacement for induced rat liver S9. Nevertheless, its use may be warranted for specific applications. These applications may include investigations of chemicals wherein species-specific differences in metabolic activation are suspected (e.g., AAs and nitrosamines).

*Objective 2:* The structural, genetic, and enzymatic characterization of cultured MutaMouse PHs.

*Outcomes:* This objective was fulfilled. **Chapter 3** comprises a thorough characterization of cultured MutaMouse PHs. This characterization included microscopic observations, immunocytchemical analyses, time-lapse imaging, karyotypic analyses, fluorescent *in situ* hybridization (FISH) analysis to confirm the presence of the λgt10 bacteriophage shuttle vector, flow cytometry-based proliferation evaluation, gene expression analysis using qPCR mouse drug metabolism arrays, and metabolic enzyme activity assessments. Collectively, these analyses demonstrated that, due to metabolic competence, normal karyotype (i.e., neither transformed nor spontaneously immortalized), proliferative capacity, and the presence of the MutaMouse transgene for rapid and reliable mutant scoring, MutaMouse PHs are well-suited for use as the basis for an *in vitro* mutagenicity assay.

*Objective 3:* Evaluation of the performance of an *in vitro* mutagenicity assessment system based on MutaMouse PHs.

*Outcomes:* This objective was fulfilled. **Chapter 4** examined the predictive capacity of the MutaMouse PH mutagenicity assay by investigating the results obtained following

exposure to a panel of thirteen known mutagens and non-mutagens, many of which are suggested by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) [D. Kirkland et al., 2008; D. Kirkland et al., 2016]. The mutagens included chemicals that are direct-acting, as well as those requiring diverse types of metabolic activation. All of the mutagens tested, save ICR 191 (i.e., 6-Chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine dihydrochloride), a direct-acting frameshift mutagen, elicited positive results. None of the four non-mutagens tested, including *tertiary* butylhydroquinone (TBHQ) and eugenol, which are known to frequently yield misleading positive results in *in vitro* assays, elicited positive results in the MutaMouse PH mutagenicity test. **Chapter 5** took the performance investigation a step further by challenging the assay with selected azo compounds, a broad class of chemicals that undergo complex metabolic activation. This study demonstrated that the applicability domain of the *in vitro* MutaMouse PHs mutagenicity assay can be extended to mutagens requiring azo reduction (i.e., Direct Black 38 and Para Red). Nevertheless, Sudan I, an azo compound that is thought to be activated through oxidative metabolism, yielded a negative response in this assay. This finding reveals a limitation that requires further investigation.

## 6.3 Thesis Novelty and Original Contributions to Scientific Knowledge

### 6.3.1 Utility of Human Liver S9 for Routine Genotoxicity Screening

**Chapter 2** scrutinised the utility and efficacy of human liver S9 for use with *in vitro* genotoxicity assays, i.e., relative to the more conventionally-used induced rat liver S9. The novel work, which constitutes the first definitive evaluation regarding the utility

of human liver S9 for routine genotoxicity screening, is especially timely due to increased interest in the use of human liver S9 and its increased availability [Ku et al., 2007]. Several earlier studies have endeavoured to investigate the utility of human liver S9 for *in vitro* genetic toxicity assessment; however, these studies generally involved relatively few lots of human liver S9, limited measures of metabolic enzyme activity, and/or a narrow range of tested compounds [Ames et al., 1973; Beaune et al., 1985; Hakura et al., 1999; Hakura et al., 2003; Hakura et al., 2005]. The work presented in **Chapter 2** is also the first to scrutinise the metabolic activity of human liver S9 preparations across the existing literature, as well as across commercially available lots of S9. Finally, to account for the paucity of data for *in vitro* mammalian cell genetic toxicity assays with human liver S9, the collected data were augmented with experimentally-generated *in vitro* micronucleus (MN) assay results comparing data obtained using human liver S9 to those obtained using induced rat liver S9. These results constitute the first known study to assess the utility of human liver S9 for the *in vitro* MN assay.

The results presented in **Chapter 2** led to several important conclusions that constitute original contributions to scientific knowledge. More specifically, the results illustrated extreme variability in mutagenic potency data obtained from bacterial reverse mutation tests conducted in the presence of human liver S9. Certain lots of human liver S9 elicited mutagenic potency values that were orders of magnitude higher than average; in some instances, these values even exceeded induced rat liver S9 values. Conversely, human liver S9 sometimes yielded negative results for noteworthy mutagens in the bacterial reverse mutation test (e.g., BaP, 2-AAF, and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine [PhIP]), whereas they were always positive in the presence

of Aroclor 1254-induced rat liver S9. Unlike Aroclor 1254-induced rat liver S9, an examination of the empirical relationship between available human liver S9 enzyme activity data and mutagenic potency did not indicate a correlation between expected enzymatic attributes (e.g., activity of CYP1A1/1A2) and mutagenic potency, thus the enzymatic attributes of human liver S9 that contribute to its ability to metabolically activate test mutagens remains unknown. Similar to the results observed in the bacterial reverse mutation assay data, human liver S9 yielded lower MN frequencies for benzo[*a*]pyrene (BaP), but higher MN frequencies for 2-AAF, relative to Aroclor 1254-induced rat liver S9. Additionally, human liver S9 was observed to elicit higher levels of cytotoxicity than induced rat liver S9, potentially due to a higher production of reactive oxygen species [D. J. Kirkland et al., 1989]. These revelations, along with the deficiencies regarding the utility of S9 with cultured mammalian cells, and the weaknesses associated with conventional assays noted in the Introduction (i.e., **Chapter 1**) of this thesis, constitute original contributions regarding the limits of existing *in vitro* genetic toxicity assays, and the necessity to develop and validate improved, alternative *in vitro* genetic toxicity assessment tools based on cells with endogenous metabolic capacity.

### 6.3.2 Development of a Metabolically Competent Mammalian Cell Mutagenicity Assay

The thesis next focused on the development and use of a metabolically-competent *in vitro* mammalian cell mutagenicity assay for identification and quantification of mutagenic hazard (i.e., the *in vitro* MutaMouse PH mutagenicity assay). Previous studies

have sought to demonstrate the utility of PHs from TGRs, including the MutaMouse and pUR288 *lacZ* Plasmid Mouse [G. Chen et al., 2010; Zwart et al., 2012; Luijten et al., 2016]. The work of Chen et al showed that mutagenicity assessment using MutaMouse PHs is possible; they demonstrated the cells' capacity to detect the mutagenicity of three chemical mutagens and a mutagenic complex mixture. However, the study did not evaluate the predictive capacity of the assay using both mutagens and non-mutagens, and, other than some routine morphological observations, the study did not characterize cultured MutaMouse PHs. This thesis presents a thorough characterization of MutaMouse PHs to showcase their advantageous attributes for use in an *in vitro* mutagenicity assay, and, by challenging the cells with both mutagens and non-mutagens, it also provides an evaluation of the predictive capacity of this assay.

Although there are other studies characterizing murine PHs *in vitro*, this information is limited and fragmented across different mouse strains. As such, the study presented in **Chapter 3** represents the most comprehensive evaluation of PHs from a specific mouse strain conducted to date. For example, work examining PHs from C57BL/6 mice, which constitutes some of the most detailed work on murine PH monolayer culture characterization available in the scientific literature, only investigated morphology, expression and protein levels of asialoglycoprotein receptor as a hepatocyte-specific marker, and temporal changes in CYP2A13 protein levels [Severgnini et al., 2012]. Further research into collagen sandwich-cultured C57BL/6 murine PHs observed temporal changes in metabolic enzyme activity and gene expression of metabolic enzymes assessed using microarrays [Mathijs et al., 2009]. Neither of these studies examined (i) murine PH proliferation in culture, (ii) markers

for other cell types to evaluate culture purity, (iii) karyotype, or (iv) enzyme inducibility. With respect to murine PHs from TGR strains, morphology, hepatocyte-specific marker expression, proliferation determined using BrdU incorporation, the activity of some metabolic enzymes, and p53 function have been assessed. However, these investigations only examined metabolic competence on a small scale (i.e., only three metabolic enzymes were scrutinised), did not perform a karyotype analysis, and did not directly observe PH doubling [Zwart et al., 2012; Luijten et al., 2016].

The thorough and unique PH characterization contained in this thesis is crucial for the test guideline (TG) validation process, i.e., as outlined by the Organization for Economic Cooperation and Development (OECD). The OECD recommends that, prior to formal intra- and inter-laboratory validation trials, a proposed assay should undergo pre-validation steps, beginning with a thorough optimization and characterization of all aspects of the test [OECD, 2005]. The characterization performed in **Chapter 3** also lends more weight to the rationale behind the development of an assay based on MutaMouse PHs.

The characteristics of MutaMouse PHs provide evidence of their utility for mutagenicity assessment. MutaMouse PH proliferation permits the fixation of mutations (i.e., due to the proliferative capacity), and the λgt10 shuttle vector permits convenient mutant frequency (MF) scoring (i.e., due to the presence of the MutaMouse transgene). These attributes, as well as MutaMouse PHs' endogenous metabolic capacity and normal phenotype/karyotype, provide advantages over conventional mutagenicity assays, such as the thymidine kinase (*Tk*) (TG 490) and *Hprt*/*xprt* (TG 476) assays [OECD, 2016a; OECD, 2016b]. This original contribution to scientific knowledge clearly indicates that a

validated mutagenicity assay based on MutaMouse PHs would be superior to conventional assays.

The measurement of cytotoxicity is critical in any *in vitro* genetic toxicity assay in order to ensure that any observed genotoxic effects are not due to non-specific cellular damage attributable to overt cellular toxicity. The accurate assessment of cytotoxicity using metrics that measure proliferation, such as relative population doubling (RPD) or relative increase in cell counts (RICC), have been shown to reduce the incidence of false, or misleading, *in vitro* positive results that can be attributed to apoptosis at cytotoxic concentrations [Fowler et al., 2012]. Accurate measurement of PH genome replication is difficult due to their polyploidy and frequent multinucleation [Guidotti et al., 2003; Duncan et al., 2010]. To address this challenge, a flow-cytometry-based ploidy-corrected nuclei enumeration method was developed and utilized to measure PH proliferation over time (**Chapter 3)**. In **Chapter 4**, this method was harnessed to develop the RINC protocol. This novel method overcomes some of the challenges associated with the use of murine PHs, and allows for the accurate, reliable measurement of cytotoxicity.

Predictive capacity is one of the criteria the OECD recommends be fully elucidated during the validation process [OECD, 2005]. With respect to predictive capacity, the *in vitro* MutaMouse PH mutagenicity assay was found to elicit positive results for 8 out of the 9 mutagens tested, and did not elicit positive results following exposures to 4 non-mutagens (**Chapter 4)**. The OECD also recommends a thorough investigation of applicability domain, i.e., the chemical classes, mechanisms of action, and ranges of responses that the test method can reliably assess. The results obtained (**Chapter 4)** revealed that the *in vitro* MutaMouse mutagenicity assay can identify

mutagens requiring diverse metabolic activation pathways, including BaP, aflatoxin $B_1$ (AFB1), 2-AAF, PhIP, 1,8-dinitropyrene (1,8-DNP), 3-nitrobenzanthrone (3-NBA), and DMN, as well as *N*-ethyl-*N*-nitrosourea (ENU) a direct-acting agent. ICR 191, however, a direct-acting mutagen that intercalates into DNA and causes frameshift mutations, yielded a negative result. Due to this unexpected negative result, it was postulated that the MutaMouse PHs may be efficiently detoxifying ICR 191, thus leading to attenuated mutagenicity in this *in vitro* system, and a gap in this assay's applicability domain. Despite this negative result, the data indicate that the *in vitro* MutaMouse PH mutagenicity assay has an excellent predictive capacity, exceeding that of conventional *in vitro* mammalian cell mutagenicity assays, as well as the *in vitro* FE1 cell mutagenicity assay (**Chapter 4)** [White et al., 2003; Maertens et al., 2017; Hanna, 2018]. No other *in vitro* mutagenicity assay using an immortalized or transformed cell line can cover this range of chemicals without the addition of an exogenous activation mixture such as induced rat liver S9.

As mentioned earlier, **Chapter 5** furthered the investigation into the applicability domain by challenging the assay with three mutagenic azo compounds: Direct Black 38, Sudan I, and Para Red. This was an important research initiative since it yielded novel information regarding the capacity of MutaMouse PHs to metabolically activate azo compounds, a class of chemicals associated with complex and diverse mechanisms of metabolic activation. The results indicated that MutaMouse PHs are capable of detecting the mutagenicity of Direct Black 38 and Para Red, two compounds that require reductive metabolism for activation. Interestingly, Sudan I, an azo compound thought to require oxidative metabolism for activation, did not elicit a mutagenic response in MutaMouse

PHs, revealing another gap in the applicability domain. It was postulated that the metabolic profile of MutaMouse PHs may be leading to greater detoxification than activation, thus resulting in a lack of mutagenic activity. Together with the characterization work performed in **Chapter 3**, the results of the validation work conducted to date contribute towards the fulfillment of test method definition, predictive capacity, and applicability domain criteria specified by the OECD. They also underscore the promise of the *in vitro* MutaMouse PH mutagenicity assay for routine use in genotoxicity assessments of new and existing chemicals.

The OECD requires that there be justification for the adoption of a new test method relative to existing TGs. Currently, the *Tk* (TG 490) and *Hprt*/*xprt* (TG 476) tests are the only other *in vitro* mammalian cell mutagenicity assays with OECD TGs [OECD, 2016a; OECD, 2016b]. As discussed in **Chapter 1**, these tests typically utilize cell lines that are transformed or spontaneously immortalized; they have unstable, aberrant karyotypes, and/or undesirable phenotypes (e.g., impaired p53 function). Additionally, the cell lines typically used for these assays are metabolically deficient, thus requiring the addition of exogenous sources of mammalian metabolic enzymes (e.g., Aroclor 1254-induced rat liver S9). As discussed in both **Chapters 1** and **2**, the use of hepatic S9 fractions, whether derived from humans or induced rats, can pose additional problems, including cytotoxicity and an incomplete representation of metabolic enzymes (e.g., predominantly CYP-mediated Phase I reactions). In contrast, the *in vitro* MutaMouse PH mutagenicity assay utilizes primary cells with normal phenotypic and karyotypic characteristics (**Chapter 3)**; moreover, cells that also express inducible metabolic enzymes, and, in the absence of exogenous S9, accurately detect a far wider range of

mutagens than the conventional *Tk* and *Hprt*/*xprt* mutagenicity assays (**Chapters 4** and **5)**. Finally, it is also important to note that the *Tk* and *Hprt*/*xprt* mutagenicity assays require laborious clone isolation and enumeration. Taking into account these time-consuming steps, the *Tk* and *Hprt*/*xprt* mutagenicity protocols can take 13 to 20 days and 15 to 22 days to complete, respectively. In contrast, including the time needed for PH isolation, culture, chemical exposure, sampling time, DNA extraction, and MF scoring, the MutaMouse PH mutagenicity assay only requires 10 days. Although further validation of the MutaMouse PH mutagenicity assay is required, the initial studies contained herein collectively constitute a substantial step forward. By demonstrating the advantages of the novel assay, its utility for *in vitro* mutagenicity assessment was definitively established.

With respect to the need for alternative *in vitro* (i.e., non-animal) tests for (geno)toxicity assessment, the relevance and originality of this work can readily be specified. The 7$^{th}$ Amendment to the European Union (EU) Cosmetics Directive, which prohibits animal-tested cosmetics and cosmetic ingredients, has led to a shift away from *in vivo* tests and towards use of novel *in vitro* assays world-wide [Adler et al., 2011; European Commission, 2009]. In addition, the document entitled *Toxicity Testing in the 21$^{st}$ Century* encourages the adoption of new high-throughput screening tools for efficient toxicity assessment and mechanism of action determination [Krewski et al., 2010]. Nevertheless, when envisioning novel assays that are aligned with the needs and priorities listed above, it is critical to keep in mind that any new assay destined for regulatory use must be aligned with the legislative requirements outlined in **Chapter 1**. The *in vitro* MutaMouse PH mutagenicity assay is one of the only newly-proposed

genetic toxicity assays that is aligned with the aforementioned paradigm shift *and* the current legislation. It requires far fewer animals than the *in vivo* TGR mutagenicity assay, is more rapid than conventional mutagenicity assays, and it directly measures chemically-induced increases in MF (i.e., an endpoint that is enshrined in current legislation).

It is important to note that some other recently developed *in vitro* assays are much higher throughput than the MutaMouse PH mutagenicity assay. However, these assays are based on DNA damage reporter systems, and they cannot assess an endpoint specified in the existing legislation (i.e., test article-induced changes in the frequency of mutations and/or chromosomal damage). For example, the ToxTracker® assay (Toxys B.V., Leiden, The Netherlands) utilizes fluorescent reporter cell lines to quantify activation of biomarkers for DNA damage, oxidative stress, and protein damage. Flow cytometric assessment of biomarker activation is used to identify genotoxicants and elucidate of mode of action [Hendriks et al., 2012]. The MultiFlow® assay (Litron Laboratories, Rochester, NY), which also employs flow cytometry, examines biomarkers indicative of clastogenicity and aneugenicity [Bryce et al., 2016]. The TGx-DDI toxicogenomic biomarker assay utilizes a 65-gene classifier to accurately differentiate DNA damage inducing (DDI) chemicals from non-genotoxic chemicals [Buick et al., 2015]. Finally, several US government agencies have collaborated on large-scale programmes (e.g., Tox21 and ToxCast) to develop and deploy other high-throughput *in vitro* reporter assays, and develop strategies to utilise *in vitro* signatures for *in vivo* hazard predictions [Huang et al., 2016; Judson et al., 2010]. However, since any changes to existing legislation will not likely be enacted in the near future, none of these assays or assay systems can currently be used for chemical safety assessment conducted in Canada, the

United States or the EU. Thus, although not high throughput *per se*, the relevance of the novel *in vitro* mutagenicity assay presented in this thesis is exemplified by its ability to assess an endpoint that is aligned with current legislative requirements.

### 6.3.3 Improved Mechanistic Understanding of Selected Azo Compounds

In addition to revealing information about the applicability domain of the *in vitro* MutaMouse PH mutagenicity assay, **Chapter 5** also presented results that offer new insight into the mechanisms of action of the mutagenic azo compounds Direct Black 38, Sudan I, and Para Red. None of these chemicals had previously been tested in a TGR mutagenicity assay, and Direct Black 38 lacked any *in vitro* genetic toxicity testing in mammalian cells. Little genetic toxicity data, either *in vitro* or *in vivo*, was available for Para Red. As such, this study generated novel *in vivo* and *in vitro* data that filled knowledge gaps regarding the genetic toxicity of these chemicals; moreover, the *in vivo* data specifically provide valuable information regarding mechanism of action and likely metabolic requirements. For example, following *in vivo* exposure, Direct Black 38 was found to be most mutagenic in the MutaMouse colon, followed by the bone marrow, glandular stomach and small intestine. The main mechanism of genotoxicity of Direct Black 38 is believed to involve the release of benzidine following azo bond cleavage mediated by intestinal microflora azoreductase activity [IARC, 2010; Cerniglia et al., 1982a; Cerniglia et al., 1982b; Bos et al., 1984; Manning et al., 1985; Bos et al., 1986]; the mutagenic effect on the gastrointestinal tract supports this mechanism of action. Sudan I was found to elicit mutagenic effects in the bone marrow and bladder of the MutaMouse. Unlike Direct Black 38, Sudan I is thought to require oxidative metabolism

mediated by either hepatic CYPs or bladder peroxidases to become DNA-reactive [Møller and Wallin, 2000; Stiborová et al., 2009]. The results in the bone marrow and bladder, combined with the absence of any effect in the colon or small intestine, again lend support to the proposed mechanism of action. Unlike Direct Black 38 and Sudan I, very little is known about the mechanism of action of Para Red, a structural analog of Sudan I, although there is some evidence to suggest that human intestinal microflora can convert Para Red into a bacterial mutagen *in vitro* [Xu et al., 2007; Aßmann et al., 1997]. Following *in vivo* exposure to Para Red, MutaMouse colon was the only tissue eliciting a mutagenic response, thus providing the first piece of evidence suggesting that intestinal azoreductase activity is required to metabolically activate Para Red *in vivo*. Overall, these results generated new information regarding the mechanism of action of these chemicals, and helped identify issues that require further study, e.g., elucidation of the precise mechanism of action of Para Red, and the causes underlying the lack of response to Sudan I in the *in vitro* MutaMouse PH mutagenicity assay.

## 6.4 Future Directions

In addition to the detailed comparative analysis regarding the utility of human liver S9 as a replacement for induced rat liver S9, the findings presented in **Chapter 2** raise some questions regarding the ability of human liver S9 to metabolically activate certain mutagens. As discussed earlier, the mutagenic potencies of AAs and DMN were generally higher in the presence of human liver S9 relative to Aroclor 1254-induced rat liver S9, although the opposite was true for every other class of chemical examined. The lower mutagenic potencies of AAs and DMN in the presence of Aroclor 1254-induced rat

liver S9 is counterintuitive, considering the extremely high metabolic enzyme activity of induced rat liver S9 relative to human liver S9, as measured by alkoxyresorufin-*O*-deethylase (AROD) activity. The AA trend has been observed in other studies [Beaune et al., 1985; Zeiger et al., 1979; Ong et al., 1980], and it was postulated (**Chapter 2)** that the different responses yielded by human and induced rat liver S9 were due to interspecies differences in the hepatic levels and activity of CYP1A1 and CYP1A2, combined with dramatic upregulation of CYP1A1 by agents such as Aroclor-1254. Indeed elevated CYP1A1 is known to lead to the detoxification of AAs in rats [Heflich and Neft, 1994]. The hypothesis that CYP1A1 level and activity underlie the AA observations could be tested by employing an antibody to specifically inhibit CYP1A1, but not CYP1A2, and comparing the mutagenic potency results obtained with human and Aroclor 1254-induced rat liver S9, both with and without inhibition. Additionally, due to the lack of correlation between human liver S9 ethoxyresorufin-*O*-deethylase (EROD) activity (i.e., CYP1A1/1A2 catalytic activity) and 2-aminoanthracine (2-AA) mutagenic potency (**Chapter 2)**, it is important that future work also compare other CYP activity values (e.g., phenacetin *O*-deethylation for CYP1A2) for human and induced rat S9. Once completed, this proposed work would permit a better understanding of the causes underlying the observed species-specific differences in the metabolic activation of mutagenic AAs.

In addition to an examination of the predictive capacity of the MutaMouse PH mutagenicity assay, **Chapter 4** also revealed a gap in the applicability domain that requires further investigation. As already noted, ICR 191, a direct-acting frameshift mutagen, elicited a negative response, and the lack of response may be related to MMR

proficiency. ICR 191 has been shown to selectively induce frameshift mutations in mismatch repair (MMR) deficient HCT116 human colon carcinoma cells, but not in MMR proficient HCT116+C3 cells [W. Chen et al., 2000]. MMR enzymes, specifically human MutSα, which is a heterodimer involving MutS protein homolog 2 (MSH2) and MutS protein homolog 6 (MSH6), have been shown to directly recognize and bind to drug-DNA cross-links, thus their activity may be crucial for the repair of ICR 191-induced lesions [Duckett et al., 1996]. There is evidence to suggest that proliferating hepatocytes have high levels of MSH2 expression relative to non-proliferating hepatocytes [Igoucheva et al., 2008]. Moreover, MSH2 and MSH6, as well as MutS protein homolog 3 (MSH3), another MMR-related protein, were found to have intermediate protein levels in mouse liver *in vivo* relative to other tissues, thus implying that freshly isolated murine PHs are likely MMR-proficient [Tomé et al., 2013]. Interestingly, ICR 191 has been shown to be mutagenic in FE1 cells, and these cells have been shown to have reduced MSH2 gene expression with respect to MutaMouse lung tissue *in vivo* [White et al., 2003; Berndt-Weis et al., 2009]. Thus, the specified gap in the applicability domain may well be related to MMR deficiency, and the notion is worthy of further investigation.

The MMR proficiency of cultured MutaMouse PHs could be investigated by examining the gene expression of key MMR enzymes (i.e., MSH2, MSH3, MSH6, etc.) using qPCR or RNA sequencing, and the levels could be compared with MutaMouse liver *in vivo*. To observe the effects of ablated MMR proficiency on ICR 191 mutagenicity in MutaMouse PHs *in vitro*, small interfering RNAs (siRNAs) targeting MSH2 or MSH6 could be employed. Alternatively, the construction of MutaMouse PH

knock-outs could be considered. The TK6 Mutants Consortium has endeavoured to investigate the roles of genes involved in DNA repair, replication, and recombination in mutation induction by generating more than 130 mutant knock-out and knock-in strains of human TK6 cells [Yasui, 2019]. Unfortunately, the use of an analogous knock-out/knock-in approach may not be feasible for MutaMouse PHs since they can only be cultured for a few days following isolation. Interestingly, other studies have demonstrated the utility of TGR strains lacking DNA repair genes for investigation of mutagenic mechanisms [van Oostrom et al., 1999; Stancel et al., 2009], and the effects of MMR deficiency on ICR 191 mutagenicity could be investigated by deriving PHs from a TGR mouse strain that lacks a key MMR gene such as MSH2 or MSH6. A knock-out TGR mouse with ablated MMR activity does not yet exist, but it could be constructed using CRISPR/Cas9-mediated gene modification technology, or by crossing a TGR such as the MutaMouse with an existing MMR knockout mouse [Lee et al., 2016; Zuo et al., 2017]. Such a mouse strain would not only be useful for examining ICR 191 mutagenicity, it would also permit investigations regarding the role of MMR in the mutagenicity of other chemical classes. Collectively, these types of studies would contribute to a better understanding of the applicability domain of the *in vitro* MutaMouse PH mutagenicity assay, and moreover, shed light on the ability of the assay to accurately detect other intercalating frameshift mutagens.

In addition to the DNA repair considerations outlined above, it is important to recognise that the negative response to ICR 191 may be related to metabolic activity. De Flora et al. demonstrated that the mutagenicity if ICR 191 mutagenicity is significantly reduced in the presence of murine S9, and that hydroxylated ICR 191 is far less potent

than the parent compound [De Flora et al., 1982]. Therefore, CYP-mediated reactions could be playing a role in detoxification, and the effect of detoxification on ICR 191 mutagenicity should be more thoroughly investigated. More specifically, the role of CYP-mediated reactions could be investigated by employing specific metabolic enzyme inhibitors, such as furafylline for CYP1A2 and ketoconazole for CYP3A, and observing any attendant increases in mutagenicity [Donato et al., 1993].

Although this thesis makes an excellent case for the use of MutaMouse PHs in an *in vitro* mutagenicity assay, more work is required to comprehensively validate the test, as stipulated by the OECD validation guidance document [OECD, 2005]. For example, although the results presented in **Chapter 4** indicate that the test has a very good predictive capacity, further study is required to better elucidate assay sensitivity and specificity. Specifically, a larger panel of both mutagenic and non-mutagenic chemicals, including *in vitro* false positive chemicals, should be examined, such as those recommended by EURL-ECVAM [D. Kirkland et al., 2008; D. Kirkland et al., 2016]. Following, or potentially concurrently during this investigation, the intra-laboratory repeatability and reproducibility of the test can be assessed by evaluating the variability of results between different operators and different dates within the same laboratory. A transferability study could then be commenced, wherein cells and protocols are transferred to a naïve lab to obtain feedback. At each of these stages, it may be necessary to further refine or optimize the assay according to the feedback received. Eventually, once the method has accumulated a sufficient amount of evidence attesting to its robustness, reliability, and transferability, a formal inter-laboratory variability study can be commenced. Each of these steps must be completed prior to the drafting and

submission of a Standard Project Submission Form (SPSF), which is required for the *in vitro* MutaMouse mutagenicity assay to be considered by the OECD for a new TG.

Transferability is a significant hurdle that must be overcome in order to accomplish the validation steps described above. The studies presented in this thesis all made use of freshly isolated MutaMouse PHs; however, to facilitate distribution to other laboratories, it is imperative that a cryopreservation protocol be developed. Luijten et al. [2016] demonstrated that thawed, cryopreserved pUR288 lacZ Plasmid Mouse PHs can be reliably used in an *in vitro* mutagenicity assay. In order to establish a similar method, MutaMouse PHs could be cryopreserved according to the Luijten protocol, and their metabolic competence and assay performance assessed. Once a reliable cryopreservation protocol is established, further intra- and inter-laboratory studies, as discussed above, can commence.

In addition to their use as a tool for *in vitro* mutagenicity assessment, MutaMouse PHs could be employed for the detection of other genetic toxicity endpoints, e.g., induction of MN as an indicator of chromosomal damage. MN assays based on rat PHs have been developed, using both manual and flow cytometric counting methods [Muller-Tegethoff et al., 1997; Eckl and Raffelsberger, 1997]. Indeed, the flow cytometry-based measures of proliferation (i.e., RINC) presented in **Chapters 3** and **4** were adapted from the techniques employed by Litron Laboratories (Rochester, NY) for their Microflow™ assay that measures MN frequency [Avlasevich et al., 2006; Bryce et al., 2007; Bryce et al., 2008]. Since the flow cytometry techniques have been optimized for MutaMouse PHs, it follows that an MN technique could readily be developed. An MN assay utilizing MutaMouse PHs would permit convenient measurement of two genotoxicity endpoints in

the same metabolically competent cells; moreover, two endpoints that are specified in existing chemical safety legislation.

Over the course of this thesis, the applicability domain of the *in vitro* MutaMouse PH mutagenicity assay has been shown to include chemicals that require oxidation, nitro-reduction, azo-reduction, and conjugation reactions for metabolic activation; however, the results presented (**Chapter 5)** raised questions concerning aspects of the applicability domain that require further study. An assessment of both metabolic enzyme gene expression and catalytic activity revealed that MutaMouse PHs have Phase I and Phase II metabolic competence. Additionally, the *in vitro* MutaMouse PH mutagenicity assay yielded positive results for chemicals requiring CYP-mediated oxidation reactions, such as BaP and AFB1. Despite these observations, Sudan I did not elicit a significant increase in MF in MutaMouse PHs exposed *in vitro*, despite the fact that it has been shown to elicit a positive response in AHH-1 and MCL-5 cells in the *in vitro Hprt* mutagenicity mutation assay [Johnson et al., 2010]. In **Chapter 5**, it was postulated that this lack of response may be due to the detoxification of Sudan I in MutaMouse PHs, potentially through the induction of CYP1A1 and NQO1. In order to better understand the utility of PHs for the evaluation of Sudan I-type azo compounds, and to gain a better understanding of potential detoxification pathways, this hypothesis could be tested through the use of CYP1A1 and NQO1 inhibitors. The continued investigation into the applicability domain of this assay will also require testing of additional well-studied compounds with existing *in vivo* and *in vitro* data, including additional azo compounds.

As discussed earlier, (geno)toxicity testing is shifting towards reliance on higher throughput methodologies. Unfortunately, neither the *in vitro* MutaMouse PH

mutagenicity assay, nor the other conventional mammalian cell mutagenicity assays, is amenable to a high-throughput format. The development of novel next generation sequencing (NGS) approaches, however, will almost certainly supersede the need for these more time-consuming assays and pave the way to higher throughput mutagenicity assessment. NGS techniques are far quicker and more cost-effective than traditional Sanger sequencing methods; however, their utility for enumerating rare chemically-induced mutations is compromised by their relatively high error rates. Thus, although NGS has been used for high-throughput, high-depth sequencing of *lacZ* mutations for mutation spectra determination, they have not been successfully used for accurate enumeration of mutations [Beal et al., 2015]. Nevertheless, new NGS approaches currently under development, such as error-corrected sequencing, are gradually reducing the error rates of NGS techniques, and bringing the notion of NGS-mediated mutagenicity assessments closer to reality [Wong et al., 2018]. More specifically, NGS technologies such as Duplex Sequencing™ (TwinStrand Biosciences, Seattle, WA), which employ advanced NGS and bioinformatics, can accurately determine the frequency of rare genetic changes (i.e., $<10^{-5}$). Indeed, the use of error-corrected sequencing for enumeration of mutations in tissues of mutagen-exposed Big Blue® rats has yielded promising results. Recent research conducted by TwinStrand Bioscience has revealed that λ *cII* MF determined using standard shuttle vector-based methods are very well correlated with MF determined using error-corrected Duplex Sequencing™. Moreover, the TwinStrand group, in collaboration with BioReliance Corporation (Rockville, MD) has observed that the Duplex Sequencing™ technology can reliably be used to enumerate mutations at a variety of endogenous loci (Bob Young, Bioreliance Corporation,

unpublished). Therefore, error-corrected NGS will likely eventually eliminate the need for TGRs, allowing assessment of mutation frequency at any locus in any tissue of any organism. This would include humans, and the technology could be used to determine MF in humans exposed to mutagenic agents in environmental and/or occupational settings. Nevertheless, PHs will still be an extremely useful genetic toxicology tool, due in large part to their normal karyotype and phenotype, and their endogenous metabolic capacity. Moreover, innovative culture techniques for PH and PH-like cells (e.g., HepaRG cells), such as co-culture and 3D culture, that maintain *in vivo*-like phenotypes and metabolic enzyme expression patterns for extended periods of time are already demonstrating considerable promise [Chan et al., 2013; Godoy et al., 2013; Ramaiahgari et al., 2017]. When combined with high fidelity NGS mutation frequency determination, they will likely prove to be even more useful in the longer term.

## 6.5 Concluding Remarks

This thesis presents an original evaluation of strategies regarding the incorporation of mammalian metabolism into *in vitro* mutagenicity assays. It starts with an investigation into the utility of human liver S9 as an exogenous source of mammalian metabolic enzymes, i.e., as a replacement for the currently advocated induced rat liver S9. Subsequently, it includes work dedicated to the development of a novel *in vitro* mutagenicity assay based on cells with endogenous metabolic capacity. Human liver S9 was shown to be inappropriate for use in routine genetic toxicity screening. With this conclusion, and the deficiencies regarding the utility of hepatic S9 in general (i.e., human- or rodent-derived) for exogenous metabolic activation of chemical mutagens, the

thesis shifted its focus towards a novel strategy utilizing cells with endogenous metabolic capacity, i.e., an *in vitro* mutagenicity assay utilizing PHs from the TGR known as MutaMouse. The thesis includes unprecedented structural, genetic, proliferative, and enzymatic analysis of cultured MutaMouse PHs, demonstrating that they are well suited for use in an *in vitro* mutagenicity assay. Their utility was assessed in a validation exercise that examined a set of test chemicals; it verified that the *in vitro* MutaMouse PH mutagenicity assay is capable of detecting mutagens that require metabolic activation via a diverse array of pathways. More specifically, the results show that the assay is able to detect mutagenic chemicals requiring oxidation, nitro-reduction, azo-reduction, and conjugation reactions for metabolic activation, without the necessity to add an exogenous source of enzymes (e.g., rodent-derived hepatic S9). This comprehensive ability to assess mutagenicity is unparalleled for an *in vitro* mammalian cell-based assay. The assay's performance was further exemplified by the lack of response for non-mutagens, including non-DNA-reactive chemicals that frequently yield positive results in other *in vitro* mammalian cell-based tests. Through a comparison of *in vivo* TGR and *in vitro* PH results for selected azo compounds, the results also provide novel information regarding the mechanism of action of such compounds. The mechanisms of action for Direct Black 38 and Sudan I were supported, and new evidence supporting reductive metabolism as a metabolic requirement for the mutagenicity of Para Red was revealed. Overall, the thesis demonstrates that a mutagenicity assay based on MutaMouse PHs, i.e., cells with endogenous metabolic competence and a transgenic reporter for convenient mutation scoring, hold great promise for routine assessments of chemical mutagenicity; moreover,

assessments of chemical mutagenicity that are aligned with current legislative requirements.

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# APPENDIX

# Appendix

## Appendix A : Supplementary Material

### A.1 Chapter 1

(none)

## A.2 Chapter 2

**Supplementary Table 2-I**: Summary of literature used to review published Ames test results

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| 2-Acetylaminofluorene | TA100 | Human | | [1-3] |
| | | Rat | Uninduced | [2] |
| | | | TCDD[a] | [3] |
| | | | PB/NF[b] | [2, 3] |
| | | | Aroclor 1254 | [4-11] |
| | TA1538 | Human | | [12-15] |
| | | Rat | Uninduced | [13] |
| | | | PB/NF | [15] |
| | | Hamster | Uninduced | [12] |
| | | Mouse | Uninduced | [12] |
| | | Pig | Uninduced | [12] |
| | TA98 | Human | | [16-18] |
| | | Rat | Uninduced | [17] |
| | | | Aroclor 1254 | [4, 6-10, 18-23] |
| | | Guinea pig | Uninduced | [17] |
| | | Rabbit | Uninduced | [17] |
| Acrylamide | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Acrylonitrile | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Aflatoxin B1 | TA100 | Human | | [1-3, 24-26] |
| | | Rat | Uninduced | [2, 24, 25] |
| | | | TCDD | [1] |
| | | | PB/NF | [2, 3] |
| | | | PCB[c] | [27] |
| | | | Aroclor 1254 | [4, 6, 22, 23, 25, 28-32] |
| | TA98 | Human | | [25, 26, 33, 34] |
| | | Rat | Uninduced | [25] |
| | | | Aroclor 1254 | [4, 7, 25, 31, 32, 35-40] |
| 2-Aminoanthracene | TA100 | Human | | [2, 3, 12, 18, 25] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | Rat | Uninduced | [2, 12] |
| | | | PB/NF | [2, 3] |
| | | | Aroclor 1254 | [5, 18, 25, 41-48] |
| | | Mouse | Uninduced | [12] |
| | | Pig | Uninduced | [12] |
| | TA1538 | Human | | [15] |
| | | Rat | PB/NF | [15] |
| | TA98 | Human | | [25, 49] |
| | | Rat | Uninduced | [25, 49] |
| | | | Aroclor 1254 | [25, 49] |
| | | Dog | Uninduced | [49] |
| | | Hamster | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| | | Monkey | Uninduced | [49] |
| | | Mouse | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| 4-Aminoazobenzene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *o*-Aminoazotoluene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 4-Aminobiphenyl | TA1538 | Human | | [12, 15] |
| | | Rat | Uninduced | [12] |
| | | | PB/NF | [15] |
| | | Hamster | Uninduced | [12] |
| | | Mouse | Uninduced | [12] |
| | | Pig | Uninduced | [12] |
| 6-Aminochrysene | TA100 | Human | | [2, 3] |
| | | Rat | Uninduced | [2] |
| | | | PB/NF | [2, 3] |
| 2-Aminofluorene | TA100 | Human | | [3, 25] |
| | | Rat | Uninduced | [25] |
| | | | PB/NF | [3] |
| | | | Aroclor 1254 | [7, 11, 25, 50-55] |
| | TA1538 | Human | | [12, 13, 15] |
| | | Rat | Uninduced | [12, 13] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | | PB/NF | [15] |
| | | Hamster | Uninduced | [12] |
| | | Mouse | Uninduced | [12] |
| | | Pig | Uninduced | [12] |
| | TA98 | Human | | [16, 25] |
| | | Rat | Uninduced | [25] |
| | | | Aroclor 1254 | [7, 11, 19, 21, 25, 35, 38, 40, 50, 51, 54-60] |
| 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| | TA98 | Human | | [61] |
| 2-Amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) | TA98 | Human | | [62] |
| 2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole acetate (MeAαC acetate) | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P2) | TA98 | Human | | [2, 62] † |
| | | Rat | Uninduced | [2] † |
| | | | PB/NF | [2, 63, 64] |
| | | | PCB | [65] |
| | | | Aroclor 1254 | [66-68] † |
| 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole acetate (Trp-P2 acetate) | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 2-Amino-3-methylimidazo(4,5-*f*)quinoline (IQ) | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| | TA98 | Human | | [2, 69-72] |
| | | Rat | Uninduced | [2, 69] |
| | | | PB/NF | [70, 71, 73] |
| | | | PCB | [74] |
| | | | Aroclor 1254 | [20, 69, 75-85] |
| 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| | TA98 | Human | | [2, 62] † |
| | | Rat | Uninduced | [2] † |
| | | | PB/NF | [2, 63, 86] |
| | | | PCB | [65] |
| | | | Aroclor 1254 | [66-68, 87-91] † |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| 1-Aminopyrene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Auramine | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 10-Azabenzo[*a*]pyrene | TA100 | Human | | [92] |
| | | Rat | PCB | [92] |
| Azathioprine | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Azobenzene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Beef extract | TA1538 | Human | | [93] |
| | | Rat | PCB | [93] |
| Benzidine | TA1538 | Human | | [12-14] |
| | | Rat | Uninduced | [12, 14] |
| | | | Aroclor 1254 | [14] |
| | | Hamster | Uninduced | [12, 14] |
| | | | Aroclor 1254 | [14] |
| | | Mouse | Uninduced | [12] |
| | | Pig | Uninduced | [12] |
| | TA98 | Human | | [49] |
| | | Rat | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| | | Dog | Uninduced | [49] |
| | | Hamster | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| | | Monkey | Uninduced | [49] |
| | | Mouse | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| Benz[*a*]anthracene | TA100 | Human | | [3, 94] |
| | | Rat | Uninduced | [94] |
| | | | PB/NF | [3] |
| | | Hamster | Uninduced | [94] |
| | | Mouse | Uninduced | [94] |
| | | Pig | Uninduced | [94] |
| Benzo[*a*]pyrene | TA100 | Human | | [1-3, 13, 70, 71, 92, 94] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | Rat | Uninduced | [2, 13, 94] |
| | | | TCDD | [1] |
| | | | PB/NF | [2, 3, 70, 71] |
| | | | PCB | [92] |
| | | | Aroclor 1254 | [4-6, 28, 48, 51, 52, 54-56, 95-97] |
| | | Hamster | Uninduced | [94] |
| | | Mouse | Uninduced | [94] |
| | | Pig | Uninduced | [94] |
| | TA1538 | Human | | [14, 98] |
| | | Rat | Uninduced | [14, 98] |
| | | | 3-MC[d] | [98] |
| | | | PB[e] | [98] |
| | | | Aroclor 1254 | [14] |
| | | Hamster | Uninduced | [14] |
| | | | Aroclor 1254 | [14] |
| | TA98 | Human | | [99] |
| | | Rat | Uninduced | [99] |
| | | | Aroclor 1254 | [99] |
| Benzo[*e*]pyrene | TA100 | Human | | [2] |
| | | Rat | Uninduced | [2] |
| | | | PB/NF | [2] |
| Chloramphenicol | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Chrysene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Congo Red | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Cyclophosphamide | TA1535 | Human | | [13] |
| Cigarette smoke condensate | TA1538 | Human | | [25, 98] |
| | | Rat | Uninduced | [25, 98] |
| | | | 3MC | [98] |
| | | | PB | [98] |
| | | | Aroclor 1254 | [25, 100] |
| Coal tar | TA98 | Human | | [99] |
| | | Rat | Uninduced | [99] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | | Aroclor 1254 | [99] |
| Dacarbazine | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 2,4-Diaminoanisole | TA98 | Human | | [16] |
| 2,4-Diamino-3-methylazobenzene | TA100 | Human | | [101] |
| | | Rat | Uninduced | [101] |
| | | | PB | [101] |
| | | | NF[†] | [101] |
| 2,4-Diamino-5-methylazobenzene | TA100 | Human | | [101] |
| | | Rat | Uninduced | [101] |
| | | | PB | [101] |
| | | | NF | [101] |
| 2,4-Diaminotoluene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Dibenz[*a,c*]anthracene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Dibenz[*a,h*]anthracene | TA100 | Human | | [3, 94] |
| | | Rat | Uninduced | [94] |
| | | | PB/NF | [3] |
| | | Hamster | Uninduced | [94] |
| | | Mouse | Uninduced | [94] |
| | | Pig | Uninduced | [94] |
| Dibenzo[*a,i*]pyrene | TA100 | Human | | [94] |
| | | Rat | Uninduced | [94] |
| | | Hamster | Uninduced | [94] |
| | | Mouse | Uninduced | [94] |
| | | Pig | Uninduced | [94] |
| 3,3'-Dichlorobenzidine 2HCl | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 1,4-Dichlorobutene-2 | TA100 | Human | | [24] |
| | | Mouse | Uninduced | [24] |
| Diethylnitrosamine | YG7108 | Human | | [2, 3] |
| | | Rat | Uninduced | [2] |
| | | | PB/NF | [2, 3] |
| *N,N*-Dimethyl-4-aminoazobenzene | TA100 | Human | | [3] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | Rat | PB/NF | [3] |
| 7,12-Dimethylbenz[*a*]anthracene | TA100 | Human | | [3, 13, 94] |
| | | Rat | Uninduced | [13, 94] |
| | | | PB/NF | [3] |
| | | Hamster | | [94] |
| | | Mouse | | [94] |
| | | Pig | | [94] |
| 7,9-Dimethylbenz[*c*]acridine | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 1,2-Dimethylhydrazine 2HCl | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Dimethylnitrosamine | TA100 | Human | | [102] |
| | | Rat | Uninduced | [102] |
| | | Hamster | Uninduced | [102] |
| | | Mouse | Uninduced | [102] |
| | | Pig | Uninduced | [102] |
| | YG7108 | Human | | [2, 3, 70] |
| | | Rat | Uninduced | [2] |
| | | | PB/NF | [2, 3, 70, 71] |
| 2,4-Dinitrotoluene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Dipropylnitrosamine | TA100 | Human | | [102] |
| | | Rat | Uninduced | [102] |
| | | Hamster | Uninduced | [102] |
| | | Mouse | Uninduced | [102] |
| | | Pig | Uninduced | [102] |
| Ethyl carbamate | TA100 | Human | | [2] |
| | | Rat | Uninduced | [2] |
| | | | PB/NF | [2] |
| Furylfuramide | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Hydrazine 2HCl | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Hydroxyethylhydrazine | TA1535 | Human | | [13] |
| 3-Methylcholanthrene | TA100 | Human | | [1, 3, 13, 25, 94] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | Rat | Uninduced | [13, 25, 94] |
| | | | PB/NF | [3] |
| | | | Aroclor 1254 | [4, 25, 36, 57, 97, 103-107] |
| | | Hamster | Uninduced | [1, 94] |
| | | Mouse | Uninduced | [94] |
| | | Pig | Uninduced | [94] |
| | TA1538 | Human | | [14] |
| | | Rat | Uninduced | [14] |
| | | | Aroclor 1254 | [14] |
| | | Hamster | Uninduced | [14] |
| | | | Aroclor 1254 | [14] |
| | TA98 | Human | | [25] |
| | | Rat | Uninduced | [25] |
| | | | Aroclor 1254 | [25] |
| 2'-Methyl-2,4-diamino-3-methylazobenzene | TA100 | Human | | [101] |
| | | Rat | Uninduced | [101] |
| | | | PB | [101] |
| | | | NF | [101] |
| 2'-Methyl-2,4-diamino-5-methylazobenzene | TA100 | Human | | [101] |
| | | Rat | Uninduced | [101] |
| | | | PB | [101] |
| | | | NF | [101] |
| 4,4'-Methylene-bis-(2-chlorobenzeneamine) | TA100 | Human | | [108] |
| | | Rat | Aroclor 1254 | [108] |
| | | Dog | Uninduced | [108] |
| | | Mouse | Uninduced | [108] |
| 4,4'-Methylenedianiline | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Methylethylnitrosamine | TA100 | Human | | [102] |
| | | Rat | Uninduced | [102] |
| | | Hamster | Uninduced | [102] |
| | | Mouse | Uninduced | [102] |
| | | Pig | Uninduced | [102] |
| 4-Methylquinoline | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| Methyl *tert*-butylether | TA102 | Human | | [109] |
| | | Rat | Aroclor 1254 | [109] |
| Metronidazole | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 1-Naphthylamine HCl | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 2-Naphthylamine | TA100 | Human | | [1, 12] |
| | | Rat | Uninduced | [12] |
| | | | TCDD | [1] |
| | | Hamster | Uninduced | [12] |
| | | Mouse | Uninduced | [12] |
| | | Pig | Uninduced | [12] |
| | TA1538 | Human | | [14] |
| | | Rat | Uninduced | [14] |
| | | | Aroclor 1254 | [14] |
| | | Hamster | Uninduced | [14] |
| | | | Aroclor 1254 | [14] |
| 4-Nitrobiphenyl | TA100 | Human | | [1] |
| | | Rat | TCDD | [1] |
| 2-Nitrofluorene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 1-Nitronaphthalene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| 1-Nitropyrene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| | TA98 | Human | | [2] |
| | | Rat | Uninduced | [2] |
| | | | PB/NF | [2] |
| 4-Nitroquinoline-*N*-oxide | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *N*-Nitrosobis(2-acetoxypropyl)amine | TA100 | Human | | [110] |
| | | Rat | Uninduced | [110] |
| | | Hamster | Uninduced | [110] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | Rabbit | Uninduced | [110] |
| *N*-Nitrosobis(2-hydroxypropyl)amine | TA100 | Human | | [110] |
| | | Rat | Uninduced | [110] |
| | | Hamster | Uninduced | [110] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110] |
| | | Rabbit | Uninduced | [110] |
| *N*-nitrosobis(2-oxopropyl)amine | TA100 | Human | | [110] |
| | | Rat | Uninduced | [110] |
| | | Hamster | Uninduced | [110] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110] |
| | | Rabbit | Uninduced | [110] |
| *N*-Nitroso-di-n-butylamine | YG7108 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *N*-nitroso-2,6-dimethylmorpholine | TA100 | Human | | [110] |
| | | Rat | Uninduced | [110] |
| | | Hamster | Uninduced | [110] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110] |
| | | Rabbit | Uninduced | [110] |
| *N*-Nitroso-di-n-propylamine | YG7108 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine | TA100 | Human | | [110] |
| | | Rat | Uninduced | [110] |
| | | Hamster | Uninduced | [110] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110] |
| | | Rabbit | Uninduced | [110] |
| *N*-nitrosomethyl(2-hydroxypropyl)amine | TA100 | Human | | [110-112] |
| | | Rat | Uninduced | [110, 112] |
| | | | 3MC | [112] |
| | | | PB | [112] |
| | | | PCB | [112] |
| | | Hamster | Uninduced | [110, 112] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| | | | 3MC | [112] |
| | | | PB | [112] |
| | | | PCB | [112] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110, 112] |
| | | | 3MC | [112] |
| | | | PB | [112] |
| | | | PCB | [112] |
| | | Rabbit | Uninduced | [110] |
| *N*-Nitroso-*N*'-methylpiperazine | TA1530 | Human | | [24] |
| | | Rat | Uninduced | [24] |
| *N*-Nitrosomorpholine | YG7108 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *N*-nitrosomethyl(2-oxopropyl)amine | TA100 | Human | | [110] |
| | | Rat | Uninduced | [110] |
| | | Hamster | Uninduced | [110] |
| | | Monkey | Uninduced | [110] |
| | | Mouse | Uninduced | [110] |
| | | Rabbit | Uninduced | [110] |
| *N*-Nitrosopiperidine | TA100 | Human | | [102] |
| | | Rat | Uninduced | [102] |
| | | Hamster | Uninduced | [102] |
| | | Mouse | Uninduced | [102] |
| | | Pig | Uninduced | [102] |
| | TA1530 | Human | | [24] |
| | | Rat | Uninduced | [24] |
| *N*-Nitrosopyrrolidine | TA100 | Human | | [102] |
| | | Rat | Uninduced | [102] |
| | | Hamster | Uninduced | [102] |
| | | Mouse | Uninduced | [102] |
| | | Pig | Uninduced | [102] |
| | TA1530 | Human | | [24] |
| | | Rat | Uninduced | [24] |
| Phenanthrene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| 1,7-Phenanthroline | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *o*-Phenylenediamine | TA98 | Human | | [49] |
| | | Rat | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| | | Dog | Uninduced | [49] |
| | | Hamster | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| | | Monkey | Uninduced | [49] |
| | | Mouse | Uninduced | [49] |
| | | | Aroclor 1254 | [49] |
| Pyrene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Pyrvinium pamoate | TA100 | Human | | [18] |
| | | Rat | Aroclor 1254 | [18] |
| | TA98 | Human | | [18] |
| | | Rat | Aroclor 1254 | [18] |
| Quercetin | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Quinoline | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Safrole | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Sodium nitrate | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Sodium nitrite | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Sterigmatocystin | TA100 | Human | | [1] |
| | | Rat | TCDD | [1] |
| Styrene | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| *o*-Tolidine | TA100 | Human | | [3] |
| | | Rat | PB/NF | [3] |
| Trypan Blue | TA98 | Human | | [3] |
| | | Rat | PB/NF | [3] |

| Compound | Strain | S9 species | Inducer | Reference(s) |
|---|---|---|---|---|
| 2,6-Xylidine | TA100 | Human | | [113] |
| | | Rat | Aroclor 1254 | [113] |
| | TA1535 | Human | | [113] |
| | | Rat | Aroclor 1254 | [113] |
| | TA1537 | Human | | [113] |
| | | Rat | Aroclor 1254 | [113] |
| | TA98 | Human | | [113] |
| | | Rat | Aroclor 1254 | [113] |

[a] TCDD, 2,3,7,8-Tetrachlorodibenzodioxin
[b] PB/NF, Phenobarbital/β-Naphthaflavone
[c] PCB, Polychlorinated biphenyls (unspecified)
[d] 3-MC, 3-Methylcholanthrene
[e] PB, Phenobarbital
[f] NF, β-Naphthaflavone
† Additional results were experimentally added for this study

**Supplementary Table 2-II**: : Summary of literature used to review other *in vitro* genotoxicity results

| Compound | In vitro assay | Cell type | Reference |
|---|---|---|---|
| 2-Acetylaminofluorene | SCE[a] | CHO[b] | [114] |
| | CA[c] | CHO | [114] |
| Aflatoxin B1 | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| | SCE | CHO | [114] |
| | | Chinese hamster fibroblasts | [116] |
| | CA | CHO | [114] |
| | | Chinese hamster fibroblasts | [116] |
| | *Hprt*[d] | CHO | [117] |
| | MLA[e] | L5178Y | [118, 119] |
| 2-Aminoanthracene | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 2-Amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P1) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 2-Amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 2-Amino-3-methylimidazo(4,5-*f*)quinoline (IQ) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P2) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) | *umu* assay | *S. typhimurium* TA1535/pSK1002 | [115] |
| Benzo[*a*]pyrene | SCE | CHO | [114] |
| | CA | CHO | [114] |
| Commercial kava samples | MLA | L5178Y | [118] |
| Coumarin | *Hprt* | CHO | [117] |
| Cyclophosphamide | SCE | Chinese hamster fibroblasts | [116] |
| | CA | Chinese hamster fibroblasts | [116] |
| Diacetyl | MLA | L5178Y | [119] |
| Diethylnitrosamine | SCE | CHO | [114] |
| | CA | CHO | [114] |
| Dimethylnitrosamine | SCE | CHO | [114] |
| | | Chinese hamster fibroblasts | [116] |
| | CA | CHO | [114] |
| | | Chinese hamster fibroblasts | [116] |
| Fern spore extracts | *In vitro* comet | Human premyeloid leukaemia (K562) cells | [120] |

| Compound | In vitro assay | Cell type | Reference |
|---|---|---|---|
| Kavalactones | MLA | L5178Y | [118] |
| Thalidomide | CA | CHO | [121] |

[a] SCE, Sister chromatid exchange
[b] CHO, Chinese hamster ovary cells
[c] CA, Chromosome aberration
[d] *Hprt*, X-linked hypoxanthine-guanine phosphoribosyltransferase
[e] MLA, Mouse lymphoma assay

**Human Liver S9 Usage Questionnaire**

1. Have you ever used human liver S9 in any *in vitro* genotoxicity test? (24 respondents)
    a. Yes (7)
    b. No (17)
2. If you do not use human liver S9 regularly, what is your reason for not using human liver S9 for routine genotoxicity testing? (24 respondants)
    a. Poor reproducibility due to large differences between lots of human liver S9, relative to induced rat liver S9. (3)
    b. Low activity of drug metabolizing enzymes in human liver S9, relative to induced rat liver S9. (2)
    c. Restrictive experimental environments do not allow the use of human liver S9 as a clinical sample. (1)
    d. Genotoxicity of metabolites are assessed using synthesized metabolites. (4)
    e. No need to evaluate the genotoxicity of human-specific metabolites. (9)
    f. Little to no information on the usefulness of human liver S9. (3)
    g. Cost. (0)
    h. Difficult to obtain the required volumes of a desired lot of human liver S9. (0)
    i. Difficult to obtain human liver S9 in a timely fashion. (0)
    j. Other (0)
3. Other than induced rat liver S9 and/or human liver S9, do you have any experience using other metabolic activation systems? (23 respondents)
    a. Rat liver microsomes (3)
    b. Human liver microsomes (3)
    c. Rat organ microsomes (other than liver) (1)
    d. Human organ microsomes (other than liver) (0)
    e. Cell line (e.g., HepG2 or HepaRG) (2)
    f. Engineered bacteria expressing CYPs or other drug-metabolizing enzymes (2)
    g. Others (Hamster S9) (2)
    h. No experience with other metabolic activation systems (14)
4. What is your opinion regarding genotoxicity assessments performed using human liver S9?

    - Poor reproducibility between lots or experiments
        - Differences between lots and lower sensitivity relative to rat S9.
    - Unstable supply

- Unknown effectiveness
    - Human S9 is just human material. It does not necessarily mean human S9 is superior than rat S9.
    - The availability of human S9 is limited in a case where the metabolite cannot be produced chemically and is not produced by rat S9.
- Lack of phase II drug-metabolizing enzymes

5. What is your opinion regarding the predictive capacity of human metabolites?
    - The prediction using human CYP enzymes, un-induced rat S9 or induced rat S9 was useful in an early development.
    - FDA draft guidance says the subfraction like S9 and microsomes may be useful to predict human metabolism in addition to the primary hepatocytes or liver slices. The metabolizing enzymes other than CYP are also important as for the genotoxic assessment.
    - Currently, iPS cells has become possible to differentiate liver cells. Although it can not be used in a regulatory sciences now, we should consider whether it in the future
6. Where do you obtain human liver S9? (Multiple answers allowed) (7 respondents)
    a. Human and Animal Bridging Research Organization (HAB) (3)
    b. KAC Company Limited (Japanese supplier) (1)
    c. BD Biosciences (1)
    d. Prepared in-house or obtained from affiliate companies in Europe or the US (0)
    e. Other (Xenotech or Nosan Corporation) (3)
7. What is the most important consideration when choosing a supplier? (Multiple answers allowed) (7 respondents)
    a. Experience with in-house studies (1)
    b. Literature in related journals and/or oral presentations at scientific meetings (3)
    c. Easily obtainable in Japan (2)
    d. Recommended by drug metabolism and pharmacokinetics department (2)
    e. Prepared in-house or obtained from affiliate companies in Europe or the US (0)
    f. Other (recommended by commercial supplier) (1)
8. Do you prefer using human liver S9 from a single donor or pooled from multiple donors? (7 respondents)

a. Single donor, with consideration given to high enzymatic activity (0)
b. Pooled from several donors (6)
c. Both S9 from single donors and S9 pooled from multiple donors (1)
d. Other (0)

9. Which tests do you perform using human liver S9? (Multiple answers allowed) (7 respondents)
    a. Ames test (3)
    b. *In vitro* cytogenetic assay (2)
    c. Mouse lymphoma assay (1)
    d. Measurement of metabolite concentrations in the Ames test (4)
    e. Measurement of metabolite concentrations in *in vitro* cytogenetic assay (6)
    f. Measurement of metabolite concentrations in the mouse lymphoma assay (3)
    g. Other (0)
10. Have you used human liver S9 in a GLP study? (7 respondents)
    a. Yes (4)
    b. No (3)
    c. In a GLP study including a non-GLP procedure (0)
11. Why did you choose to use human liver S9? (7 respondents)
    a. Voluntarily to assess human-specific metabolites (1)
    b. Regulators asked to assess human-specific metabolites (0)
    c. To synthesize a human metabolite that cannot be synthesized by rat liver S9 (2)
    d. To add to the weight of evidence given that previous tests using induced rat liver S9 were positive (3)
    e. To fulfill a request from a sponsor (0)
    f. Other ( To compare the results with human S9 with that in bacteria expressing human CYP) (1)
12. What drawbacks have you encountered when using human liver S9? (Multiple answers allowed) (7 respondents)
    a. Pre-treatment of human liver S9 (e.g., defatting) (0)
    b. Differences between lots for drug-metabolizing enzyme activities and for individual test results (2)
    c. Differences between experiments for drug-metabolizing enzyme activities and for individual test results (0)
    d. Selection of appropriate positive controls (0)
    e. Selection of doses for positive controls (4)
    f. Growth inhibition or cytotoxicity caused by human liver S9. (0)
    g. Other (new protocols required for quality control, delays obtaining S9) (2)
13. What result did you obtain using human liver S9? (7 respondents)

a. Negative (result using induced rat liver S9 was unknown) (0)
b. Positive (result using induced rat liver S9 was unknown) (0)
c. Negative (result using induced rat liver S9 was negative) (2)
d. Negative (result using induced rat liver S9 was positive) (1)
e. Positive (result using induced rat liver S9 was negative) (0)
f. Positive (result using induced rat liver S9 was positive) (2)
g. Human liver S9 produced the target metabolite(s) more than induced rat liver S9 (0)
h. Human liver S9 produced the target metabolite(s) less than induced rat liver S9 (0)
i. Other (Ames test using human S9 was negative, the result was the same as rat S9. However a chromosome aberration test using human S9 positive unlike the test using rat S9 for negative) (2)

14. Will you conduct a test with human liver S9 in the future? (7 respondents)
    a. Yes (6)
    b. No (1)
15. In which situations will you choose to use human liver S9 in *in vitro* assays? (6 respondents)
    a. A test using human liver S9 was negative, thus contributing to the development of a candidate chemical (1)
    b. The synthesis of a metabolite is difficult and required human liver S9 (2)
    c. To follow up a positive result from a test with induced rat liver S9 as part of a weight of evidence approach (2)
    d. Other (to assess the genotoxicity of human specific metabolite) (1)
16. Why would you choose not to use human liver S9 in an *in vitro* genotoxicity assay? (1 respondent)
    a. Because the target metabolite was not produced by human liver S9 (0)
    b. Because the regulator does not accept the use of human liver S9 as relevant due to the lower activity of drug-metabolizing enzymes, relative to induced rat liver S9 (0)
    c. Due to the poor reproducibility of human liver S9 between lots (0)
    d. Due to the poor reproducibility of human liver S9 between experiments (0)
    e. Other – Organization no longer has a requirement to conduct the test with human S9 (1)

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## A.3 Chapter 3

**Supplementary Table 3-I.** Primary and secondary antibodies used for immunocytochemical analyses

| Antibody | Target | Host | Clonality | Conjugate | Dilution | Positive control cell line |
|---|---|---|---|---|---|---|
| *Primary antibodies* | | | | | | |
| Abcam ab19194 | Mouse serum albumin | Goat | Polyclonal | No | 1/200 | HepG2 |
| Abcam ab14047 | Human cytokeratin 18 (mouse reactivity) | Chicken | Polyclonal | No | 1/100 | HepG2 |
| Abcam ab53119 | Human cytokeratin 19 (mouse reactivity) | Rabbit | Polyclonal | No | 1/100 | HepG2 |
| Cell Signaling Technology 9854 | Human vimentin (mouse reactivity) | Rabbit | Monoclonal | Alexa Fluor® 488 | 1/500 | A549 |
| Abcam ab15200 | Human desmin (mouse reactivity) | Rabbit | Polyclonal | No | 1/100 | C2C12 |
| Abcam ab60343 | Mouse F4/80 | Rat | Monoclonal | FITC | 1/100 | RAW 264.7 |
| *Secondary antibodies* | | | | | | |
| ThermoFisher Scientific A11058 | Goat IgG | Donkey | Polyclonal | Alexa Fluor® 594 | 1/2000 | NA |
| ThermoFisher Scientific A11039 | Chicken IgY | Goat | Polyclonal | Alexa Fluor® 488 | 1/2000 | NA |
| ThermoFisher Scientific A21441 | Rabbit IgG | Chicken | Polyclonal | Alexa Fluor® 488 | 1/2000 | NA |

**Supplementary Table 3-II**: Settings for the electrospray ion sources used for the acquisition of testosterone, 16β-hydroxytestosterone, 6β-hydroxytestosterone, and 7-hydroxycoumarin data with a Waters Xevo TQMS, and acquisition of 7-hydroxycoumarin sulphate and 7-hydroxycoumarin glucuronide data with a Waters TQS.

| Parameter | Setting Xevo TQMS | Setting TQS |
|---|---|---|
| Capillary voltage (kV) | 3.5 | 0.7 |
| Source temperature (°C) | 150 | 150 |
| Desolvation gas temperature (°C) | 500 | 650 |
| Desolvation gas flow (L/h) | 1000 | 1200 |
| Cone gas flow (L/h) | 100 | 150 |
| Collision gas flow (mL/min) | 0.17 | 0.15 |

**Supplementary Table 3-III**: Mass spectrometric detection parameters optimized via Multiple Reaction Monitoring (MRM) methods using Waters QuanOptimise. ES- electrospray ionization. + indicates positive ion.

| Compound ID | Ionisation mode | Transition | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| Testosterone | ES+ | 289.53 > 109.08 | 42 | 28 |
| 16β-hydroxytestosterone | ES+ | 305.17 > 96.91 | 28 | 22 |
| 6β-hydroxytestosterone | ES+ | 305.14 > 268.98 | 28 | 16 |
| 7-hydroxycoumarin | ES+ | 162.98 > 107.02 | 28 | 22 |
| 7-hydroxycoumarin sulphate | ES- | 240.81 > 160.85 | 35 | 15 |
| 7-hydroxycoumarin glucuronide | ES+ | 338.80 > 162.90 | 35 | 15 |
| Diclofenac | ES+ | 296.05 > 214.14 | 22 (TQMS)<br>10 (TQS) | 32 (TQMS)<br>30 (TQS) |
| Reserpine | ES+ | 609.37 > 195.09 | 46 (TQMS)<br>66 (TQS) | 36 (TQMS)<br>34 (TQS) |

**Supplementary Table 3-IV**: UPLC gradient profile used for analysis of testosterone, 16β-hydroxytestosterone, 6β-hydroxytestosterone and 7-hydroxycoumarin.

| Parameter | Setting | |
|---|---|---|
| Column | Kinetex XB-C18 100A 50 x 2.1 mm, 2.6 µm | |
| Column temperature | 40 °C | |
| Flow rate | 0.7 mL/min | |
| Injection volume | 1 µL (testosterone, 16β-hydroxytestosterone)<br>2 µL (6β-hydroxytestosterone, 7-hydroxycoumarin) | |
| Mobile phase A | 0.01% Formic acid in water (v/v) | |
| Mobile phase B | 0.01% Formic acid in acetonitrile (v/v) | |
| Gradient profile | Time (minutes) | Mobile phase B (%) |
| | 0.00 | 5 |
| | 0.20 | 5 |
| | 1.20 | 95 |
| | 1.80 | 95 |
| | 1.85 | 5 |
| | 2.00 | 5 |
| Retention time | Testosterone | 1.05 minutes |
| | 16β-hydroxytestosterone | 0.91 minutes |
| | 6β-hydroxytestosterone | 0.85 minutes |
| | 7-hydroxycoumarin | 0.76 minutes |
| | Diclofenac | 1.16 minutes |
| | Reserpine | 0.89 minutes |

**Supplementary Table 3-V**: UPLC gradient profile used for analysis of 7-hydroxycoumarin sulphate and 7-hydroxycoumarin glucuronide

| Parameter | Setting | |
|---|---|---|
| Column | Acquite BEH-C18 100A 50 x 2.1 mm, 1.7 µm | |
| Column temperature | 40 °C | |
| Flow rate | 0.8 mL/min | |
| Injection volume | 2 µL | |
| Mobile phase A | 0.1% Formic acid in water (v/v) | |
| Mobile phase B | 0.1% Formic acid in acetonitrile (v/v) | |
| Gradient profile | Time (minutes) | Mobile phase B (%) |
| | 0.00 | 2 |
| | 0.25 | 2 |
| | 1.00 | 98 |
| | 1.50 | 98 |
| | 1.60 | 2 |
| Retention time | 7-hydroxycoumarin glucuronide | 0.66 minutes |
| | 7-hydroxycoumarin sulphate | 0.73 minutes |
| | Diclofenac | 1.04 minutes |
| | Reserpine | 0.84 minutes |

**Supplementary Table 3-VI**: Genes included in the Qiagen Mouse Drug Metabolism RT2 profiler PCR array. Genes shaded in green are housekeeping genes for normalization and genes shaded in grey are qPCR controls.

| Symbol | Description |
|---|---|
| Abcb1a | ATP-binding cassette, sub-family B (MDR/TAP), member 1A |
| Abcb1b | ATP-binding cassette, sub-family B (MDR/TAP), member 1B |
| Abcb4 | ATP-binding cassette, sub-family B (MDR/TAP), member 4 |
| Abcc1 | ATP-binding cassette, sub-family C (CFTR/MRP), member 1 |
| Aoc1 | Amiloride binding protein 1 (amine oxidase, copper-containing) |
| Adh1 | Alcohol dehydrogenase 1 (class I) |
| Adh4 | Alcohol dehydrogenase 4 (class II), pi polypeptide |
| Adh5 | Alcohol dehydrogenase 5 (class III), chi polypeptide |
| Ahr | Aryl-hydrocarbon receptor |
| Alad | Aminolevulinate, delta-, dehydratase |
| Aldh1a1 | Aldehyde dehydrogenase family 1, subfamily A1 |
| Alox12 | Arachidonate 12-lipoxygenase |
| Alox15 | Arachidonate 15-lipoxygenase |
| Alox5 | Arachidonate 5-lipoxygenase |
| Apoe | Apolipoprotein E |
| Arnt | Aryl hydrocarbon receptor nuclear translocator |
| Asna1 | ArsA arsenite transporter, ATP-binding, homolog 1 (bacterial) |
| Blvra | Biliverdin reductase A |
| Blvrb | Biliverdin reductase B (flavin reductase (NADPH)) |
| Ces1g | Carboxylesterase 1G |
| Ces2c | Carboxylesterase 2C |
| Chst1 | Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1 |
| Comt | Catechol-O-methyltransferase |
| Cyb5r3 | Cytochrome b5 reductase 3 |
| Cyp11b2 | Cytochrome P450, family 11, subfamily b, polypeptide 2 |
| Cyp17a1 | Cytochrome P450, family 17, subfamily a, polypeptide 1 |
| Cyp19a1 | Cytochrome P450, family 19, subfamily a, polypeptide 1 |
| Cyp1a1 | Cytochrome P450, family 1, subfamily a, polypeptide 1 |
| Cyp1a2 | Cytochrome P450, family 1, subfamily a, polypeptide 2 |
| Cyp27b1 | Cytochrome P450, family 27, subfamily b, polypeptide 1 |
| Cyp2c29 | Cytochrome P450, family 2, subfamily c, polypeptide 29 |
| Cyp2e1 | Cytochrome P450, family 2, subfamily e, polypeptide 1 |
| Cyp3a11 | Cytochrome P450, family 3, subfamily a, polypeptide 11 |
| Cyp3a44 | Cytochrome P450, family 3, subfamily a, polypeptide 44 |
| Cyp4b1 | Cytochrome P450, family 4, subfamily b, polypeptide 1 |
| Ephx1 | Epoxide hydrolase 1, microsomal |
| Ephx2 | Epoxide hydrolase 2, cytoplasmic |
| Faah | Fatty acid amide hydrolase |
| Fbp1 | Fructose bisphosphatase 1 |
| Gad1 | Glutamic acid decarboxylase 1 |
| Gad2 | Glutamic acid decarboxylase 2 |
| Gckr | Glucokinase regulatory protein |
| Ggt1 | Gamma-glutamyltransferase 1 |
| Gpi1 | Glucose phosphate isomerase 1 |
| Gpx1 | Glutathione peroxidase 1 |
| Gpx2 | Glutathione peroxidase 2 |

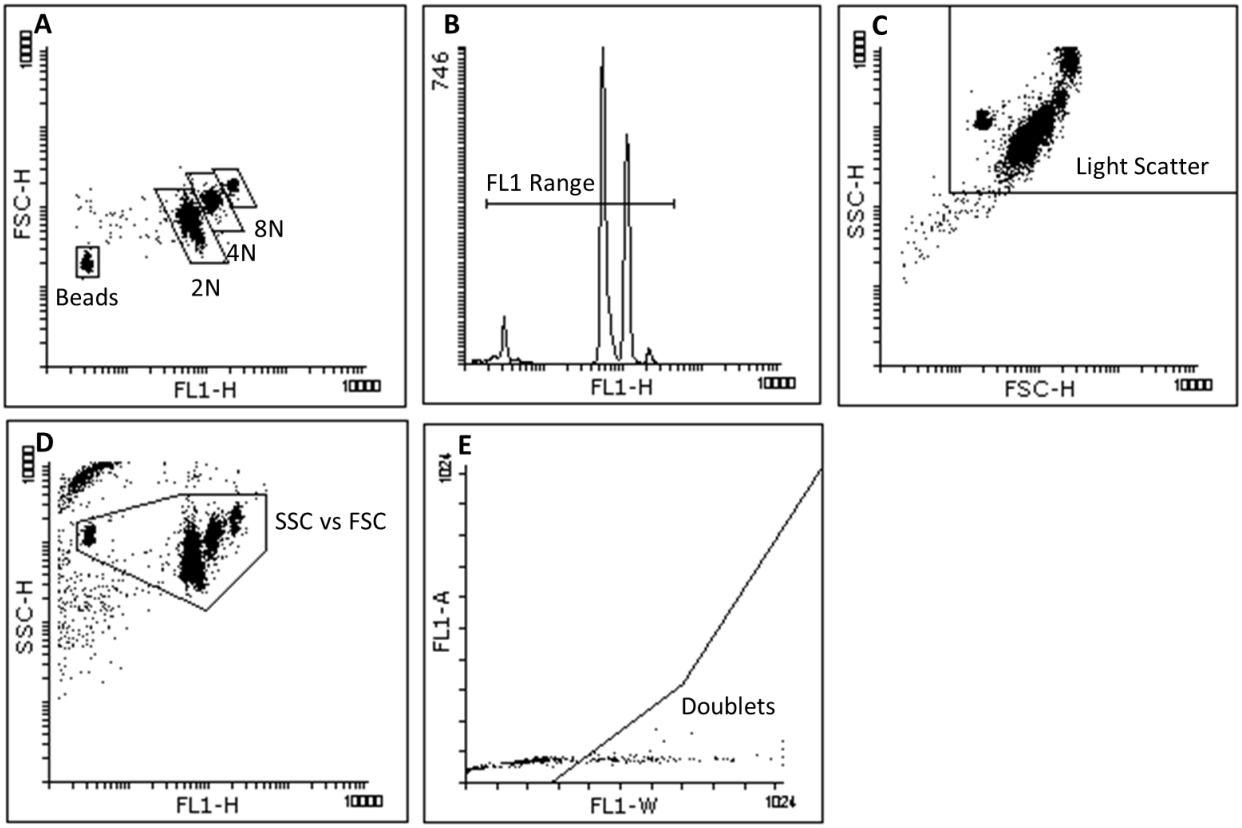
| | |
|---|---|
| Gpx3 | Glutathione peroxidase 3 |
| Gpx5 | Glutathione peroxidase 5 |
| Gsr | Glutathione reductase |
| Gsta1 | Glutathione S-transferase, alpha 1 (Ya) |
| Gsta3 | Glutathione S-transferase, alpha 3 |
| Gsta4 | Glutathione S-transferase, alpha 4 |
| Gstm1 | Glutathione S-transferase, mu 1 |
| Gstm2 | Glutathione S-transferase, mu 2 |
| Gstm3 | Glutathione S-transferase, mu 3 |
| Gstm4 | Glutathione S-transferase, mu 4 |
| Gstm5 | Glutathione S-transferase, mu 5 |
| Gstp1 | Glutathione S-transferase, pi 1 |
| Gstt1 | Glutathione S-transferase, theta 1 |
| Gstz1 | Glutathione transferase zeta 1 (maleylacetoacetate isomerase) |
| Hk2 | Hexokinase 2 |
| Hsd17b1 | Hydroxysteroid (17-beta) dehydrogenase 1 |
| Hsd17b2 | Hydroxysteroid (17-beta) dehydrogenase 2 |
| Hsd17b3 | Hydroxysteroid (17-beta) dehydrogenase 3 |
| Lpo | Lactoperoxidase |
| Mgst1 | Microsomal glutathione S-transferase 1 |
| Mgst2 | Microsomal glutathione S-transferase 2 |
| Mgst3 | Microsomal glutathione S-transferase 3 |
| Mpo | Myeloperoxidase |
| Mt2 | Metallothionein 2 |
| Mt3 | Metallothionein 3 |
| Mthfr | 5,10-methylenetetrahydrofolate reductase |
| Nat1 | N-acetyl transferase 1 |
| Nat2 | N-acetyltransferase 2 (arylamine N-acetyltransferase) |
| Nos3 | Nitric oxide synthase 3, endothelial cell |
| Nqo1 | NAD(P)H dehydrogenase, quinone 1 |
| Pklr | Pyruvate kinase liver and red blood cell |
| Pkm | Pyruvate kinase, muscle |
| Pon1 | Paraoxonase 1 |
| Pon2 | Paraoxonase 2 |
| Pon3 | Paraoxonase 3 |
| Snn | Stannin |
| Srd5a1 | Steroid 5 alpha-reductase 1 |
| Srd5a2 | Steroid 5 alpha-reductase 2 |
| Actb | Actin, beta |
| B2m | Beta-2 microglobulin |
| Gapdh | Glyceraldehyde-3-phosphate dehydrogenase |
| Gusb | Glucuronidase, beta |
| Hsp90ab1 | Heat shock protein 90 alpha (cytosolic), class B member 1 |
| MGDC | Mouse Genomic DNA Contamination |
| RTC | Reverse Transcription Control |
| PPC | Positive PCR Control |

**Supplementary Table 3-VII.** The fold-changes in gene expression in MutaMouse primary hepatocytes over time for 84 murine metabolism. Expression measured using the Qiagen Mouse Drug Metabolism RT2 profiler PCR array. Red-shaded cells indicate a significant fold-increase, whereas green-shaded cells indicate a significant fold-decrease ($p \leq 0.05$). Grey shading indicates lack of signal or a Ct value above the cut-off of 35.

| | 0 h | 2 h | 8 h | 24 h | 48 h |
|---|---|---|---|---|---|
| Abcb1a | 1.00 | **1.73** | **3.31** | **7.70** | **2.07** |
| Abcb1b | 1.00 | **20.46** | **190.96** | **5764.58** | **1474.46** |
| Abcb4 | 1.00 | 1.05 | 0.69 | **0.23** | **0.08** |
| Abcc1 | 1.00 | **2.93** | **7.37** | **15.25** | **13.59** |
| Aoc1 | 1.00 | 0.70 | ND[a] | ND | 0.27 |
| Adh1 | 1.00 | 1.16 | **0.77** | **0.04** | **0.02** |
| Adh4 | 1.00 | 0.97 | **0.22** | **0.16** | **0.13** |
| Adh5 | 1.00 | 1.29 | 0.94 | **0.91** | **0.48** |
| Ahr | 1.00 | **1.93** | **1.78** | **2.56** | 0.86 |
| Alad | 1.00 | 1.02 | **0.62** | 0.78 | **0.33** |
| Aldh1a1 | 1.00 | 0.89 | 0.59 | **2.74** | 1.69 |
| Alox12 | 1.00 | 2.64 | 0.45 | 1.12 | **2.49** |
| Alox15 | 1.00 | 2.43 | 2.62 | 3.77 | 0.97 |
| Alox5 | 1.00 | 1.19 | **3.27** | **6.09** | **10.46** |
| Apoe | 1.00 | 1.04 | **0.79** | **0.49** | **0.13** |
| Arnt | 1.00 | **1.70** | 1.43 | **2.95** | **2.12** |
| Asna1 | 1.00 | 1.19 | 1.36 | **5.23** | **3.74** |
| Blvra | 1.00 | 1.01 | **1.26** | **4.11** | **1.91** |
| Blvrb | 1.00 | 1.26 | **4.06** | **7.72** | 1.37 |
| Ces1g | 1.00 | 0.94 | 0.57 | **0.22** | **0.01** |
| Ces2c | 1.00 | 1.12 | 0.92 | **0.73** | **0.23** |
| Chst1 | 1.00 | 3.70 | 2.13 | **6.25** | 3.35 |
| Comt | 1.00 | 1.28 | 0.73 | 1.26 | 1.01 |
| Cyb5r3 | 1.00 | 1.16 | 1.17 | 1.27 | 0.45 |
| Cyp11b2 | ND | ND | ND | ND | ND |
| Cyp17a1 | 1.00 | 1.01 | **0.54** | **0.11** | **0.00** |
| Cyp19a1 | ND | ND | ND | ND | ND |
| Cyp1a1 | 1.00 | 6.43 | **16.66** | **4.06** | 1.77 |
| Cyp1a2 | 1.00 | 1.07 | **0.61** | **0.26** | **0.02** |
| Cyp27b1 | ND | ND | ND | ND | ND |
| Cyp2c29 | 1.00 | 1.11 | 0.96 | **0.33** | **0.01** |
| Cyp2e1 | 1.00 | 1.00 | **0.67** | **0.19** | **0.00** |
| Cyp3a11 | 1.00 | 1.05 | 0.75 | 0.24 | **0.00** |
| Cyp3a44 | 1.00 | 1.32 | 0.97 | **0.37** | **0.00** |
| Cyp4b1 | 1.00 | 1.07 | 1.19 | **1.74** | 0.70 |
| Ephx1 | 1.00 | 1.25 | 0.96 | **3.00** | **3.78** |
| Ephx2 | 1.00 | 0.83 | **0.61** | **0.26** | **0.20** |
| Faah | 1.00 | 0.89 | **0.55** | **0.34** | **0.16** |
| Fbp1 | 1.00 | 1.07 | **0.81** | **0.22** | 0.03 |
| Gad1 | 1.00 | 1.82 | 0.75 | 0.94 | 4.58 |
| Gad2 | 1.00 | ND | ND | ND | ND |
| Gckr | 1.00 | 1.04 | 0.90 | **0.51** | **0.02** |
| Ggt1 | 1.00 | 1.08 | ND | 1.48 | 1.35 |
| Gpi1 | 1.00 | **1.60** | **2.52** | **5.06** | **2.61** |
| Gpx1 | 1.00 | 1.57 | 1.05 | 1.03 | **0.49** |

| | | | | | |
|---|---|---|---|---|---|
| Gpx2 | 1.00 | 7.41 | 8.65 | **35.11** | **16.12** |
| Gpx3 | 1.00 | 1.91 | **9.84** | **126.35** | **65.21** |
| Gpx5 | 1.00 | ND | ND | ND | 1.35 |
| Gsr | 1.00 | 1.75 | **3.93** | **15.28** | **5.04** |
| Gsta1 | 1.00 | 43.34 | **2612.45** | **5323.62** | **471.67** |
| Gsta3 | 1.00 | 1.03 | **0.67** | **0.13** | **0.04** |
| Gsta4 | 1.00 | 1.28 | **1.48** | **1.58** | 0.83 |
| Gstm1 | 1.00 | 1.26 | **1.31** | 0.77 | **0.17** |
| Gstm2 | 1.00 | 1.24 | 1.24 | 0.88 | **0.20** |
| Gstm3 | 1.00 | 1.16 | 1.47 | **0.51** | **0.02** |
| Gstm4 | 1.00 | 1.17 | 0.76 | **0.30** | **0.14** |
| Gstm5 | 1.00 | 1.53 | **2.17** | **3.70** | **1.91** |
| Gstp1 | 1.00 | 1.33 | **8.73** | **15.65** | **4.82** |
| Gstt1 | 1.00 | 1.25 | 0.76 | **0.40** | **0.19** |
| Gstz1 | 1.00 | 0.99 | 0.72 | **0.18** | **0.05** |
| Hk2 | 1.00 | **5.16** | 1.83 | **15.81** | **29.01** |
| Hsd17b1 | 1.00 | 0.37 | **1.77** | **1.93** | 1.64 |
| Hsd17b2 | 1.00 | 1.12 | **0.77** | 0.90 | **0.51** |
| Hsd17b3 | 1.00 | **2.25** | 1.88 | ND | **0.20** |
| Lpo | 1.00 | ND | ND | ND | 2.52 |
| Mgst1 | 1.00 | 1.11 | 0.98 | 0.55 | **0.21** |
| Mgst2 | ND | ND | ND | ND | ND |
| Mgst3 | 1.00 | 1.26 | **1.47** | 2.20 | 1.08 |
| Mpo | 1.00 | 0.39 | 0.76 | 0.36 | 0.67 |
| Mt2 | 1.00 | **39.54** | **198.73** | **7.08** | 2.19 |
| Mt3 | 1.00 | **7.71** | **325.24** | **476.17** | **39.89** |
| Mthfr | 1.00 | 0.78 | 0.69 | **6.25** | 1.92 |
| Nat1 | 1.00 | 0.65 | **0.08** | **0.14** | **0.21** |
| Nat2 | 1.00 | 0.72 | 1.53 | **3.62** | 0.99 |
| Nos3 | 1.00 | 2.31 | 1.75 | 0.62 | 0.60 |
| Nqo1 | 1.00 | 1.14 | **7.19** | **5.74** | 0.95 |
| Pklr | 1.00 | 1.11 | 0.79 | 0.64 | 0.43 |
| Pkm | 1.00 | 1.58 | **3.98** | **21.31** | **24.49** |
| Pon1 | 1.00 | 1.06 | 0.67 | **0.33** | **0.06** |
| Pon2 | 1.00 | 1.12 | 0.99 | **3.95** | **2.30** |
| Pon3 | 1.00 | 1.19 | 0.90 | **2.40** | **1.93** |
| Snn | 1.00 | 0.91 | 1.97 | **8.02** | **20.58** |
| Srd5a1 | 1.00 | 1.01 | 0.39 | 0.66 | **0.20** |
| Srd5a2 | 1.00 | 1.19 | **0.41** | 1.47 | **1.73** |

[a] ND, not determined




**Supplementary Figure 3-1**: Histogram and dot plots of primary MutaMouse hepatocyte nuclei illustrating the gates used to discriminate bead and nuclei populations from noise and spurious events. Events displayed and scored (panel A) were required to fall within the FL1 range (panel B), the light scatter region (panel C), the SSC vs FSC region (panel D), and the region that excludes doublets (panel E). The resulting FSC versus FL1 dot plot (panel A) displays a distinct bead population, as well as three populations representing 2n, 4n, and 8n nuclei.

**Supplementary Video 3-1.** Time-lapse imaging of MutaMouse primary hepatocytes in culture. The imaging begins 2 hours post-isolation and continues for 120 hours. Individual images were captured at 10 minute intervals at 10X magnification using bright-field imaging.

## A.4 Chapter 4

**Supplementary Table 4-I.** Summary of the $BMD_{100}$ values, including 90% confidence intervals (i.e., BMDL and BMDU values) for all positive *lacZ* mutant frequency (MF) data using both the exponential and Hill models from the MutaMouse primary hepatocyte (PH) assay.

| Chemical | $BMD_{100}$ (μg/mL) | | BMDL (μg/mL) | | BMDU (μg/mL) | |
|---|---|---|---|---|---|---|
| | Exponential | Hill | Exponential | Hill | Exponential | Hill |
| 3-NBA | 0.15 | 0.15 | 0.0689 | 0.0689 | 0.373 | 0.363 |
| BaP | 0.3 | 0.3 | 0.184 | 0.184 | 0.446 | 0.446 |
| AFL | 0.47 | 0.47 | 0.193 | 0.193 | 1.47 | 1.47 |
| 1,8-DNP | 0.97 | 0.97 | 0.39 | 0.39 | 2.62 | 2.44 |
| 2-AAF | 1.9 | 1.9 | 0.964 | 0.964 | 3.96 | 3.96 |
| PhIP | 2.6 | 2.6 | 1.05 | 1.05 | 7.8 | 7.05 |
| DMN | 7.6 | 7.6 | 3.18 | 3.18 | 21.5 | 16.8 |
| ENU | 76 | 76 | 36.6 | 36.6 | 178 | 170 |

**Supplementary Table 4-II.** Summary of the enzymes required for metabolic activation of the chemicals tested and the presence of these enzymes in MutaMouse primary hepatocytes (PHs) *in vitro*.

| Chemical | Enzymes required | Presence in MutaMouse PHs[a] | | Reference |
|---|---|---|---|---|
| | | Activity[b] | Expression[c] | |
| BaP | CYP[d]1A1 | Yes | Yes | [Jeffrey, 1985; Bauer et al., 1995; Kim et al., 1998] |
| | CYP1A2 | Yes | Yes | |
| | CYP3A | Yes | Yes | |
| | Epoxide hydrolase | ND | Yes | |
| AFB1 | CYP1A2 | Yes | Yes | [Gallagher et al., 1984] |
| | CYP3A | Yes | Yes | |
| 2-AAF and PhIP | CYP1A1 | Yes | Yes | [Schut and Snyderwine, 1999; Heflich and Neft, 1994; Cai et al., 2016] |
| | CYP1A2 | Yes | Yes | |
| | SULT[e] | Yes | Yes | |
| | NAT[f] | ND | Yes | |
| | UGT[g] | Yes | ND | |
| 1,8-DNP | NQO1[h] | ND | Yes | [IARC, 2014] |
| | NAT | ND | Yes | |
| 3-NBA | NQO1 | ND | Yes | [IARC, 2014; Arlt et al., 2003; Arlt et al., 2005] |
| | NAT | ND | Yes | |
| | SULT | Yes | Yes | |
| DMN | CYP2E1 | ND | Yes | [Chowdhury et al., 2012; Yamazaki et al., 1992; Hoffmann and Hecht, 1985] |

[a] Data from [Cox et al., 2018]
[b] Activity measured by EROD for CYP1A1/1A2 and LC-MS/MS for CYP3A, SULT and UGT
[c] Gene expression measured by RT-qPCR
[d] CYP, cytochrome P450
[e] SULT, sulfotransferase
[f] NAT, *N*-acetyltransferase
[g] UGT, UDP-glucuronosyltransferase
[h] NQO1, NA(D)PH dehydrogenase, quinone 1

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## A.5 Chapter 5

(none)

## A.6 Chapter 6

(none)