

The Genetic Toxicity of Polycyclic Aromatic
Hydrocarbons:
A Cross-Tissue, Multi-Endpoint Study in the Transgenic
MutaMouse

by

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are produced via the incomplete combustion of organic matter. They are ubiquitously present in the environment, and human exposures typically involve complex PAH mixtures in complex matrices (e.g., soil, urban air). Many PAHs are genotoxic carcinogens; exposures can augment cancer risk and reliable risk assessment of PAH mixtures is a regulatory concern. There is a paucity of *in vivo* genotoxicity information for most PAHs and PAH mixtures. Risk assessment of PAH mixtures assumes dose addition (i.e., additive, incremental contributions from each PAH); however, there is a lack of evidence to support this assumption. This thesis assessed the *in vivo* genotoxicity of 9 PAHs and 6 PAH mixtures following sub-chronic oral exposure of transgenic MutaTMMouse (i.e., adduct and *lacZ* mutant frequency across 5 tissues). The results revealed that PAHs and PAH mixtures induce significant levels of genetic damage; the mixtures induced very high levels of damage and mutations. Differences in the nature and magnitude of the effects in individual tissues appear to be related to the processes that govern PAH metabolism and the processing of genetic damage (e.g., repair and translesion synthesis). Scrutiny of the dose addition assumption revealed more-than-additive effects in tissues proximal to the exposure route (i.e., intestine, liver), but less-than-additive effects in distal tissues (i.e., bone marrow); however, discrepancies between the experimentally-observed and predicted responses were typically small (i.e., within 5-fold). Comparisons of cross-tissue patterns in adduct and mutant frequencies revealed that the frequency of the former is generally inversely related to that of the latter. This appears to be related to the experimental design, and the influence of repair and replication on adduct and mutant

frequency. The BMD approach was employed to estimate genotoxic (i.e., adduct) potency and mutagenic (i.e., *lacZ* mutant) potency for all agent-tissue combinations. The results demonstrate that the mutagenic potency of PAHs and PAH mixtures is empirically related to genotoxic potency; moreover, that there is cross-tissue and cross-compound congruence in the processing of PAH-induced damage. The results obtained significantly advance existing knowledge regarding the genotoxic hazards of PAHs and PAH mixtures; moreover, the empirical relationships between genetic toxicity endpoints.

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List of Abbreviations

3Rs	Reduction, refinement, and replacement
AhR	Aryl hydrocarbon receptor
AKR	Aldo-keto reductase
BaA	Benzo(<i>a</i>)anthracene
BaP	Benzo(<i>a</i>)pyrene
BbF	Benzo(<i>b</i>)fluoranthene
Bd	Bladder
BDM	Bioassay-derived method
BER	Base excision repair
BghiP	Benzo(<i>g,h,i</i>)perylene
BkF	Benzo(<i>k</i>)fluoranthene
BM	Bone marrow
BMD	Benchmark dose
BMDL	Benchmark dose 90% lower confidence limit
BMDU	Benchmark dose 90% upper confidence limit
BMR	Benchmark response
BPDE	Benzo(<i>a</i>)pyrene-7,8-dihydrodiol-9,10-epoxide
BW	Body weight
CALA	Canadian Association for Laboratory Accreditation
CAS	Chemical abstract service
CCME	Canadian Council of Ministers of the Environment
CED	Critical effect dose
CEDL	Critical effect dose lower confidence interval
CEDU	Critical effect dose upper confidence interval
CEPA	Canadian Environmental Protection Act
CHRY	Chrysene
CI	90% Confidence intervals on the BMD
CMP	Chemicals Management Plan
COMSIG	Causes of Mutation SIGNatures

CT	Coal tar
CTRL	Control group
CYP	Cytochrome P450
DBahA	Dibenzo(<i>a,h</i>)anthracene
DBaP	Dibenzo(<i>a,l</i>)pyrene
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DQRA	Detailed quantitative risk assessment
DRZ	Diagonal radioactive zone
ECHA	European Chemicals Agency
EDTA	Ethylenediaminetetraacetic acid
EF	Exposure factor
EFSA	European Food Safety Authority
EH	Epoxide hydrolase
ELCR	Excess lifetime cancer risk
EMS	Ethyl methanesulphonate
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
Eq	Equivalent
EROD	Ethoxyresorufin- <i>O</i> -deethylase
FBS	Fetal bovine serum
FCSAP	Federal Contaminated Sites Action Plan
FS	Forestomach
GG-NER	Global genomic nucleotide excision repair
GI	Gastrointestinal
GS	Glandular stomach
GST	Glutathione- <i>S</i> -transferase
GTTC	Genetic Toxicology Technical Committee
HC	Health Canada
HESI	Health and Environmental Sciences Institute
HHRA	Human health risk assessment
HR	Homologous recombination

IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonisation
ILSI	International Life Sciences Institute
INDENO	Indeno(1,2,3- <i>c,d</i>)pyrene
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
Kd	Kidney
K_{ow}	Octanol-water partition coefficient
Lg	Lung
LI	Labelling index
LOAEL	Lowest observed adverse effect level
LOGEL	Lowest observed genotoxic effect level
Lv	Liver
MF	Mutant frequency
MGP	Manufactured gas plant
MGMT	O ⁶ -methylguanine DNA methyltransferase
MI	Mitotic index
Mix	Mixture
MN	Micronucleus
MOA	Mode of action
MOE	Margin of exposure
MPR	Mutagenic potency ratio
NC3R	National Centre for the Replacement, Refinement, and Reduction of Animals in Research
NCE	Normochromatic erythrocyte
NER	Nucleotide excision repair
NGS	Next generation sequencing
NOAEL	No observed adverse effect level
NOGEL	No observed genotoxic effect level
NQO1	NAD(P)H quinone oxidoreductase 1
NSERC	National Society and Research Council of Canada

nt	Nucleotide
OECD	Organization for Economic Cooperation and Development
OR	Origin
P450	Cytochrome P450
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PDE	Permitted daily exposure
PEF	Potency equivalency factor
PEF-M	Potency equivalency factor-method
pfu	Plaque forming units
Pgal	Phenyl- β -D-galactoside
PoD	Point of departure
Pol	Polymerase
RBC	Red blood cell
RET	Reticulocyte
RIVM	National Institute for Public Health and the Environment
ROS	Reactive oxygen species
RPF	Relative potency factor
SDS	Sodium dodecyl sulfate

Statement of Contributions

Chapter 2: Tissue-specific In Vivo Genetic Toxicity of Nine Polycyclic Aromatic Hydrocarbons Assessed Using the MutaTMMouse Transgenic Rodent Assay

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DNA adduct analysis.....	Volker Arlt
Micronucleus assay.....	Litron Laboratories
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Chapter 3: Oral Exposure to Commercially Available Coal Tar-Based Pavement Sealcoat Induces Murine Genetic Damage and Mutations

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Chapter 4: The Genetic Toxicity of Complex Mixtures of Polycyclic Aromatic Hydrocarbons: Evaluating Dose-Additivity in a Transgenic Mouse Model

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Chapter 5: Empirical Analysis of BMD Metrics in Genetic Toxicology Part II: In Vivo Potency Comparisons to Promote Reductions in the Use of Experimental Animals for Genetic Toxicity Assessment

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<i>Pig-a</i> experiment animal exposures and scoring.....	Stephen Dertinger Jeffrey Bemis
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Chapter 6: Benchmark Dose Analyses of Multiple Genetic Toxicity Endpoints Permit Robust, Cross-Tissue Comparisons of Muta™ Mouse Responses to Orally-delivered Benzo[a]pyrene

Submitted to: Health Canada internal review

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Chapter 7: Quantitative relationships between mutagenic and genotoxic potency in Muta™Mouse tissues following oral exposures to polycyclic aromatic hydrocarbons

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Appendix A: In Vitro mammalian mutagenicity of complex polycyclic aromatic hydrocarbon mixtures in contaminated soils

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Appendix B: Cancer risk assessment of polycyclic aromatic hydrocarbon contaminated soils determined using bioassay-derived levels of benzo[*a*]pyrene equivalents

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CHAPTER ONE

1 Chapter: General Introduction

1.1 Genetic Damage and Disease

Genetic damage, that is chemical or structural changes to DNA, can result in mutations or chromosomal aberrations, which in turn may contribute to the pathogenesis of human disease. Whether the damage occurs in germ cells, somatic cells, or mitochondria, as well as *when* it occurs, will determine the nature of the disease. It is well known that germ cell mutations are responsible for a variety of heritable diseases [Michaelson et al., 2012; Xu et al., 2011; Yuen et al., 2015], as well as spontaneous, idiopathic miscarriages [Singer and Yauk, 2010; Egozcue et al., 2000], and there are currently nearly 8,000 genes in the human gene mutation database for which mutations have been linked to inherited disease (e.g., cystic fibrosis, sickle-cell disease) [Stenson et al., 2003]. Mutations in mitochondrial DNA can cause maternally inherited diseases associated with mitochondrial dysfunction (e.g., Leigh syndrome, mitochondrial myopathy) [Wallace, 1999; Wallace, 2010]. Somatic mutations early in gestation can cause somatic mosaicism, with tissues arising from the mutant cell being genetically distinct from the remainder of the somatic cells in the developed fetus [Erickson, 2003; Erickson, 2010; Meier et al., 2016; Campbell et al., 2015]. Somatic mosaicism that disrupts the function of a critical gene has been implicated in several diseases (e.g., neurofibromatosis I, Cornelia de Lange syndrome, paroxysmal nocturnal hemoglobinuria) [Campbell et al., 2015; Erickson, 2010]. Structural alterations of the genome, including deletions, copy number variants, and inversions have also been causally linked to disease (e.g., genomic diseases/disorders such as Charcot-Marie-Tooth disease type 1A, Pelizaeus-Merzbacher disease, spinal muscular atrophy) [Lupski, 2015;

Lupski, 2009]. However, the most extensively studied relationship between genetic damage and disease relates to the involvement of somatic mutations in cancer. It is well recognized that somatic mutation is causally linked to the development and progression of many types of cancers, a concept that was first proposed by Theodore Boveri in 1929 [Boveri, 2008].

1.1.1 Mutations and cancer

For the 2015 calendar year the Canadian Cancer Society estimated the national incidence of new cancer cases at 196,400, with 78,000 cancer-related deaths [Canadian Cancer Society, 2015]. Somatic mutation is invariably linked to cancer, and is considered an enabler of tumour establishment and progression [Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011]. More specifically, an accumulation of mutations in critical genes such as tumour suppressor genes or proto-oncogenes can lead to uncontrolled cell proliferation, replicative immortality, and the capacity to evade apoptosis, all of which can contribute to the establishment of a neoplastic mass that may ultimately gain its own blood supply and invade neighbouring tissues (i.e., become metastatic). Although intrinsic risk factors (e.g., inherited genes, number of stem cell divisions of a tissue) also contribute to cancer risk [Tomasetti and Vogelstein, 2015], extrinsic risk factors such as environmental exposures to agents that cause genetic damage are also critically important [Wu et al., 2016; Alexandrov et al., 2016]. Genetic damage can occur due to endogenous errors associated with DNA replication and endogenous metabolism, but may also be induced by chemicals, ionizing radiation (e.g., X-rays), and biological agents such as viruses (e.g., the human papilloma virus). Chemical mutagens comprise the largest category of genotoxic agents, including

endogenous compounds such as reactive oxygen species, and exogenous compounds found in the external environment.

1.1.2 Environmental mutagens

Mutagenic and carcinogenic compounds are ubiquitous, and environmental exposures are unavoidable. Chemical mutagens were first described by Auerbach and colleagues [Auerbach et al., 1947], and there are currently numerous recognized classes. The sources of chemical mutagens can be natural (e.g., forest fire and volcanic emissions, fungal and plant metabolites) or anthropogenic (e.g., synthetic industrial chemicals, combustion emissions). High temperature combustion emissions, including emissions from forest fires, coke production, metal refining and founding, fossil fuel combustion (e.g., coal, diesel, gasoline, etc.), coal gasification, and cigarette smoking are all major sources of environmental mutagens such as nitroarenes, heterocyclic amines, nitrosamines, and polycyclic aromatic hydrocarbons (PAHs) [Stout et al., 2002].

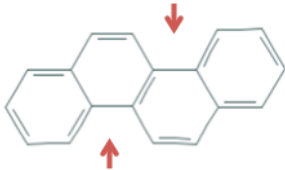
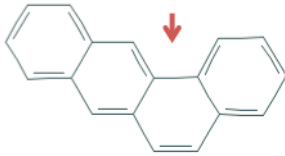
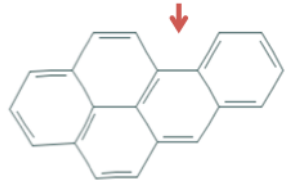
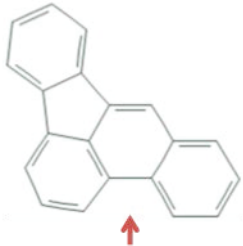
1.2 Polycyclic Aromatic Hydrocarbons – An Important Group of Environmental Mutagens

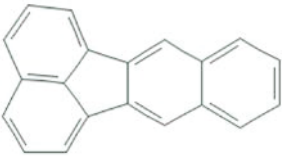
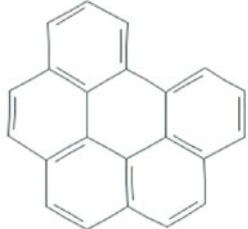
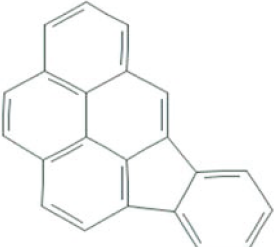
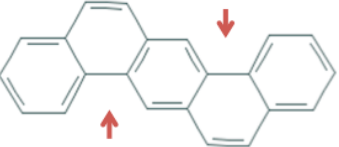
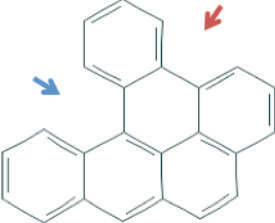
PAHs are a group of over 100 organic compounds formed from at least two fused benzene (i.e., aromatic) rings. Homocyclic, unsubstituted PAHs are stable, neutral, non-polar, lipophilic compounds containing only carbon and hydrogen. PAHs can be either alternant (i.e., composed of only 6-sided rings) or non-alternant (i.e., containing a 5-sided ring, as well as 6-sided rings), and they can contain a bay- or fjord-region, which influences their genotoxic potency, as described below. PAHs are lipophilic, with fairly high octanol-water partition coefficients (i.e., K_{OW}), which increases with the number of rings present. Low molecular weight PAHs such as naphthalene are more volatile,

whereas higher molecular weight PAHs have lower vapour pressures and can become strongly sorbed to particles in soil, water, and air. Heterocyclic PAHs are frequently referred to as polycyclic aromatic compounds (PACs), and include N-, O-, and S-heterocyclics. Additional types of substituted PAHs include oxy-PAHs, nitroarenes, and aromatic amines. The PAHs examined in this thesis are 4-ring PAHs such as chrysene (CHRY), and benz[*a*]anthracene (BaA), 5-ring PAHs such as benzo[*a*]pyrene (BaP), dibenz[*a,h*]anthracene (DBaA) benzo[*k*]fluoranthene (BkF), and benzo[*b*]fluoranthene (BbF), and 6-ring PAHs such as benzo[*g,h,i*]perylene (BghiP), indeno[1,2,3-*c,d*]pyrene (INDENO), and dibenzo[*a,l*]pyrene (DBaP) (also known as dibenzo[*def-p*]chrysene¹). An overview of the physical and structural characteristics of these PAHs is found in **Table 1-I.**

¹ Many PAHs were recently re-named according to updated IUPAC rules. This thesis refers to the PAHs according to their common names; nevertheless, changes in the naming convention are acknowledged (see Ehrenhauser et al. [2015])

Table 1-I. Chemical and structural characteristics of the 9 polycyclic aromatic hydrocarbons examined in this thesis

Polycyclic Aromatic Hydrocarbons	CAS #	Molecular formula	Molecular weight (g/mol)	Log ₁₀ K _{ow}	Chemical structure	Structural features
Chrysene (CHRY)	218-01-9	C ₁₈ H ₂₂	228.294	5.73		Alternant, bay-region
Benzo[a]anthracene (BaA)	53-55-3	C ₁₈ H ₁₂	228.294	5.79		Alternant, bay-region
Benzo[a]pyrene (BaP)	50-32-8	C ₂₀ H ₁₂	252.316	6.13		Alternant, bay-region
Benzo[b]fluoranthene (BbF)	205-99-2	C ₂₀ H ₁₂	252.316	6.60		Non- alternant, bay-region

Benzo[<i>k</i>]fluoranthene (BkF)	207-08-9	C ₂₀ H ₁₂	252.316	6.84		Non-alternant
Benzo[<i>g,h,i</i>]perylene (BgHiP)	191-24-2	C ₂₂ H ₁₂	276.338	6.63		Alternant, non-classical bay-region
Indeno[1,2,3- <i>cd</i>]pyrene (INDENO)	193-39-5	C ₂₂ H ₁₂	276.338	6.70		Non-alternant
Dibenz[<i>a,h</i>]anthracene (DBaH)	53-70-3	C ₂₂ H ₁₄	278.354	6.75		Alternant, bay-region
Dibenzo[<i>a,l</i>]pyrene (DBaIP)	191-30-0	C ₂₄ H ₁₄	302.376	7.71		Alternant, fjord-region, bay-region

1.2.1 Complex polycyclic aromatic hydrocarbon mixtures in the environment

PAHs are always found in the environment in complex mixtures; moreover, in complex environmental matrices such as soil, air, and water. Common sources of environmental PAH exposures include diesel exhaust, air pollution, cigarette smoke, charred meats, combustion emissions from fossil fuels, coal tar, and coal tar creosote [IARC., 2015; IARC., 2014; IARC., 2010b; IARC., 2012], with occupational exposures also occurring in industries related to coal combustion and distillation, coke production, iron and steel founding, aluminum and aluminum anode production, paving and roofing, among others [IARC., 2010b]. Degradation and metabolic products of PAHs, including PACs, are also commonly present in these mixtures.

1.2.2 History of polycyclic aromatic hydrocarbons - discovery and identification

PAHs have an important place in the history of environmental and chemical carcinogens. In 1775 Dr. Percival Pott, a British surgeon, made the first observation of an occupational disease association when he noted that chimney sweeps had a high frequency of scrotal tumours, which was later determined to be a result of their exposure to soot (a complex PAH mixture) [Pott and Earle, 1819; Potter, 1963]. In the early 19th century, it was observed that there were high incidences of cancer associated with occupational exposures to coal tar [Phillips, 1983], and in 1915, the Japanese researchers Yamagiwa and Ichikawa were the first to successfully induce tumours experimentally, by painting rabbit ears with coal tar [Yamagiwa and Ichikawa, 1918]. Attempts to isolate and characterize the carcinogenic compounds in coal tar were led by the British researcher E. L. Kennaway, where they successfully determined that coal tar must contain PAHs [Kennaway, 1924], and demonstrated that tars of a PAH (DBahA) and its

3-methyl derivative both induced tumours when painted on rabbits – the first experimental demonstration of a pure chemical carcinogen [Kennaway and Hieger, 1930]. Finally, in 1933, BaP was isolated and identified as the major carcinogenic constituent of coal tar [Cook et al., 1933].

1.2.3 Polycyclic aromatic hydrocarbons are mutagenic carcinogens

Several PAHs, particularly the larger 5- and 6-ring compounds are mutagens as well as human carcinogens, as declared by the International Agency for Research on Cancer (IARC) [IARC., 2010b; IARC., 1983]. IARC has classified one PAH (BaP) as a known human carcinogen (Group 1), three PAHs as probable human carcinogens (Group 2A), and eleven PAHs as possible human carcinogens (Group 2B) [IARC., 1983; IARC., 2010b] (See **Table 1-II** for a summary of IARC classifications and target organs in rodents). In comparison with BaP and several other PAHs evaluated by IARC, very little is known about the toxicological properties of PAH mixtures [IARC., 2010b]. The sections below focus on toxicological properties of BaP, the most heavily researched PAH.

Table 1-II. The IARC classifications and tumour target organs for the 9 polycyclic aromatic hydrocarbons studied in this thesis. Source: IARC [2010b].

Polycyclic Aromatic Hydrocarbons	IARC Classification	Tumour Target Organs
Benzo[a]pyrene	Group 1	<u>Oral</u> Mouse: lung, forestomach, liver, lymphoreticular system, oesophagus, tongue Rat: forestomach, mammary gland <u>Dermal</u> Mouse: Skin tumours <u>Inhalation</u> Hamster: tumours in upper respiratory tract (nose, larynx, trachea) and upper digestive tract (pharynx, oesophagus, forestomach). <u>Intratracheal Instillation</u> Mouse: lung Rat: lung Hamster: respiratory tract
Dibenz[a,h]anthracene	Group 2A	<u>Dermal</u> Mouse: skin <u>Intraperitoneal</u> Mouse: lung <u>Intrapulmonary</u> Rat: lung <u>Intravenous</u> Mouse: lung <u>Intratracheal Instillation</u> Hamster: lung
Dibenzo[a,l]pyrene	Group 2A	<u>Dermal</u> Mouse: skin, lung, lymphomas of spleen, lymphomas of multiple organs <u>Intraperitoneal</u> Mouse: lung, liver, kidney, intestine, ovaries, skin
Benzo[a]anthracene	Group 2B	<u>Dermal</u> Mouse: skin papilloma <u>Intraperitoneal</u> Mouse: lung, liver

Benzo[<i>b</i>]fluoranthene	Group 2B	<u>Intraperitoneal</u> Mouse: lung, liver <u>Intrapulmonary</u> Rat: lung
Benzo[<i>k</i>]fluoranthene	Group 2B	<u>Intraperitoneal</u> Mouse: lung, liver <u>Intrapulmonary</u> Rat: lung
Indeno[1,2,3- <i>cd</i>]pyrene	Group 2B	<u>Intraperitoneal</u> Mouse: lung <u>Intrapulmonary</u> Rat: lung
Chrysene	Group 2B	<u>Intraperitoneal</u> Mouse: lung, liver <u>Intrapulmonary</u> Rat: lung
Benzo[<i>g,h,i</i>]perylene	Group 3	None*

* Only inadequate or negative results were available

1.2.4 Human exposure and toxicokinetics

Humans are exposed to PAHs through a variety of routes. As mentioned above, many occupations are associated with enhanced exposures to PAH mixtures. Some examples of non-occupational exposure include exposures via oral ingestion of PAH-containing food, house dust, and water, inhalation of air pollution, diesel exhaust, combustion emissions from residential wood heating, and cigarette smoke, and dermal contact with coal-tar sealed pavement (and runoff from these sealed surfaces), and rainwater [IARC., 2010b]. Oral exposure via dietary ingestion is considered the major route of exposure for the general population (i.e., non-smokers who are not occupationally exposed) [Butler et al., 1993; Van Rooij et al., 1994; Phillips, 1999]. A review of the literature by Ramesh et al. [2004] found that dietary intake of PAHs in the USA ranged from 0.04 – 2.8 µg/person/day, and in Austria, was as high as 28 µg/person/day [Ramesh et al., 2004]. Additionally, there is substantial oral exposure resulting from the inhalation of particle-bound PAHs, as mucociliary clearance can return inhaled particles to the mouth where they can be swallowed [Sun et al., 1982].

As indicated by their relatively high K_{OW} values, PAHs are sparingly soluble in water. In contrast, they are soluble in lipids and non-polar solvents (e.g., hexane, octanol, etc.), and they can readily cross the lipid membranes of mammalian cells [IARC., 2010b]. PAHs are able to be widely distributed throughout the body. For an oral exposure, PAHs are transported to the liver following absorption from the length of the gastro-intestinal (GI) tract. From the liver, the parent compounds or metabolites can either be returned to the intestine via enterohepatic circulation [Miller and Ramos, 2001; Ramesh et al., 2004], or they may reach the systemic circulation, allowing for distribution to more distal tissues

(e.g., bone marrow, lung, etc.). Metabolites of PAHs are found in both the faeces and urine, with the larger molecular weight PAHs being primarily excreted through the bile (i.e., feces), and smaller molecular weight PAHs being primarily excreted through the urine.

1.2.5 Metabolism

PAHs must be metabolically transformed in order to be both detoxified and excreted, and metabolic transformation can result in conversion to DNA reactive substances (i.e., genotoxicants). Once absorbed, PAHs are rapidly metabolized to several different chemical classes, including epoxides, phenols, dihydrodiols, dihydrodiol epoxides, and quinones, in order to become more water soluble substances that can be conjugated to endogenous compounds in preparation for excretion [IARC., 2010b]. These initial steps of PAH metabolism are carried out by a variety of enzymes including the cytochrome P450 (CYP) mono-oxygenases (i.e., members of the CYP1A, CYP1B, CYP2B, CYP2C, and CYP3C families), aldo-keto-reductases (AKRs) (i.e., AKR1C1, AKR1C2, AKR1C3, AKR1C4, AKR1A1), NAD(P)H quinone oxidoreductase 1 (NQO1), and epoxide hydrolase (EH). Metabolites are then frequently conjugated with sulfate (i.e., via sulfotransferase; SULT), glutathione (i.e., via glutathione-S-transferase; GST), and UDP-glucuronic acid (i.e., via UDP-glucuronosyltransferase; UGT) in order to further increase their solubility for excretion [IARC., 2010b]. Intermediate metabolites include some potent electrophiles that can readily react with DNA thereby causing genetic damage that may result in permanent sequence changes (i.e., mutations) (See **Figure 1.1a**).

1.2.6 PAH conversion to DNA-reactive metabolites

A major pathway for conversion of PAHs to DNA-reactive metabolites involves the production of dihydrodiol-epoxides via metabolism by CYP1 isozymes and EH [Shimada, 2006] (**Figure 1.1b**). CYP1A1 and CYP1B1 are the major enzymes involved in the diol-epoxide pathway, although CYP1A2 can catalyze the initial oxidation reaction of PAHs as well, resulting in a ring epoxide [Shimada, 2006; Shimada and Fujii-Kuriyama, 2004; Xue and Warshawsky, 2005]. Epoxides may then be hydrated by EH catalysis, forming dihydrodiols, which can be further metabolized via P450 enzymes (e.g., CYP1A1, CYP1B1) to form dihydrodiol epoxides: genotoxic metabolites that react with macromolecules such as DNA and protein. These dihydrodiol epoxides are potent electrophiles that form a covalent link with a nucleotide, creating a DNA adduct [Parkinson, 2001; Shimada, 2006]. In the case of BaP, the major reactive metabolite is BaP-7,8-dihydrodiol-9,10-epoxide (BPDE), which typically covalently binds to the N2 of deoxyguanosine [Hess et al., 1997; Jennette et al., 1977]. The four possible stereoisomers that can be formed vary in their genotoxic potency, with the (+)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide isomer being both the most potent and the predominant adduct-forming BaP metabolite in mammalian systems [IARC., 2010b] (**Figure 1.1c**).

PAHs that are especially mutagenic frequently contain a bay- (e.g., BaP, DBaA) or fjord-region (e.g., DBaP), which are both sterically-hindered (see **Table 1-I**). If an epoxide forms in either of these regions, the steric hindrance results in increased stability of the diol-epoxide [IARC., 2010b]. The increased stability of the genotoxic metabolite in turn contributes to the higher potency of the diol-epoxide. Fjord-regions are even more sterically-hindered than bay-regions, resulting in higher stability than diol-epoxides

formed in bay-regions [Glatt et al., 1991], and likely contributing to the greater genotoxic potency of DBaP in comparison with BaP [Binková et al., 2000; Cavalieri et al., 1991; Higginbotham et al., 1993].

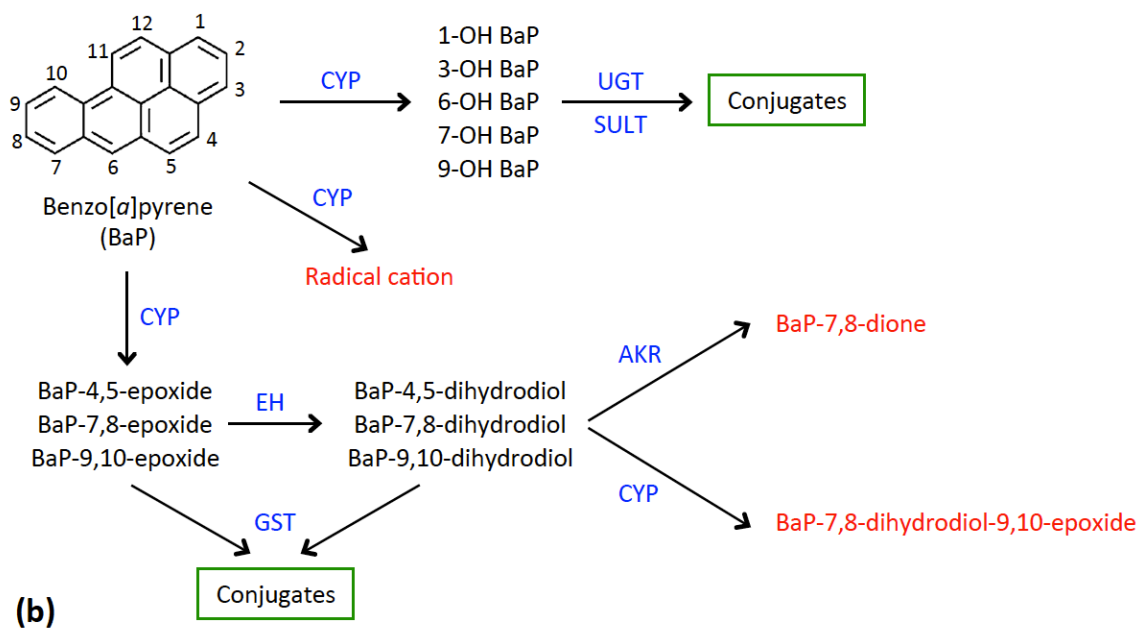
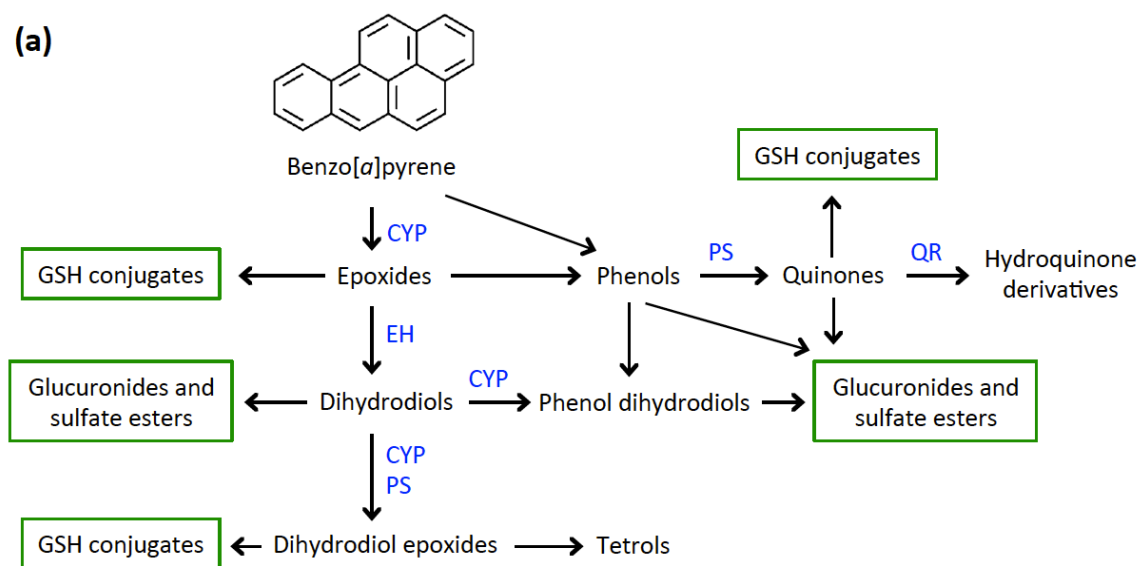
Expression of P450 enzymes varies across tissues; CYP1A2 is a major hepatic P450 enzyme, whereas CYP1A1 and 1B1 are primarily expressed extra-hepatically in several murine tissues including lung and small intestine [Choudhary et al., 2003; Hart et al., 2009; Renaud et al., 2011; Zhang et al., 2003]. CYP1A1 is generally present only at low levels unless its expression is induced via aryl hydrocarbon receptor (i.e., AhR) agonism, whereas CYP1B1 is constitutively expressed in certain tissues, and a substantial amount of CYP1A2 activity is present in liver. Both of these enzymes can also be upregulated following induction via AhR agonism [Nebert et al., 2004].

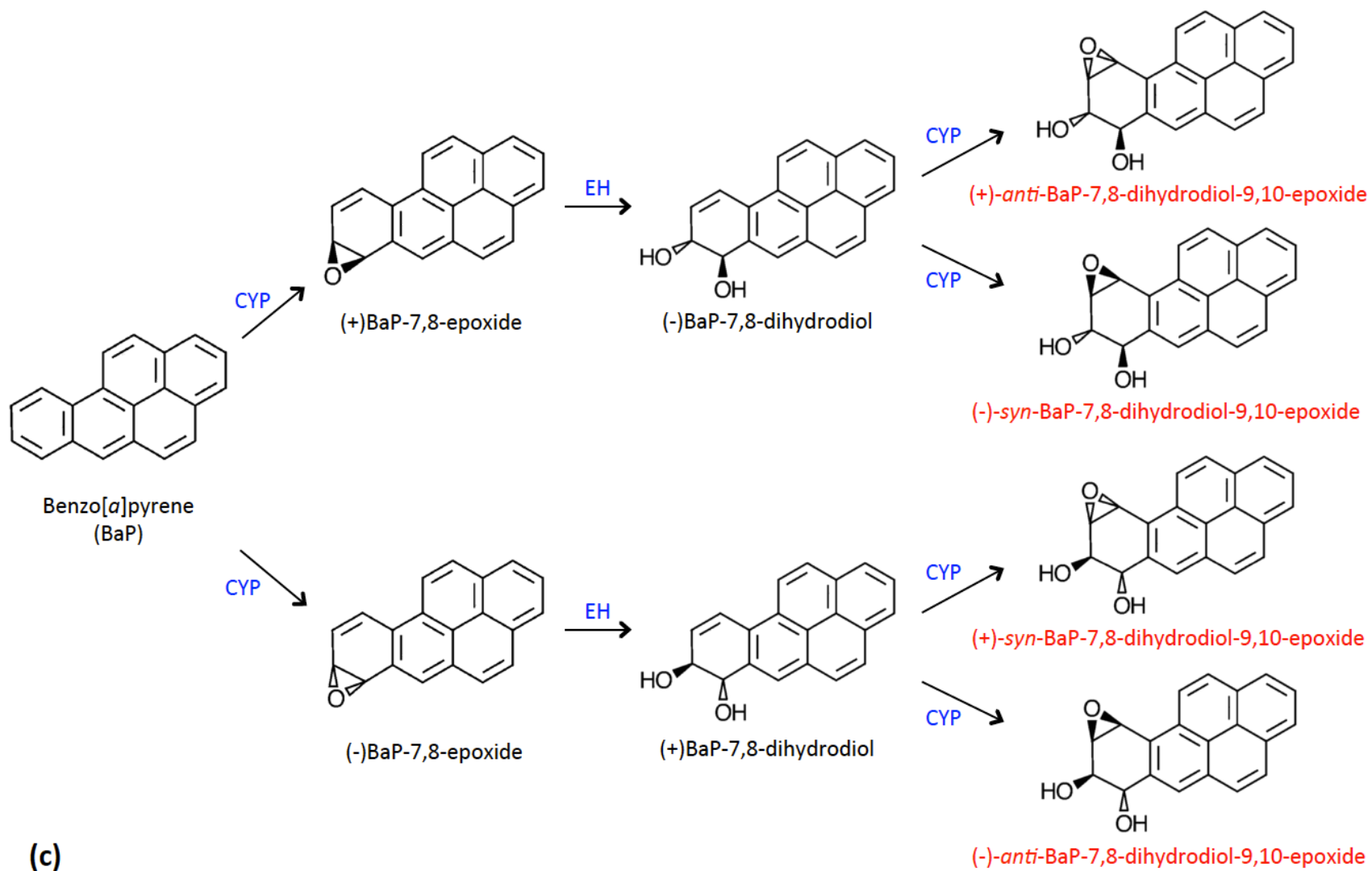
Two other metabolic pathways are also known to result in the generation of genotoxic metabolites: the radical cation pathway and the *ortho*-quinone pathway [IARC., 2014]. The radical cation pathway is a one-electron oxidation that forms radical cations of BaP, resulting in depurinating adducts on adenine and guanine that lead to abasic sites [IARC., 2014]. The *ortho*-quinone pathway begins with the generation of the PAH-dihydrodiol (as with the diol-epoxide pathway) that is metabolised by AKRs to a catechol, which can then be oxidized to produce *ortho*-quinones [Penning, 2014; Penning et al., 1996] (**Figure 1.1b**). The *ortho*-quinones may then form covalent adducts with DNA nucleotides, or may become caught in a persistent redox cycle, generating reactive oxygen species (ROS), which are also DNA-damaging agents [Penning et al., 1996; Penning, 2014].

In summary, for an oral exposure, there is GI tract exposure of the parent PAHs, which can be transported to the liver following absorption from the length of the GI tract. Most of the absorbed compound will then likely be metabolised and detoxified; however, a portion can also be metabolically *bioactivated* by the liver and GI tract (e.g., small intestine, stomach) [Choudhary et al., 2003], resulting in the formation of DNA adducts in these tissues. From the liver, the parent compound or metabolites can either be returned to the intestine via enterohepatic circulation, resulting in re-exposure of these tissues and/or excretion [Miller and Ramos, 2001; Ramesh et al., 2004], or they may reach the systemic circulation, allowing for distribution to more distal tissues.

Figure 1.1. (a) A general schema for the metabolism of benzo[*a*]pyrene (BaP), a prototypical mutagenic PAH; **(b)** The metabolic activation of BaP by CYPs; **(c)** The stereoisomers formed by the metabolic conversion of BaP to its genotoxic metabolite BaP-7,8-dihydrodiol-9,10-epoxide. The (+)-*anti*-BaP-7,8-dihydrodiol-9,10-epoxide is the most potent of the four stereoisomers. Modified from IARC Monographs vol. 92 [2010b]. CYP: cytochrome P450; EH: epoxide hydrolase; PS: prostaglandin H synthase; QR: quinone reductase; GSH: glutathione; UGT: uridine 5'-diphosphate-glucuronosyltransferase; SULT: sulfotransferase; AKR: aldo-keto reductase; GST: glutathione-*S*-transferase.

(a)





1.2.7 DNA repair and translesion bypass of PAH-induced genetic damage

DNA adducts are important precursors of mutations; however, not all DNA adducts contribute to the establishment of permanent sequence changes. In order for a mutation to occur, the DNA adduct must escape detection by the cell's repair machinery and cause an error to be made in the nucleotide incorporated opposite the lesion during replication. A detailed discussion of DNA repair mechanisms is beyond the scope of this thesis; nevertheless, it is appropriate to briefly describe repair processes that are equipped to handle PAH-induced genetic damage.

PAH dihydrodiol-DNA adducts are primarily repaired through nucleotide excision repair (NER) [Iyama and Wilson, 2013; Braithwaite et al., 1998; Hess et al., 1997]. NER operates through both global genome repair (GG-NER) and transcription-coupled repair (TC-NER). GG-NER repairs damage throughout the genome, whereas TC-NER repairs damage on actively transcribed regions of the genome, specifically on the transcribed strand [Marteijn et al., 2014; Iyama and Wilson, 2013]. In GG-NER, the bulky adduct is recognized by the DNA repair machinery (i.e., XPC in a complex with RAD23 and CETN2, which binds to the single-stranded DNA on the un-damaged strand) due to the distortion in the double-helix caused by the adduct [Marteijn et al., 2014]. Adducts are detected differently for TC-NER, where the adduct causes transcription stalling, which increases the affinity of CSB for RNA Pol II, triggering the formation of the CSA-CSB complex that renders the lesion at the stalled site accessible for DNA repair [Marteijn et al., 2014; Foustieri and Mullenders, 2008]. Following the damage recognition step, the GG- and TC-NER pathways are essentially identical and involve the same proteins [Iyama and Wilson, 2013]. Once the damage is recognized, TFIIH is

recruited to the damage site, and binds to the pre-excision site along with XPG. The CAK sub-complex then dissociates from the TFIIH complex, and the TFIIH helicase activity unwinds the double helix surrounding the lesion, with subunit XPD verifying the presence of lesions along with the XPB subunit, while XPA binds to the single-stranded DNA on the damaged strand [Marteijn et al., 2014]. Single stranded binding protein (i.e., RPA) is recruited to coat the undamaged strand. XPA then recruits the XPF-ERCC1 heterodimer, which creates an incision 5' to the lesion. This in turn activates XPG, which creates an incision 3' to the lesion, thereby excising the lesion and creating a gap that is 22-30 nucleotides long [Marteijn et al., 2014]. PCNA is directly loaded by XPF-ERCC1 following the 5' incision, which recruits DNA Pol δ , DNA Pol κ , or DNA Pol ϵ to fill the gap [Marteijn et al., 2014]. The newly synthesized DNA is then ligated (i.e., by DNA ligase 1 or DNA ligase 3), and the repair is complete. GG-NER takes place independent of the cell cycle [Iyama and Wilson, 2013; Fagbemi et al., 2011]; however, as an added safeguard for maintaining the integrity of the genome, damaged cells will arrest at cell cycle checkpoints (i.e., G₁, G₂, M). The G₁ checkpoint is particularly important in preventing damage induced by PAHs, as cells with damaged DNA will arrest at this checkpoint in order to provide time for DNA repair to take place before proceeding to S-phase, where damage could result in permanent sequence changes [Sancar et al., 2004]. Under heavy levels of damage, the cell may instead be directed toward programmed cell death (i.e., apoptosis), instead of surviving with damage and passing on potentially critical mutations. This is clearly not a perfect system, as we are able to detect both DNA adducts and mutations in cells following exposure to mutagenic compounds such as PAHs.

Should BPDE adducts persist, they may be bypassed by a process known as translesion synthesis (TLS); however, this may occur in an error-free, or error-prone manner. When a BPDE adduct is encountered during replication, it will result in stalling of the replication fork, as high-fidelity replicative polymerases cannot replicate past this blockage [Jha et al., 2016]. The polymerase will then dissociate, and typically a Y-class polymerase will be recruited to replicate past the bulky adduct (i.e., carry out TLS) [Prakash et al., 2005; Liu et al., 2016; Yang and Woodgate, 2007]. There are at least two types of Y-class polymerases that are recognized to carryout TLS across BPDE deoxyguanosine adducts; Pol κ , which does so in an error-free manner (i.e., correctly inserting deoxycytidine monophosphate) [Liu et al., 2014; Jha et al., 2016; Yang and Woodgate, 2007], and Pol η , which does so in an error-prone manner (i.e., incorrectly inserting adenosine monophosphate) [Christmann et al., 2016]. The circumstances under which Pol κ and Pol η are employed are not currently understood. There is also some evidence that base excision repair may be involved in the repair of damage induced by PAH-DNA adducts (likely those that result in AP-sites), and this process may play a larger role in the repair of lesions induced by some weaker PAHs such as chrysene [Braithwaite et al., 1998].

In summary, PAH-dihydrodiol-epoxides exert their mutagenic effects by covalently binding to a nucleotide, causing a DNA adduct. If this damaged DNA is not excised and repaired prior to DNA replication, and if error-prone TLS takes place, then adduction can cause permanent DNA sequence changes such as deletions, insertions, or base-pair substitutions (i.e., mutations). PAHs may also generate oxidative damage and abasic sites, both of which can cause mutations. As mentioned, mutations in critical genes

can lead to uncontrolled cell proliferation and replicative immortality, contributing to tumour formation and establishment [Cairns, 1998; Josephy, 1997].

1.2.8 PAH mixtures, priority PAHs, and the chemical specific approach to hazard and risk assessment

Humans are rarely exposed to individual PAHs, but rather to complex mixtures of PAHs in environmental media such as soil, water, and air. Environmental mixtures of PAHs also contain PACs, many of which are also toxic and mutagenic [Lundstedt et al., 2003; Rosenkranz and Mermelstein, 1983; Watanabe et al., 2003; Møller et al., 1985]. Several PAH-containing complex environmental mixtures are IARC Group 1 carcinogens, including diesel exhaust [IARC., 2014], air pollution [IARC., 2015], tobacco smoke [IARC., 2012; IARC., 2004], and coal tar [IARC., 2010a]. Due to the known toxicity of PAHs, several regulatory agencies have identified a number of PAHs as important environmental pollutants. In accordance with amendments to the Clean Water Act, in the late 1970s the United States Environmental Protection Agency (US EPA) designated sixteen PAHs as priority pollutants (i.e., priority PAHs) [Keith and Telliard, 1979; Keith, 2015]. The US EPA also designates seven PAHs as B2 carcinogens (i.e., probable human carcinogens) [Schoeny and Poirier, 1993]. Under the Canadian Environmental Protection Act (CEPA), five PAHs have been designated as probable human carcinogens [CEPA., 1994], and the Canadian Council of Ministers of the Environment (CCME) designates eight PAHs as carcinogens [CCME., 2010].

Complex environmental mixtures, including PAH mixtures in complex environmental matrices, are especially challenging from a risk assessment perspective. Most available toxicological data are for single compounds; moreover, no two

environmental mixtures are exactly the same, and the identity, concentrations, and toxicity of compounds within the mixtures are largely unknown. Finally, there is a paucity of data regarding possible interactions between the known mixture components. Each of these issues complicates hazard/risk assessment.

To address these challenges, governmental organizations such as the US EPA [USEPA, 2000] and Health Canada [Health Canada, 2010] have published guidance documents for conducting human health risk assessments of chemical mixtures. Although the preferred approach is to examine the actual mixture in question, it is unrealistic to toxicologically characterize all environmental mixtures. As a result, a chemical-specific, dose-addition model is recommended for compounds such as PAHs that are presumed to act via a common mode-of-action. This approach calculates the total hazard or risk of a mixture as the incremental sum of the contributions from a small number of targeted PAHs. In Canada, these targeted PAHs are the eight CCME carcinogenic PAHs [CCME., 2010], and in the United States, they are the seven B2 carcinogenic PAHs [Schoeny and Poirier, 1993]. Importantly, this approach does not take into consideration toxic effects of non-targeted PAHs, nor PACs that may be present in the mixture. Moreover, it assumes that the targeted PAHs act in an additive manner, whereby the total activity of the mixture is the incremental sum of the contributions from each targeted component. There is a paucity of data in the literature to support the additivity assumption, and additional research is required to support the approach currently employed for the assessment of complex PAH mixtures. Risk assessment approaches for PAHs mixtures are described in more detail in **Chapter 4**.

1.3 Human Health Risk Assessment of Environmental Chemicals

As already noted, a number of adverse health effects, including cancer, are empirically and mechanistically associated with exposures to environmental mutagens and/or teratogens. Prevention of mutations and mutation-related diseases, or the reduction of the likelihood of mutations, is a major focus in regulatory toxicology. Regulatory bodies in many countries, including Canada and the United States, use a multi-stage process for chemical risk assessment. The terminology employed and organization of the steps involved are often slightly different across assessment types, organizations, and countries; however, the risk assessment framework laid out by the US EPA is also generally applicable to the process for chemical risk assessment used in Canada. The process includes (1) hazard assessment, (2) dose-response assessment, (3) exposure assessment, and (4) risk characterization [USEPA, 2005]. Throughout the risk assessment process, a weight-of-evidence approach is employed, whereby all relevant information is considered, including uncertainties. The hazard assessment and dose-response assessment stages are within the scope of this thesis, and further described below.

Hazard assessment involves determining whether a compound has the ability to harm humans (sometimes referred to as hazard identification), and defining the adverse endpoint of concern/critical effect. This is carried out by reviewing test results from a variety of assays for different toxicological endpoints, including genetic toxicity. Information generated during this stage is also used for mode-of-action determination.

Dose-response assessment involves characterization of the dose-response relationship to determine a point-of-departure (PoD) metric that, when suitably adjusted for extrapolation uncertainties, yields a human dose limit associated with negligible risk

of adverse health effect. The PoD metrics typically used relate to the most sensitive, relevant endpoint (i.e., the adverse endpoint of concern/critical effect) defined at the hazard assessment stage. The PoD is typically the no observed adverse effect level (NOAEL) or the benchmark dose (BMD), but alternatively may be the lowest observed effect level (LOAEL). The LOAEL is the first dose at which there is a statistically significant increase in response relative to the control, whereas the NOAEL, which is preferred, is the dose below the LOAEL. The BMD is determined differently. The entire range of doses is modelled to determine the best function that describes the dose-response relationship, with the BMD being the interpolated dose that elicits the benchmark response (BMR), a pre-determined small increase in response (e.g., BMD₁₀ is the dose at which there is a 10% increase in response above the concurrent control) (**Figure 1.2**).

Most toxic effects are assumed to have a threshold; that is, there is a dose below which the risk of adverse outcome is considered negligible. Consequently, the PoDs noted above (i.e., the NOAEL, LOAEL or BMD) are used to estimate exposure limit values for risk assessment. This approach is employed for carcinogens with a non-genotoxic mode-of-action. In contrast, the dose-response for genotoxic carcinogens is assumed to be linear back from tested doses to zero, with all non-zero doses associated with a finite risk of effect. Consequently, linear extrapolation from the PoD is employed, with the resulting slope between the PoD and zero dose termed the “slope factor”. The slope factor is used to estimate the dose associated with an acceptable level of excess lifetime cancer risk.

Regulation of genotoxic compounds on the basis of potential carcinogenicity, rather than mutagenicity *per se*, assumes that there is no dose below which the risk of

adverse effect is considered negligible. Consequently, although assessment of genetic toxicity is an integral part of the hazard assessment stage of human health risk assessment, the data are generally used in a qualitative manner to merely identify compounds that are potential carcinogens. In other words, the results of genetic toxicity studies are not typically used for dose-response analysis and PoD determination; moreover, not used to determine human exposure limits associated with negligible effects. However, the genetic toxicology community worldwide is increasingly questioning the validity and utility of a regulatory paradigm for genotoxic compounds that is based solely on potential carcinogenicity, with the attendant assumption of a linear response from zero dose (i.e., inability to determine a dose associated with negligible risk).

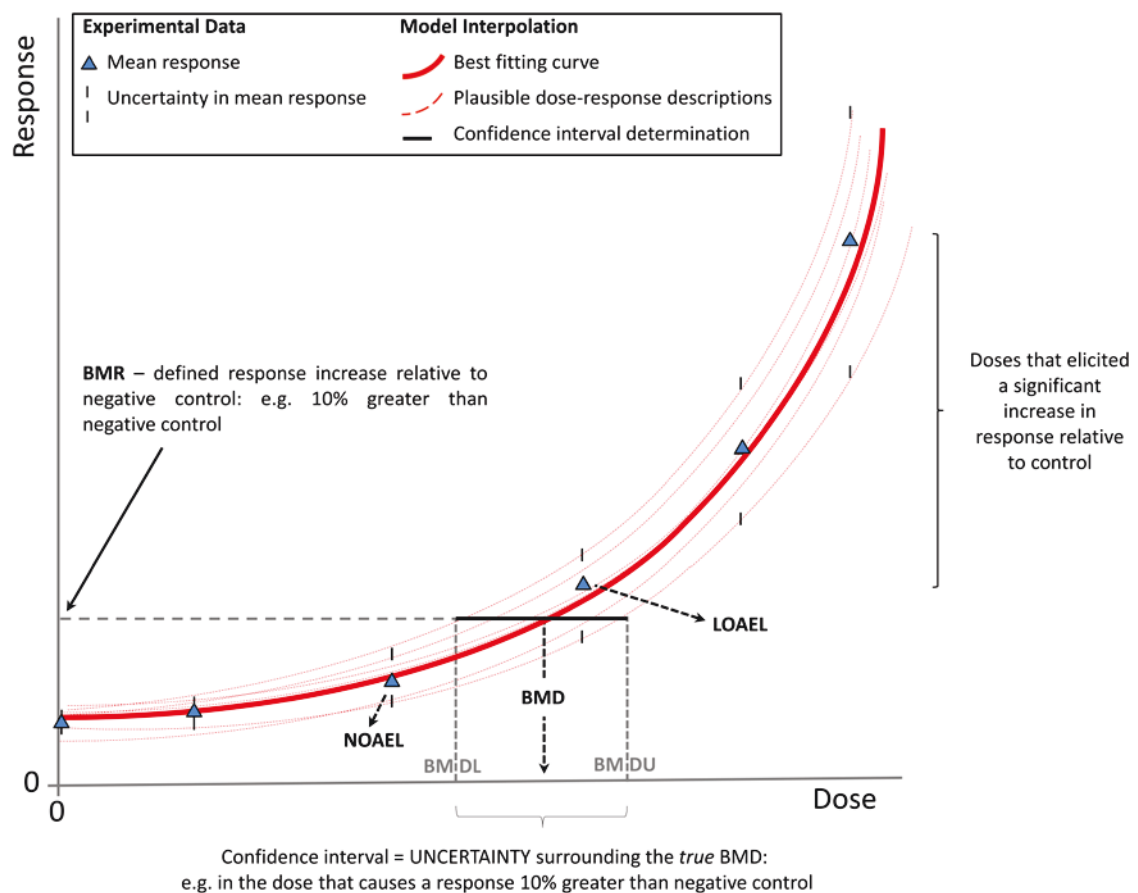


Figure 1.2. Schematic representation of the benchmark (BMD) approach for quantitative analysis of dose-response data. The BMD is the dose that elicits the benchmark response (BMR), that is, a pre-specified increase in response relative to the control (e.g., 10%). The upper (BMDU) and lower (BMDL) confidence limits on the BMD indicate the level of uncertainty surrounding the BMD estimate. NOAEL and LOAEL values are also indicated. The LOAEL is the first dose at which there is a statistically significant increase in response relative to the control, whereas the NOAEL is the dose below the LOAEL. Modified from Wills et al. [2016].

1.3.1 *In vitro* and *in vivo* tests to identify chemical mutagens

Regulatory genetic toxicology testing in Canada, the United States, Europe, and Japan began in the 1970s-1980s [MacGregor et al., 2015a], when it was recognized that there is a need to screen chemicals for their ability to cause genetic damage [Gollapudi et al., 2013]. In Canada and the United States, the genetic toxicology test battery, which is employed in the hazard assessment stage, is generally comprised of an *in vitro* bacterial test for gene mutation, an *in vitro* mammalian cell test for gene mutation or chromosome damage, and an *in vivo* mammalian assay for chromosome damage [SOR/2005-247 NSNR, 2005; FDA, 2007] or gene mutation [SOR/2005-247 NSNR, 2005]. In Canada, the assays employed to evaluate chemicals in commerce depend on the assessment program and the annual production or import volume of the chemical [SOR/2005-247 NSNR, 2005]. The assays commonly used include the Ames/*Salmonella* reverse mutation assay [OECD, 1997a], the mouse lymphoma *tk* gene mutation assay [OECD, 1997c], the *in vitro* mammalian micronucleus (MN) assay [OECD, 2010], the mammalian erythrocyte MN assay [OECD, 1997b], and the bone marrow chromosomal aberration assay [OECD, 2016].

Prior to 2011, there was no OECD (Organisation for Economic Co-operation and Development) test guideline for a readily available mammalian *in vivo* gene mutation assay. As a result, the *in vivo* test employed in the standard test battery generally examined chromosome damage in hematopoietic tissue (i.e., erythrocyte micronucleus assay, bone marrow micronucleus assay or chromosomal aberration assay); however (i) not all mutagens are clastogens, and (ii) the target for mutation or chromosome damage may not be haematopoietic tissue. Researchers attempted to resolve this “target tissue

issue” by developing assays that allow examination of genotoxic endpoints in the tissue that is suspected to be a target for induced genetic damage. However, many of these assays assess genetic damage that is potentially reversible (e.g., strand breaks, DNA adducts, unscheduled DNA synthesis), and do not assess induced *bona fide* mutational events. The few existing *in vivo* gene mutation assays were/are not frequently used as they are extremely laborious and require large numbers of animals (e.g., the *in vivo* *Hprt* mutation assay). To fill the gap in the genetic toxicity regulatory test battery, transgenic rodent *in vivo* gene mutation assays were developed in the late 1980’s [Gossen et al., 1989]. A test guideline for the transgenic rodent somatic and germ cell mutation assays was approved by the OECD in July 2011 [OECD, 2013], thus providing tools for assessment of induced *in vivo* mutations across many tissues. More recently, other mammalian *in vivo* gene mutation assays have been developed. However, like the aforementioned *in vivo* chromosome damage endpoints, they are largely restricted to hematopoietic tissues (e.g., the *Pig-a* assay).

1.3.2 Transgenic rodent *in vivo* gene mutation assays

Transgenic rodents (TGRs) are extremely useful and powerful genetic toxicology tools. They can detect induced mutant frequency *in vivo*, in any target tissue including germ cells. Consequently, they are also extremely useful for understanding tissue-specific genotoxic responses. The TGR systems employ a mutation target contained within a shuttle vector or plasmid that can readily be excised following extraction of genomic DNA from the tissue of interest. The vector or plasmid containing the transgenic target is recovered from genomic DNA, and scored following infection of host bacteria and biochemical or thermal selection of transgene mutants.

There are several TGR systems, including both mouse and rat. The MutaTMMouse system, which was utilized for the analyses presented herein, is described in more detail below. There are two BigBlue[®] mice (C57BL/6 and B6C3F1) and one BigBlue[®] rat (F-344), all of which contain the λ LIZ α shuttle vector. The λ LIZ α vector contains the *lacI* gene that can be scored for mutants via a colorimetric assay, as well as the lambda bacteriophage *cII* gene, which can be scored via a temperature-driven positive selection assay. The *lacZ* plasmid mouse (C57BL/6) has a pUR288 plasmid that contains *lacZ* as the mutation target, which can be scored using a chemically-driven positive selection assay. The *gpt* delta mouse (C57BL/6J) and rats (Sprague Dawley and F-344) have a λ EG10 shuttle vector that carries *gpt* as the mutation target, with one selection method for point mutations, and a second selection method for large deletions (i.e., deletions in the *red/gam* genes). A thorough overview of the various TGR assays is available in the OECD detailed review paper [Lambert et al., 2009].

1.3.2.1 The MutaTMMouse system

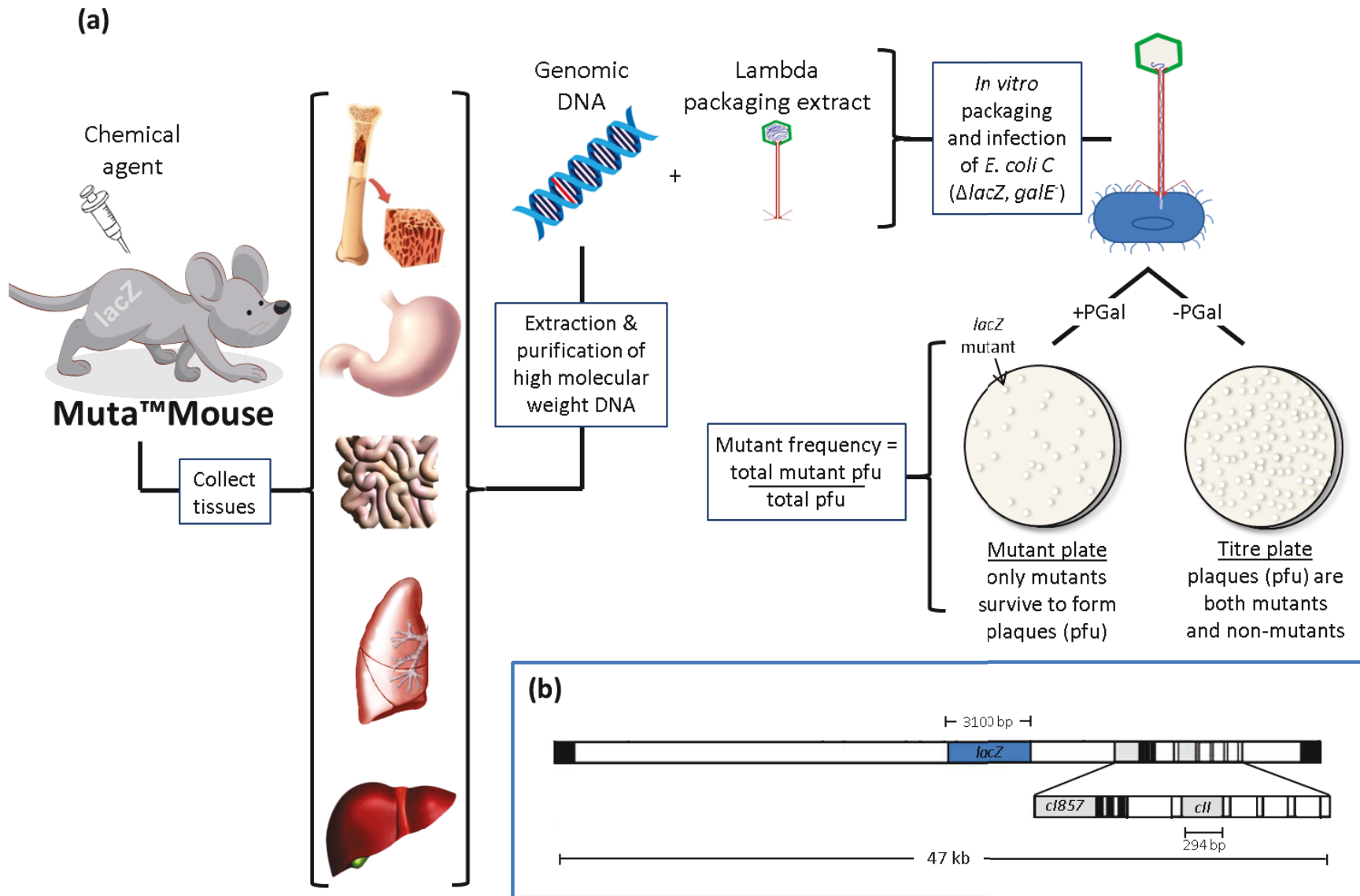
The MutaTMMouse TGR is a CD2F1 transgenic mouse that contains a bacterial *lacZ* gene (3100 bp) as a mutation target. The target transgene is contained in a lambda vector construct known as λ gt10 (47 kb) [Gossen et al., 1989]. The TGR mice were produced by microinjection of an embryo resulting from a female BALB/C and male DBA/2 cross, and strain 40.6 was selected. Subsequent genetic characterization showed that strain 40.6 (i.e., the MutaTMMouse) contains 29 ± 4 concatenated copies of the λ gt10 vector stably incorporated into chromosome 3 in a head-to-tail manner [Blakey et al., 1995; Shwed et al., 2010]. The system has been shown to effectively detect base-pair substitution and frameshift mutations, as well as small deletions [Lambert et al., 2005].

The *lacZ* gene encodes β -galactosidase, a gene product that is amenable to positive selection of mutants using *Escherichia coli galE⁻ lacZ* and the PGal (phenyl- β -D-galactoside) positive selection system [Gossen et al., 1992]. In this system *lacZ galE⁻* host bacteria that are infected with a functional copy of *lacZ* will convert the selective agent (i.e., PGal) to galactose and then UDP-galactose, but will be unable to convert this toxic metabolite to non-toxic UDP-glucose. Thus, in the presence of a wild type copy of *lacZ*, a toxic metabolite is formed that prevents the formation of plaques. In contrast, host bacteria infected with a non-functional mutant copy of *lacZ* will not be able to initiate the metabolism of galactose, there will be no build-up of the toxic metabolite (i.e., UDP-galactose), and the infected cells survive to form plaques. Additionally, mutant frequency can be determined at the *cII* locus (294 bp) via a temperature-sensitive positive selection assay [Lambert et al., 2009]. A schematic overview of the positive selection assay for *lacZ* is presented in **Figure 1.3**. The ability to also score mutants at the *cII* locus is very convenient, as it allows for comparison with *lacZ* mutant frequency, and the fact that it is a 10-fold smaller gene than *lacZ* makes it convenient for Sanger sequencing. Additionally, a method was recently developed that allows for efficient mutation spectrum analysis in MutaTMMouse by next-generation sequencing of the *lacZ* gene following PGal positive selection of mutants [Beal et al., 2015].

The performance of the MutaTMMouse TGR assay has been tested with a wide range of known mutagens with various mechanisms-of-action (e.g., BaP, *N*-ethyl-*N*-nitrosourea, acrylamide), and it has demonstrated high sensitivity (i.e., the ability of an *in vivo* genetic toxicity assay to return a positive response for a genotoxic carcinogen) in several target tissues (see Lambert et al. [2009]). Additionally, many other genotoxicity

assays, such as the mammalian erythrocyte micronucleus assay [OECD, 1997b], the *Pig-a* endogenous gene mutation assay [Phonethepswath et al., 2008; Dertinger et al., 2011], and the ^{32}P -postlabelling assay for DNA adducts [Phillips and Arlt, 2007; Phillips and Arlt, 2014] can easily be integrated with a TGR assay [Lemieux et al., 2011]. The integrated assessment of multiple toxicological endpoints in various tissues maximizes the data that can be obtained from a single animal study, thus reducing the need for additional animal testing, and providing valuable data for mode-of-action determination.

Figure 1.3. (a) A schematic of the steps involved in carrying out the MutaTMMouse transgenic rodent assay; **(b)** the λ gt10 shuttle vector containing the *lacZ* gene and the *cII* gene, both of which can be used as a mutation target in positive selection assays. Part (b) modified from Lambert et al. [2005]. PGal: phenyl- β -D-galactoside; pfu: plaque forming units; bp: base pairs; kb: kilobases.



1.4 Quantitative Analyses of Genetic Toxicity Data

Despite the fact that interpretation of genetic toxicity test results is generally restricted to qualitative hazard identification, there is increasing interest in employing quantitative analyses of genetic toxicity dose-response data to derive metrics that can be used to establish human exposure limits or MOEs (Margins of Exposure), thereby supporting human health risk assessments and regulatory decisions [White and Johnson, 2016]. The sections below provide an overview of the events leading to this paradigm shift.

1.4.1 Genetic toxicity – a brief history of regulatory screening

The aforementioned genetic toxicity screening batteries developed in the early 1970's were initially concerned with the prevention of heritable mutations in humans, rather than cancer [Zeiger, 2004; DHEW, 1969; Drake et al., 1975; Crow, 1968; Flamm et al., 1977; NRC, 1983]. The requirement for quantitative *in vivo* data “from relevant animal model systems” to be used for determining “virtually safe or tolerable levels of exposure” was also acknowledged [Flamm et al., 1977], and early assessment strategies involving quantitative assessments were employed to address concerns about heritable effects. However, this was all occurring around the time that Bruce Ames and colleagues published a seminal paper declaring that “Carcinogens are Mutagens”, and describing the Ames test (i.e., the *Salmonella* mammalian microsome reverse mutation assay) as a screening tool capable of correctly identifying carcinogenic compounds [Ames et al., 1973]. The Ames test's predictive ability was met with a great deal of enthusiasm. It was assumed that carcinogenic compounds are relatively rare, and therefore, that a genetic toxicity test battery could effectively identify them and screen them out [Gollapudi et al.,

2013; MacGregor et al., 2015a]. This resulted in a shift from the previous quantitative emphasis on germ cell damage and heritable mutations, to cancer as the main health consequence of concern with qualitative screening and mutagen identification used for regulatory decision-making [MacGregor et al., 2015a]. A tiered testing strategy was then implemented, as it was considered the most effective approach for evaluating large numbers of chemicals. Genetic toxicology test batteries were implemented with a qualitative “screen and bin” approach (i.e., hazard identification) that identifies mutagens that are likely carcinogens. Thus, genetic toxicity endpoints have traditionally been inextricably linked to carcinogenicity, and not recognized as independent, relevant (i.e., *bona fide*) toxicological endpoints for risk assessment and regulatory decision-making.

1.4.2 Recognition of mutation as a *bona fide* toxicological endpoint

As mentioned previously, the genetic toxicology community increasingly recognizes that exposure to mutagenic compounds can result in a host of diseases, such as somatic mosaic diseases and syndromes, mitochondrial disorders, genomic diseases and disorders, and congenital defects resulting from germ cell mutations, in addition to cancer. As a result, mutagenicity is now being increasingly recognized as a *bona fide* toxicological endpoint for regulatory decision-making [Thybaud et al., 2007; MacGregor et al., 2015a; MacGregor et al., 2015b; Gollapudi et al., 2013; Johnson et al., 2014; DNH&W, 1993; White and Johnson, 2016]. This recognition supports the use of quantitative dose-response analysis to determine PoD metrics that can be employed for risk assessment and regulatory decision-making. Moreover, it is now acknowledged that it is mechanistically plausible that low doses of genotoxic agents may not result in adverse effects due to the host’s ability to maintain homeostasis via specific mechanisms

that include upregulation of detoxification enzymes and DNA repair [MacGregor et al., 2015a; Gollapudi et al., 2013; Johnson et al., 2014; Johnson et al., 2012; Jenkins et al., 2010; Zair et al., 2011; Elhajouji et al., 2011; Lynch et al., 2003]. The recognition that compensatory mechanisms are likely able to maintain homeostasis at low doses provides a foundation for quantitative analyses that determine a dose below which the probability of genetic damage is negligible. Interestingly, although the quantitative assessment of genetic toxicity data for regulatory purposes is only recently starting to be implemented, the assertion that genotoxicity itself is a *bona fide* toxicological endpoint upon which chemicals should be regulated was already recognized by Health Canada, as stated in regulatory documents back in the 1980s and 1990s [DNH&W/DOE, 1988; DNH&W, 1993; Health Canada, 1996], making Health Canada an important pioneer in this paradigm shift.

1.4.3 The Viracept incident

The first thorough application of quantitative analysis of genetic toxicity test results for human health risk assessment relates to the accidental contamination of a batch of nelfinavir mesylate tablets (i.e., Viracept) with the known mutagen ethyl methanesulphonate (EMS) [Müller and Gocke, 2009; Müller et al., 2009]. The F. Hoffmann-La Roche Company (i.e., Roche) was then faced with the challenge to quickly determine whether the amount of impurity present in the tainted tablets could adversely harm patients. A rodent carcinogenicity study was clearly not suitable as they take over 2 years to complete. Instead, Roche utilized the transgenic rodent MutaTMMouse, and conducted a subchronic oral study with analysis of induced mutant frequency in several tissues. They then employed quantitative analysis of the results to determine a PoD (i.e.,

the no observed genotoxic effect level (NOGEL) for *lacZ* mutants in the GI tract of MutaTMMouse), which was then used, along with the human exposure level, to calculate the *safety factor* (i.e., ratio of the NOGEL to the human exposure level). They determined that there was a 454-fold safety factor between what would be expected to induce adverse effects in mice, and the patients' maximum exposure level. This safety factor, along with detailed absorption, distribution, and metabolism data for EMS in multiple species, convinced regulatory agencies that the amount of EMS present in the tablet would result in negligible risk to those who had taken tablets from the contaminated batch [Lutz, 2009].

1.4.4 Genotoxicity point-of-departure metrics for human health risk assessment and regulatory decision-making

Following the Viracept incident, several researchers and international working groups evaluated a variety of statistical approaches for quantitative analyses of genetic toxicity dose-response data, and determination of PoD metrics for risk assessment and regulatory decision-making. A number of quantitative approaches for assessing dose-response relationships in genetic toxicology studies were examined by the Genetic Toxicology Technical Committee (GTTC) of the Health and Environmental Sciences Institute (HESI), which is part of the International Life Sciences Institute (ILSI) [Gollapudi et al., 2013; Johnson et al., 2014]. The working group evaluated several metrics, including the NOGEL (i.e., a NOAEL for genetic toxicology endpoints), BMD, BPD (breakpoint dose), and the STD (slope transition dose). The works of MacGregor et al. [2015a; 2015b], Gollapudi et al. [2013] and Johnson et al. [2015] noted that the BMD-approach, which has been extensively used for other toxicological endpoints

[Crump, 1984], is the most suitable method for determining a PoD to be used to define human exposure limits. Moreover, the BMD is also preferable over other metrics as it involves analysis of the entire dose-response function, and is generally more flexible in terms of the number of doses required to obtain a reasonably precise PoD estimate. The precision of BMD estimates is readily determined by comparing BMDUs (upper confidence limits) and BMDLs (lower confidence limits).

Following the aforementioned evaluations of various PoD metrics and selection of BMD as the most pragmatic, several international consortia and working groups outlined an approach whereby quantitative dose-response analysis could be employed to calculate human exposure limits and/or margins of exposure. These metrics provide regulatory authorities with the ability to use *in vivo* genetic toxicity dose-response data for risk assessment of genotoxic compounds [MacGregor et al., 2015a].

1.5 Thesis Overview

1.5.1 Rationale

PAHs are ubiquitous environmental contaminants, and humans are readily exposed to PAHs as complex environmental mixtures (e.g., coal tar), in complex environmental matrices (e.g., soil). However, the vast amount of toxicological data for PAHs relates to high dose studies of individual compounds. Moreover, beyond BaP, existing research has only examined genetic damage induced by PAHs in a limited number of tissues across a limited number of endpoints, and very few data sets exist that has examined the induction of mutations by PAHs *in vivo*. The TGR Muta™Mouse assay allows for *in vivo* mutation detection in essentially any tissue of interest, and is easily integrated with other assays for genetic damage. The major goals of this thesis are to (1)

employ the MutaTMMouse system to thoroughly examine the *in vivo* genetic toxicity of several genotoxic PAHs, and complex PAH-containing mixtures, across a number of tissues and endpoints, and (2) employ quantitative analyses of the results to evaluate differences in tissue-specific effects across compounds, to examine empirical relationships between genotoxic endpoints, and to evaluate the genotoxic effects of PAH mixtures. More specifically, the thesis aimed to: (1) scrutinise the tissue-specific genotoxic responses of 9 individual PAHs (**Chapter 2**); (2) assess the tissue-specific genetic toxicity of complex PAH mixtures, and moreover, scrutinise the assumption of component additivity that is routinely employed for regulatory human health risk assessment of complex PAH mixtures (**Chapters 3,4**); (3) employ recently-developed quantitative methods (i.e., BMD-combined covariate modelling) to analyse genetic toxicity dose-response data, thereby providing improved BMD precision that permits robust genotoxic potency ranking across experimental covariates (**Chapters 5, 6**); and finally, (4) employ the BMD approach to examine empirical relationships between the ability of PAHs and PAH mixtures to induce the formation of bulky DNA adducts and *lacZ* mutations (**Chapters 6, 7**).

1.5.2 Chapter objectives and hypotheses

Chapter 2

Hypothesis: Following an oral exposure, proximal tissues (e.g., small intestine, stomach, liver) will be more sensitive to PAH-induced genetic damage (i.e., yield a positive response for a genotoxic carcinogen) in comparison with haematopoietic tissues.

Objective:

- ✓ To evaluate the ability of the Muta™Mouse TGR *lacZ* mutation assay to correctly return a positive result (i.e., sensitivity) for selected carcinogenic PAHs with a mutagenic mode-of-action.
- ✓ To compare the sensitivity of the TGR *lacZ* endpoint across tissues, to the sensitivity of an endpoint that is commonly used for regulatory assessment of genotoxicity (i.e., peripheral blood MN assay).
- ✓ To critically examine the pattern of genetic damage in selected tissues in order to scrutinise the relative influence of distribution, metabolism, and tissue-specific repair on the genetic toxicity of orally-delivered PAHs.

Importance: This study is the largest sub-chronic *in vivo* genetic toxicity analysis of PAHs. In many instances, this is the first evaluation of PAHs for some endpoints examined, and for some compounds, the first *in vivo* mutagenicity assessment, and the first demonstration of a positive result for a specific endpoint and/or tissue. The results presented clearly demonstrate the utility of the Muta™Mouse TGR assay for reliable *in vivo* mutagenicity assessment of suspected carcinogens with a mutagenic mode-of-action; in particular, for substances that require metabolic conversion to DNA-reactive metabolites (e.g., PAHs). Moreover, that the test can successfully identify *in vivo* mutagens that are not detected using other tests more commonly used for regulatory assessments of new and existing chemicals.

Chapter 3

Hypothesis: Oral murine exposure to an extract of a commercially available coal tar-based driveway sealcoat will induce a statistically significant increase in genetic damage in multiple tissues.

Objective:

- ✓ To assess genetic toxicity in mice induced by oral exposure to coal tar-based driveway sealcoat extract by examining the frequency of DNA adducts and *lacZ* mutants in 5 somatic tissues, as well as micronuclei in blood.

Importance: This is the first study to demonstrate mammalian *in vivo* genetic toxicity induced by coal tar-based driveway sealcoat. This study demonstrated that the largest induction of genetic toxicity occurred in the site-of-contact tissues, rather than haematopoietic tissues, which could not have been shown using other hazard identification and assessment assays.

Chapter 4

Hypothesis: The genetic toxicity of orally-delivered PAH mixtures will be equivalent to the sum of the incremental contributions from the targeted mixture components (i.e., the mixture components will act in an additive manner).

Objective:

- ✓ To examine the *in vivo* genetic toxicity of 3 complex PAH mixtures, and 3 matched synthetic PAH mixtures in 5 different somatic tissues following sub-chronic oral exposure.

- ✓ In order to determine whether PAHs in complex and synthetic mixtures act in an additive manner, to compare the induced *in vivo* genetic toxicity results from the complex mixtures with those from the matched synthetic PAH mixtures, as well as those expected under an assumption of additivity (i.e., by employing the concentrations and potencies of each targeted PAHs).
- ✓ To scrutinize the tissue-specific differences in *in vivo* genotoxic responses across 5 somatic tissues induced by oral exposures to PAH mixtures.
- ✓ To compare BaP-equivalent concentrations calculated using the traditional additive method to those calculated using a novel bioassay-based approach (i.e., the bioassay-derived method or BDM).

Importance: Additivity of targeted mixture components is a major assumption used in the risk assessment of PAH mixtures, yet there is little data to support this assumption, and indeed the entire additivity paradigm. By examining *in vivo* mutations induced by complex and synthetic PAH mixtures, across multiple tissues, and comparing the results with those obtained for individual PAHs under an identical study design, the study was able to examine the pervasive assumption of additivity.

Chapter 5

Hypothesis: By simultaneously conducting dose-response analyses across experimental variables such as compound or sex, the BMD-covariate approach can increase the precision of genetic toxicity BMD estimates (i.e., reduce BMD confidence intervals); resultant metrics and confidence limits permit potency ranking across *in vivo* covariates (e.g., compound, tissue, sampling time).

Objective:

- ✓ To demonstrate how the combined BMD-covariate approach can be used to improve the analyses and interpretation of *in vivo* genetic toxicity dose-response data (i.e., to provide smaller 90% BMD CIs), compared with the traditional BMD-approach.
- ✓ To employ these metrics to conduct potency comparisons across *in vivo* covariates (e.g., compound, tissue, sampling time).

Importance: Using the BMD-covariate approach the study illustrates how empirical comparisons of dose-response data across experimental covariates (e.g., compound, sex) can permit more effective use of dose-response data and improved BMD precision, permitting attendant potency ranking of test articles and/or formulation of explicit mechanistic hypotheses. In addition, the study illustrates how dose-response data for large-scale studies (i.e., numerous doses) can be used to improve the precision of BMDs derived from datasets with far fewer doses.

Chapter 6

Hypothesis: BMD analyses of MutaTMMouse genetic toxicity dose-response data related to sub-chronic, oral BaP exposure, and subsequent comparisons of BMD estimates across tissues and endpoints, permits statistical delineation of tissue sensitivities within an endpoint and empirical relationships between endpoints.

Objective:

- ✓ To generate high-quality genotoxicity data for an extended dose-range of BaP in order to rigorously examine dose-response relationships across multiple tissues and endpoints.
- ✓ To employ the BMD-approach to determine the sensitivity of selected tissues to four types of BaP-induced genetic damage (i.e., to rank sensitivity to across tissues for a given endpoint).
- ✓ To employ the BMD-approach to examine empirical relationships between endpoints.

Importance: This study demonstrated that the BMD-approach permits robust comparisons of responses across tissues and endpoints, providing valuable information that adds to our mechanistic understanding of how BaP induces an array of genetic damage across several tissues. BMD rankings within an endpoint, and empirical comparisons across endpoints, contribute to an improved quantitative understanding of tissue-specific, chemically-induced genetic damage, and this knowledge can provide a foundation for the selection of tissues, endpoints and BMRs for use in human health risk assessments.

Chapter 7

Hypothesis: The ability of PAHs and PAH mixtures to elicit permanent DNA sequence changes (i.e., mutations) is positively correlated with their ability to induce genetic damage (i.e., formation of bulky DNA adducts).

Objective:

- ✓ To assess the frequency of bulky DNA adducts across 5 tissues for 15 test articles (i.e., 9 PAHs and 6 PAH-containing mixtures).
- ✓ Using previously published *lacZ* mutant frequency dose-response data for 15 test articles (i.e., 9 PAHs and 6 PAH-containing mixtures), and the newly generated DNA adduct dose-response data, examine empirical relationships between the tissue-specific BMDs (i.e., the BMD₁₀₀) for bulky DNA adducts and matched BMDs for induction of *lacZ* mutants.

Importance: This study demonstrated a statistically significant quantitative relationship between mutagenic potency and genotoxic potency when all tissues were analysed together; therefore the results demonstrate that the PAH potencies for these two endpoints are empirically related. When each tissue was examined independently, a significant relationship was observed for liver, glandular stomach, and lung, with no significant differences in the mutation-adduct potency ratio between tissues (i.e., slopes), thereby indicating that PAH-induced genetic damage is processed in a similar manner across several tissues. There is a paucity of quantitative information regarding the relationships between induction of DNA adducts and induction of mutations, and the results constitute an important contribution to evaluations regarding the utility of DNA damage frequency for human health risk assessment.

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CHAPTER TWO

2 Chapter: Tissue-specific *In Vivo* Genetic Toxicity of Nine Polycyclic Aromatic Hydrocarbons Assessed Using the Muta™Mouse Transgenic Rodent Assay

2.1 Preamble: Authors, Affiliations, and Style

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Style: This chapter was prepared according to the style guide for *Toxicology and Applied Pharmacology*.

2.2 Abstract

Test batteries to screen chemicals for mutagenic hazard include several endpoints regarded as effective for detecting genotoxic carcinogens. Traditional *in vivo* methods primarily examine clastogenic endpoints in hematopoietic tissues. Although this approach is effective for identifying systemically distributed clastogens, some mutagens may not induce clastogenic effects; moreover, genotoxic effects may be restricted to the site of contact and/or related tissues. An OECD test guideline for transgenic rodent (TGR) gene

mutation assays was released in 2011, and the TGR assays permit assessment of mutagenicity in any tissue. This study assessed the responses of two genotoxicity endpoints following sub-chronic oral exposures of male MutaTMMouse to 9 carcinogenic polycyclic aromatic hydrocarbons (PAHs). Clastogenicity was assessed via induction of micronuclei in peripheral blood, and mutagenicity via induction of *lacZ* transgene mutations in bone marrow, glandular stomach, small intestine, liver, and lung. Additionally, the presence of bulky PAH-DNA adducts was examined. Five of the 9 PAHs elicited positive results across all endpoints in at least one tissue, and no PAHs were negative or equivocal across all endpoints. All PAHs were positive for *lacZ* mutations in at least one tissue (sensitivity = 100%), and for 8 PAHs, one or more initial sites of chemical contact (i.e., glandular stomach, liver, small intestine) yielded a greater response than bone marrow. Five PAHs were positive in the micronucleus assay (sensitivity = 56%). Furthermore, all PAHs produced DNA adducts in at least one tissue. The results demonstrate the utility of the TGR assay for mutagenicity assessment, especially for compounds that may not be systemically distributed.

2.3 Introduction

In 1973 Ames and colleagues published a scientific paper proclaiming “Carcinogens are Mutagens” [Ames et al., 1973]. Since then, a flurry of publications has debated the validity of this statement. Currently, the genetic toxicology community acknowledges that 65 to 90% of rodent carcinogens will elicit a positive response in at least one of the standard regulatory assays for genetic toxicity [Zeiger, 1998; Kirkland et al., 2005; Waters et al., 2010; Hernandez et al., 2009].

Genetic toxicology test batteries are generally effective for detecting carcinogenic compounds, and the sensitivity of the three-test battery is quite high (i.e., 85-90%, depending on the assays included) [Kirkland et al., 2005]. However, not all chemicals fit this paradigm, and there is increasing recognition of response inconsistency. *In vivo* "false negatives" are known rodent carcinogens that fail to induce a positive response in an *in vivo* genotoxicity assay and may be the result of either (i) a lack of target tissue exposure, or (ii) endpoint incompatibility, or potentially both. With respect to the former, the standard test battery often examines hematopoietic tissue; however, the target tissue for mutation or chromosome damage may be a solid organ such as liver or the gastrointestinal tract. With respect to the latter, *in vitro* tests may assess the induction of mutations, whereas the *in vivo* tests often assess clastogenicity. Yet, mutagenic compounds do not necessarily induce chromosome damage, and examining only clastogenicity *in vivo* may result in a missed positive response induced by a mutagenic carcinogen. By examining mutagenicity both *in vitro* and *in vivo*, it may be possible to resolve some *in vivo* false negatives.

Researchers have attempted to resolve problems related to a lack of target tissue exposure by designing assays that assess genotoxicity in the tissue that is suspected to be a target for induced genetic damage or carcinogenesis. Examples include unscheduled DNA synthesis in liver [OECD, 1997b], the comet assay for DNA strand breaks in various target tissues [Collins, 2004; JaCVAM, 2012; OECD, 2013; Rothfuss et al., 2010], and the liver micronucleus (i.e., MN) assay [Suzuki et al., 2009; Suzuki et al., 2004; Suzuki et al., 2005; Takasawa et al., 2010; Takasawa et al., 2013]. Several *in vivo* mammalian gene mutation assays exist, however, most are not well suited to regulatory

use because they are labour intensive, require large numbers of animals, and are prohibitively costly (e.g., the *in vivo* *Hprt* mutation assay). A novel endogenous gene mutation assay based on the *Pig-a* gene was more recently developed (Bryce et al., 2008); however, it is also currently restricted to haematopoietic tissue, and will require validation before it can be adopted for routine use.

Tests for gene mutations in TGRs (transgenic rodents) may be capable of resolving the issues leading to false negative results since they can detect induced transgene mutations *in vivo* in almost any target tissues. An OECD test guideline for the TGR somatic and germ cell gene mutation assays (OECD test guideline #488) was approved on July 28, 2011 [OECD., 2013]. The Muta™Mouse TGR assay has been tested with a wide range of known mutagens, and it has accurately returned positive results in several target tissues (for a review, see Lambert et al., 2005 & Lambert et al., 2009). Additionally, it can be combined with other assays that detect cytogenetic damage, such as the peripheral blood MN assay [OECD, 1997a]. This is advantageous, as it is difficult to combine the comet assay, which can also provide tissue-specific indications of DNA damage, with other genotoxicity endpoints (e.g., TGR mutations, MN, or *Pig-a* mutations) as the timing of sample collection for comet cannot be accommodated in the OECD test guidelines or recommended protocols for the other endpoints [OECD, 2013]. A previous study published by our group simultaneously examined the frequency of transgene (*lacZ*) mutations, *Pig-a* mutations, and MN induced in Muta™Mouse by oral exposure to benzo(*a*)pyrene (i.e., BaP), a prototypical polycyclic aromatic hydrocarbon (i.e., PAH) [Lemieux et al., 2011].

PAHs are a ubiquitous group of combustion-derived organic compounds containing at least two fused benzene rings. Several PAHs, particularly the larger five- and six-ring compounds, are mutagens in mammals and other vertebrates, and are classified by the International Agency for Research on Cancer (i.e., IARC) as carcinogens [IARC., 2010b; IARC., 1983]. IARC currently lists one PAH (i.e., BaP) as a known human carcinogen (Group 1), three PAHs as probable human carcinogens (Group 2A), and eleven PAHs as possible human carcinogens (Group 2B) [IARC., 1983; IARC., 2010b].

PAHs must be metabolically transformed to become DNA reactive. The major mutagenic pathway of PAH activation is via the production of DNA-reactive dihydrodiol-epoxides via CYP1 isozymes and epoxide hydrolase. The PAH-dihydrodiol-epoxides exert their mutagenic effect by covalently binding to a nucleotide, thereby forming a bulky DNA adduct. If these adducts are mis-repaired they can cause permanent DNA sequence changes (i.e., mutations); mutations in critical genes can lead to uncontrolled cell proliferation and replicative immortality, eventually leading to the establishment of neoplasia [Cairns, 1998; Josephy, 1997].

Cytochrome P450 1a1 (i.e., CYP1A1) is the major enzyme responsible for catalyzing the initial oxidation of PAHs; however, other P450 isozymes such as CYP1B1 and CYP1A2 are also capable of catalyzing oxidative reactions [Xue and Warshawsky, 2005; Shimada and Fujii-Kuriyama, 2004; Shimada et al., 2002]. CYP1A2 is a major hepatic P450 isozyme, whereas CYP1A1 and 1B1 are primarily expressed extra-hepatically in several murine tissues including lung and small intestine [Zhang et al., 2003; Hart et al., 2009; Renaud et al., 2011; Choudhary et al., 2003]. CYP1A1 is

generally present only at low levels unless its expression is induced via aryl hydrocarbon receptor (i.e., AhR) agonism, whereas CYP1B1 is constitutively expressed in certain tissues, and a substantial amount of CYP1A2 activity is present in liver. Both of these enzymes can also be upregulated following induction via AhR agonism [Nebert et al., 2004].

A second PAH-activation pathway, known as the radical cation pathway, involves one-electron oxidation that produces PAH radical cations that contribute to the formation of depurinating adducts. A third activation pathway involves the PAH-dihydrodiol intermediate produced by CYP1 metabolism and further metabolism by aldo-keto reductases (AKRs) to produce a catechol that can undergo oxidation to generate *o*-quinones. The resulting *o*-quinones can form covalent DNA adducts or undergo redox cycling that generates oxidative DNA damage. This third pathway is known as the *o*-quinone or AKR pathway [Penning, 2014].

To expedite assessments of PAH-contaminated matrices (e.g., contaminated soil, urban air particulates) regulatory agencies have identified a select number of PAHs that are routinely the focus of concern and control (i.e., the US EPA 16 priority PAHs). The United States Environmental Protection Agency (US EPA) designates seven priority PAHs as B2 carcinogens (i.e., probable human carcinogens) [Keith and Telliard, 1979]. The *Canadian Environmental Protection Act* (CEPA) identifies five PAHs as probable human carcinogens [CEPA., 1994]. Few studies have examined the *in vivo* genotoxic potency of the aforementioned B2 carcinogens; an assessment of their relative potency across a number of tissues would permit an evaluation of existing *in vivo* endpoints for

genotoxicity assessment, and the identification of critical tissues for effective hazard identification and assessment.

The objectives of this study were to evaluate the ability of the MutaTMMouse TGR *lacZ* mutation assay to correctly return a positive result in various tissues for selected carcinogenic PAHs, and to compare the sensitivity of the TGR response to another regulatory genotoxicity endpoint (i.e., peripheral blood MN assay). Nine genotoxic PAHs were selected for this study (**Table 2-I**); they are either listed as IARC Group 1 and 2 carcinogens, are US EPA B2 carcinogens, or were previously found to be genotoxic in MutaTMMouse FE1 cells [IARC., 2010b; Lemieux et al., 2015b; Keith and Telliard, 1979]. BghiP is classified as Group 3 by IARC due to inadequate evidence to evaluate its carcinogenicity in experimental animals; however numerous studies have shown that BghiP can induce the formation of stable adducts (IARC 2010), and it returns a positive mutation response in MutaTMMouse FE1 cells. Therefore, it was also included in the current study. The PAHs examined herein are benz(*a*)anthracene (BaA; IARC Group 2B), dibenz(*a,h*)anthracene (DBaA; IARC Group 2A), benzo(*b*)fluoranthene (BbF; IARC Group 2B), chrysene (CHRY; IARC Group 2B), benzo(*k*)fluoranthene (BkF; IARC Group 2B), indeno(1,2,3-*c,d*)pyrene (INDENO, IARC Group 2B), and DBaP (IARC Group 2A; also known as dibenzo(*def,p*)chrysene), and BghiP (IARC Group 3). The previously published results for BaP (IARC Group 1)[Lemieux et al., 2011; Labib et al., 2012], the liver *lacZ* mutation and DNA adduct data for DBaA [Malik et al., 2013], as well as lung *lacZ* mutation and DNA adduct data recently published by Labib et al. [2015] (for all compounds except DBaP) are also included in our analyses.

2.4 Materials & Methods

2.4.1 Animal treatment

Adult male MutaTMMouse (strain 40.6) specimens were individually housed in a microVENT ventilated rack (Allentown Inc., Allentown, NJ) on a 12-h light/12-h dark cycle. Animals received standard rodent chow (2014 Teklad Global standard rodent diet) and water *ad libitum* for the duration of the study. Animals were administered BaP, BaA (Sigma-Aldrich, Oakville, ON, Canada), DBaA, BbF, CHRY, BkF, INDENO, BghiP, and DbalP (Cambridge Isotopes, Tewksbury, MA) dissolved in highly refined olive oil (Sigma-Aldrich), and administered at 0.005 ml/g body weight. Compound purity, Chemical Abstract Service (CAS) numbers, doses and animal ages at the commencement of each study, as well as data source (i.e., from this study or previously published) are listed in **Table 2-I**. The doses were administered daily by oral gavage for 28 consecutive days. Doses were selected based on preliminary range-finding experiments for each compound, and the selected doses did not elicit overt signs of toxicity. There were five animals in each dose group and the vehicle control group. As per OECD guideline #474, 2-days following the last dose (i.e., one day prior to necropsy), all animals were bled via the saphenous or facial vein in order to obtain peripheral blood for the MN assay [OECD, 1997a]. All animals were euthanized at 3 days post-dosing [OECD., 2013] via cardiac puncture under isoflurane anaesthesia, followed by cervical dislocation and chest cavity opening. The femurs were removed and the bone marrow was flushed out, pelleted via brief centrifugation, and flash frozen in liquid nitrogen. The small intestine and glandular stomach were flushed with PBS and flash-frozen. The liver and lung were removed and flash frozen. All tissues were stored at -80°C. Mice were bred, maintained, and treated in

accordance with the Canadian Council for Animal Care Guidelines, and the protocols were approved by Health Canada's Animal Care Committee.

Table 2-I. Description of chemicals, doses, animal ages at the commencement of exposures, and data sources for the analyses presented in this work

Compound	CAS #	Purity	Dose (mg/kg BW/day)			Animal Age	Data Source
			Low	Med	High		
BaP	50-32-8	99%	25	50	75	25 weeks	Lemieux et al., 2011, Labib et al., 2012
BaA	53-55-3	99%	20	40	80	10 weeks	This study*
DBahA	53-70-3	≥ 98%	6.25	12.5	25	10 weeks	Malik et al., 2013 (lacZ and DA in Lv), this study*
BbF	205-99-2	≥ 98%	25	50	100	16 weeks	This study*
CHRY	218-01-9	≥ 98%	20	40	80	14 weeks	This study*
BkF	207-08-9	≥ 98%	25	50	100	11 weeks	This study*
INDENO	193-39-5	≥ 98%	12.5	25	50	13 weeks	This study*
BghiP	191-24-2	≥ 98%	6.25	12.5	25	10 weeks	This study*
DBaIP	191-30-0	≥ 98%	0.2	0.6	2	12 weeks	This study

DA: DNA adducts; Lv: liver; *mutation and DA data for lung only published in Labib et al., (2015)

2.4.2 Genomic DNA isolation for lacZ mutation scoring and PAH-DNA adduct analysis

Murine tissues were prepared for total genomic DNA isolation in the following manner:

Bone Marrow: Thawed bone marrow was combined with 5-ml ice cold lysis buffer (1 mM Na₂EDTA, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% SDS (w/v)) and incubated overnight at 37°C with gentle shaking.

Liver: The right lobe of the liver was thawed and homogenized in TMST (50 mM Tris, pH 7.6, 3 mM magnesium acetate, 250 mM sucrose, 0.2 % Triton X-100) using a Dounce tissue grinder. The homogenized tissue was then pelleted by centrifugation for 6 min at 800 x g (4°C) and washed twice in TMST. The pellet was then re-suspended in 5 ml lysis buffer and incubated overnight at 37°C with gentle shaking.

Small Intestine: Tissue from the jejunum was slit open and the mucus layer and intestinal contents were removed by rinsing the tissue in ice-cold buffer (75 mM KCl, 20 mM EDTA) via repeated aspiration (i.e., 3-4 times) in and out of a 1-ml needleless syringe. The epithelial lining was then removed from the supporting tissue by repeatedly (10 times) forcing the tissue in and out of the syringe in fresh buffer. The sample was then centrifuged for 10 min at 2800 rpm (4°C), re-suspended in 5 ml lysis buffer, and incubated overnight at 37°C with gentle shaking.

Lung: Lung tissue (right lobe) was minced and placed in a sterile tube containing PBS. The tissue was then de-gassed by applying a vacuum to the top of the tube. The tubes were then centrifuged and re-suspended in 5 ml lysis buffer for an overnight incubation at 37°C with gentle shaking.

Glandular Stomach: Stomach tissue was opened and flushed with PBS, and then scraped using the edge of a scalpel blade. The scraped tissue was transferred to a glass Dounce tissue grinder and homogenized. The homogenized tissue was combined with 5 ml lysis buffer and incubated for 1 hour at 37°C with gentle shaking. RNase A (0.1 mg/ml) was then added and samples incubated overnight at 37°C with gentle shaking.

Genomic DNA was isolated from all lysed tissues using a phenol/chloroform extraction procedure described previously [Douglas et al., 1994; Vijg and Douglas, 1996]. Isolated DNA was dissolved in 100 µl TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) and stored at 4°C until use.

2.4.3 Mutant frequency analysis

The PGal (phenyl-β-D-galactoside) positive selection assay was used for the determination of *lacZ* mutant frequency in DNA samples from bone marrow, liver, lung, glandular stomach, and small intestine, as previously described [Lambert et al., 2005; Vijg and Douglas, 1996; Gossen et al., 1992]. Mutant frequency was calculated as the ratio of mutant plaque forming units (i.e., pfu) to total pfu.

2.4.4 PAH-DNA adduct analysis

The nuclease P1 enrichment version of the ³²P-postlabelling assay was used to determine DNA adduct frequency in DNA samples from bone marrow, liver, lung, glandular stomach, and small intestine. The procedure was performed as described in Phillips and Arlt (2014) and Arlt et al. (2008), with modifications as described in Malik et al. (2013).

2.4.5 Micronucleus (MN) assay

MicroFlow[®] kits (Litron Laboratories, Rochester, NY) were used for enumeration of micronucleated reticulocytes (RET) and normochromatic erythrocytes (NCE). Briefly, approximately 60 µl of peripheral blood was immediately combined with 350 µl of anticoagulant. Blood samples were then fixed by transferring to ice-cold methanol and stored at -80°C. After 3-5 days, fixed blood samples were then centrifuged, rinsed, and transferred to a long-term storage solution. Coded specimens were shipped to Litron Laboratories (Rochester, NY) for analysis. Micronuclei were scored in NCEs (i.e., MN-NCEs) and RETs (i.e., MN-RETs) by flow cytometry via a 3-colour labelling method described in [Dertinger et al., 2004; Torous et al., 2001].

2.4.6 Data analysis

The *lacZ* and MN dose-response data were analysed in SAS v.9.1 (SAS Institute, Cary, NC) using Poisson regression. The data were fit to the model $\log(E(Y_i)) = \log t_i + \beta x_i$, where $E(Y_i)$ is the expected value for the i th observation, β is the vector of regressions coefficients, x_i is a vector of covariates for the i th observation, and t_i is the offset variable used to account for differences in observation count period (e.g., total pfu). The offset (e.g., natural log of pfu) was given a constant coefficient of 1.0 for each observation, and log-linear relationships between mutant count and test article concentration were specified by a natural log link function.

Genotoxic potencies were calculated for each endpoint in each tissue as the slope of the linear portion of the dose-response functions. BaP MN (in blood), as well as DNA adducts and *lacZ* data in bone marrow, liver, small intestine and glandular stomach were previously published in Lemieux et al. (2011). Lung data were previously published in

Labib et al. (2012). DBahA *lacZ* mutant frequency data for liver only were previously published in Malik et al. (2013), and lung *lacZ* and DNA adduct data for BaA, DBahA, BbF, CHRY, BkF, INDENO, and BghiP were recently published in Labib et al.[2015]. These data were re-analysed in the manner stated above to permit direct comparison to the results generated specifically for this study.

2.5 Results

2.5.1 DNA Adducts

For simplicity, the DNA adduct data were only examined in a binary manner (i.e., positive or negative) to investigate target tissue exposure to reactive metabolites (**Table 2-II**). The adduct data will be analyzed and discussed in more detail (i.e., quantitative analyses) in a separate manuscript. Six of the 9 PAHs (i.e., BaP, BaA, DBahA, BbF, BkF, and DBaP) induced significant increases in DNA adduct frequencies in all 5 tissues examined (i.e., glandular stomach, small intestine, liver, lung, bone marrow). CHRY elicited a positive response in all tissues except bone marrow. INDENO only elicited a positive response in liver and lung. BghiP only elicited a positive response in lung. Interestingly, lung was the only tissue to show a significant response for all 9 PAHs.

Table 2-II. PAH-DNA adduct result across all 5 tissues for each PAH examined

Compound	Glandular Stomach	Small Intestine	Liver	Lung	Bone Marrow
BaP	+	+	+	+	+
BaA	+	+	+	+	+
DBahA	+	+	+	+	+
BbF	+	+	+	+	+
CHRY	+	+	+	+	-
BkF	+	+	+	+	+
INDENO	-	-	+	+	-
BghiP	-	-	-	+	-
DBC	+	+	+	+	+

2.5.2 *lacZ* Mutagenicity

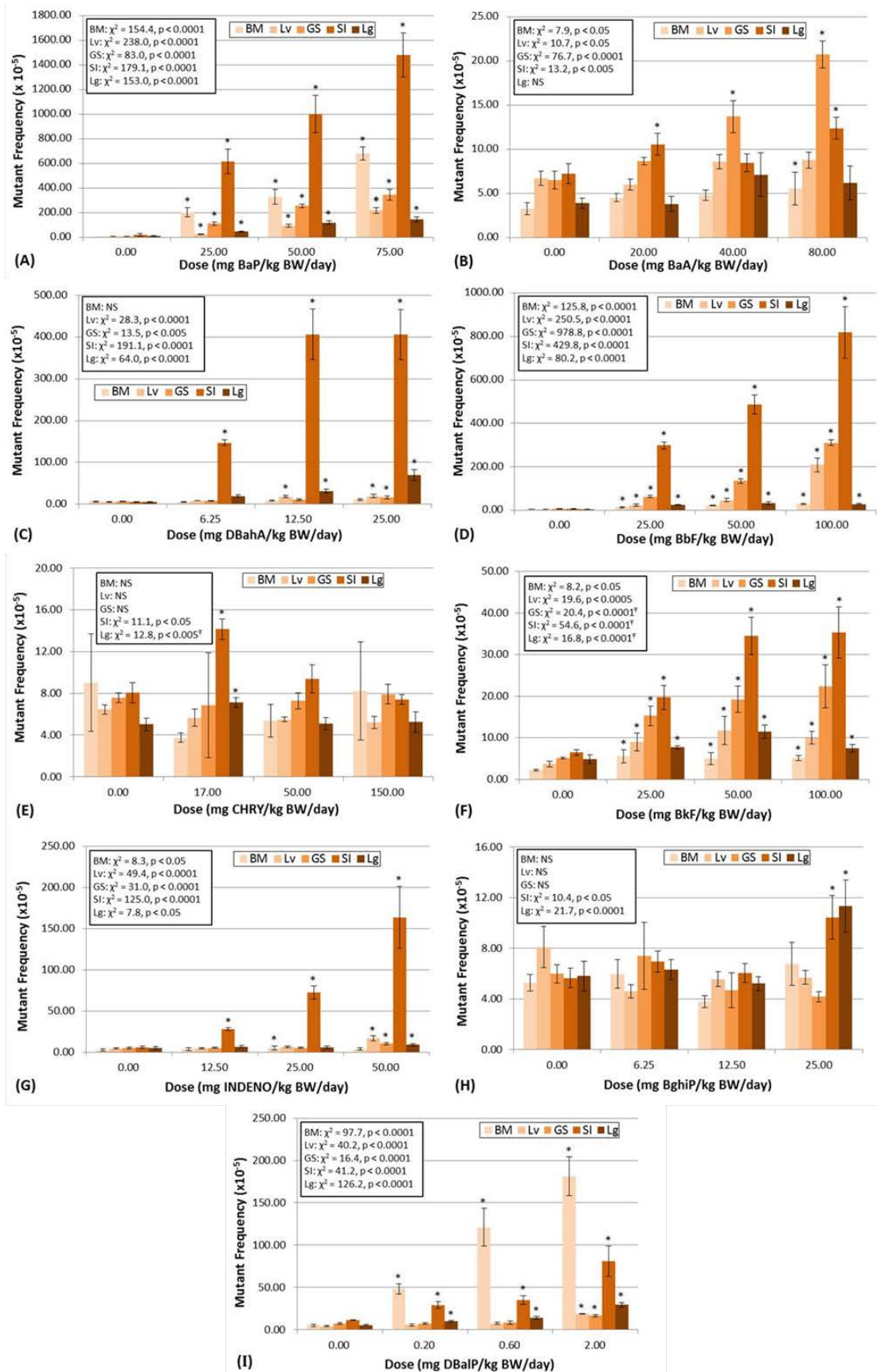
The Muta™Mouse TGR assay was able to correctly identify the selected PAHs as *in vivo* mutagens, and all 9 PAHs elicited positive responses in at least one tissue (**Table 2-III**). BaP, BbF, BkF, INDENO, and DBaP elicited significant increases in *lacZ* mutant frequency in all 5 tissues examined (**Table 2-III; Figure 2.1A, D, F, G, I**). BaA and DBaA elicited a positive response for 4 out of the 5 tissues, with negative responses for lung and bone marrow, respectively (**Table 2-III; Figure 2.1 B, C**). CHRY and BghiP elicited positive responses in only 2 of the 5 tissues examined, both were negative in glandular stomach, liver, and bone marrow (**Table 2-III; Figure 2.1E, H**). For all PAHs examined, with the exception of DBaP, the fold-increase in mutant frequency (i.e., over control) for site of contact or related tissues (i.e., small intestine, glandular stomach, liver) was higher than for bone marrow (**Figure 2.1**). The largest fold-increase in mutant frequency for BaP, DBaA, BbF, CHRY, BkF, and INDENO was observed for small intestine (**Figure 2.1A, C, D, E, F, G**). For BaA, only glandular stomach yielded a larger fold-change than small intestine (**Figure 2.1B**), and for BghiP, only lung yielded a larger fold-change than small intestine (**Figure 2.1H**). For DBaP, the largest fold-change over control was obtained for bone marrow (**Figure 2.1I**). For BbF, BkF, and INDENO (**Figure 2.1D, F, G**), bone marrow results showed the smallest fold-change over control, and for DBaA, CHRY, and BghiP, the bone marrow responses were negative (i.e., no significant increase) (**Figure 2.1C, E, H**).

Table 2-III. Genotoxic response for *lacZ* mutagenicity results in glandular stomach (GS), small intestine (SI), liver (Lv), lung (Lg), and bone marrow (BM), and micronucleus (MN) results in reticulocytes (RETs) and normochromatic erythrocytes (NCEs). Sensitivity for each tissue is also presented

Compound	<i>lacZ</i>					MN	
	GS	SI	Lv	Lg	BM	RETs	NCEs
BaP	+++	+++	+++	+++	+++	+	++
BaA	+	+	WP	-	+	-	-
DBahA	+	+++	+	+++	-	+	+
BbF	+++	+++	+++	++	++	+	+
CHRY	-	+	-	WP	-	-	-
BkF	+	++	+	+	+	+	+
INDENO	+	+++	+	+	+	-	-
BghiP	-	+	-	+	-	-	-
DBaIP	++	+	++	+	+++	++	+
Total Pos	7	9	7	8	6	5	5
Total Neg	2	0	2	1	3	4	4
Sensitivity	78%	100%	78%	89%	67%	56%	56%

+: Dose-response, 1.5-5-fold above background; ++: Dose-response, 5-10-fold above background; +++: Dose-response, >10-fold above background; WP (weak positive): dose-response, but below 1.5-fold above background; -: Negative.

Figure 2.1. MutaTMMouse *lacZ* mutagenicity results for 9 PAHs in bone marrow (BM), liver (Lv), glandular stomach (GS), small intestine (SI), and lung (Lg). Plots show results for **(A)** benzo(*a*)pyrene, **(B)** benz(*a*)anthracene, **(C)** dibenz(*a,h*)anthracene, **(D)** benzo(*b*)fluoranthene, **(E)** chrysene, **(F)** benzo(*k*)fluoranthene, **(G)** indeno(1,2,3-*c,d*)pyrene, **(H)** benzo(*g,h,i*)perylene, and **(I)** dibenzo(*a,l*)pyrene. Mutant frequencies $\pm$ standard error are displayed for each tissue and dose. Statistical results for the overall dose-response relationship are presented for each tissue. Results of the custom contrasts for each dose vs. control are marked with an *, indicating significance at $p < 0.05$. F: dose-response data were truncated prior to X^2 analysis. NS indicates not significant.

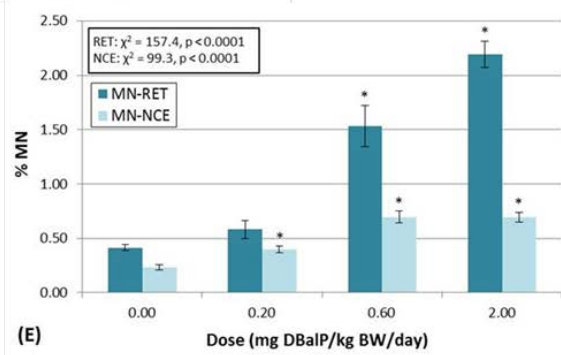
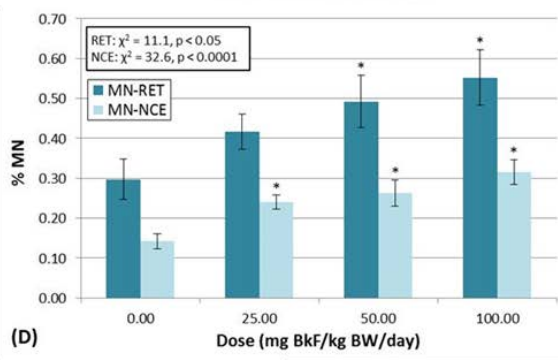
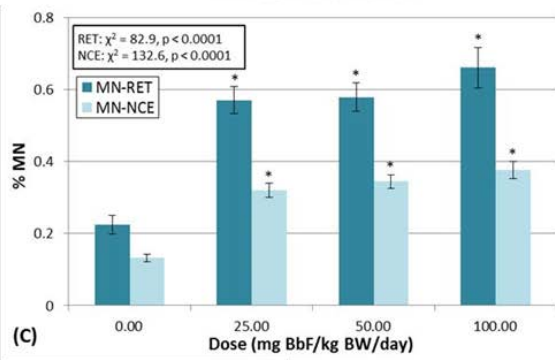
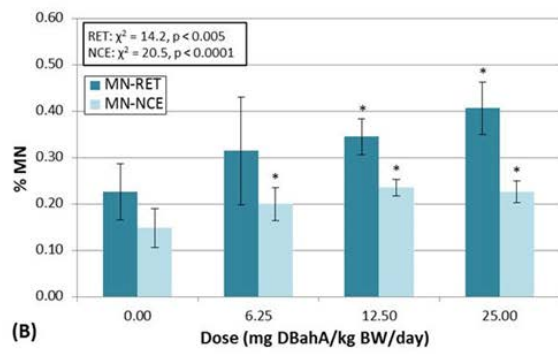
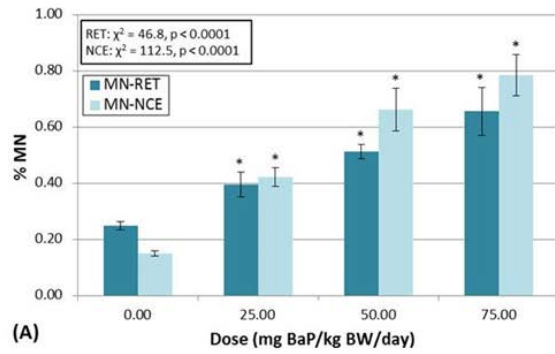


2.5.3 Micronucleus (MN) Frequency

BaP, DBaA, BbF, BkF, and DBaP elicited statistically significant positive results in the MN assay for both RETs and NCEs (**Table 2-III, Figure 2.2A-E**). BaA, CHRY, INDENO, and BghiP did not elicit significant positive responses for the MN assay (**Table 2-III**). Larger fold-changes over control were consistently seen in RETs, versus NCEs, with the sole exception being BaP (**Figure 2.2A**). This highlights the importance of scoring micronuclei in the immature red blood cell population (i.e., RETs), which captures the effects of recent damage.

CHRY, BaA, and INDENO are all IARC Group 2B carcinogens for which experimentally-induced genetic damage is well documented; however, the results obtained herein indicate an inability of these compounds to induce MN. This is noteworthy and concerning since the results support the contention that the MN assay in peripheral blood, which is routinely employed for regulatory screening, has a limited ability to detect PAH-induced genetic damage.

Figure 2.2. Micronucleus (MN) frequency results for the 5 PAHs that elicited positive responses in this assay in reticulocytes (RETs) and normochromatic erythrocytes (NCEs). Plots show results for (A) benzo(*a*)pyrene, (B) dibenz(*a,h*)anthracene, (C) benzo(*b*)fluoranthene, (D) benzo(*k*)fluoranthene, and (E) dibenzo(*a,l*)pyrene. Percent micronucleated blood cells (i.e., % MN) $\pm$ standard error are displayed for each tissue and dose. Statistical results for the overall dose-response relationship are present for both tissues. Results of the custom contrasts for each dose vs. control are marked with an *, indicating significance at $p < 0.05$.



2.5.4 Potency comparisons

The potency (i.e., slope of the linear portion of the dose-response function) of each compound was ranked by tissue for each endpoint (i.e., 1 = most potent, 5 = least potent) (**Table 2-IV**). For the *lacZ* mutation endpoint, the highest potency (i.e., rank = 1) was observed in small intestine for 6 out of 9 PAHs (i.e., BaP, DBaH, BbF, CHRY, BkF, INDENO). Potencies for small intestine were second highest (i.e., rank = 2) for the remaining 3 compounds (i.e., BaA, BghiP, DBaP). Conversely, 3 compounds failed to elicit a positive response in bone marrow (i.e., DBaH, CHRY and BghiP), and bone marrow responses were least potent (i.e., rank = 5) for an additional 3 compounds (i.e., BbF, BkF, INDENO). Interestingly, DBaP elicited the most potent response (i.e., rank = 1) in bone marrow. Effects on glandular stomach and lung showed intermediate potency for all compounds, with potency ranks ranging from 1-5, whereas responses in the liver were the least potent after bone marrow, with potency ranks ranging from 2-4. A mean relative potency score was calculated for each tissue, across all compounds, in order to provide a general potency ranking for PAHs in the tissues examined. The most potent response was observed in small intestine (1.3), followed by glandular stomach (2.9), lung (3.0), liver (3.3), and finally bone marrow (3.7).

For the MN endpoint, the most potent responses were observed in RETs for all compounds that yielded a significant positive response (i.e., DBaH, BbF, BkF, DBaP), with the sole exception of BaP (**Figure 2.2**). BaP yielded a more potent response in NCEs (**Figure 2.2**), resulting in mean potencies of 1.2 for RETs, and 1.8 for NCEs.

Table 2-IV. Relative ranking of *lacZ* mutagenic potency for each compound in glandular stomach (GS), small intestine (SI), liver (Lv), lung (Lg), and bone marrow (BM), as well as micronucleus (MN) induction response in reticulocytes (RETs) and normochromatic erythrocytes (NCEs). The last row shows mean rank across all compounds for a given tissue/endpoint.

Compound	<i>lacZ</i>					MN	
	GS	SI	Lv	Lg	BM	RETs	NCEs
BaP	3	1	4	5	2	2	1
BaA	1	2	3	-	4	-	-
DBahA	4	1	3	2	-	1	2
BbF	2	1	3	4	5	1	2
CHRY	-	1	-	2	-	-	-
BkF	2	1	4	3	5	1	2
INDENO	3	1	2	4	5	-	-
BghiP	-	2	-	1	-	-	-
DBaIP	5	2	4	3	1	1	2
Mean Rank	2.9	1.3	3.3	3.0	3.7	1.2	1.8

Potencies are ranked 1-5. 1: highest potency for each compound per assay. 5: lowest potency for each compound per assay. -: negative response.

2.5.5 Cross-tissue comparisons

For the Muta™Mouse *lacZ* mutation endpoint BaP elicited the largest fold-change increase over control across all 5 tissues, which is not surprising since BaP is known to be a potent mutagen and carcinogen and the only PAH declared by IARC as a Group 1 human carcinogen. Unlike most other PAHs, BaP induced a potent *lacZ* response in bone marrow. DBaP also elicited a positive *lacZ* mutagenicity response in all tissues. Again, this is not surprising since DBaP is an extremely potent *in vivo* mutagen. It is noteworthy that, in contrast to the results for the other tested PAHs, the most potent response for this compound was observed in bone marrow. This suggests that there is substantial systemic distribution of DBaP, which is then metabolically converted in bone marrow to a reactive metabolite (e.g., DBaP-11,12-diol-13,14-epoxide). In comparison with BaP, DBaP elicited a smaller fold-change increase for most tissues; however, due to the extreme toxicity of DBaP (i.e., as observed in preliminary range-finding experiments), it was necessary to select doses that are far lower than those used for BaP (i.e., highest responses for DBaP observed at doses almost 40-fold lower than BaP).

The fact that DBaA elicited a positive response in all tissues except bone marrow is concerning since regulatory assessments often only examine hematopoietic tissue. It seems reasonable to assert that reactive DBaA metabolites do not reach the bone marrow, and therefore are unable to induce mutations in this tissue. However, DBaA did elicit positive responses for the MN endpoint in RETs and NCEs, therefore systemic exposure of reactive metabolites is likely. Indeed, systemic exposure of the reactive metabolite was confirmed by quantification of DBaA-induced bulky adducts in bone marrow at all three doses. The fact that stable, bulky adducts do not appear to have

been converted to stable mutations in a rapidly proliferating tissue such as bone marrow may be an indication that there is sufficient capacity for repair of DNA lesions or error-free translesion DNA synthesis. The positive MN response for DBahA may have been induced by secondary metabolites, such as reactive oxygen species, that did not contribute to the formation of mutations in the bone marrow.

CHRY is a relatively weak carcinogen, causing lung and liver cancer in mice following *i.p.* administration, and lung cancer in rats following intrapulmonary administration [IARC., 2010b]. IARC recently declared CHRY a Group 2B carcinogen (i.e., possibly carcinogenic to humans), which constitutes an upgrade from the earlier Group 3 declaration. In our study, CHRY only elicited a clear positive response in small intestine and a weak positive response in lung (i.e., significant dose response, but response at high dose below 1.5-fold above control). The *lacZ* mutation assay results suggest that lung was exposed to reactive CHRY metabolites; indeed CHRY-DNA adducts were detected in lung. In comparison, a lack of *lacZ* and MN responses in bone marrow and peripheral blood, respectively, suggest that haematopoietic tissue was not exposed. This was confirmed by a lack of CHRY-induced DNA adducts in bone marrow.

BkF and BbF, which are both IARC Group 2B carcinogens, elicited positive responses across all tissues, with the greatest *lacZ* mutant frequency induction occurring in the small intestine. Both BbF and BkF induced a positive response in the MN assay.

INDENO elicited positive *lacZ* mutagenicity responses across all tissues (i.e., including bone marrow), but failed to induce positive MN responses in RETs or NCEs, nor did the results reveal measureable adducts in bone marrow. Given the lack of bulky adducts in bone marrow, it is unlikely that INDENO is being metabolized via the diol-

epoxide pathway in this tissue. Alternatively, the positive bone marrow response may result from oxidative DNA damage produced via the *o*-quinone activation pathway.

BaA, which is also classified as an IARC Group 2B carcinogen, induced a significant positive response in the *lacZ* mutagenicity assay in all tissues except lung. The compound elicited a weak positive response in the liver.

BghiP, which is classified by IARC as Group 3 (i.e., not classifiable due to limited or inadequate data in humans and experimental animals), elicited a positive response in small intestine and lung. Similar to the results obtained for DBahA, the lack of positive response in bone marrow is problematic from a regulatory point of view since assessments are most commonly based on results for hematopoietic tissue. BghiP also failed to elicit a positive MN response in both RETs and NCEs; therefore, it is possible that there is no systemic distribution of activated metabolites. Indeed, DNA adducts were not detected in the bone marrow of BghiP-exposed animals. However, since a positive *lacZ* response was observed in the lung, activated metabolites are either reaching the lung via pulmonary circulation, or BghiP is being metabolically activated in the lung itself.

2.5.6 Assay Sensitivity

Table 2-III summarises the tissue-specific responses of the PAHs examined herein, and the response patterns therein are briefly outlined below. Importantly, the MutaTMMouse *lacZ* mutation assay was more sensitive for all tested tissues (i.e., correct identification of a genotoxic carcinogen) in comparison with the MN assay in either RETs or NCEs (**Table 2-III**). All of the PAHs investigated in this study elicited significant positive responses in the MutaTMMouse *lacZ* mutation assay in at least one tissue.

For the *lacZ* mutagenicity assay, small intestine was the most sensitive tissue, with a positive response for all 9 PAHs tested (100%) (**Table 2-III**). Small intestine is a site-of-contact tissue for an oral exposure, which may contribute to its high sensitivity for this assay. Lung, a remote tissue for which responses were consistently lower in comparison to site of contact, was the second most sensitive tissue (i.e., 89%). Lung is the only common tumour site for all 9 PAHs, and therefore is a toxicologically-relevant tissue for an oral exposure. The only compound that did not elicit a positive response in lung was BaA. Seven of the 9 PAHs examined elicited positive responses in both glandular stomach and liver (78%). CHRY and BghiP were both negative in these tissues, and also negative in bone marrow. In the context of this study the glandular stomach constitutes the site of first contact, with compounds interacting with stomach epithelium prior to contact with the small intestine or liver. It is reasonable to assert that the enzymatic activity and/or residence time in the stomach may not be sufficient to achieve adequate conversion of the compounds into DNA-reactive metabolites that elicit bulky adducts and transgene mutations. Bone marrow was the least sensitive tissue for induction of *lacZ* mutations, with only 6 of 9 compounds eliciting significant positive responses; DBahA, CHRY, and BghiP were all negative. As already noted, lack of responses for known mutagenic carcinogens are potentially a result of ineffective target tissue exposure to the reactive metabolite.

The sensitivity of the MN assay was equal for RETs and NCEs; with both endpoints returning a positive response for 5 out of the 9 PAHs examined (56%).

From a regulatory evaluation point of view it is interesting to consider the results as though the study only examined hematopoietic tissues (i.e., bone marrow, blood),

which would be the case if the MN assay alone was employed. The results would have suggested that CHRY and BghiP are not *in vivo* genotoxicants, despite the fact that both of these compounds induced significant positive responses in other Muta™Mouse tissues. If the study had examined the Muta™Mouse TGR endpoint in bone marrow only, we would have also missed the positive response for DBahA, an IARC Group 2A carcinogen that elicited significant positive responses in all other tissues examined and response induction levels more than 75- and 12-fold above control for small intestine and lung, respectively. Finally, if we had only examined induced increases in MN frequency in peripheral blood (i.e., RETs and NCEs), we would have missed significant positive responses for BaA, INDENO, CHRY, and BghiP. BaA and INDENO elicited positive *lacZ* mutagenicity responses in several tissues, including bone marrow. The fact that these compounds are unable to elicit reliable positive responses in hematopoietic tissues raises a concern regarding their ability to be detected using standard regulatory assays for genetic toxicity, and highlights the importance of examining site-of-contact tissues and known sites of tumor formation, either alone or in combination with hematopoietic tissues.

2.6 Discussion

This study employed the Muta™Mouse TGR system to examine the *in vivo* genetic toxicity of 9 PAHs. The compounds are all mutagenic carcinogens in animal models, and, with the exception of BghiP, are IARC Group 1, 2A, or 2B human carcinogens. It is well-known that the primary mode of action underlying PAH-induced carcinogenicity is genotoxicity, and key events leading to the adverse outcome (i.e.,

tumors) include the conversion of stable adducts to point mutations or small insertions/deletions [IARC., 2010b]. Indeed, all 9 PAHs elicited significant positive responses in at least one tissue in the MutaTMMouse *lacZ* mutation assay, with 5 out of the 9 PAHs eliciting a positive response in all 5 tissues, thereby demonstrating not only their mutagenic potential, but also the ability of this assay to correctly identify genotoxic carcinogens.

Several PAHs have also been shown to induce micronuclei as a result of clastogenicity [Warshawsky et al., 1995; Crofton Sleight et al., 1993; Glatt et al., 1990; Abramsson Zetterberg et al., 2013; Whong et al., 1994; Nishikawa et al., 2005; He and Baker, 1991; Schober et al., 2006]. Interestingly, only 5 of the 9 PAHs examined here elicited significant positive responses in the MN assay, indicating that a complementary assay to detect *in vivo* mutagenic activity is necessary to prevent “false negatives”.

The MN results in RETs consistently elicited larger fold-changes over control, as well as greater potency values in comparison with the results for NCEs. RETs only make up ~10% of circulating red blood cells and their life span is only 1-2 days prior to their maturation into NCEs. Thus, by examining RETs we are able to see evidence of the most recent damage [Clark and Korst, 1969]. As they mature, the mutant RETs begin to accumulate as mutant NCEs, which have a life span of 38-46 days in mice [Horky et al., 1978]. Therefore, the lower response in NCEs is likely to be due to dilution; in other words, whereas all RETs were recently exposed, only a subset of the sampled NCEs were exposed during the 28-day treatment period. Since affected cells accumulate, a longer sampling time (i.e., > 2 days post-exposure) would have increased the magnitude of the MN frequency response for NCEs (i.e., relative to RETs).

In this study BaP, DBaP, BbF, and BkF were positive across all tissues for both endpoints. A number of positive results for BaP-exposed TGRs have been previously reported for several tissues including small intestine, lung, liver, bone marrow, and spleen in Big Blue® mouse and rat, Muta™Mouse, *gpt* delta mouse, *Dlb-1* congenic mice, and the *lacZ* plasmid mouse [Leavitt, 2008; Boerrigter, 1999; Delker et al., 2008; Shane et al., 1997; Monroe et al., 1998; Skopek et al., 1996; Horibata et al., 2013; Brooks et al., 1999]. BaP has previously been shown to induce positive MN results in several tissues/cell types including skin, peripheral blood erythrocytes, and bone marrow [Nishikawa et al., 2005; Abramsson Zetterberg et al., 2013; Lemieux et al., 2011]. DBaP has previously been tested in TGR assays, and a significant increase in *lacI* mutant frequency was reported in Big Blue® mouse lung following an *i.p.* administration [Leavitt, 2008], as well as a significant increase in *cII* mutant frequency in Big Blue® mouse tongue, following a topical application of the oral cavity [Chen et al., 2013]. Our group recently published positive DNA adduct and *lacZ* results in spleen and bone marrow, as well as positive MN results in peripheral blood, following a 3-day oral exposure [Chepelev et al., 2015]. The current study is the first study that examined DBaP MN induction and mutagenicity following a sub-chronic oral exposure, and the first report of its mutagenicity in glandular stomach, small intestine, and liver. Interestingly, DBaP has been demonstrated to be much more carcinogenic than BaP in animal models, and perhaps the most carcinogenic of all PAHs tested to date [Cavalieri et al., 1991; Cavalieri et al., 2005; Higginbotham et al., 1993; Chen et al., 2013]. Surprisingly, there are no previous reports of TGR or MN results for BbF and BkF.

In our study, DBahA failed to elicit a positive TGR response for bone marrow, yet it was positive in the MN assay. The only previous report of TGR assay results for DBahA, which was published by our group and incorporated into the analyses presented herein, show a positive response in liver [Malik et al., 2013]. Previous publications examining DBahA genetic toxicity reported positive results for micronucleus induction in skin, bone marrow, spleen, and lung [Whong et al., 1994; Zhong et al., 1995; Nishikawa et al., 2005]; however the current study appears to be the first to demonstrate a positive MN response in blood.

The results obtained indicate that INDENO, BaA, and BghiP were negative in the MN assay. INDENO elicited a positive response in the TGR assay for all tissues examined, whereas BaA was positive in the TGR assay in all tissues except lung, and BghiP only elicited a positive TGR response in small intestine and lung. There are no previous reports of TGR results for any of these three compounds. In addition, to our knowledge there are no reports in the literature regarding the ability of INDENO or BghiP to induce MN *in vitro* or *in vivo*; however, several studies have documented that BaA induces MN in skin, bone marrow, spleen, and lung [Nishikawa et al., 2005; Zhong et al., 1995; Whong et al., 1994].

CHRY elicited positive TGR results for small intestine and lung only, and was negative in the MN assay. Only one study has previously reported positive TGR assay results for CHRY. Following an *i.p.* exposure of MutaTMMouse, significant elevations in *lacZ* mutant frequencies were observed in liver, spleen, lung, kidney, bone marrow, and colon [Yamada et al., 2005]. Our study did not observe positive results in liver or bone marrow; however, the Yamada et al. (2005) study used an *i.p.* administration, which

would contribute to elevated, direct exposures of organs in the peritoneum (e.g., liver). To our knowledge, the literature only contains a single report of a CHRY-induced increase in MN; He and Baker (1991) documented a significant increase in hairless mouse skin following topical administration [He and Baker, 1991]. However, a similar study by Nishikawa (2005) failed to detect an increase in MN in the same species and tissue following topical treatment [Nishikawa et al., 2005].

Small intestine was not only the most sensitive among the tissues examined, but also displayed the largest induction of *lacZ* mutant frequency (i.e., the most potent response). It has previously been demonstrated that inducible levels of *Cyp1a1* gene expression in the small intestine are relatively high, which is not the case for most other tissues [Choudhary et al., 2003; Renaud et al., 2011]. In the epithelium of the GI tract, maximum induction levels of CYP1A1 have been shown to be 3-10 times greater than CYP1B1, which in turn, have been shown to be 3-10 times greater than CYP1A2 [Uno et al., 2008]. Studies with *Cyp1a1/1a2/1b1* single, double, and triple knockout mice have highlighted and clarified the roles of these enzymes in metabolising BaP and distributing BaP-metabolites following oral exposures (reviewed in [Nebert et al., 2013]). Knocking out *Cyp1a1* expression results in increased expression of *Cyp1b1*, concomitant bioactivation of BaP, and adenocarcinoma formation in the proximal small intestine. In contrast, BaP treated *Cyp1b1* knockout were healthy [Shi et al., 2010b]. The relatively high levels of inducible CYP1A1 appear to be especially important in the detoxification of BaP in the GI tract, whereas CYP1B1 is implicated in the conversion of BaP to DNA-damaging metabolites [Nebert et al., 2013]. Following an oral exposure, compounds such as PAHs are absorbed from the small intestine and transported to the liver, where they

undergo oxidative metabolism. This metabolism contributes to clearance of the parent compound, but also in the formation of DNA adducts and mutations in the liver itself. Additionally, as PAH metabolites have been previously demonstrated to undergo enterohepatic circulation, the small intestine would likely be re-exposed to reactive metabolites, and this process would contribute to increased levels of DNA damage that can, in turn, contribute to an increased frequency of mutations [Ramesh et al., 2004; Miller and Ramos, 2001]. The combination of *in situ* metabolism in the small intestine, coupled with re-exposure to reactive metabolites produced in the liver and the high mitotic index of the epithelial lining of the small intestine, likely led to the high levels of transgene mutations observed herein.

Although the liver is the major site of PAH metabolism, both CHRY and BghiP failed to induce significant increases in *lacZ* mutations in this tissue. Liver cells generally have a much lower mitotic index than parenchymal cells in the other examined tissues [Edwards and Klein, 1961; White et al., Provisionally accepted]; therefore, for less potent chemicals, a 28-day exposure regime with a 3-day sampling time may not be sufficient to allow for conversion of DNA damage to *lacZ* mutations. Both CHRY and BghiP are relatively weak mutagens/carcinogens, with BghiP being classified as IARC Group 3, and CHRY being recently upgraded from Group 3 to 2B. If slowly proliferating tissues such as liver are deemed to be of particular importance, the OECD protocol for TGR assays indicates that a longer sampling time (i.e., 28-days post-exposure) may be more appropriate [OECD., 2013].

The *lacZ* mutagenic potency values for glandular stomach were penultimate with a sensitivity of 78%. For an oral exposure the stomach is the first site-of-contact tissue,

and although the metabolic capacity of stomach epithelium is low in comparison with liver, the magnitude of the response highlights the importance of examining tissues deemed relevant to the exposure route. Only CHRY and BghiP, two of the weakest PAHs examined, failed to elicit a positive response in this tissue.

The fact that *lacZ* mutagenic activities were observed across all 5 tissues, including remote tissues such as bone marrow and lung, indicates that several PAHs are capable of being metabolized and converted into DNA-reactive substances at remote tissues *in situ*, or alternatively, that the active metabolites are being systemically distributed. Lung was the second most sensitive tissue for the TGR assay, returning a positive result for all compounds except BaA. Both *Cyp1a1* and *Cyp1b1* are highly expressed in the mouse lung [Choudhary et al., 2003; Renaud et al., 2011; Arlt et al., 2015]; thus, lung has the ability to carry out *in situ* conversion of PAHs to reactive diol-epoxides that would form bulky adducts. This also appears to be the case for DBahA, CHRY, and BghiP, which failed to elicit positive *lacZ* results in bone marrow, but elicited positive results for lung. The negative BaA result for lung is troubling since BaA-DNA adducts were detected in this tissue; however, due to the relatively low mitotic index of most lung parenchymal cells, DNA replication may not be sufficient to fix a detectable level of mutations during the exposure and sampling period employed here [White et al., Provisionally accepted].

In this study, bone marrow was the least sensitive tissue for the TGR assay, and the lowest potency *lacZ* responses were also observed in bone marrow, with DBahA, CHRY, and BghiP failing to elicit positive responses. Additionally, CHRY, BaA, INDENO, and BghiP were unable to induce MNs in peripheral blood. The negative *lacZ*

mutation and MN responses in hematopoietic tissue from CHRY- and BghiP-exposed animals may be the result of insufficient exposure of the target tissue to reactive metabolites. This is likely due to the lack of sufficient distribution of the parent compound to bone marrow and/or peripheral blood via systemic circulation. As mentioned, following an oral exposure, induction of GI tract *Cyp1a1* appears to be especially important for the detoxification of BaP, and this likely holds true for several PAHs. Thus, following an oral exposure to PAHs that are effective AhR agonists, upregulation of *Cyp1a1* expression in the GI tract/liver will contribute to enhanced clearance, leaving little parent compound for systemic distribution to bone marrow where reactive metabolites can be generated *in situ* via catalysis by isozymes such as CYP1B1. This supposition is supported by the aforementioned knock-out studies that showed increased damage at peripheral tissues if *Cyp1a1* expression is knocked out either globally or in the GI tract itself; moreover, the effect is reversed by also knocking out *Cyp1b1* [Shi et al., 2010a; Uno et al., 2006]. However, it should be emphasized that the PAHs examined are not equipotent AhR agonists. In this study, BaP and DBaP induced the most potent *lacZ* and MN responses in bone marrow/blood, and interestingly, BaP is a relatively poor AhR agonist in comparison to most of the other PAHs examined. Furthermore, it is not clear whether DBaP is an AhR ligand at all [Machala et al., 2001; Ziccardi et al., 2002], and if it is not, one would not expect induction of *Cyp1a1* expression in the GI tract, leaving more parent compound available to be metabolized in bone marrow by the constitutively expressed *Cyp1b1*. This deduction is supported by our previous DBaP study where we observed significant bone marrow cytotoxicity that resulted in a substantial decrease in the percentage of circulating RETs following a 3-day

exposure [Chepelev et al., 2015]. In contrast to the aforementioned toxicokinetic processes that likely modify the *in vivo* responses to BaP and DBaP, effective AhR agonism, which has been observed for BkF, DBaH, and INDENO [Ziccardi et al., 2002], and upregulation of *Cyp1a1* expression in the GI tract, would likely contribute to weak or negative bone marrow responses. Indeed, this is what was observed.

The results obtained failed to show a detectable increase in DNA adducts in bone marrow from CHRY-, BghiP-, and INDENO-treated animals, which supports the lack of exposure of hematopoietic tissue to reactive metabolites. However, a significant increase in adduct formation in the bone marrow of BaA-treated animals indicates that circulation of this compound did occur, and that the bone marrow was exposed to reactive, DNA-damaging metabolites. This is confirmed by the positive *lacZ* mutagenicity response in bone marrow. Therefore, the aforementioned negative MN results may result from the inability of the mutagenic metabolites to produce chromosome breaks and MN.

MNs are produced during nuclear division when whole chromosome or acentric chromosome or chromatid fragments lag behind at anaphase. This can result from mis-repair (or lack of repair) of double-strand breaks, or simultaneous excision repair of damaged or incorrect bases on opposite strands [Fenech et al., 2011]. PAHs do not primarily cause double strand breaks, but rather exert their mutagenic effect via formation of bulky adducts that are repaired via nucleotide excision repair. Although it has been well documented that several PAHs (i.e., BaP, DBaH) are capable of inducing MN [Zhong et al., 1995], it is possible that not all PAHs are capable of causing double strand breaks that contribute to MN formation.

DBahA was one of the 3 compounds that failed to elicit a positive *lacZ* mutagenicity response in the bone marrow. As already noted, lack of responses for known mutagenic carcinogens are potentially indicative of ineffective target tissue exposure. However, for DBahA, a positive response in bone marrow was obtained for the adduct frequency and MN endpoints, indicating that the haematopoietic tissue was exposed to reactive metabolites. However, the tissue levels of reactive metabolites were likely not sufficient to overwhelm damage repair processes that would inhibit the establishment of mutations. Alternatively, it is possible that the positive MN response for DBahA resulted from a secondary metabolite (e.g., reactive oxygen) that did not contribute to the formation of mutations in the bone marrow. It has previously been shown that an alternate metabolic pathway via dihydrodiol dehydrogenase leads to the auto-oxidation of PAH metabolites to *o*-quinones, which can undergo redox cycling that generates DNA-damaging ROS and depurinating adducts [Smithgall et al., 1988; Penning et al., 1996; Miller and Ramos, 2001; IARC., 2014].

The hematopoietic tissue results for INDENO are interesting since adducts were not detected in bone marrow, and the MN assay result was negative; however, we did observe a significant increase in *lacZ* mutations. Given the lack of bulky adducts, it is unlikely that the reactive metabolites are reaching the bone marrow via systemic circulation. A likely alternative cause of the observed *lacZ* mutations, which would not contribute to the formation of bulky INDENO-DNA adducts, is reactive oxygen species (ROS) generated via the aforementioned *o*-quinone pathway. Some PAHs, especially those with higher molecular weights (e.g., INDENO), have been found to have greater redox activity that can readily lead to ROS formation and oxidative DNA damage, among

other types of cellular injury [Jeng et al., 2011]. Oxidative DNA damage cannot be detected using the ^{32}P -postlabelling methodology employed in this study.

Several published studies have previously examined the *in vivo* genetic toxicity of PAHs; however, the vast majority of these studies assessed responses following *i.p.* administrations (i.e., no first-pass metabolism), which is generally not considered useful for regulatory decisions that must consider the most likely route of receptor exposure. The most common route of human PAH exposure is oral, followed by inhalation, and dermal. This study is the first to employ an identical oral exposure regime to simultaneously evaluate induction of transgene (*lacZ*) mutations in various tissues and MN formation in peripheral blood following TGR exposures to 9 PAHs. This type of study is essential to critically evaluate the utility of TGR tools for regulatory genetic toxicity assessment (i.e., assay sensitivity), to compare and contrast TGR results across various tissues (OECD #488), and perhaps more importantly, to benchmark TGR responses against the MN endpoint in peripheral blood (OECD #474).

As noted, the current study concurrently examined induced *lacZ* mutations in 5 tissues, and the frequency of MN in RETs and NCEs, following sub-chronic oral administration of 9 PAHs. The evaluation of both of these endpoints was conducted in accordance with the protocols for dosing and sample collection recommended in the relevant OECD guidelines (i.e., OECD #474 & 488), and this work demonstrates that the two complementary endpoints can be integrated into a single sub-chronic study, thus providing concurrent assessment of mutation and chromosome damage. Although the study was restricted to a single compound class (i.e., PAHs), the results nonetheless underscore the utility of simultaneous genetic toxicity assessment in multiple tissues

across multiple endpoints (i.e., mutation and cytogenetic damage). Additional research is required to determine whether the observed sensitivity of the TGR mutagenicity endpoint is generally applicable, and additionally, whether specificity is equally suitable.

Interestingly, detailed reviews of the TGR endpoints have previously demonstrated the ability of the MutaTMMouse *lacZ* mutagenicity assay to return positive results for a wide range of genotoxic compounds, including carcinogens that failed to elicit positive responses in the *in vivo* MN assay. For example, Oxazepam (7-Chloro-3-hydroxy-5-phenyl-1,3-dihydro-1,4-benzodiazepin-2-one), a carcinogen that failed to elicit MN *in vitro* and *in vivo*, was shown to elicit a positive response in a TGR mutagenicity assay [Lambert et al., 2009]. Indeed, we are currently expanding this type of analysis and employing the MutaTMMouse system to examine the *in vivo* mutagenicity of carcinogens that failed to elicit positive clastogenicity responses *in vivo* (i.e., false negatives).

Overall, with respect to the tissues examined in this study, the MutaTMMouse *lacZ* mutagenicity assay was more sensitive (i.e., correct identification of mutagenic carcinogens) than the peripheral blood MN assay; moreover, for the MutaTMMouse endpoint, bone marrow was the least sensitive of the tissues examined. Small intestine, a site-of-contact tissue for oral exposure, was the most sensitive tissue, with the responses therein constituting the largest observed increases in mutant frequency for the PAHs examined. The results of this study call into question the reliability of only examining tissues that are remote from the site of contact (e.g., blood or bone marrow) when assessing the genotoxic hazard of substances for which the primary route of exposure is oral. Moreover, following up an *in vitro* positive result for mutation with an *in vivo* clastogenicity assay is not necessarily effective, and may even lead to the generation of *in*

vivo false negatives. This is particularly important for carcinogens (e.g., PAHs) that must be metabolically converted into DNA-reactive metabolites, since the reactive metabolites may not be systemically distributed and/or may only be generated *in situ* in remote tissues (e.g., bone marrow). As noted, phenomena such as AhR agonism in site of contact and remote tissues can control the nature of the tissue exposure (e.g., type and level of reactive metabolites), and the effects manifested therein that are indicative of sensitivity.

2.7 Conclusion

It is evident from the results presented that a dynamic interplay between several phenomena govern tissue-specific induction of genetic toxicity endpoints including DNA damage (i.e., adduct frequency), mutagenicity (i.e., *lacZ* transgene mutations), and clastogenicity (i.e., MN). Specifically, genetic damage in a target tissue appears to be controlled by dynamic processes that regulate (i) absorption, tissue-specific metabolism and systemic distribution of both metabolites and parent compounds, (ii) tissue-specific damage response and DNA repair capacity, and (iii) tissue-specific cellular proliferation.

The results presented clearly demonstrate the utility of the MutaTMMouse TGR assay for reliable *in vivo* mutagenicity assessment of suspected carcinogens with a mutagenic mode of action; in particular, for substances that require metabolic conversion to DNA-reactive metabolites (i.e., PAHs). Furthermore, the low sensitivity of the peripheral blood MN and the *lacZ* mutagenicity responses in bone marrow (i.e., limited ability to identify mutagenic carcinogens) highlights the need to examine site-of-contact tissues in addition to hematopoietic tissues, particularly for weak mutagens. Simultaneous enumeration of induced *lacZ* mutations in several tissues and micronuclei in peripheral

blood, comprises complementary components of a highly sensitive system for the detection of mutagenic carcinogens, and we are currently extending the type of analyses presented herein to encompass prioritised environmental matrices contaminated with complex mixtures of combustion-derived PAHs (e.g., contaminated soil, coal-tar amended consumer products).

2.8 References

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CHAPTER THREE

3 Chapter: Oral Exposure to Commercially Available Coal Tar-Based Pavement Sealcoat Induces Murine Genetic Damage and Mutations

3.1 Preamble: Authors, Affiliations, and Style

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3.2 Abstract

Coal tar (CT) is a thick black liquid produced as a by-product of coal carbonization to produce coke or manufactured gas. It is comprised of a complex mixture of polycyclic aromatic compounds, including a wide range of polycyclic aromatic hydrocarbons (PAHs), many of which are genotoxic and carcinogenic. CT is used in some pavement sealants (also known as sealcoat), which are applied to pavement in order to seal and beautify the surface. Human exposure is known to occur not only during application, but also as a result of the weathering process, as elevated levels of PAHs have been found in settled house dust in residences adjacent to CT-sealed surfaces. In this study we examined the genotoxicity of an extract of a commercially available CT-based

sealcoat in the transgenic MutaTMMouse model. Mice were orally exposed to 3 doses of sealcoat extract daily for 28-days. We evaluated genotoxicity by examining: (1) stable DNA adducts and (2) *lacZ* mutations in bone marrow, liver, lung, small intestine, and glandular stomach, as well as (3) micronucleated red blood cells. Significant increases were seen for each endpoint and in all tissues. The potency of the response differed across tissues, with the highest frequency of adducts occurring in liver and lung, and the highest frequency of mutations occurring in small intestine. The results of this study are the first demonstration of mammalian genotoxicity following exposure to CT-containing pavement sealcoat. This work provides *in vivo* evidence to support the contention that there may be adverse health effects in mammals, and potentially in humans, from exposure to coal tar.

3.3 Introduction

Driveway sealants (i.e., sealcoats) are widely used across North America by homeowners and commercial property owners to beautify and protect asphalt and concrete surfaces. There are two main formulations of sealcoat products used in North America: aqueous emulsions containing 10-35% coal tar (CT) and aqueous emulsions containing a similar level of asphalt (i.e., bitumen) [Mahler et al., 2005]. Crude CT used in the production of the former is a thick black liquid, generated as a by-product of coal carbonization to produce coke or manufactured gas. CT-based sealcoats generally contain a refined material known as RT-12 (i.e., ASTM D490, Road Tar 12), which is derived from high-temperature CT pitch, the material that remains after removal of distillation

products such as light and medium oils from crude CT. RT-12 is the most viscous of 12 refined products derived from high-temperature coal-tar pitch.

CT is well recognized as a multi-organ carcinogen in experimental animals [Culp et al., 1998; Goldstein et al., 1998; Robinson et al., 1987], and CT and CT pitch are both known human carcinogens [IARC., 1985; IARC., 2010b; IARC., 2010a]. They contain a complex mixture of polycyclic aromatic compounds (PACs), including a wide range of polycyclic aromatic hydrocarbons (PAHs), many of which have the ability to damage DNA (i.e., are genotoxic), as well as cause mutations and cancer [IARC., 1983; IARC., 2010b]. In contrast, asphalt ('bitumen' in Europe), which is similar in appearance to CT, is a by-product of petroleum refining and contains only low concentrations of genotoxic and carcinogenic PAHs. In addition, according to IARC's 2011 evaluation, there is only limited evidence that bitumens are animal carcinogens [IARC., 2011]. The concentration of priority PAHs (i.e., the 16 PAHs designated by the US EPA as priorities for concern and control [Keith and Telliard, 1979]) in CT-based sealcoats is more than 1000-fold greater than in asphalt-based products [Mahler et al., 2012]. Although usage data for CT-based sealcoats in Canada are not available, it is estimated that 320 million litres of CT-based sealcoat are used annually in the United States (US) [Scoggins et al., 2009]. An estimated 82 to 100 kilotonnes of CT pitch is produced annually in Canada (unpublished data).

Sealcoat is applied to surfaces by either rolling/spreading or spraying. Human exposure is known to occur during the application process as well as from volatilization while drying. Subsequent exposure to particulate material can also occur from surface weathering. Two hours following product application, volatile PAH concentrations in air

sampled at 0.03 m above CT-sealed lots are 5000-fold greater than those above unsealed lots [Van Metre et al., 2012]. In addition, PAH concentrations in water runoff from CT-sealed lots were shown to be ~65-fold higher than in runoff from asphalt-sealed lots, and a study of 40 US lakes determined that CT sealcoat was likely the greatest contributor to elevated levels of sediment PAHs [Mahler et al., 2005; Van Metre and Mahler, 2010]. Finally, PAH levels in settled house dust (i.e., SHD) collected from apartments adjacent to CT-sealed lots were found to be 25-fold higher than SHD samples from apartments adjacent to asphalt-sealed lots, a difference that has been translated into significant elevations in excess lifetime cancer risk [Mahler et al., 2010; Williams et al., 2013]. A ban on CT-based pavement sealcoats was implemented in the city of Austin, TX in November, 2005, and subsequent analysis of priority PAH levels in dated lake sediment cores showed a 44% decline in mean levels from 1998-2005 to 2006-2012 [Van Metre and Mahler, 2014]. Recently, in an effort to reduce PAH loading to local waterways via storm water discharge, other municipalities (e.g., Washington, DC) have implemented similar bans [District of Columbia, 2013].

The toxicity of CT-based sealcoats and runoff has been demonstrated in several aquatic organisms. Studies of *Ceriodaphnia dubia*, *Pimephales promelas* [Mahler et al., 2015] and *Xenopus laevis* [Bryer et al., 2006] exposed to CT-sealcoat runoff, and freshwater macroinvertebrates [Bryer et al., 2010] exposed to CT-sealcoat in soil showed increased mortality relative to controls. Eastern newts exposed to CT-sealcoat in sediment also demonstrated signs of toxicity [Bryer et al., 2010]. Additionally, a study by Kienzler et al. [2015] that employed the comet assay, showed increased DNA damage in a piscine liver cell line exposed to diluted CT sealcoat runoff.

Collectively, the aforementioned studies and reviews indicate that crude CT is mutagenic and carcinogenic, and known to contain a wide range of mutagenic and carcinogenic PAHs. Moreover, emissions from pavements treated with CT-based sealcoat products, which can contribute to elevated levels of PAHs in air, SHD, soil, and aquatic sediments, are toxic and/or genotoxic to a variety of organisms. As such, CT-based sealcoats constitute an environmental and human health hazard. However, although the genetic toxicity and carcinogenicity of CT and PAHs have been well documented [Culp et al., 1998; IARC., 1983; IARC., 2010b], the mammalian genotoxicity of commercially available CT-based sealcoats has hitherto not been investigated.

In the current study, we have used the MutaTMMouse system to examine the genetic toxicity of an extract of a commercially available CT-based sealcoat. We have previously demonstrated the ability to integrate several genotoxicity endpoints into a single *in vivo* murine study by employing the transgenic MutaTMMouse system [Lemieux et al., 2011]. Additionally, we have shown the reliability of the study design to detect induced mutations in multiple MutaTMMouse tissues following oral exposures to several PAHs [Long et al., 2016]. In addition, we have employed an *in vitro* version of the MutaTMMouse mutagenicity assay to assess the mutagenic activity of complex PAH-containing extracts from CT-contaminated soils [Lemieux et al., 2015a; Lemieux et al., 2015b]. Herein we report the genotoxicity observed in mice as a result of daily exposure to an extract of CT-based sealcoat, including the frequency of stable DNA adducts and mutations found in bone marrow, liver, lung, small intestine, and stomach, as well as the frequency of chromosome damage in peripheral blood cells.

3.4 Materials & Methods

3.4.1 Driveway Sealcoat Handling and Extraction

All chemicals used for the extraction were analytical grade and obtained from EMD Chemicals (Gibbstown, NJ, USA). A 15-L pail of CT-based sealcoat was purchased from a local home improvement retailer. The product is a water-based CT emulsion that, according to the Material Safety Data Sheet for the product, contains 10-30% high-temperature CT pitch (CAS # 65996-93-2) by weight. The sealcoat was thoroughly homogenized and aliquots of the homogenized material were dispensed onto several large glass petri dishes to air dry for 48-hours. This drying process resulted in a total dry weight of 25% of the bulk sealcoat (i.e., water content ~75%). Approximately 10 g of dry sealcoat was then scraped from the surface of the petri dishes, weighed on an analytical balance, and transferred to a coffee grinder, where it was ground into a fine powder. The resulting powder was combined with 25 g of attapulgus clay (Forcoven Products Inc., Humble, TX), and extracted using a method adapted from Wise et al. [1988] that permits removal of solvent-insoluble hydrocarbons (e.g., asphaltenes) and isolation of the complex, PAH-containing fraction for chemical and toxicological analyses. Briefly, the sealcoat/clay mixtures were applied to three 10 cm diameter open columns each packed with 300 g of attapulgus clay conditioned with 10% dichloromethane (DCM) in pentane prior to the extraction. The following amount of dried CT sealcoat was applied to each column: (a) 10.47 g, (b) 10.11 g, and (c) 10.05 g. The columns were eluted with 1.8 L of 10% DCM in pentane, and the eluate was combined into a single vial and evaporated under ultra-pure nitrogen until a viscous dark brown liquid remained that could no longer be reduced. The final volume of the sealcoat

eluate was 5.85 ml. A small aliquot (100 µl) was removed for chemical analyses, and the remainder used to prepare dosing solutions by first sonicating the eluate and then diluting the material in an appropriate quantity of highly refined olive oil (Sigma-Aldrich Canada, Oakville, ON). Doses were prepared based on the mg equivalent (eq) weight of the crude (i.e., wet) sealcoat.

3.4.2 Chemical Analysis by GC-MS

The PAH content of the sealcoat concentrate was determined using GC-MS according to US EPA Method 8270D; the analyses were conducted by a commercial laboratory accredited by the Canadian Association for Laboratory Accreditation (CALA), the National Institute of Standards and Technology, and the National Voluntary Laboratory Accreditation Program. Briefly, the samples were warmed and diluted with dichloromethane and an aliquot analysed on an Agilent 6890N gas chromatograph with a split/splitless injector and Restek Rxi-5Sil MS column (30 mm x 0.25 mm ID, film thickness 0.25 µm, Chromatographic Specialties, Brockville, ON). Target analytes were identified and quantified using an Agilent 5973N mass spectrometer operating in SIM (selected ion monitoring) mode. The concentrations of a standard panel of 19 PAHs were determined and results expressed as mg PAH/kg sealant-eq. This includes the 16 priority PAHs, as well as seven PAHs designated by the US EPA as class B2 carcinogens (i.e., probable human carcinogens) [Schoeny and Poirier, 1993].

3.4.3 Animal Treatment

Adult MutaTMMouse (strain 40.6) males (18-weeks of age) were randomly assigned to a dose group and individually housed in microVENT ventilated racks (Allentown Inc., Allentown, NJ). The mice received standard rodent chow (2014 Teklad

Global standard rodent diet) and water *ad libitum* for the duration of the study, and were maintained on a 12-hour light/12-hour dark cycle. The dosing solution was administered at 0.005 ml/g body weight (BW). The sealcoat extract was dissolved in highly refined olive oil and prepared at the following doses: 1974, 3949, 7897 mg crude sealcoat eq./kg BW/day. There were 5 mice in each dose group, including the control group (20 animals in total). Sealcoat extract was administered via oral gavage daily for 28-days, followed by a 3-day sampling time (i.e., no additional doses) prior to necropsy, according to OECD test guideline # 488 [OECD., 2013]. Two days after the final dose, blood was obtained from the facial vein for micronucleus frequency determination. Animals were euthanized via cardiac puncture under isoflurane gas anaesthesia, followed by cervical dislocation and chest cavity opening. The glandular stomach and small intestine were rinsed with phosphate-buffered saline (PBS) (Thermo-Fisher, Waltham, MA) to remove partially digested food and flash frozen along with liver and lung. The bone marrow was flushed from both femurs with PBS, pelleted, and flash frozen. All tissues were stored at -80 °C. Mice were bred, maintained, and treated in accordance with the Canadian Council for Animal Care Guidelines and the protocols were approved by the Health Canada Ottawa Animal Care Committee.

3.4.4 DNA Extraction

Murine tissues were prepared for genomic DNA extraction as described in Long et al. [2016]. Briefly, prepared tissues were suspended in freshly prepared ice-cold lysis buffer (1 mM Na₂EDTA, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% SDS (w/v)) and incubated overnight at 37 °C with gentle shaking. Genomic DNA was isolated from lysed tissues using a phenol/chloroform extraction procedure described previously [Douglas et

al., 1994; Vijg and Douglas, 1996]. Isolated DNA was dissolved in 100 µl TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) and stored at 4 °C until use.

3.4.5 Analysis of DNA Adducts by ³²P-Postlabeling

The nuclease P1 enrichment version of the thin-layer chromatography (TLC) ³²P-postlabeling assay was used to determine DNA adduct formation in bone marrow, liver, lung, glandular stomach, and small intestine. The procedure was performed as described previously [Wohak et al., 2014; Krais et al., 2015]. As in prior studies [Longhin et al., 2013; Molina et al., 2013; Siddens et al., 2012; Kim et al., 2011], total DNA adduct levels were measured in the diagonal radioactive zone (DRZ) area of the TLC plates and were considered representative of PAH-DNA and other aromatic/hydrophobic adducts resistant to nuclease P1 digestion. The method provides a summary measure of a complex mixture of adducts present in the postlabeling chromatograms. The results were expressed as DNA adducts/10⁸ nucleotides.

3.4.6 Analysis of *lacZ* Mutations by the PGal Positive Selection Assay

The PGal (phenyl-β-D-galactoside) positive selection assay was used for the determination of *lacZ* mutant frequency in DNA samples from bone marrow, liver, lung, glandular stomach, and small intestine, as previously described [Gossen et al., 1992; Lambert et al., 2005; Vijg and Douglas, 1996]. Mutant frequency was calculated as the ratio of mutant plaque forming units (i.e., pfu) to total pfu.

3.4.7 Analysis of Peripheral Blood Micronuclei by Flow Cytometry

MicroFlow[®] kits (Litron Laboratories, Rochester, NY) were used for enumeration of micronucleated reticulocytes (RET) and normochromatic erythrocytes (NCE). Briefly, approximately 60 µl of peripheral blood was immediately combined with 350 µl of

anticoagulant. Blood samples were then fixed by transferring to ice-cold methanol and stored at -80°C . After 3-5 days, fixed blood samples were then centrifuged, rinsed, and transferred to a long-term storage solution. Coded specimens were shipped to Litron Laboratories (Rochester, NY) for analysis. Micronuclei were scored in NCEs (i.e., MN-NCEs) and RETs (i.e., MN-RETs) by flow cytometry using a 3-colour labeling method described in [Dertinger et al., 2004; Torous et al., 2001].

3.4.8 Statistical Analysis

The *lacZ* and MN dose-response data were analysed in SAS v.9.1 (SAS Institute, Cary, NC) by Poisson regression and a Type 3 chi-squared analysis. The data were fit to the model $\log(E(Y_i)) = \log t_i + \beta x_i$, where $E(Y_i)$ is the expected value for the i th observation, β is the vector of regressions coefficients, x_i is a vector of covariates for the i th observation, and t_i is the offset variable used to account for differences in observation count period (e.g., total pfu). The offset (e.g., natural log of pfu) was given a constant coefficient of 1.0 for each observation, and log-linear relationships between mutant count and test article concentration were specified by a natural log link function. Post-hoc custom contrasts based on the asymptotic chi-square distribution of the likelihood ratio statistic were conducted to compare each dose group with the control. Should visual examination suggest the need to truncate the top dose, data were re-fit without the top dose, and if this resulted in an improved chi-squared value, then the truncated results were retained, and this was used for potency determination (see below).

The potency of the responses for each significant tissue/endpoint combination was determined using ordinary least-squares linear regression and expressed as the slope of

the linear portion of the dose-response function. Individual animal genotoxicity results are available from the author upon request.

3.5 Results

3.5.1 Analysis of sealcoat and sealcoat extract

Chemical analysis of the CT-based sealcoat revealed that there is 44.2 g (4.4% w/w) of the US EPA priority PAHs per kg of sealcoat, including 8.66 g (0.87% w/w) of US EPA class B2 PAHs (i.e., probable human carcinogens), and 1.91 g (0.19% w/w) of benzo(*a*)pyrene (BaP) (**Table 3-I**). Concentrations in the sealcoat extract are expressed in terms of sealcoat equivalents (eqs), which were calculated based on the amount of crude sealcoat that was extracted and submitted for chemical analysis. Additionally, we calculated the total amount of PAHs present in a 15-L pail of CT-based sealcoat (i.e., the format available for retail purchase). The results indicate that a 15-L pail contains more than 750 g of priority PAHs and 147 g of B2 carcinogenic PAHs, including 32 g of BaP (**Table 3-I**).

Table 3-I. The concentrations of polycyclic aromatic hydrocarbons (PAHs) in CT-based driveway sealcoat extract (expressed per unit weight of crude wet sealcoat), as well as the total amount of PAHs per 15-L pail (i.e., the commercially available size). The chemical abstract service number (i.e., CAS #) for each PAH is provided.

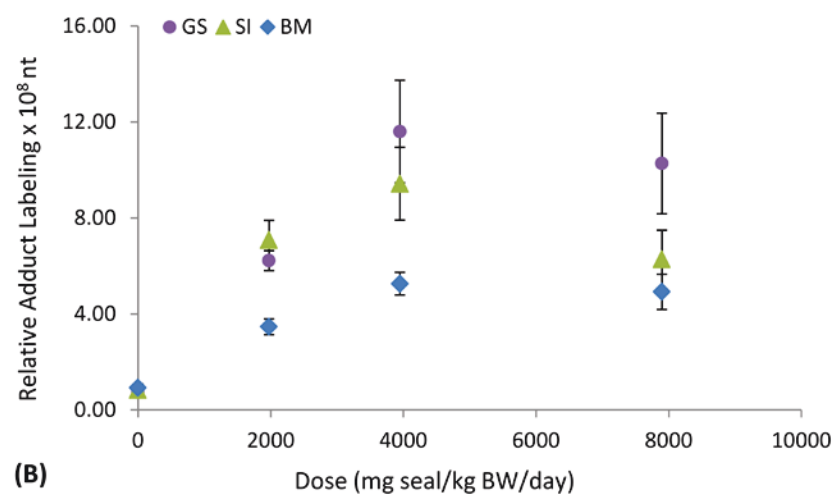
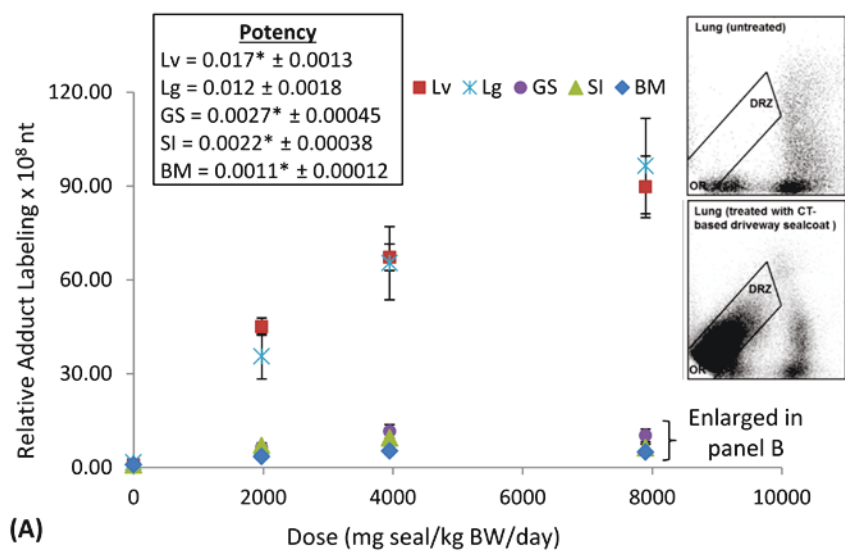
Polycyclic Aromatic Hydrocarbons	CAS #	Sealcoat (mg PAH/kg sealcoat)	Per 15-L pail (g)
Acenaphthene [†]	83-32-9	2,433	41.30
Acenaphthylene [†]	208-96-8	BDL	N/A
Anthracene [†]	120-12-7	1,582	26.85
Benzo[a]anthracene ^{†*}	53-55-3	1,325	22.50
Benzo[a]pyrene ^{†*}	50-32-8	1,906	32.35
Benzo[b]fluoranthene ^{†*}	205-99-2	1,407	23.89
Benzo[g,h,i]perylene [†]	191-24-2	958	16.26
Benzo[k]fluoranthene ^{†*}	207-08-9	784	13.30
Biphenyl	92-52-4	87	1.48
Chrysene ^{†*}	218-01-9	2,409	40.89
Dibenz[a,h]anthracene ^{†*}	53-70-3	151	2.56
Fluoranthene [†]	206-44-0	8,706	147.78
Fluorene [†]	86-73-7	1,867	31.69
Indeno[1,2,3-cd]pyrene ^{†*}	193-39-5	682	11.58
1-Methylnaphthalene	90-12-0	201	3.42
2-Methylnaphthalene	91-57-6	351	5.96
Naphthalene [†]	91-20-3	725	12.32
Phenanthrene [†]	85-01-8	11,850	201.15
Pyrene [†]	129-00-0	6,820	115.76
TOTAL		44,242	751.01
Total 16 US EPA priority pollutant PAHs		43,603	740.16
Total US EPA B2 carcinogenic PAHs		8,663	147.06

†: designated a US EPA priority pollutant; *: designates a US EPA B2 carcinogen; eq: equivalents; BDL: below detection limit

3.5.2 DNA Adduct Frequency

Bulky DNA adduct frequency, determined using ^{32}P -postlabeling analyses, was employed to document tissue-specific exposures to DNA-damaging agents in the administered sealcoat extract. The results revealed statistically significant increases in DNA adduct frequency relative to controls in all tissues examined (**Figure 3.1**). Furthermore, damage frequency increased in a dose-dependent manner, with potency (i.e., magnitude of response increase per unit dose increase) varying across the tissues examined. Analyses of the dose-response relationships revealed the most potent response (i.e., highest slope) for the liver (maximum response up to 102-fold over control), followed by the lung (maximum response up to 60-fold over control), glandular stomach (maximum response up to 14-fold over control), small intestine (maximum response up to 8-fold over control), and finally the bone marrow (maximum response up to 5-fold over control).

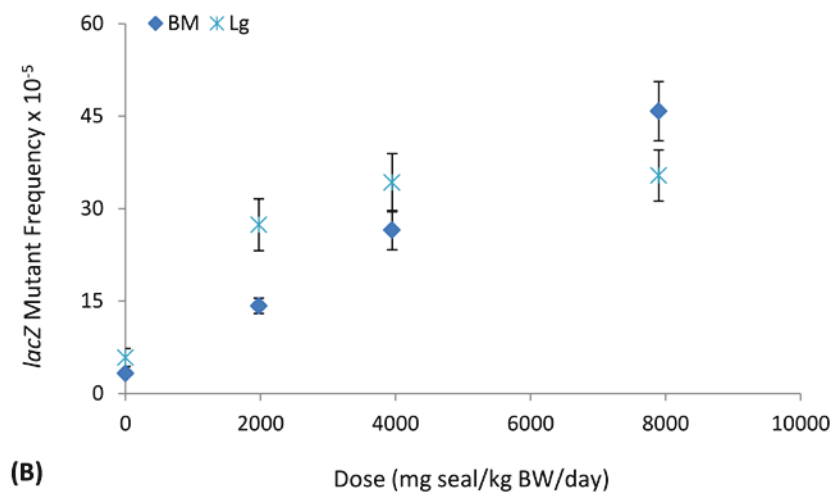
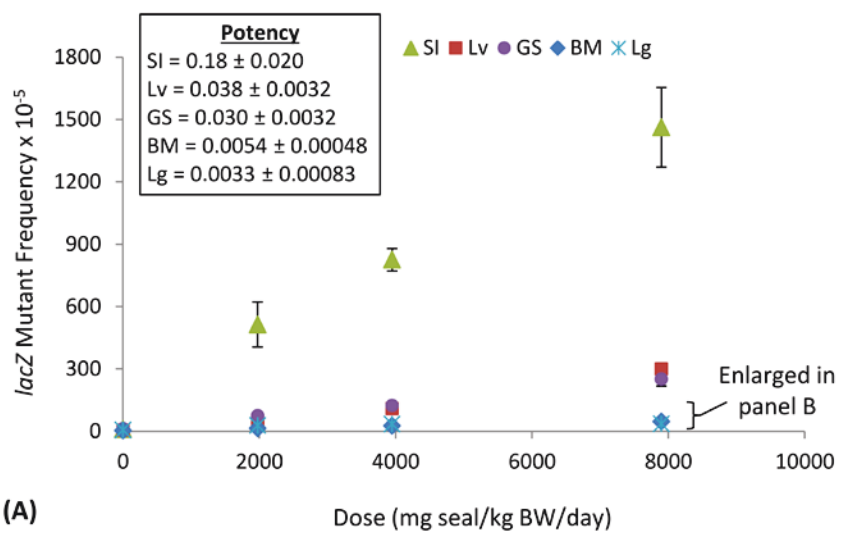
Figure 3.1. Relative adduct labeling (DNA adducts per 10^8 nucleotides (nt)) in tissues from MutaTMMouse orally exposed to CT-based driveway sealcoat (seal). Total frequency of adducts was determined from the diagonal radioactive zone (DRZ). **(A)** Dose response-data and potency (i.e., the slope of the linear portion of the dose-response function) is presented for each tissue. Lv: liver, Lg: lung, GS: glandular stomach, SI: small intestine, BM: bone marrow. * indicates where data were truncated during statistical analysis, therefore the mutagenic potency is a result of fewer than 4 dose groups. **(B)** Restricted y-axis scale to better display dose-response data for GS, SI, and BM. Inserts: Representative autoradiographic profiles of DNA adducts in lungs from untreated mice or mice subchronically exposed to CT-based driveway sealcoat (these profiles are representative of adduct profiles obtained with DNA from other mouse tissues including liver, glandular stomach, small intestine and bone marrow) . Solvent conditions for the separation of PAH-derived DNA adducts using thin-layer chromatography were as follows: D1, 1.0 M sodium phosphate, pH 6.0; D3, 3.5 M lithium-formate, 8.5 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea. The origins (OR), at the bottom left corner, were cut off before imaging.



3.5.3 *lacZ* Mutant Frequency

The frequency of *lacZ* mutants was assessed in 5 tissues. The results revealed a statistically significant response for each tissue with dose-dependent increases in mutant frequency relative to controls and variations in potency across the tissues examined (**Figure 3.2**). The most potent response (i.e., highest slope) was observed in the small intestine (maximum response up to 154-fold above control), followed by the liver (maximum response up to 51-fold above control), glandular stomach (maximum response up to 17-fold above control), bone marrow (maximum response up to 14-fold above control), and finally lung (maximum response up to 6-fold above control). The fact that the highest responses were seen in the site-of-contact and related tissues emphasizes the importance of examining these tissues following an oral administration.

Figure 3.2. The *lacZ* mutant frequency in tissues from MutaTMMouse orally exposed to CT-based driveway sealcoat (seal). **(A)** Dose-response data and potency (i.e., the slope of the linear portion of the dose-response function) is presented for each tissue. SI: small intestine, Lv: liver, GS: glandular stomach, BM: bone marrow, Lg: lung. **(B)** Restricted y-axis to better display dose-response data for BM and Lg.



3.5.5 Micronucleus Frequency

The frequency of micronucleated red blood cells was assessed as a measure of clastogenicity in hematopoietic tissue. Statistically significant increases in micronucleated RETs and NCEs were observed relative to controls, with a 2.3-fold greater potency in RETs (i.e., a potency of 0.000082 in RETs versus 0.000036 in NCE) (**Figure 3.3**). The results emphasize the importance of scoring RETs to examine the effects of recent damage.

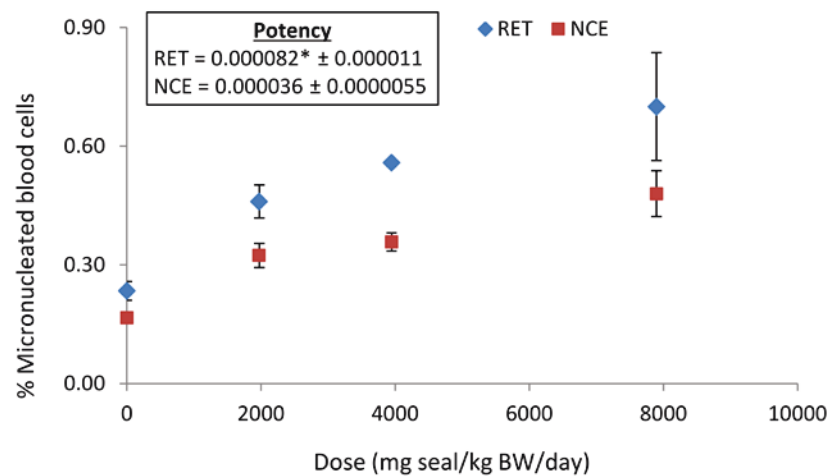


Figure 3.3. The frequency of micronuclei in peripheral blood from Muta™Mouse orally exposed to CT-based driveway sealcoat (seal). Potency (i.e., the slope of the linear portion of the dose-response function) is presented for each tissue. RET: reticulocytes, NCE: normochromatic erythrocytes. * indicates where data were truncated during statistical analysis, therefore the mutagenic potency is a result of fewer than 4 dose groups.

3.7 Discussion

This study used a 28-day repeat-dose oral exposure of transgenic MutaTMMouse to assess the multi-organ genotoxicity of a PAH-containing extract of a commercially available CT-based driveway sealcoat. The results obtained clearly indicate that oral exposure to the purified extract induces both significant increases in DNA damage (i.e., adducts) and mutations (i.e., *lacZ*) across several tissues, as well as chromosome damage (i.e., MN) in peripheral blood cells.

Chemical analysis of the crude CT-based sealcoat examined here revealed that the product contains over 4% PAHs by weight, with approximately 0.9% US EPA B2 PAHs. In comparison with available information on other CT-based sealcoats, the product examined in this study contained a lower concentration of PAHs. For example, in a study by Bryer et al. [2006], the sealcoat contained 23% PAHs by weight, with 5% B2 PAHs. Bommarito et al. [2010] reported that the CT-based sealcoat examined in their study was approximately 8% PAHs by weight, and nearly 3% B2 PAHs. It is important to note that crude CT, CT pitch and products derived from CT pitch contain complex mixtures of PAHs, with their relative composition concentration varying widely with source and type of material (e.g., crude CT versus high-temperature pitch). Additionally, sealcoats such as that examined here can contain 10-35% CT by weight and the final PAH content of the product will depend on the CT concentration in each batch. Although the PAH content of CTs will vary according to the type of material (i.e., crude or refined) and its source, the material generally used for the production of CT-based pavement sealants is a derivative of CT pitch known as RT-12. Although one might expect some consistency in the PAH content of CT-based sealcoat products containing RT-12, the source of the crude CT and

the exact RT-12 concentration in each sealcoat batch (i.e., 10-35% by weight) affects the final PAH content of the sealcoat [Mahler and Van Metre, 2011]. Moreover, the certificate of analysis for the National Institute of Standards and Technology Standard Reference Material 1597 (i.e., a complex mixture of PAHs from CT), which employed the Wise et al. [1988] method also used here, notes the presence of other polycyclic aromatic compounds including sulphur heterocyclics, along with PAHs. This is consistent with our earlier work [Lemieux et al., 2008; Lundstedt et al., 2006] that employed a similar approach to prepare PAH-containing extracts from CT-contaminated soils, and noted that extracts can contain *N*- and *S*-heterocyclics, in addition to homocyclic PAHs, all of which may contribute to the overall genotoxicity of the mixture.

The most potent DNA adduct response was observed in liver and lung, followed by glandular stomach. This pattern of CT-induced DNA adducts across murine tissues following oral exposure is similar to what was observed in the CT study of Culp and Beland [1994]. More specifically Culp and Beland assessed bulky DNA adduct frequency in several mouse tissues following dietary administration of CT for 21-days, and noted that levels were highest in lung, followed by liver and forestomach [Culp and Beland, 1994]. The presence of stable DNA adducts in a particular tissue indicates that the tissue was exposed to DNA-reactive metabolites (e.g., PAH-diol-epoxides). This can occur via CYP1-mediated metabolism within the target tissue, and/or alternatively, metabolism in the gastrointestinal system followed by systemic circulation of reactive metabolites and tissue delivery. For example, the reactive form of BaP (i.e., benzo(*a*)pyrene-diol-epoxide; BPDE) has been identified in serum following an oral exposure to BaP [Ginsberg and Atherholt, 1989]; however, due to the highly reactive nature of these metabolites, it is

more likely that the production of DNA-reactive metabolites occurs *in situ* in each tissue. Indeed, it has previously been demonstrated in several mouse models that the tissues examined here have constitutive or inducible levels of CYP1A1, 1A2, and/or 1B1, all of which are known to convert PAHs to DNA-reactive metabolites [Arlt et al., 2015; Nebert et al., 2013; Choudhary et al., 2003; Nebert et al., 2004; Renaud et al., 2011; Uno et al., 2008]. Moreover, our lab group has previously demonstrated induction of CYP1A1 and 1B1 in MutaTMMouse lung and liver following an identical oral exposure regime to several individual PAHs [Labib et al., 2015].

The most potent response for induction of *lacZ* mutations was observed in the small intestine, followed by liver, glandular stomach, bone marrow, and lung. For an oral exposure route, glandular stomach and small intestine are both initial site-of-contact tissues, with liver being closely related; whereas, both bone marrow and lung require distribution of the sealcoat compounds and/or compound metabolites via systemic and/or pulmonary circulation. These results support the contention that the production of DNA-reactive metabolites occurs *in situ*. It is therefore not unexpected that the highest level of response induction was observed for site-of-contact and related tissues (i.e., stomach, liver, small intestine) prior to complete metabolism, detoxification and excretion. Nebert et al. [2013] demonstrated the importance of metabolism in small intestine and liver in the initial detoxification of orally-delivered BaP. Efficient detoxification in the gastrointestinal system, and subsequent excretion of conjugates via the bile, would reduce the amount of compound and/or compound metabolite available for delivery to distal tissues. This contention is consistent with the high levels of CYP1 isozymes that have been observed in liver and small intestine (i.e., CYP1A2 in the liver, CYP1A1/1B1 in

small intestine) [Shi et al., 2010b; Uno et al., 2008; Shi et al., 2010a]. These isozymes are capable of converting PAHs into DNA-reactive metabolites, thus contributing to elevated mutagenic responses in these tissues.

Despite significant increases in genotoxic damage across all endpoints and tissues, the results show clear differences in the tissue-specific patterns of DNA adduct and *lacZ* mutation induction. As noted, the most potent adduct responses were observed in liver and lung; whereas, for *lacZ* mutations, the most potent response was observed for small intestine, with the least potent response observed in lung. Although the precise cause of this discrepancy remains to be determined, it seems reasonable to assert it relates to tissue-specific differences in repair capacity and cellular proliferation rate. Conversion of DNA damage to permanent sequence changes (i.e., mutations) is controlled by a complex series of dynamic processes that control damage processing and repair, and cellular proliferation, and although a discussion of the mechanistic nuances of the process is beyond the scope of this work, it is interesting to note that cells in the lung and liver have relatively low proliferation rates in comparison with the other tissues examined herein (unpublished data). This would be expected to limit the conversion of adducts to mutations; moreover, tissue-specific differences in damage processing and repair can be expected to augment or diminish the establishment of mutations. Although most cells in liver and lung tissues have low mitotic indices, lung has been shown to have an elevated capacity for error-free lesion bypass that could contribute to a reduction in the conversion of adducts to mutations [Velasco-Miguel et al., 2003]. More specifically, Ogi et al. [2002] noted that expression of the translesion DNA polymerase Pol κ is AhR-dependant (i.e., PAH-inducible), and Bi et al. [2005] noted that Pol κ is required for recovery from

BPDE-induced cell cycle checkpoint. Thus, although the precise causes of the observed cross-tissue patterns in induced DNA adduct and mutation levels remain to be determined, dynamic differences in tissue specific metabolism, cellular growth, and DNA damage and repair likely play key roles.

Exposure to the sealcoat extract induced a significant increase in the frequency of micronucleated RETs and NCEs, although the fold-increase in event frequency is much lower in comparison with the *lacZ* mutation and DNA adduct endpoints. It is well recognized that the primary mode of action for genotoxic PAHs is induction of mutations via formation and mis-repair of bulky adducts; however, PAHs have also been previously shown to induce clastogenic effects and elevated levels of chromosomal damage [Lemieux et al., 2011; Abramsson Zetterberg et al., 2013; Crofton Sleight et al., 1993; Glatt et al., 1990; He and Baker, 1991; Nishikawa et al., 2005; Schober et al., 2006; Warshawsky et al., 1995; Whong et al., 1994]. The results obtained showed significant elevations in micronuclei, which are thought to be produced during cellular replication following DNA double strand breaks [Fenech et al., 2011]. These results are consistent with a previous study by our group that employed an identical study design to evaluate the genotoxicity of 8 priority PAHs. The results of that study showed that 4 of the priority PAHs examined (i.e., BaP, dibenz(*a,h*)anthracene, benzo(*b*)fluoranthene, and benzo(*k*)fluoranthene) induced significant increases in the frequency of micronucleated red blood cells [Long et al., 2016].

The product instructions for the sealcoat examined in this study indicates that a 15-L pail contains sufficient material to cover an area of 20 to 49 m², and additionally, that it is a “1-year sealcoat” that should be reapplied annually. Thus, for homeowners

with a typical double-car driveway (e.g., $\sim 36 \text{ m}^2$) at least a full pail of sealcoat would be required for annual pavement maintenance, and therefore annual application of total priority PAHs for a typical household would exceed 750 g, including 147 g of US EPA B2 carcinogenic PAHs and 32 g of BaP. Despite the level of PAHs in coal tar-based sealcoat, genotoxic hazard, and the recommended application amount and frequency, the risk of adverse human health effect depends on the potential for exposure, as well as the route, level, and duration of exposure. Clearly, the magnitude of human exposure to the product, and by extension the PAHs in the product, will depend on the magnitude and frequency of contact with the product (e.g., during application), contact with the pavement surface, and contact and/or oral ingestion of PAH-contaminated particulate material (e.g., pavement dust and house dust) from weathered pavement. Although currently available information does not permit accurate determination of total human exposure to PAHs in the product or materials derived from the product, studies such as that by Williams et al. [2013] estimated the level of exposure (in ng BaP equivalents/kg BW/day) of individuals inhabiting residences adjacent to surfaces treated with CT-based sealcoats. Moreover, Williams et al. estimated the excess lifetime cancer risk associated with non-dietary ingestion of PAH-contaminated soils and SHDs collected in close proximity to CT-sealed pavements, and determined that values can exceed 1×10^{-4} [Williams et al., 2013], a level that exceeds the essentially negligible cancer risk for contaminated site risk assessment [Health Canada., 2012].

Published studies that investigated the murine carcinogenicity of CT have examined crude high-temperature CT rather than the aforementioned refined material that is used in the production of CT-based sealcoat products [Culp et al., 1998; Goldstein et

al., 1998]. Nonetheless, it is interesting to compare the results of the Culp et al. [1998] and Goldstein et al. [1998] studies to those obtained here. A 2-year rodent cancer bioassay involving B6C3F1 mice fed CT-amended food found carcinomas in liver, lung, forestomach, and small intestine, with the highest tumour incidence observed in small intestine followed by lung [Culp et al., 1998; Goldstein et al., 1998]. This tumour induction pattern corresponds with the results of this study (i.e., highest levels of mutations and/or adducts in lung, liver and intestine). Despite the facts that not all mutagens are necessarily carcinogens and that the current study examined an extract of a CT-containing pavement sealcoat rather than crude CT, the cross-tissue, multi-endpoint responses observed herein, and the correspondence between genotoxicity herein and earlier carcinogenicity results, strongly suggest that oral exposure to CT-based sealcoat products would also induce rodent carcinomas in multiple tissues in longer-term studies. This is especially concerning, since numerous studies by Mahler, van Metre, and colleagues [Mahler et al., 2010; Williams et al., 2013; Van Metre et al., 2012; Metre et al., 2008] have clearly demonstrated a potential for human exposure to mutagenic carcinogens in environmental samples (e.g., air, SHD, soil) collected in the vicinity of pavements treated with CT-based sealcoats.

3.8 Conclusion

CT-based sealcoat products contain high levels of priority PAHs, including several mutagenic carcinogens, and previous work has demonstrated that environmental samples derived from sealed pavements (e.g., runoff water) or collected in the vicinity of surfaces treated with CT-based sealcoats are similarly contaminated with priority PAHs

and induce toxic/genotoxic effects in a range of organisms. Moreover, earlier work has documented the potential for human exposure via contact with PAH-contaminated environmental matrices such as soil and SHD collected near surfaces treated with CT-based sealcoats. The results obtained in this study clearly demonstrate that CT-based pavement sealcoat extract has the ability to induce significant increases in genetic damage, including DNA adducts, mutations and chromosomal damage, in several tissues of orally-exposed mice. Although it is not possible to quantitatively translate the magnitude of the observed rodent responses into effects that may occur in humans, the work provides conclusive identification of *in vivo* mammalian genotoxic hazard from oral exposure to a PAH extract from coal tar-based driveway sealcoat.

3.9 References

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CHAPTER FOUR

4 Chapter: The Genetic Toxicity of Complex Mixtures of Polycyclic Aromatic Hydrocarbons: Evaluating Dose-Additivity in a Transgenic Mouse Model

4.1 Preamble: Authors, Affiliations, and Style

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4.2 Abstract

This study evaluates the risk assessment approach currently employed for polycyclic aromatic hydrocarbon (PAH)-contaminated media, wherein carcinogenic hazards are evaluated using a dose-addition model that employs potency equivalency factors (PEFs) for targeted carcinogenic PAHs. Here, MutaTMMouse mice were sub-chronically exposed to PAH mixtures (*p.o.*), and mutagenic potency (MP) values were determined for five tissues. Predicted additive mixture MPs were generated by summing the products of the concentrations and MPs of the individual targeted PAHs; values were

compared to the experimental MPs of the mixtures to evaluate additivity. Additionally, the PEF-determined BaP-equivalent concentrations were compared to those determined using a bioassay-derived method (BDM) (i.e., an additivity-independent approach). In bone marrow, mixture mutagenicity was less-than-additive and the PEF-method provided higher estimates of BaP-equivalents than the BDM. Conversely, mixture mutagenicity in site-of-contact tissues (e.g., small intestine) was generally more-than-additive and the PEF-method provided lower estimates of BaP-equivalents than the BDM. Overall, this study demonstrates that additive predictions of mixture mutagenic potency based on the concentrations and potencies of a small number of targeted PAHs results in values that are surprisingly close to those determined experimentally, providing support for the additive assumption employed for human health risk assessment of PAH mixtures.

4.3 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds produced by the incomplete combustion of organic material. PAH exposures characteristically involve complex mixtures in complex environmental matrices such as contaminated soil, urban air, vehicle exhaust, tobacco smoke, or contaminated water. Several PAHs have been shown to be genotoxic and/or carcinogenic in experimental animals, as well as carcinogenic to humans^{1,2}. Benzo(*a*)pyrene (BaP), the prototypical PAH, has been classified by the International Agency for Research on Cancer (IARC) as a Group 1 human carcinogen, with several additional PAHs listed as probable or possible human carcinogens (i.e., Group 2a or 2b)¹. Additionally, several PAH-containing complex

mixtures are Group 1 IARC carcinogens, including diesel exhaust³, air pollution⁴, tobacco smoke^{5,6}, and coal tar (CT)⁷.

PAHs induce somatic mutations in mammalian cells via DNA-binding of activated metabolites, subsequent incorrect or inadequate removal/repair of lesions, and fixation of mutations during replication³. Somatic mutations have been mechanistically and empirically linked to cancer, and genetic damage is considered an enabler of carcinogenesis^{8,9}. Reduction of exposures to known or suspected carcinogens, including PAHs and PAH mixtures, has been a focus of public health organisations for several decades, and a recent high-impact publication has re-emphasized the importance of environmental carcinogen exposures in determining the overall cancer risk¹⁰. Thus, efficient and effective assessment of the risks posed by PAH mixtures is critical for identifying exposure scenarios that can increase risk to human health; and moreover, interventions that can reduce risk.

Hazard and risk assessments of complex environmental mixtures, including PAH mixtures in complex environmental matrices, are challenging since most available toxicological data pertain to individual compounds. Additionally, no two environmental mixtures are exactly the same, and the identity and toxicological properties of hazardous compounds in the mixtures remain largely unknown. Finally, there remains a paucity of data regarding possible interactions between known mixture components. Each of these issues hampers reliable hazard/risk assessment.

To address these challenges, governmental organizations such as the US Environmental Protection Agency¹¹ and Health Canada¹² have published guidance documents for conducting human health risk assessments of chemical mixtures. Although

examination of the actual mixture in question is ideal and preferred, it is unrealistic to toxicologically characterize all environmental mixtures. Consequently, a dose-addition model is recommended, which calculates the total hazard or risk of a mixture as the incremental sum of the contributions from a small number of targeted components.

The dose-addition model for PAHs employs several empirical assumptions. First, it is assumed that the genotoxic mixture components, when combined, act in a strictly additive manner. Unfortunately, there is a paucity of data to support the validity of this statement. Second, the model implies that the total hazard/risk posed by the mixture is effectively represented by the contributions from a handful of targeted chemicals that are included on governmental lists of prioritised pollutants, in this case the 7-8 PAHs that are probable, possible, or known human carcinogens^{1, 13, 14}. To critically evaluate these assumptions for genotoxic PAHs, several studies have scrutinized the genotoxicity of complex PAH mixtures and simplified synthetic PAH mixtures. Unfortunately, the vast majority of these studies were conducted *in vitro*, and only examined highly simplified (e.g., binary) mixtures. A more thorough overview of PAH mixtures research is presented in our earlier works¹⁵⁻¹⁷.

To estimate the incremental lifetime cancer risk posed by a PAH mixture, the dose-addition method assesses the concentrations of each targeted PAH, and then, using the appropriate compound-specific potency equivalency factors (PEFs), converts the concentration of each targeted PAH into equivalents of BaP, the reference PAH. The total BaP-equivalent concentration in the mixture is then calculated as the sum of the incremental contributions from each carcinogenic PAH. This PEF-driven assessment method (PEF-M) is appealing and convenient since it evaluates a small number of

targeted PAHs, and does not require toxicological data for the specific mixture. However, regulatory agencies have recognized and highlighted limitations of human health risk assessment approaches based on a few PAHs that employ the assumption that doses scaled to BaP are additive^{11, 18, 19}. A more thorough overview of PAH mixtures risk assessment approaches is presented in our earlier works¹⁵⁻¹⁷.

The current study aims to critically evaluate the assumption of dose additivity, as well as to determine whether contributions from other mixture components affect the observed mixture effects. Rodent cancer bioassays are prohibitively costly and, for a single chemical, take over two years to complete; therefore, since PAHs are carcinogens with a mutagenic mode-of-action¹, we chose to employ *in vivo* mutagenicity as a surrogate endpoint for cancer. Previously, we published two studies that examined the mutagenic potency (MP) (i.e., the linear slope of the dose-response function) of 8 targeted PAHs deemed carcinogenic by the Canadian Council of Ministers of the Environment (i.e., CCME carcinogenic PAHs)²⁰, PAH-contaminated soil fractions, and matching synthetic mixtures that only contain the CCME carcinogenic PAHs in proportions identical to their presence in the soil fractions. The first study employed a bacterial gene mutation assay¹⁵ and the later work used an *in vitro* mammalian cell gene mutation assay based on an immortalised cell line derived from the transgenic MutaTMMouse^{16, 17}. In the bacterial study, we found less-than-additive mixture results¹⁵, and in the *in vitro* study, we found both less-than-additive and more-than-additive mixture results, all of which were within 2-fold of the additive prediction with a single exception¹⁶. In the current study, our aim was to expand on our earlier works by examining PAH mixture additivity across several tissues using an *in vivo* model.

Recently, we determined the *in vivo* MP of the eight CCME carcinogenic PAHs²⁰, as well as the MP of a purified CT-based driveway sealcoat (CT-seal)²¹ following sub-chronic MutaTMMouse oral exposures. The data presented in these studies, plus new *in vivo* assessments of two CT preparations (i.e., CT-1 and CT-2), form the basis of the current follow-up study that employs *in vivo* genetic toxicity data to complete our evaluations of the dose-additivity assumption for PAHs. More specifically, we critically evaluate the additivity paradigm by comparing observed MP values across five tissues to those predicted using the incremental contributions of each PAH²⁰. In order to evaluate possible contributions of other compounds present in the complex mixtures studied, we compared the complex mixture results to results obtained with synthetic mixtures only containing the eight CCME carcinogenic PAHs.

Additionally, in order to examine the PEF-M for PAH mixtures, we previously developed a bioassay-derived method (BDM) that assesses the actual mutagenic activity of the complex mixture, and expresses that activity relative to the activity of BaP^{15, 17}. This method calculates BaP-equivalent concentrations without employing a chemical-specific assumption of additivity, and can be used as a tool to compare with the BaP-equivalent concentrations determined using the PEF-method. The use of the term “bioassay-derived method” refers to the fact that the BDM uses bioassay data for the actual mixture being evaluated. In contrast, the PEF-M does not. We employed this approach herein to scrutinise the assumption of dose-additivity underlying the human health risk assessment of PAH-containing mixtures.

4.4 Materials & Methods

4.4.1 Coal tar extraction

CT-1, provided by the Electric Power Research Institute, is a dense non-aqueous phase liquid from a former manufactured gas plant (MGP) in the United States. CT-2, provided by the Canadian National Research Council, is from a Canadian coke production facility. Each CT sample was thoroughly mixed until homogenous, and an aliquot was removed and extracted in an identical manner as the CT-seal²¹, according to a method adapted from Wise *et al.*²². An aliquot of the extract was set aside for chemical analyses, and the remainder was used to prepare dosing solutions by first sonicating the eluate, and then diluting it in highly-refined olive oil (Sigma-Aldrich Canada, Oakville, ON). Dosing solutions, in mg equivalent (eq) weight of crude CT per mL, were prepared weekly and stored in amber glass vials.

4.4.2 PAH content of coal tars by GC-MS

The PAH content of the CTs was determined via GC-MS according to USEPA Method 8270D. Analyses were conducted by a commercial laboratory as described previously²¹. The concentrations of a standard panel of 19 PAHs (including the 8 CCME carcinogenic PAHs¹⁸) were expressed as mg PAH/kg CT.

4.4.3 Synthetic mixture preparation

Using the results of the chemical analyses, synthetic mixtures were prepared to match the composition of CCME carcinogenic PAHs in both CT-extracts, as well as the CT-seal extract. Synthetic mixture-1 (Syn-1) was prepared to match CT-1, Syn-2 was prepared to match CT-2, and Syn-3 was prepared to match CT-seal. Three concentrations

of each mixture were prepared weekly in amber glass vials and dissolved in an appropriate volume of highly refined olive oil.

4.4.4 Animal treatment

Adult male MutaTMMouse mice were used for all *in vivo* exposures. As dietary intake provides the main source of human exposure to PAHs (i.e., for non-smokers who are not occupationally exposed)^{1,23}, an oral route of exposure was selected for this study. The dosing solutions were administered by oral gavage daily for 28 days, with a 3-day post-exposure sampling time as per OECD test guideline #488²⁴. The administered doses of the complex mixtures were: 250, 500, and 1000 mg CT-eq/kg body weight (BW)/day of CT-1, 700, 1400, and 2100 mg CT-eq/kg BW/day of CT-2, 1974, 3949, and 7897 mg CT-seal-eq/kg BW/day of the CT-seal. The doses of the synthetic mixtures were: 1092, 2184, 4368 mg CT-eq/kg BW/day for Syn-1; 700, 1400, and 2100 mg CT-eq/kg BW/day for Syn-2, and 1974, 3949, and 7897 CT-seal-eq/kg BW/day for Syn-3. Mice were euthanized and necropsied as previously described²⁰, and glandular stomach (GS), small intestine (SI), liver, (Lv), bone marrow (BM), and lung (Lg) were immediately harvested and stored at -80°C^{20,21}. GS was used instead of forestomach as we were unable to obtain sufficient high-quality DNA from the latter. Mice were bred, maintained, and treated according to the Canadian Council for Animal Care Guidelines, and the Health Canada Animal Care Committee approved the protocols.

4.4.5 Genomic DNA isolation

Tissue samples were digested as described previously²⁰. Genomic DNA was isolated from lysed tissues using a phenol/chloroform extraction procedure described

previously^{25, 26}. Isolated DNA was dissolved in TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) and stored at 4°C.

4.4.6 Mutant frequency analysis

The PGal (phenyl-β-D-galactoside) positive selection assay was used to determine the *lacZ* mutant frequency (ratio of mutant plaque forming units (pfu) to total pfu) in DNA samples from GS, SI, Lv, BM, and Lg as previous described²⁶⁻²⁸.

4.4.7 Statistical analysis

The *lacZ* dose-response data were analysed in SAS v.9.1 (SAS Institute, Cary, NC) by conducting a Type 3 chi-squared analysis, and employing Poisson regression to examine the significance of the overall response, with post-hoc custom contrasts as described previously²⁰. Should visual examination of the dose-response suggest the need to truncate the data series (i.e., remove the top dose), the restricted data were reanalysed. If reanalysis resulted in an improved chi-squared value, the truncated results were retained and used for MP determination, as described below.

4.4.8 Experimental and additive mutagenic potency

MPs for the *lacZ* endpoint, which are defined as the slope of the linear portion of the dose-response functions, were determined using ordinary least-squares linear regression for the complex and synthetic mixtures in all tissues (expressed as mutant frequency x 10⁻⁵/mg CT (or CT-seal)-eq/kg BW/day). The observed MP values for individual PAHs in each tissue, expressed as mutant frequency x 10⁻⁵/mg PAH/kg BW/day, were recently published by our group²⁰ (**Supp. Table 4-I**). These were employed in our additivity calculation along with the measured PAH concentrations in the complex mixtures (in mg PAH/mg CT or CT-seal-eq). The additive MPs of the PAH

mixtures, expressed as mutant frequency $\times 10^{-5}$ /mg CT (or CT-seal)-eq/kg BW/day, were calculated as the sum of the contributions from each targeted PAH according to

Equation 1.

$$[1] \quad \begin{aligned} &\text{Total Additive Mutagenic Potency of PAH Mixture} = \\ &\sum_{i=1}^n \text{Experimental potency of PAH}_i \times \text{PAH}_i \text{ Concentration in Mixture} \\ &\text{for targeted PAHs 1 through } n \end{aligned}$$

As with our previous *in vitro* work, statistical comparisons of the calculated additive MPs with corresponding experimentally-observed mixture MPs were not conducted due to the large variance associated with the predicted values attributable to the variances associated with the contributions of individual PAHs to the total predicted MP²⁹. The tissue-matched MP of each synthetic PAH mixture was compared to that of the corresponding complex mixture using the two-tailed Student *t*-test ($p \leq 0.05$).

4.4.9 BaP-equivalent concentrations: Potency equivalency factor method and bioassay-derived method

Routine assessment of incremental lifetime cancer risk for oral exposure to PAH-contaminated complex matrices (e.g., soil) requires quantitative information pertaining to (1) the level of PAH contamination (i.e., the concentrations of targeted PAHs), (2) PEFs to convert PAH concentrations to a BaP-equivalent concentration, (3) ingestion rate, (4) exposure duration, (5) lifespan, (6) body weight, and (7) the BaP oral cancer slope factor. For this study, we only calculated the BaP-equivalent concentrations, as the remaining factors would be constants for a given PAH mixture. The PEF-M (**Equation 2**), which assumes dose-additivity, calculates total BaP-equivalents for each of the examined

mixtures as the sum of the products of PAH concentration and their compound-specific cancer PEFs. The maximum possible PEF ranges were calculated using the highest and lowest published PEF values (derived from *in vivo* data only) for each PAH, obtained from 13 sources^{14, 30-41} cited in¹⁸, and two additional sources^{42, 43} (**Supp. Table 4-II**).

PEF-method:

$$[2] \quad \text{BaP-equivalent concentration} = \sum_{i=1}^n C_i \times \text{PEF}_i$$

for targeted PAHs 1 through n

In contrast, the BDM approach (equation 3 below), which provides tissue-specific BaP-equivalents for each of the examined mixtures, calculates total BaP-equivalents (in mg BaP/mg CT or (CT-seal)-eq) as the ratio of the experimental MutaTMMouse MP of a mixture (as mutant frequency $\times 10^{-5}$ /mg CT (or CT-seal)-eq/kg BW/day) to the MutaTMMouse MP of BaP (as mutant frequency $\times 10^{-5}$ /mg BaP/kg BW/day) in the same tissue (**Equation 3**). By definition, this approach does not assume additivity of prioritised mixture components.

Bioassay-derived-method:

$$[3] \quad \text{BaP-equivalent concentration} = \frac{\text{Mutagenic Potency}_{\text{mixture}}}{\text{Mutagenic Potency}_{\text{BaP}}}$$

The BDM was employed to calculate BaP-equivalents for the 3 complex and 3 synthetic mixtures, which were then compared with those calculated using the traditional PEF-M.

4.5 Results

4.5.1 PAH content of the coal tar and coal tar-based sealcoat

PAH concentrations in the CTs and CT-seal are summarised in **Supp. Table 4-III**. The results of the chemical analysis of CT-1 revealed that it is composed of 28% analysed PAHs by weight, including 6.0% CCME carcinogenic PAHs, and nearly 1% BaP. Analysed PAHs made up 19% of CT-2, with 1.8% of the material being CCME carcinogenic PAHs, and 0.30% BaP. Analysed PAHs made up 4.4% of CT-seal, with 0.96% being CCME carcinogenic PAHs, and 0.19% BaP.

4.5.2 The *lacZ* mutagenic potency of the complex and synthetic PAH mixtures

All six mixtures induced a significant increase in *lacZ* mutant frequency in all tissues examined (**Supp. Figure 4.1, Supp. Table 4-IV**). Across all mixtures, the most potent response was consistently observed in SI. The second most potent response was observed in Lv for all complex mixtures, and in GS for all synthetic mixtures. The least potent responses were observed in Lg for CT-2, CT-seal, Syn-1, and Syn-2, and in BM for CT-1. Lg and Lv displayed the least potent responses for Syn-3.

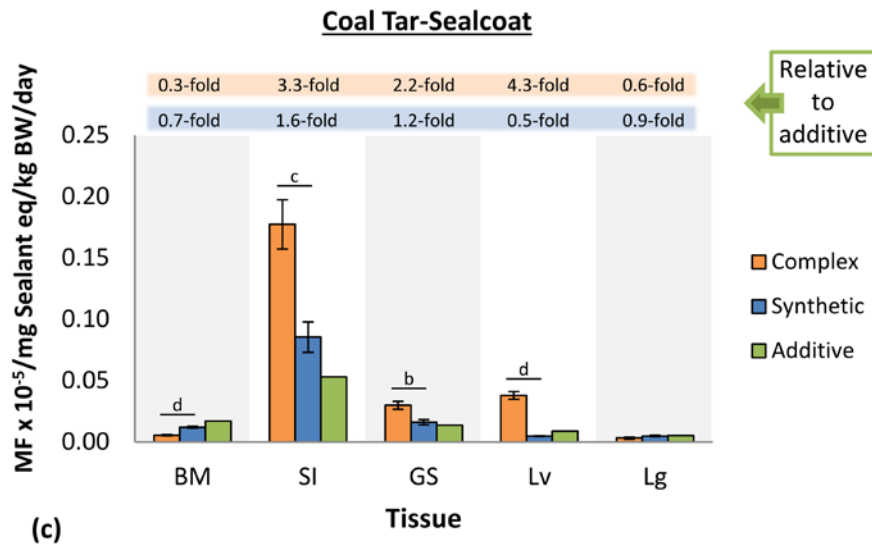
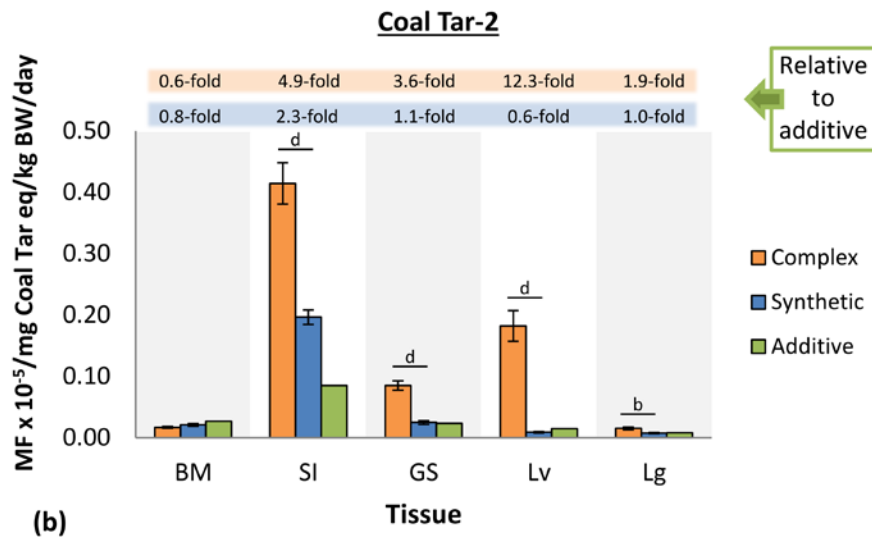
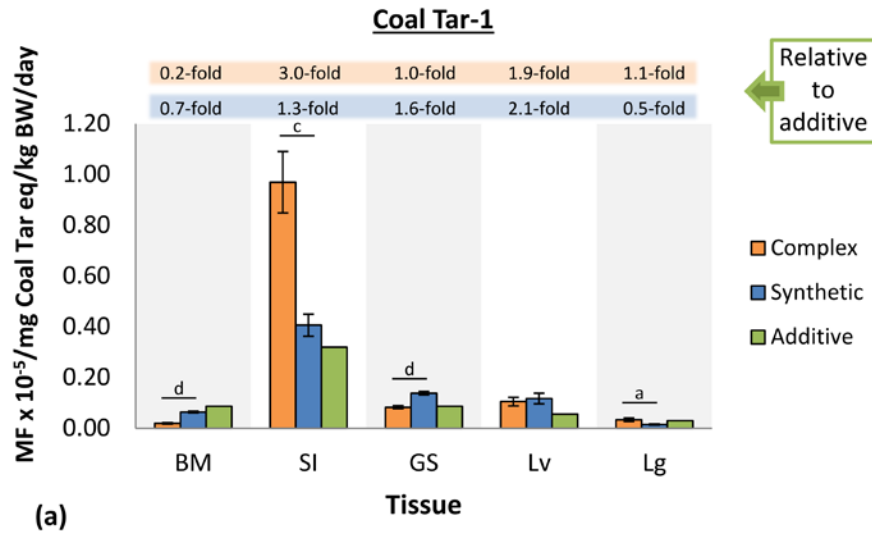
Among the complex mixtures, the highest MP was observed for CT-1 in SI (i.e., 2-fold and 5-fold higher than CT-2 and CT-seal, respectively). However, this was not the case for each tissue (e.g., CT-2 MP in Lv was 1.8-fold higher than CT-1 Lv). CT-seal consistently yielded the least potent responses for all tissues. Among the synthetic mixtures, Syn-1 exhibited the highest potency in each tissue, in comparison with the other two synthetic mixtures. The lowest potencies across all tissues were consistently observed for Syn-3.

4.5.3 Experimental versus additive mutagenic potency

Under an assumption of additivity, the predicted MPs of each PAH mixture in each tissue were calculated as the sum of the contributions from each targeted PAH (i.e.,

Equation 1). These calculated values were compared with the experimental MPs for the complex and synthetic PAH mixtures, across all tissues (**Figure 4.1**). Comparisons of the complex and synthetic mixture response with the additive (i.e., calculated) response can be used to assess the likelihood that the mixture components interact and/or the mixture contains additional unidentified mutagens. Results for SI indicate that the potencies of the mixtures are more-than-additive in all cases, with the MP of the complex mixture yielding responses 3.0-fold to 4.9-fold more-than-additive, and the synthetic mixtures yielding responses 1.3-fold to 2.3-fold more-than-additive. In all cases the discrepancies from additivity is SI are less than 5-fold. Conversely, for BM, the mixture results are less-than-additive, with the potency of the complex mixture yielding responses that were 0.2-fold to 0.6-fold less-than-additive, and the synthetic mixtures yielding responses that were 0.7-fold to 0.8-fold less-than-additive. Lg results yielded experimental MP values that were the closest to the additive prediction, ranging from 0.6-fold to 1.9 fold for the complex mixtures, and 0.5-fold to 1.0-fold for the synthetic mixtures. Some responses in GS results were also close to the additive prediction (1.0-fold for CT-1, 1.1-fold for Syn-2, 1.2-fold for Syn-3) with other responses exceeding the additive predictions by up to 3.6-fold (i.e., CT-2). In Lv, the complex mixtures also consistently exceeded the additive prediction; CT-1 was 1.9-fold more-than-additive, CT-3 was 4.5-fold-more-than-additive, and CT-2 had the greatest overall deviation from additivity, at 12.3-fold. Interestingly, the matched synthetic mixture for CT-2 (i.e., Syn-2) elicited a response that is less than the additive prediction (0.6-fold), as did Syn-3 (0.5-fold); whereas Syn-1 elicited a response that was 2.1-fold more-than-additive.

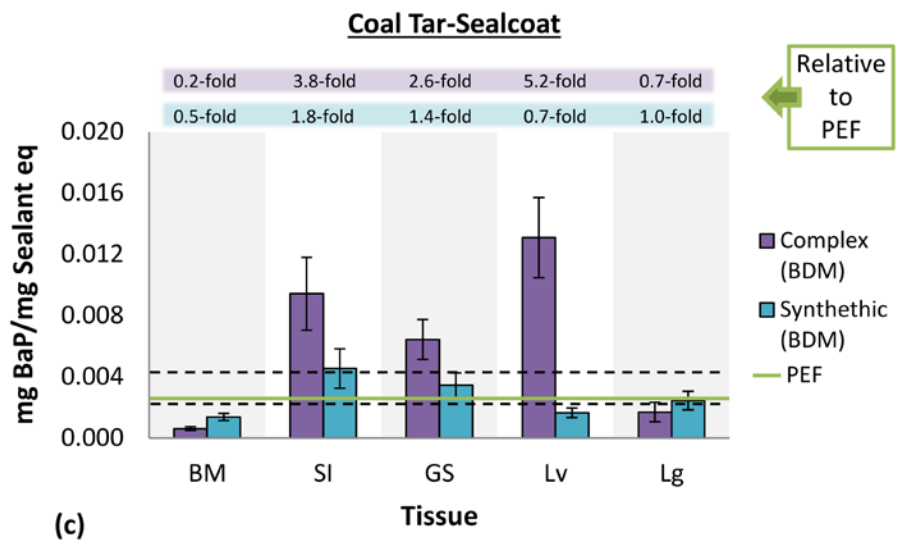
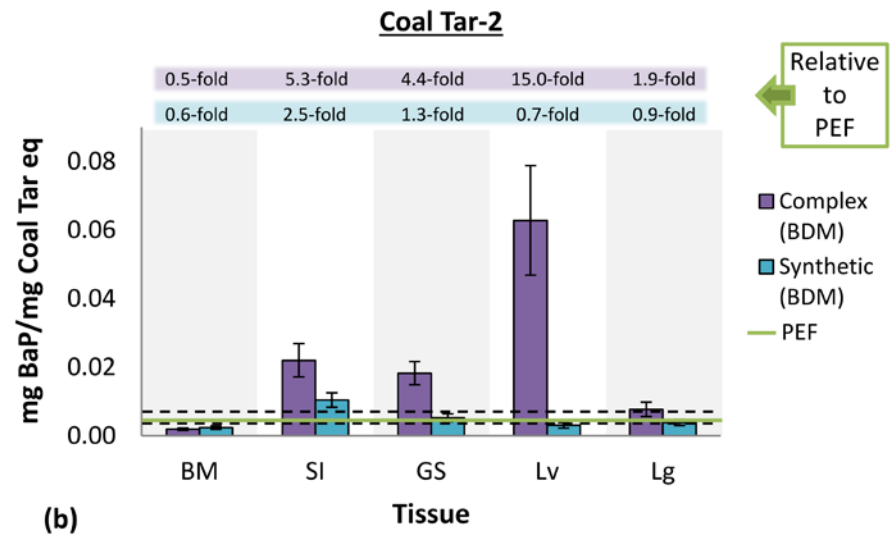
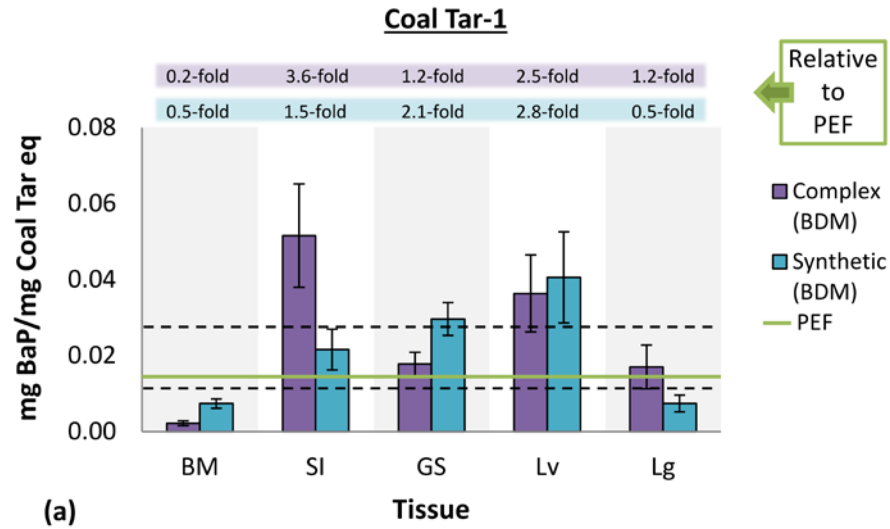
Figure 4.1. Experimental and additive *lacZ* mutagenic potency values for bone marrow (BM), small intestine (SI), glandular stomach (GS), liver (Lv), and lung (Lg) elicited by oral exposures to **(a)** coal tar-1 (CT-1) and synthetic PAH mixture-1 (Syn-1), **(b)** coal tar-2 (CT-2) and synthetic PAH mixture-2 (Syn-2), and **(c)** coal tar-based driveway sealcoat (CT-seal) and synthetic PAH mixture-3 (Syn-3). Experimentally observed potencies for complex (orange bars) and synthetic mixtures (blue bars) are compared with those calculated under the assumption of additivity according to Equation 1 (green bars) (i.e., using the concentrations of the 8 CCME carcinogenic PAHs in the mixtures, and each PAH's experimentally determined tissue-specific mutagenic potency). Fold-changes compare mutagenic potency of complex (in orange) and synthetic (in blue) mixtures to the additive prediction. Statistically significant differences between complex and synthetic mixtures are indicated above the bars (a: $p < 0.05$, b: $p < 0.01$, c: $p < 0.001$, d: $p < 0.0001$). MF: mutant frequency.



As the synthetic mixtures only contain the 8 CCME carcinogenic PAHs, comparisons of the experimental responses for the complex mixtures with the responses for the matched synthetic mixture in the same tissue can be used to investigate whether there are contributions from additional unidentified mutagens present in the complex mixtures. Comparing the complex mixture MPs to the MPs of the matching synthetic mixtures revealed the following: for CT-1, statistically significant differences for all tissue-sample combinations except liver, for CT-2, statistically significant differences for all tissues except BM, and for CT-Seal, statistically significant differences for all tissues except Lg. Where significant differences existed, the complex mixture response deviated more from additivity than the synthetic mixture in 10 out of 12 observations. For example, in SI, both complex and synthetic mixtures were more-than-additive, with the complex mixture MPs consistently exceeded the values for the matched synthetic mixtures; whereas in BM, both complex and synthetic mixtures were less-than-additive, with complex mixtures MPs consistently below the matched synthetic mixtures. In all cases, the MPs for the matched complex and synthetic mixtures deviated from additivity in the same direction, except for Lv CT-2 and CT-seal, and Lg CT-1.

Overall, experimental MPs were within one order of magnitude (i.e., 5-fold in either direction) of the additive predictions, with the exception of CT-2 in Lv, where the observed MP was 12.3-fold greater than the additive prediction. Additionally, although both less-than-additive and greater-than-additive responses were observed, the latter were more common, especially in site-of-contact tissues such as SI and GS.

Figure 4.2. BaP-equivalent concentrations for **(a)** coal tar-1 (CT-1) and synthetic PAH mixture-1 (Syn-1), **(b)** coal tar-2 (CT-2) and synthetic PAH mixture-2 (Syn-2), and **(c)** coal tar-based driveway sealcoat (CT-seal) and synthetic PAH mixture-3 (Syn-3). BaP-equivalent concentrations were determined using the bioassay-derived method (i.e., BDM) for both complex (purple bars) and synthetic mixtures (turquoise bars) using *lacZ* mutagenicity data for bone marrow (BM), small intestine (SI), glandular stomach (GS), liver (Lv), and lung (Lg), and the values are compared with the additive potency equivalency factor method (i.e., PEF-M)-derived value (green line). Dotted lines represent the maximum and minimum PEF-M values, which were calculated using the highest and lowest PEFs for each PAH obtained from the literature (**Supp. Table 4-II**). Fold-changes compare the BaP-equivalent concentrations, calculated using the BDM for the complex (in purple) and synthetic (in turquoise) mixtures, to the BaP-equivalent concentration calculated using the PEF-M.



4.5.4 Determination of BaP-equivalent concentrations

BaP-equivalents for each mixture were calculated using two methods: the traditional (additive) PEF-M, which calculates one BaP-equivalent concentration per mixture, and the experimentally-based BDM, which permits calculation of BaP-equivalent values for each tissue (**Figure 4.2**). For each mixture investigated, the BDM-derived, tissue-specific BaP-equivalents were compared to the corresponding PEF-derived values, the ranges in the latter being determined using maximum and minimum PEF values collected from the literature. This exercise was carried out in order to scrutinise the assumption of dose-additivity underlying the PEF-M.

Since the PEF-M is based on the assumption of additivity, the results of this analysis are quite similar to those presented in **Figure 4.1**. The discrepancy between the BDM and the PEF-M values was generally greater for the complex mixtures than for the synthetic mixtures (i.e., 12/16 cases). For all three complex mixtures, the BDM yielded BaP-equivalents that were consistently greater than the PEF-M-derived values for both SI (3.6- to 5.3-fold greater than PEF-M) and Lv (2.5- to 15.0-fold compared with PEF-M). For all three of the synthetic mixtures, the BDM also yielded values that were greater than the corresponding PEF-M BaP-equivalents for SI (1.5- to 2.5-fold); however, only for Syn-2 is the lower standard error (SE) range of the BDM BaP-equivalent higher than the upper limit of the PEF-M value range (**Figure 4.2b**). Conversely, for BM the BDM consistently yielded values that were lower than the PEF-M BaP-equivalents for both the complex and synthetic mixtures (0.2- to 0.6-fold). For GS, the BDM yielded BaP-equivalents that were greater than the PEF-M values for all 6 mixtures; however, the

lower SE of the BDM value only exceeded the upper limit of the PEF-M range for CT-2 and CT-seal (4.4- and 2.6-fold). The smallest deviations from the BDM were observed for Lg, ranging from 0.5- to 1.9-fold of the PEF-M values.

Although the BDM BaP-equivalent concentrations were both greater-than and less-than the corresponding PEF-M BaP-equivalents depending on the tissues examined, they were more frequently higher relative to the traditional additive PEF-M values, especially for SI and GS. Nevertheless, all values were approximately within one order of magnitude (i.e., 5-fold in either direction) with the notable exception of CT-2 in Lv (15-fold).

4.6 Discussion

Previous studies have examined the carcinogenicity of CT, and we previously reported the *in vivo* genotoxicity of the CT-seal. However, this is the first report regarding the use of a TGR mutagenicity assay to investigate multi-organ, genotoxic effects induced by oral exposure to CT. We used this newly generated data, along with the published CT-seal results and MPs for the individual PAHs, to examine the assumption of dose-additivity that is commonly employed for human health risk assessment of complex PAH mixtures. It is important to note that cancer is a complex disease that develops over time, involving a number of different processes and phenomena. These include chemically induced proliferation⁴⁴, tumour promotion resulting from arylhydrocarbon-receptor (AhR)-agonism⁴⁵, as well as genetic damage. Nevertheless, since PAHs are recognized to be carcinogens with a mutagenic mode-of-action¹, and due to the fact that it would be prohibitively costly and time consuming to

use cancer bioassays to test the underlying theory of additivity, our goal was to determine whether the mutagenic activity of PAHs in mixtures is in fact additive.

CT-1 had only 1.5-fold higher levels of analysed PAHs than CT-2, although the levels of CCME carcinogenic PAHs and BaP were almost 4-fold greater in CT-1, emphasizing the heterogeneity of PAH composition of CTs. The CTs examined here, which showed a range of PAH concentrations and proportions, are fairly comparable to the four CTs examined by Weyand *et al.*,⁴⁶ and the two CT-composite mixtures examined by Culp *et al.*^{44, 47-49}. The Weyand study examined CTs that contained 4.3 – 28% PAHs by weight, with CCME carcinogenic PAHs at 0.89 – 2.5%, and BaP at 0.17 – 0.64%. The Culp studies examined two CTs that were a composite of either seven or three CTs from MGP sites, both of which had lower total PAH levels than CT-1 and CT-2 (8.4% and 11%), but they were comparable to CT-2 for CCME PAHs (1.2% and 1.8%) and BaP (0.18% and 0.28%). As with our study, the relative proportions of the PAHs in the CT samples examined in the aforementioned studies varied between samples. As discussed in Long *et al.*²¹, the PAH levels in the CT-seal examined here are lower in comparison with other CT-based sealcoats reported in the literature.

The pattern of *lacZ* MPs across tissues for the two CTs and the three synthetic mixtures (e.g., highest in SI) was consistent with what was previously observed for the CT-seal, as well as for the individual PAHs^{20, 21}. The results of this study are also in line with published rodent genotoxicity results showing that oral exposures to CT induced significant elevations in DNA adducts in several tissues including Lg, forestomach (FS), SI, and Lv^{46, 47, 50-53}. CT is also a known multi-organ carcinogen (oral route of exposure), inducing tumours in FS, SI, Lv, and Lg^{44, 48, 49}.

The tissue-specific pattern of relative responses was fairly consistent across the PAH mixtures examined, with the results suggesting that the actual effect of the mixtures at site-of-contact and related tissues (SI, GS, Lv) are generally greater than predicted (i.e., more-than-additive), especially for SI. In contrast, the effect on a more distal tissue such as BM can be substantially less than that predicted using the additive paradigm. Nevertheless, it is important to note that the experimental MPs are all within an order of magnitude (and within 5-fold in either direction) of the additive prediction with the exception of Lv for CT-2. Interestingly, there is a larger divergence from additivity associated with increased mixture complexity (i.e., greater deviations from additivity were observed for complex mixtures, in comparison with the deviations from additivity observed for the matched synthetic mixtures). More specifically, when comparing the discrepancy from additivity between the matched complex and synthetic mixtures, a greater discrepancy from additivity was observed for the complex mixture for 10 of the tissue-sample 15 pairs. For example, SI mixture responses were consistently more-than-additive, while BM mixture responses were consistently less-than-additive, but in both cases the complex mixture deviated from additivity more so than the synthetic mixture. Deviations from additivity with increased mixture complexity were observed in both directions, depending on the tissue analysed, and we believe this may be related to tissue-specific differences in metabolism, as discussed below.

Elevated complex mixture responses may be due to contributions from additional mixture components not present in the defined synthetic mixtures, and, by definition, not include in the additive MP calculation. In addition to the analysed PAHs (**Supp. Table 4-III**), the PAH-containing extracts from the CTs likely contain a variety of other

mutagenic, unsubstituted, homocyclic PAHs. For example, dibenzo[*a,l*]pyrene (a.k.a. dibenzo[*d,e,f-p*]chrysene) is an extremely potent rodent carcinogen and mutagen (e.g., 9-fold more potent than BaP in the MutaTMMouse TGR assay²⁰), and was found in an extract of CT-based driveway sealcoat⁵⁴. Additionally, studies examining DNA adducts in mouse tissues following oral exposure to CT noted that the primary adduct in lung was from 7H-benzo[*c*]fluorene, a PAH not included in routine chemical analyses, nor in the synthetic mixtures examined here^{52, 55}. Analysis of an extract of a CT standard reference material prepared in the same manner as our CT extracts lists concentrations of 30 different PAHs²², and a recent study found 23 parent PAHs, 11 high molecular weight PAHs, and a number of PAH derivatives in extracts from two CT-based driveway sealcoats⁵⁴. Thus, contributions of mutagenic PAHs that have not been highlighted for concern and control can be expected. Indeed, they may play a role in elevated complex mixture responses in comparison with the matched synthetic mixture and the additive predictions.

Another likely reason for the greater-than-additive responses of the complex PAH mixtures in SI, GS, and Lv is the tissue-specific induction of cytochrome P450 (CYP) isozymes via AhR-agonism by PAHs present only in the CTs/CT-seal. PAHs primarily exert their genotoxic effect by forming bulky adducts on purines in DNA¹, but in order for PAHs to covalently bind to DNA, they must be metabolically transformed into reactive electrophiles. Following an oral exposure, PAHs such as BaP bind the AhR, and induce the transcription of metabolic genes such as *Cyp1a1/1a2/1b1* in various tissues⁵⁶. Up-regulation of CYPs by compounds present in the complex, but not the synthetic, mixtures, and that are not necessarily mutagenic themselves, could contribute to

increased oxidative metabolism and bioactivation of the mutagenic PAHs in complex mixtures, and a concomitant increase in genotoxic effect. SI is known to have high inducible levels of CYP1A1, unlike many other tissues such as BM or GS^{57, 58}. Moreover, non-mutagenic PAHs such as phenanthrene and pyrene can up-regulate metabolic enzymes, which may lead to increased bioactivation of genotoxic components in a complex mixture. Culp *et al.*⁵¹ found that BaP-DNA adduct levels in FS were 6-fold higher than expected based on the amount of BaP in the CT, suggesting that other components of the CT are enhancing the metabolic activation of BaP and increasing the rate of adduct formation. Similarly, the more-than-additive responses to synthetic mixtures in several tissues may also result from increased AhR induction by mixtures; that is the PAHs in the synthetic mixtures that are less mutagenic than BaP²⁰ may contribute to upregulation of AhR-regulated CYPs and increased bioactivation of the more mutagenic PAHs in the mixture.

BM showed a reversed trend compared with other tissues, with the complex mixture response being lower than the matched synthetic mixture for all 3 mixture pairs. Although BM expresses *Cyp1b1*, it is not a major site of PAH metabolism. Furthermore, for an oral exposure, BM is not a site-of-contact tissue; rather, it requires systemic circulation of the PAHs and/or activated metabolites. The lower responses in this tissue may be due to saturation of BM metabolic machinery by components of the complex mixtures.

As CYPs are also important for metabolic detoxification and clearance, increased metabolic capacity in site-of-contact tissues at very high doses may lead to higher levels of *in situ* damage (e.g., in SI), as well as increased detoxification (and clearance), with a

concomitant lower amount of parent compound available for systemic circulation. The pattern of tissue-specific responses is similar to what we observed in our single-PAH MutaTMMouse studies²⁰, which we interpreted in the context of the work by Nebert *et al.* that examined the consequences of oral BaP exposures in CYP knockout mice⁵⁶. They demonstrated the critical importance of intestinal *Cyp1a1* in determining the toxicological effects of oral exposure to BaP. More specifically, in wild type mice oral doses of BaP were effectively metabolized and eliminated without apparent toxicity; however, in *Cyp1a1* knock-outs high doses of BaP induced immunotoxicity and death. At lower doses, *Cyp1a1* knock-outs developed adenocarcinoma in the proximal SI⁵⁶. In our case, we believe that in comparison with single chemical exposures, oral exposure to a complex mixture of PAHs results in increased local expression of metabolic enzymes in the SI and Lv, a concomitant increase in detoxification (as well as some *in situ* genotoxicity), and an overall reduction in distal genotoxic effects due to reduced circulating parent compounds. The Nebert *et al.* results⁵⁶, and those presented herein, highlight the critical importance of tissue-specific metabolic capacity on the overall genotoxicity of PAHs and PAH mixtures.

The observed higher MPs for synthetic mixtures in BM in comparison with the matched complex mixture, is consistent with our *in vitro* results in MutaTMMouse FE1 cells¹⁶. In contrast, SI consistently displayed higher MPs for all complex mixtures relative to the matched synthetic mixture, and Lv, GS, and Lg exhibited higher MPs for complex mixtures for 2 of the 3 mixture pairs. Thus, with the exception of BM, our *in vivo* results do not correspond with *in vitro* observations, which is probably a consequence of significant differences in metabolic capacity between the *in vitro* test

system and most tissues *in vivo*. Although FE1 cells upregulate *Cyp1a1/1a2/1b1* in response to BaP exposure^{59, 60}, saturation of PAH metabolic capacity in cultured FE1 cells was suggested by the observation that more simplified synthetic mixtures (i.e., mixture of 5 carcinogenic PAHs) consistently elicited a greater response than matched synthetic mixtures containing all 16 priority PAHs¹⁶. In contrast, saturation of metabolism in tissues with high metabolic capacity is less likely.

We employed a bioassay-based approach (i.e., BDM) to determine BaP-equivalents in PAH mixtures, and compared the results with BaP-equivalents determined using the traditional PEF-M. Similar to our earlier *in vitro* work, we are not recommending the BDM as an alternative to the PEF-M, but simply as a tool to scrutinise the assumption of dose-additivity underlying the human health risk assessment of PAH-containing mixtures.

Conclusions about the significance of differences between the PEF and BDM results presented herein, and their relevance to cancer risk, are predicated on a quantitative relationship between carcinogenic potency and MP. In our previous work, we tested this assumption by examining the empirical relationship between relative *in vitro* MP (i.e., relative to BaP) in MutaTMMouse FE1 cells and relative carcinogenic potency (i.e., the PEFs). The results revealed a highly significant relationship across 5 PAHs ($r^2 = 0.98$, F ratio = 77.0, $p < 0.0004$)¹⁷. A more general analysis of the empirical relationship between *in vivo* TGR MP and tissue-matched carcinogenic potency (N=15) also noted a significant correlation ($r^2=0.59$), which was further improved ($r^2=0.79$) when micronucleus (i.e., chromosome damage) data were also included (N=18)⁶¹. Lastly, although the data across tissues and compounds are limited, additional tissue-specific

analyses of the PAH results presented herein revealed significant relationships between carcinogenic potency and TGR potency for 3 of the 5 tissues examined (i.e., SI, Lg, BM).

Like the results of our experimental versus additive MP analysis, the differences between the PEF-M-derived BaP-equivalents and the BDM-derived BaP-equivalents are, with one exception (i.e., CT-2 Lv), less than one order of magnitude; and moreover, for all remaining instances except CT-2 SI and CT-seal Lv, less than 5-fold. The pattern of deviations between BaP-equivalents determined using the BDM as compared to the PEF-M follows that observed for the comparison of additive MP versus experimentally determined MP. These similarities are not unexpected since the PEF-M approach assumes additivity, and the BDM approach is based on experimental mixture responses. It is important to note that the additive PEF-M, which is traditionally used for cancer risk assessment, does not allow for variations across tissues since each PAH has a single PEF value. Thus, the PEF-M approach will yield risk values that, according to our BDM result, underestimate the risk posed to certain tissues. Currently there are no organ-specific PEFs for PAHs, however should they become available in the future it may be useful to re-examine the data presented here.

Each jurisdiction employs a different set of “targeted” PAHs and PEFs, and this will impact the result of the additive determination of BaP-equivalents. Consequently, we conducted a literature review to identify maximum and minimum PEFs for our selected PAHs in order to calculate the possible PEF range for the determination of BaP-equivalents. When examining the BDM BaP-equivalents, 7 of the 15 synthetic mixture observation SEs and 10 of the 15 complex mixture observations SEs fall outside of the PEF range.

Evaluation of the cancer risk implications of the PEF-M-BDM comparisons requires careful considerations of cancer risk assessment methodologies, and the guidelines for cancer risk assessments of PAH mixtures. If, given the aforementioned quantitative relationships between *in vivo* PAH mutagenicity and PAH carcinogenicity, we assume that the BDM values based on the *in vivo* mutagenicity data are reflective of carcinogenicity, then from a human health risk perspective, low PEF-M BaP-equivalents values (i.e., relative to BDM values), are cause for concern. In particular, concern is warranted for SI, GS, and Lv where the discrepancies reach 5-fold and beyond. In such cases, the results suggest that risks estimated based on PEF-M-derived BaP-equivalents may be too low (i.e., not sufficiently conservative). However, the extent of the differences between the PEF-M and BDM approaches (i.e., one order of magnitude) can be viewed as small given the range of assumptions and uncertainties commonly employed for human health risk assessment of complex mixtures^{11, 14, 18}. Interestingly, regulatory authorities such as the CCME assert that since animal studies have inherent uncertainties, “a difference of less than one order of magnitude would typically be considered quite minor for the purpose of acceptability of a model”¹⁸. Furthermore, the CCME recommends inclusion of an additional safety factor when dealing with CT-contaminated matrices such as soil. More specifically, the CCME risk assessment guideline states that the BaP PEF scheme under-predicts laboratory measured potency, and inclusion of an additional 3-fold safety factor for CT- or creosote-contaminated soils is recommended¹⁸. Use of this safety factor brings the BDM and the PEF-M estimations even closer.

The ability to investigate tissue-specific responses is an important advantage of the current work, relative to our earlier *in vitro* analyses. The *in vitro* work demonstrated that the mutagenic potency of the complex, PAH-containing soil fractions were lower than the corresponding additive predictions for 6 of 10 soils, and higher than the additive prediction for 4 of 10 soils. Moreover, potency values were within 2-fold of the additive prediction with only 1 exception¹⁶. This is very much in line with our *in vivo* work, where complex PAH-containing mixtures elicited responses that were both below and above the additivity predictions, depending on the tissue; and were all within 5-fold of the additive prediction with only 1 exception. The modest differences between the PEF-M- and the BDM –derived BaP-equivalent values determined in the two studies is remarkable considering that the PEF-M-derived value is based on only 8 of the priority PAHs that were selected by the USEPA in the late 1970s⁶². Taken together, these two studies provide good evidence that assessments based on a small number of targeted, carcinogenic PAHs can provide reasonable estimates of BaP-equivalents.

In summary, our *in vivo* mutagenicity analyses of PAH mixtures revealed deviations from the underlying assumption of additivity for both complex and synthetic PAH mixtures, with deviations that are greater than additive for tissues that are proximal to the exposure route (i.e., SI, GS, Lv), and less-than-additive for tissues that are remote from the exposure route (i.e., BM). Moreover, there is generally a larger divergence from additivity with increased mixture complexity. The results suggest that mechanisms governing the magnitude of genetic damage elicited by orally-delivered PAH mixtures appears to be different from those governing the effects of individual PAHs, and these differences likely result from alterations of processes that regulate metabolism. Though

guarded, we contend that the results obtained can be extended to determinations of BaP equivalents that would be employed for human health risk assessment. Overall, this study demonstrates that additive predictions of mutagenic activity based on the concentrations and potencies of a small number of targeted PAHs results in values that are surprisingly close to those determined experimentally, providing support for the additive assumption employed in the human health risk assessment of PAH mixtures.

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CHAPTER FIVE

5 Chapter: Empirical Analysis of BMD Metrics in Genetic Toxicology

Part II: *In Vivo* Potency Comparisons to Promote Reductions in the Use of Experimental Animals for Genetic Toxicity Assessment

5.1 Preamble: Authors, Affiliations, and Style

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Style: This chapter was prepared according to the style guide for *Mutagenesis*.

5.2 Abstract

Genotoxicity tests have traditionally been used only for hazard identification, with qualitative dichotomous groupings being used to identify compounds that have the capacity to induce mutations and/or cytogenetic alterations. However, there is increasing interest in employing quantitative analysis of *in vivo* dose-response data to derive point-of-departure (PoD) metrics that can be used to establish human exposure limits or MOEs (Margins of Exposure), thereby supporting human health risk assessments and regulatory

decisions. This work is an extension of our companion paper on *in vitro* dose-response analyses and outlines how the combined benchmark dose (BMD) approach across included covariates can be used to improve the analyses and interpretation of *in vivo* genetic toxicity dose-response data. Using the BMD-covariate approach we show that empirical comparisons of micronucleus frequency dose-response data across multiple studies justifies dataset merging, with subsequent analyses improving the precision of BMD estimates and permitting attendant potency ranking of seven clastogens. Similarly, empirical comparisons of *Pig-a* mutant phenotype frequency data collected in males and females justified dataset merging across sex. This permitted more effective scrutiny regarding the effect of post-exposure sampling time on the mutagenicity of *N*-ethyl-*N*-nitrosourea observed in reticulocytes and erythrocytes in the *Pig-a* assay. The BMD-covariate approach revealed tissue-specific differences in the induction of *lacZ* transgene mutations in MutaTMMouse specimens exposed to benzo[*a*]pyrene (BaP), with the results permitting the formulation of mechanistic hypotheses regarding the observed potency ranking. Lastly, we illustrate how historical dose-response data for assessments that examined numerous doses (i.e., induced *lacZ* mutant frequency across ten doses of BaP) can be used to improve the precision of BMDs derived from datasets with far fewer doses (i.e., *lacZ* mutant frequency for three doses of dibenz[*a,h*]anthracene). Collectively, the presented examples illustrate how innovative use of the BMD approach can permit refinement of the use of *in vivo* data; improving the efficacy of experimental animal use in genetic toxicology without sacrificing PoD precision.

5.3 Introduction

Genetic toxicity testing is an integral part of safety assessments for new and existing substances (e.g., industrial chemicals, therapeutic products, food additives, etc.), with the aim to minimise the risk of adverse human health effects mediated by genetic damage. Genotoxicity tests have traditionally been used for hazard identification only, with qualitative dichotomous groupings being applied to identify compounds that have the capacity to induce effects such as mutation and alterations in chromosome structure or number. However, there is increasing interest in employing quantitative analysis of *in vivo* dose-response data to derive metrics that can be used to establish human exposure limits or MOEs (Margins of Exposure), thereby supporting human health risk assessments and regulatory decisions.

The first rigorous use of quantitative analysis of genetic toxicity test results for human health risk assessment relates to the accidental contamination of a batch of nelfinavir mesylate tablets (i.e., Viracept) with the known mutagen ethyl methanesulphonate (EMS). Here, the results of *in vivo* mutagenicity assessment in the transgenic rodent MutaTMMouse were used to establish a regulatory limit (i.e., PDE or Permitted Daily Exposure), and this value was used to determine that the likelihood of adverse genetic effects in patients receiving the medication was negligible (1,2). Subsequently, several research and international working groups have evaluated a variety of statistical approaches for quantitative analyses of *in vivo* genetic toxicity dose-response data and determination of PoD (point of departure) metrics, such as the benchmark dose (BMD), to define exposure limits (e.g., tolerable daily intake (TDI) or PDE, etc.) or MOEs for regulatory decision-making (3-8).

The works of MacGregor et al., Gollapudi et al. and Johnson et al. noted that the BMD approach, which has been extensively used for other toxicological endpoints, is the most suitable PoD metric for defining human exposure limits and/or MOE values; and methods for routine determination and application of BMD values are already available for application in genetic toxicology (3,4,6,8,9). As noted in Part I (8), our companion paper in this issue, BMD analyses of *in vitro* data can be used for potency ranking and to support mode of action (MOA) evaluation. However, although quantitative approaches to permit the use of *in vitro* results for human health risk assessment are under development, they are not sufficiently established to permit routine use in a regulatory context (10,11). In contrast, when combined with reliable exposure information and/or appropriate adjustments for cross-species extrapolation, inter-individual variability, and effect severity, *in vivo* dose-response data and attendant PoD metrics can readily be employed to define exposure limits (e.g., PDE, TDI, etc.) and/or MOEs for quantitative risk evaluation and regulatory decision-making (4,7,8).

Although *in vitro* assays are critical for most regulatory programs used by OECD (Organization of Economic Co-operation and Development) member countries to evaluate the safety of various consumer products and pesticides, *in vivo* assays are also regularly employed and/or required, with the requirements for *in vivo* testing varying according to the type of product, production volume and/or intended use. For example, most new pesticide submissions in Canada require *in vivo* toxicity studies (12). In addition, in accordance with international guidelines such as ICH S2(R1), evaluations of therapeutic products also routinely require *in vivo* toxicity assessments (13,14), including assessments for residual solvents and potential impurities. With respect to industrial

chemicals, *in vivo* test systems are generally used only to evaluate substances that exceed specified production thresholds. For example, Canadian New Substances Notification Regulations for chemicals and polymers require *in vivo* genetic toxicity assessment only when production amounts exceed 10,000 kg per year (15). This is similar to the European Chemicals Agency (ECHA) approach (16,17).

Despite the aforementioned requirements for *in vivo* testing, guidelines for the evaluation of consumer products discourage unnecessary use of experimental animals; moreover, several regulatory authorities (e.g., ECHA) and industrial sectors actively embrace the 3Rs principles that promote Reduction, Refinement and Replacement of *in vivo* animal testing wherever possible (18). Recent reports by Slob outline how quantitative dose-response analysis using the BMD approach support the 3Rs principles for toxicity assessment; more specifically, emphasising that the BMD approach, which is more precise than the no observed adverse effect level (NOAEL) approach, can provide more information from the same number of experimental animals or similar information from fewer animals (19,20).

The work presented here, which is an extension of the Part I (8) companion paper on *in vitro* dose-response analyses, outlines how the combined BMD-covariate approach can be used to improve the analyses and interpretation of *in vivo* genetic toxicity dose-response data. Using the BMD-covariate approach we illustrate how empirical comparisons of dose-response data across experimental covariates such as sex, tissue or study can permit more effective use of dose-response data and improved BMD precision, permitting attendant potency ranking of test articles and/or formulation of explicit mechanistic hypotheses. In addition, we illustrate how historical dose-response data for

assessments that examined numerous doses can be used to improve the precision of BMDs derived from new datasets with far fewer doses. Collectively, we illustrate how innovative use of the BMD approach in genetic toxicology can permit reductions in the use of experimental animals, thus improving assessment efficiency without sacrificing precision. The approach outlined herein sets the stage for routine quantitative dose-response analyses of genetic toxicity endpoints for human health risk assessment (HHRA).

5.4 Materials & Methods

5.4.1 Data obtained from the published literature

The *in vivo* dose-response data utilised in **Figures 5.1, 5.2 and 5.4** (panel A only), for the micronucleus (MN), phosphatidylinositol glycan-class A (*Pig-a*) mutation, and transgenic rodent (TGR) mutation assays, were collected from the published literature. The MN data used to assess study reproducibility were collected from papers published by Litron Laboratories (Rochester, NY, USA) describing combination studies whereby *in vivo* cytogenetic damage analyses were performed in the same animals evaluated for *Pig-a* gene mutations (21-23). Similarly, the *Pig-a* mutant phenotype data used to examine sex-specific differences in responses were collected by Litron Laboratories (24). Briefly, these studies used ~7-week old male and female Sprague-Dawley rats (CrI:SD) with test articles administered for either 3- or 28-days via oral gavage at 10 mL/kg/body weight (BW)/day at 24 h intervals. Peripheral blood was collected from the tail vein with micronucleated reticulocytes (MN-RETs), *Pig-a* mutant phenotype red blood cell (RBC CD59-) and reticulocyte (RET CD59-) frequencies determined by flow cytometry

according to the *in vivo* MicroFlow[®] and MutaFlow[®] kit instructions described elsewhere (25-27). Note that whereas some studies evaluated MN-RET frequencies at two time points, the statistical analyses described herein are restricted to day 4 blood samples; that is blood collected 24 h after the final treatment in the 3-day repeat-dose regimen. The TGR MutaTMMouse *lacZ* mutation data for dibenz[*a,h*]anthracene (DBahA) was recently published by Long et al. (28). Animal exposures, DNA extractions and *lacZ* mutant frequency scoring were carried out in an identical manner to the *MegaBaP* study described below, however only three doses of DBahA (Cambridge Isotopes, Tewksbury, MA) were used at 6.25, 12.5, and 25 mg/kg/BW/day (5 animals per dose-group, 20 animals total including control).

5.4.2 MegaBaP MutaTMMouse Study

The benzo[*a*]pyrene (BaP) experiment designed to study induced transgene mutant frequency across tissues, and to increase precision in the combined DBahA/BaP analysis was conducted at Health Canada (Ottawa, ON, Canada). Adult male MutaTMMouse were individually housed in microVENT ventilated racks (Allentown Inc., Allentown, NJ) and maintained on a 12-h light, 12-h dark cycle. Animals were fed standard rodent chow (2014 Teklad Global standard rodent diet) and received water *ad libitum*.

MutaTMMouse specimens were exposed daily for 28-days via oral gavage to 10 doses of BaP (Sigma-Aldrich Canada, Oakville, ON, Canada) ranging from 0.1 – 50 mg/kg/BW/day dissolved in highly-refined olive oil (Sigma-Aldrich) and administered at 0.005 mL/g body weight. A concurrent vehicle control was also carried out. There were 7 animals per dose group, with 14 animals in the control group (i.e., 84 animals total). A 3-

day sampling period was employed following the last dose, as per OECD guideline #488 (29). Animals were anesthetized with isoflurane, and euthanized via cardiac puncture followed by opening of the chest cavity. Liver, lung, and bone marrow flushed from both femurs were flash frozen in liquid nitrogen. Glandular stomach and small intestine were rinsed with PBS and flash frozen. Tissues were stored at -80°C until use. Mice were bred, maintained, and treated in accordance with the Canadian Council for Animal Care Guidelines, and Health Canada's Animal Care Committee approved the protocol.

Bone marrow, liver, small intestine, glandular stomach, and lung were prepared for overnight lysis as described previously (28). High molecular weight DNA was purified and isolated using standard phenol-chloroform extraction and ethanol precipitation as described previously (30). Precipitated DNA was re-dissolved in 50-100 µl of TE buffer (10 mM Tris, 1 mM EDTA pH 7.6) and stored at 4°C until use. The *lacZ* mutant frequency was scored using the phenyl-β-D-galactoside (PGal) positive selection assay as previously described (31-33). Mutant frequency (MF) was calculated as the ratio of mutant plaque forming units (pfu) to total pfu.

5.4.3 Benchmark Dose Analyses

Benchmark dose (BMD) analyses were carried out using PROAST version 50.9 (<http://www.proast.nl>). Dose-response data were analysed using one (exponential) or both (exponential and the Hill) nested model families recommended by the European Food Safety Authority for the assessment of continuous data (34). Where combined analyses were used, dose-response relationships were analysed using the factor discriminating the subgroups as a covariate (e.g., compound, sex, tissue, study, etc.). PROAST uses the likelihood ratio test to arrive at the optimal model where more

complex models with additional parameters are only accepted if the difference in log-likelihood exceeded the critical value at $p < 0.05$ (35). PROAST outputs designate potency (i.e., the BMD) and its two-sided 90% confidence interval (i.e., the BMDL and BMDU) as the Critical Effect Dose (CED), CEDL and CEDU, respectively, for each level of the covariate.

Using the BMD-covariate approach, the model parameters that require estimation for each subgroup, and those which may be considered as constant across subgroups, can be established for a combined dataset. In general the combined analyses presented here assumed that the shape parameters (i.e., the maximum response or parameter c , log-steepness or parameter d) were equal for all subgroups, while the background response (parameter a), potency (parameter b) and within-group variation (var) were tested for subgroup dependence (35). Model fits for each of the subgroups in each analysis are presented in **Suppl. Figures 5.1-5.5**, and were used to visually evaluate the validity of the assumption of conserved shape. This approach was preferred to statistical testing as tests on shape parameters have been shown to be overly sensitive to non-random errors that are ubiquitous in experimental data. Importantly, even minor non-random errors in the data could lead to rejection of shape parameter constancy due to the relatively high statistical power in a combined dataset; whereas in reality, small differences among shape parameters between subgroups can only have a small impact on BMD confidence interval coverage (35).

The benchmark response (BMR) (i.e., the Critical Effect Size or CES in PROAST notation) used in the presented analyses was 100% (i.e., a doubling in response relative to control). This value of CES was chosen as it is commonly used for the assessment of

genotoxicity data, and because it tended to lie within the range of observation in the datasets considered herein. The BMDL and BMDU values represent the lower and upper bounds of the two-sided 90% confidence interval of the BMD (36), respectively, with the difference between the BMDU and the BMDL defining the width of the confidence interval, and therefore, its precision. As established in the aforementioned companion paper (8), confidence interval plots, arranged using the geometric midpoint of the BMDL-BMDU interval, were employed to visually compare potencies across levels of examined covariates while taking estimation uncertainty into account (21).

5.5 Results & Discussion

Our companion paper in this issue (8) demonstrated that comparing BMD confidence intervals (e.g., distance between the BMDL and BMDU), as opposed to just comparing BMD values, comprises a robust way to study differences in potency (see conceptual explanation of BMD, **Part I, Figure 5.1** (8)). It is essential to consider confidence intervals since they delineate the range in which we are most assured that the true BMD lies, and reflects the *uncertainty* (e.g., due to random sampling errors in the data) of the underlying dose-response relationship(s). Consequently, we can only conclude that dose-response relationships (e.g., belonging to different compounds) are less or more potent when their BMD confidence intervals do not overlap. When overlaps do occur, the underlying data simply do not provide enough information to determine how potencies differ.

5.5.1 BMD-covariate analyses to assess study reproducibility and rank compound potency

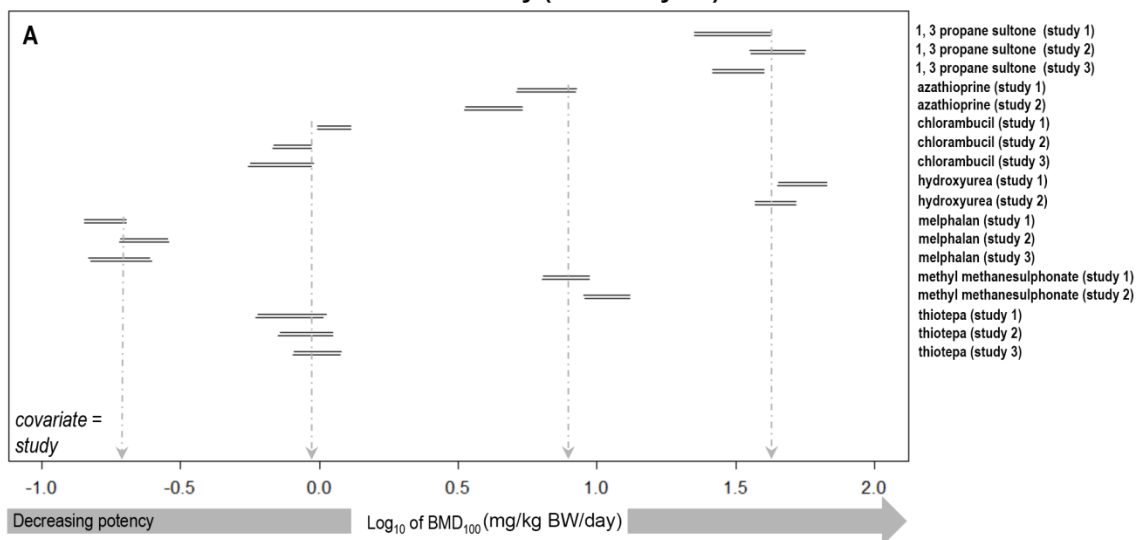
Applying these concepts, the combined BMD-covariate method using both exponential and Hill models (37) was used to analyse dose-response relationships for the *in vivo* micronucleus endpoint across seven clastogenic compounds, with multiple studies carried out for each compound (**Suppl. Figure 5.1**). Initially, the factor study was included as a covariate (i.e., 18 sub-datasets), and the results showed that despite the fact that studies were conducted across different litters and dose ranges, BMD confidence intervals clustered for each same chemical. Each cluster of study-specific BMDs (i.e., same chemical) typically spanned a factor of ~1.5, suggesting good reproducibility (**Figure 5.1A**). The confidence intervals were also sufficiently precise to delineate potency differences (indicated by arrows) and determine potency rankings (i.e., most to least potent, left-to-right side of the plot in the order melphalan > chlorambucil or thiotepa > azathioprine or methyl methanesulphonate > 1, 3-propane sultone or hydroxyurea).

Based on the finding of good reproducibility across studies, the analysis was continued using only compound as a covariate (**Suppl. Figure 5.2**). In this way, all data and different dose groups collected across the constituent studies contributed to the result for each compound. This approach improved (i.e., narrowed) each compound's BMD confidence interval (**Figure 5.1B**), thus increasing precision in the BMD estimates and permitting a more precise potency ranking (now ordered by geometric midpoint) in the order melphalan > chlorambucil or thiotepa > azathioprine > methyl methanesulphonate > 1,3-propane sultone > hydroxyurea. In this analysis, distinct (i.e., non-overlapping)

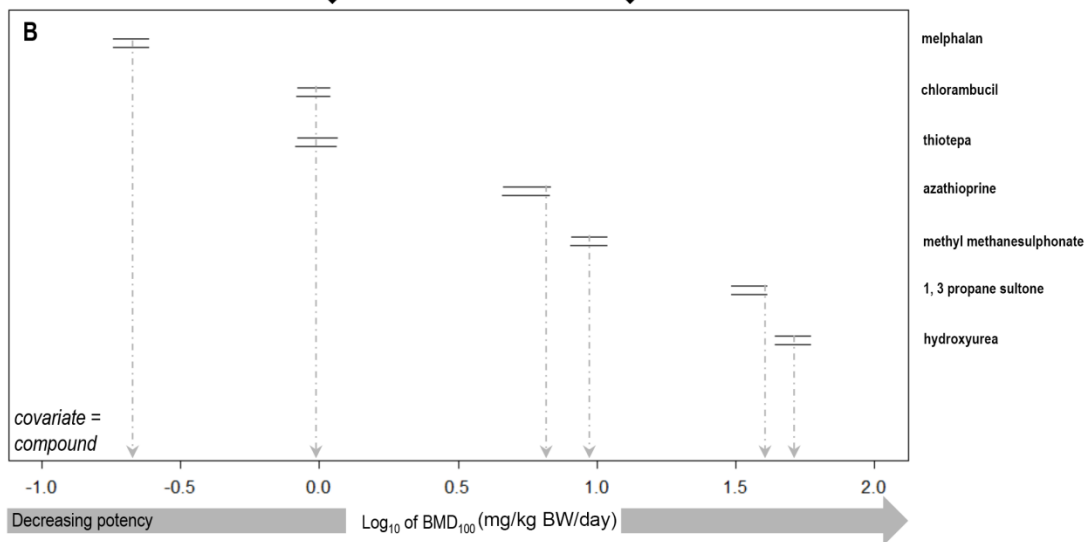
potencies were found for six compounds (indicated by arrows), with only the overlap between chlorambucil and thiotepa remaining unresolved. Accordingly, visualising confidence intervals across studies is a useful tool for interpreting reproducibility. Where reproducibility supports the merging of data from separate studies, better resolution of potency differences provides robust, comparative potency rankings across compounds (or other covariates), thus improving the utility of *in vivo* dose-response data in an HHRA context (3-7). Although substantiation by analysis of many more compounds would be needed, the BMD confidence interval-based approach to potency ranking could provide a good starting point for the establishment of ‘compound groupings’ to facilitate data gap filling via read across. For example, evidence of potency similarity (e.g., chlorambucil and thiotepa), along with supporting information on structural and chemical characteristics and/or MOAs, could form the basis for data-gap filling using read-across approaches (see more detailed discussion in Part I (8)) (38-42). Successful implementation of such initiatives would have positive repercussions with respect to cost, time and animal use for toxicological assessments in support of regulatory evaluations. Similarly, using the approach to combinatorially analyse historical data, or to combine multiple smaller datasets (e.g., with study as covariate), BMDs could be established with sufficient precision to eliminate the need for study repeats.

Figure 5.1. Combined BMD-covariate dose-response analysis for the *in vivo* micronucleus endpoint (in reticulocytes) to examine study reproducibility and to rank the potency of seven clastogenic compounds (21-23). **(A)** The upper panel shows the two-sided 90% confidence intervals for the BMD₁₀₀ values for each of the multiple studies (study 1, study 2, etc.) carried out for each compound using the exponential (top interval) and Hill (bottom interval) models, where study was included as covariate. Four compounds can be resolved from one another in terms of potency as indicated by vertical arrows (i.e., non-overlapping confidence intervals between compounds). As the analyses showed good study reproducibility (i.e., clustered confidence intervals for each compound), the analysis was repeated **(B)** in the lower panel by combining study datasets and treating compound as the sole covariate. Improved BMD precision (i.e., narrower confidence intervals) permitted six of the seven compounds to be resolved from one another and ranked in terms of potency (vertical arrows). The underlying dose-response data and fitted model curves are shown in **Supp. Figure 5.1 – 5.2**.

BMD Confidence Intervals: In Vivo Micronucleus Assay (reticulocytes)



REANALYSIS:
compound as covariate



5.5.2 Sex-specific differences in *Pig-a* assay dose-response data

The *in vivo* *Pig-a* mutation assay relies on the fact that the *Pig-a* gene is X-linked, thus there is only one copy in males and one functional copy in females (i.e., due to lyonisation). An inactivating mutation in the single *Pig-a* copy therefore results in a mutant phenotype that can readily be scored via flow cytometry (43-45). Despite the existence of X-chromosome inactivation in females, most *in vivo* *Pig-a* work has been carried out in male animals, leading to the suggestion that corroborative results in females comprises an important data-gap (9). A further important consideration in the *Pig-a* assay is the sampling time after treatment, as cell turnover rate through erythropoiesis is known to affect mutant cell frequencies (46).

Labash et al. (24) recently published results investigating sex-specific differences in the *Pig-a* mutation assay. Seven-week old male and female rats were exposed for 3 days to *N*-ethyl-*N*-nitrosourea (ENU) before sampling RBCs and RETs on days 15, 29 and 46. Below, we present a reanalysis of these data to assess the applicability of the BMD-covariate method for investigating sex- and time-dependent differences in response. The results are presented using the exponential model only, as the Hill model resulted in nearly identical BMD confidence intervals.

BMD analysis using sex as a covariate revealed that, for each sampling day, RET and RBC male and female responses could be adequately described using a single curve (**Figure 5.2A**). In each instance just one parameter for within-group variation (V), background (a), potency (b), max-response (c) and log-steepness (d) was needed to describe the response in both sexes; with the exception of day 15 RETs, where inclusion of sex-specific parameterisation of within-group variation (denoted ‘M5-V’ in the

PROAST output for model 5 fitting with covariate-dependent V) yielded a significant improvement in model fit between sexes. This indicated a statistically significant increase in within-group variation between males and females in RETs on this single sampling day only. Given the consistent absence of differences between sexes in all other cases, this pattern may be due to three data points being outliers in the controls and lowest dose (see **Figure 5.2A**).

Reanalysis of the combined data (i.e., sex ruled out as a meaningful covariate) using day as a covariate suggested that sampling time is unimportant for RETs, but covariate-dependent for RBCs (**Suppl. Figure 5.3**). Instead of presenting the confidence intervals from these combined analyses, as they may be viewed as too optimistic since the data arise from the same animals analysed across sampling days, the confidence intervals from the previous analysis (**Figure 5.2A**) (i.e., independently analysed by sampling day) are shown in **Figure 5.2B**. Concordantly, the largely overlapping confidence intervals for RETs suggest the mutant phenotype is more stably expressed over time for this cell type compared to RBCs, where distinct (i.e., non-overlapping) intervals between sampling days were established. From a biological perspective, this is not unexpected since RETs have a lifespan of only 1-2 days prior to their maturation into RBCs (47). The RET population is therefore undergoing constant turnover with persistently elevated mutant phenotype RET frequencies likely being the result of mutations in erythroid precursors or hematopoietic stem cells that have self-renewal capacities. As RETs mature into mutant phenotype RBCs, which have a lifespan of up to 46 days in mice (48), the proportion of mutant RBC increases with time. This slower turnover of RBCs relative to RETs has been previously shown to influence mutant

phenotype frequency in the *Pig-a* assay (46). Our BMD analysis supports this observation and suggests that choice of sampling day for the *Pig-a* assay is more important for RBCs than RETs.

In interpreting the *Pig-a* analyses it is nonetheless important to note that the RET confidence intervals, particularly for days 15 and 29, are quite wide relative to those obtained for RBCs (**Figure 5.2B**). Here it should be noted that the statistical assumptions of normality and variance homogeneity, which are usually applicable to the continuous *Pig-a* response metric, were violated for the RET data (i.e., log-scale variances showed mild deviations from normality, see **Suppl. Figure 5.3**). This likely relates to the very low mutant fractions measured in the control and first dose-group; and moreover, the ability to evaluate approximately 50-times more RBCs than RETs (i.e., 150 million versus 3 million, respectively), which may have affected the precision of the continuous frequency estimation. In follow-up work we will investigate the extent to which these factors could compromise the coverage of the calculated BMD confidence intervals for response metrics based on rare genetic events. If more data were available here for RETs, it is conceivable that significant differences in BMD confidence intervals across RET sampling days could be identified (i.e., similar to that noted for RBCs). At this point however, our results do not indicate that different results will be obtained when sampling RETs at any time between day 15 and 46.

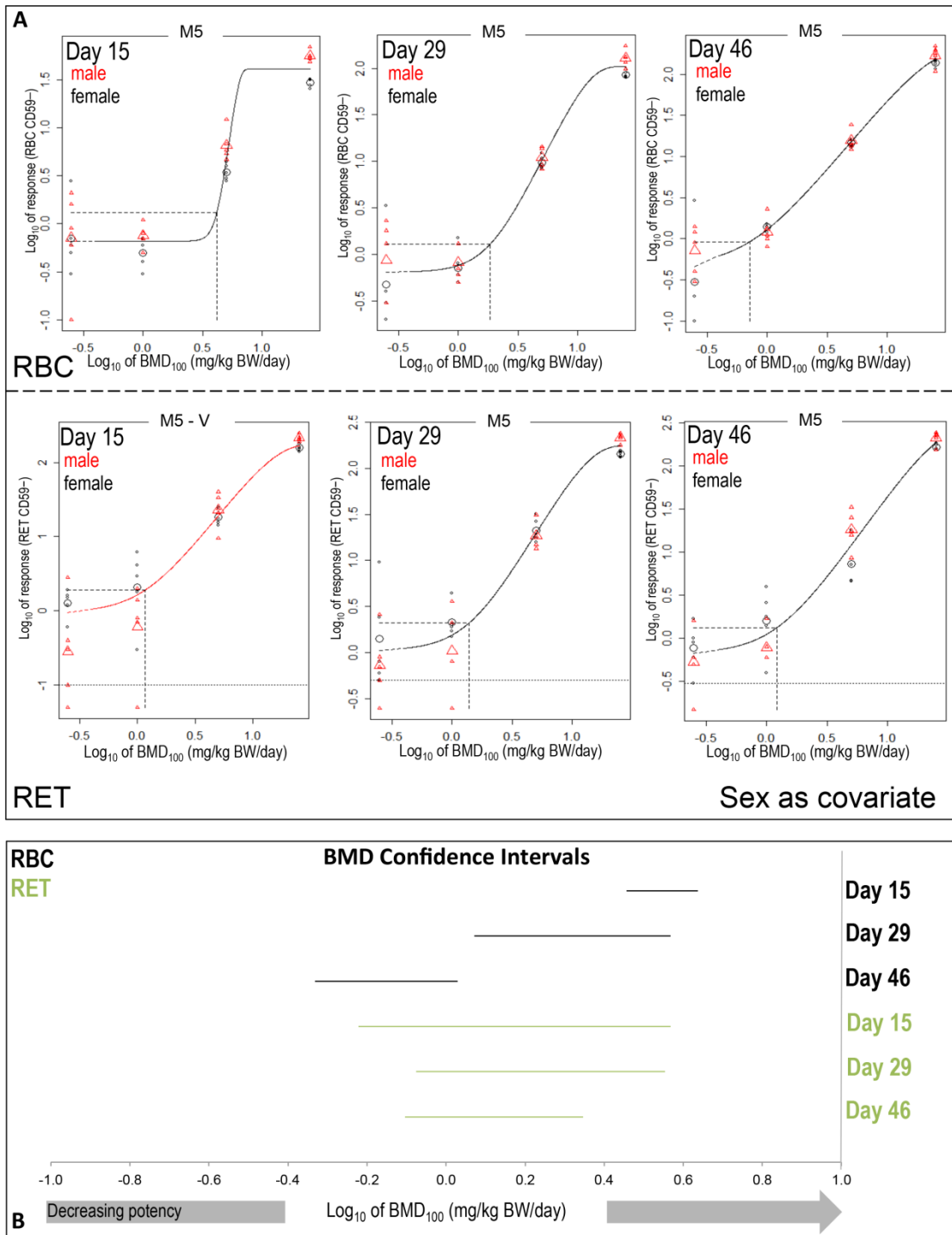
In terms of sex-specificity, the BMD analysis supports the Labash et al. (24) conclusion of no evidence for a difference between male and female responses to a prototypical mutagen (i.e., ENU). The combined BMD analysis permitted efficient examination of the data relative to the pairwise tests typically used; as the same model

was fitted to both sexes simultaneously allowing determination of which parameters were sex-dependent. This approach is advantageous since shared model parameters are estimated from data combined across both sexes, thus providing smaller confidence intervals and concomitant improvements in BMD precision (20,37). Further, all animals used in the study contribute to the statistical comparisons during dose-response modelling; whereas only information from animals in the two respective dose groups under comparison are utilised in traditional pairwise analyses (e.g., two-way analysis of variance (ANOVA) approaches) (20).

It should be noted that the aforementioned results are also consequential with respect to the historical bias in genetic toxicology whereby only males are examined when sex-specific differences in metabolism are not expected. The analytical approach employed here supports the inclusion of both males and females in the same experimental study, and when there is no support for sex as an influential covariate, data can be combined for BMD determination without loss of statistical power. In turn, when sex does have an impact on the dose-response, the loss of statistical power is limited relative to pairwise testing as the complete dose-response curve and all animals in the study contribute to BMD estimates. Importantly, with respect to cost-efficacy and 3Rs initiatives, this approach requires no/few additional animals while improving subsequent regulatory decisions since information related to unexpected sex-specific differences may still be determined (24,49).

Figure 5.2. Examining sex-specific differences in *Pig-a* mutant red blood cell (RBC CD59-) and reticulocyte (RET CD59-) frequencies using non-linear regression analysis in PROAST. Male and female rats were exposed to *N*-ethyl-*N*-nitrosourea (ENU) via oral gavage at 24-h intervals for three consecutive days before sampling on post-exposure days 15, 29 and 46 (24). **(A)** Separate BMD₁₀₀ analyses for RBCs and RETs for each sampling day using the exponential model and sex as a covariate. **(B)** BMD₁₀₀ confidence intervals from each of the analyses conducted in **(A)** showing the effect of sampling day on mutant phenotype frequency.

BMD Analyses:
***In Vivo* Pig-A Assay (red blood cells / reticulocytes)**



5.6 Tissue-specific responses to benzo[a]pyrene contribute to mode-of-action determination

Oral exposure of MutaTMMouse specimens to BaP caused significant increases in *lacZ* mutant frequencies for all five tissues examined. To better understand mutation induction across tissue, response sensitivity was considered using independent BMD analyses that treated data for each tissue individually (**Suppl. Figure 5.4**), i.e., rather than in combination using tissue as a covariate. This approach avoids the risk of compromised BMD confidence intervals due to the possible correlation of response across tissues harvested from the same animal.

Inspection of the BMD confidence intervals revealed that mutation induction is tissue specific, and ranked according to sensitivity in the order small intestine > bone marrow > glandular stomach or lung > liver (**Figure 5.3**). The exposure regime employed (i.e., oral gavage) results in the stomach and small intestine being the initial site-of-contact tissues. Although a potent response in the stomach might therefore be expected, glandular stomach sensitivity was lower than small intestine, and the more distally-exposed bone marrow. Several *CYP1* isozymes, which are known to initiate the conversion of BaP to its DNA-reactive metabolite benzo[a]pyrene-diol-epoxide, are known to be expressed in the small intestine (50-55), and the sensitivity ranking (i.e., small intestine > stomach) likely relates differences in tissue-specific generation of DNA-reactive metabolites. Moreover, BaP undergoes enterohepatic circulation (56,57), a process that permits BaP metabolites to be absorbed from the small intestine and transported to the liver, the metabolic powerhouse of the body, before secretion back into the small intestine via the bile. This process can occur multiple times, allowing further

activation of BaP and BaP metabolites, and multiple re-exposures of the small intestine resulting in bulky adduct formation and mutation induction that likely account for the relatively high sensitivity of this tissue.

In addition to tissue-specific metabolism, tissue-specific differences in DNA repair and DNA replication will also affect tissue-specific conversion of genetic damage into permanent sequence changes (i.e., mutation fixation). Although the precise role of tissue-specific damage processing in determining the level of BaP-induced mutations has yet to be delineated, it is increasingly clear that mitotic index can augment or diminish tissue-specific responses by altering the efficacy of mutation fixation. For example, the mitotic indices of intestinal epithelia (58-61) and bone marrow cells are at least an order of magnitude greater than that of cells in slower proliferating tissues such as liver and lung (unpublished data, 62). This difference would be expected to translate into more effective conversion of stable DNA adducts to mutations in intestine and bone marrow, and concomitantly lower BMDs for these tissues, as seen here (unpublished data).

The BaP results (**Figure 5.3**) show differences in tissue-specific BMD values that span an order of magnitude. This difference is large enough to influence calculated human exposure limit values (e.g., PDE, TDI, etc.) or MOE values. Although there are currently no guidelines or generally accepted methods for the use of genetic toxicity PoDs for the determination of human exposure limits, the PDE for EMS published by Muller and Gocke (2) was based on the lowest NOEL from the Gocke et al. (63) MutaTMMouse 28-day repeat dose oral exposure (i.e., GI tract and bone marrow, 25 mg/kg/BW/day) (13). Thus, there is already precedence for the use of tissue-specific responses in transgenic rodents for the determination of human exposure limits or MOEs

that can be employed by governmental evaluators, and furthermore, a similarly conservative approach for other agents might routinely employ the lowest tissue-specific BMD. However, the implications of this approach for regulatory decisions and risk management would need to be scrutinised prior to general acceptance of an approach based on lowest *in vivo* genetic toxicity BMD.

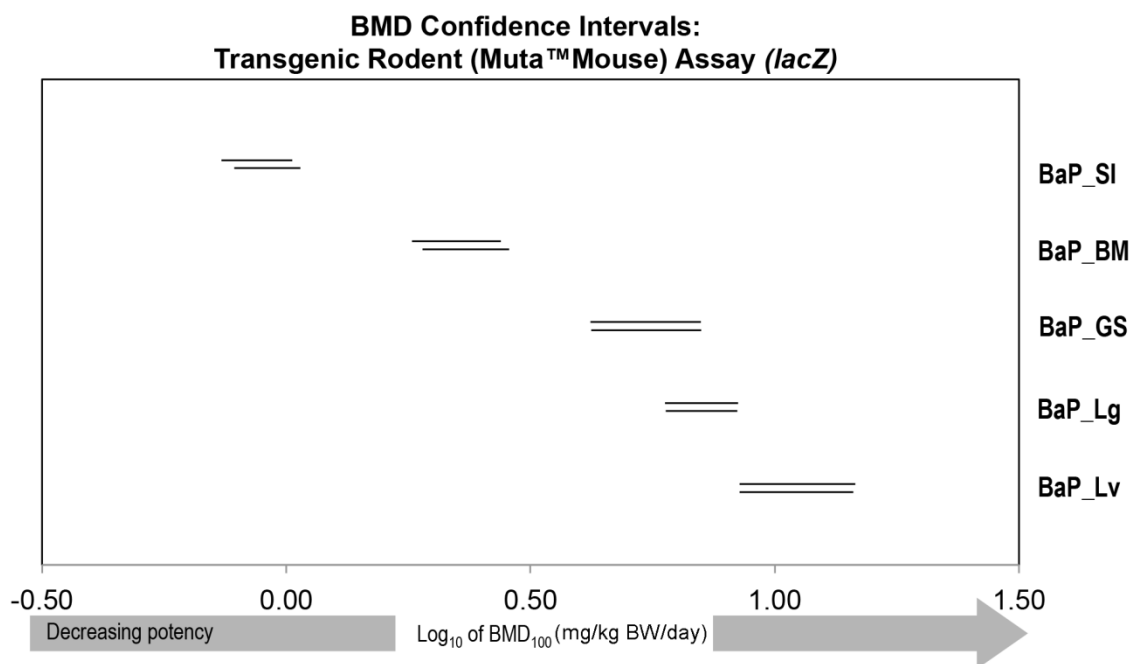


Figure 5.3. Tissue-specific two-sided 90% confidence intervals from BMD₁₀₀ analyses of Muta™Mouse *lacZ* mutant frequency responses to 28-day repeat-dose oral exposure of benzo[*a*]pyrene (BaP). Dose-response datasets were analysed independently across five tissues: small intestine (SI), bone marrow (BM) glandular stomach (GS), lung (Lg) and liver (Lv). For each tissue, the top confidence interval relates to the fitted exponential model, the bottom to the Hill model. The underlying dose-response data and fitted models are shown in **Supp. Figure 5.4**.

5.6.1 Increasing BMD precision using historical data

Slob and Setzer (35) demonstrated that fitting a dose-response model to combined datasets from multiple compounds can result in more precise BMD estimates for each compound (i.e., reduced BMDU-BMDL ratios). This occurs when all compounds in the combined analysis have similar dose-response shapes after correcting each for x- and y-axis scaling (i.e., making model parameter a (background) and b (potency) covariate dependent). Constant values for the model's shape parameters (maximum response (parameter c) and log-steepness (parameter d)) can then be used in the fitting of all dose-response curves, which improves BMD precision as all datasets in the combined analysis contribute to the estimation of these shared parameters. Importantly, a large meta-analysis of historical data (35) supported this approach by showing that dose-response shapes, after this correction, are typically highly similar across compounds for the same endpoint and study design.

Applying this principle, we investigated whether the 'MegaBaP' dose-response data (i.e., ten doses plus control, $\geq$ seven animals per dose-group, **Figure 5.3**) could be used to improve BMD precision for another polycyclic aromatic hydrocarbon (PAH), in this case DBahA (28). The studies shared identical designs with the exception that the DBahA study included only three doses plus control with $\leq$ five animals per dose-group. First, BMD analyses for DBahA were carried out independently across four tissues (lung, small intestine, liver, glandular stomach) and the resulting confidence intervals plotted (**Figure 5.4A**). The analysis was then repeated using the combined BMD-covariate approach with compound as a covariate (**Figure 5.4B**). Analyses were carried out pairwise by tissue (e.g., BaP-lung versus DBahA-lung in one combined analysis, etc.),

thereby avoiding the potential that responses across different tissues from the same animal are correlated.

The combined analysis yielded narrower DBahA confidence intervals (i.e., improved BMD precision) for small intestine, lung and glandular stomach, but remained similar for liver. The combined approach also resolved confidence interval overlaps observed for DBahA in the independent analysis (i.e., between liver and glandular stomach and between lung and small intestine, **Figure 5.4** vertical arrows). Therefore, the tissue sensitivity ranking from the combined approach better identified tissue-specific differences in mutation induction, thus contributing improved information for MOA delineation. It should be noted that the potency ranking for DBahA (i.e., small intestine > lung > liver > glandular stomach) follows a different order than that observed for BaP (i.e., **Figure 5.3**, small intestine > glandular stomach or lung > liver). This is because during the combined analysis (**Figure 5.4B**), it is maximum response and log-steepness (i.e., *shape*) information, and not *potency*, which are shared between BaP and DBahA. A detailed discussion regarding the toxicokinetic and/or toxicodynamic phenomena that may underlie tissue-specific MutaTMMouse responses to DBahA, BaP and several other PAHs is available in Long et al. (28). Nonetheless, it is important to emphasise that the BMD-covariate analyses presented can permit the formulation of explicit hypotheses to account for the difference in cross-tissue BMD rankings for multiple compounds, as well as the cross-tissue patterns observed for each individual compound (see above, **Figure 5.3**).

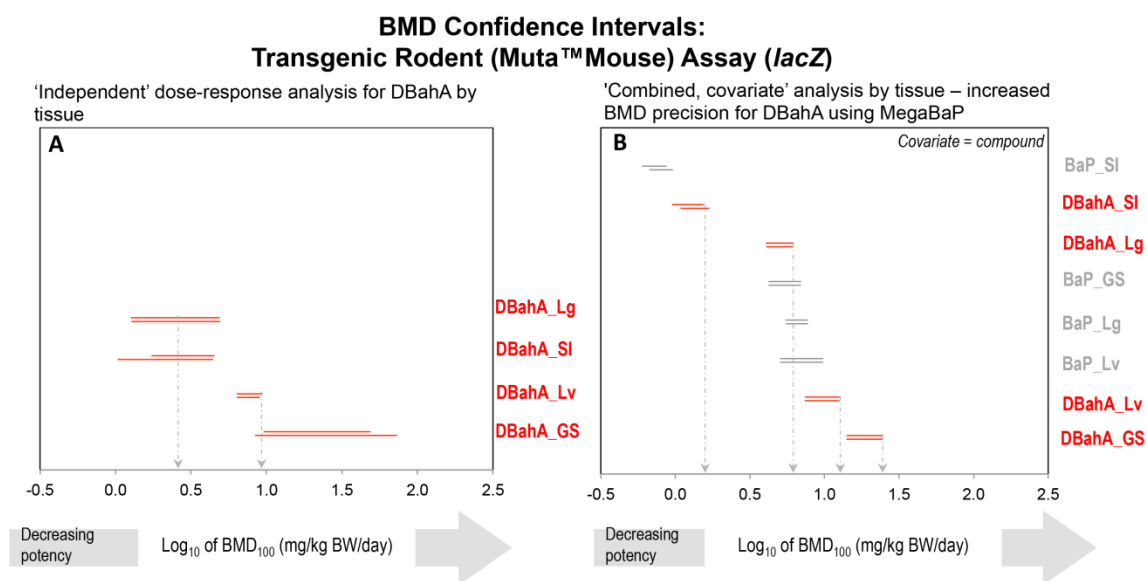


Figure 5.4. Two-sided 90%-confidence intervals of BMD₁₀₀ values from the analyses of Muta™Mouse *lacZ* mutant frequency responses to dibenz[*a,h*]anthracene (DBahA) or benzo[*a*]pyrene (BaP). DBahA (28) dose-responses were first analysed independently (**A**) across four tissues: small intestine (SI), glandular stomach (GS), lung (Lg), and liver (Lv). For each tissue, the top confidence interval relates to the fitted exponential model and the bottom to the Hill model. (**B**) Analyses were then carried out with the DBahA and BaP data for each tissue combined, using compound as a covariate. The underlying dose-response data and fitted models are shown in **Supp. Figure 5.5**.

Since the aforementioned Slob and Setzer study demonstrated that dose-response shape appears to be conserved across compounds for the same endpoint/study design (35), the MegaBaP data could feasibly be used to improve precision for any newly-collected data for the same TGR study design. This approach could thus be harnessed to make better use of data from pilot or dose range-finding studies. For example, if a small dose range-finding study was carried out using three doses and only two animals per dose group, combined analysis with MegaBaP could significantly improve BMD precision or permit extrapolation to determine the optimal dose range for a chosen CES (19,20). Thus, this approach could significantly reduce the use of experimental animals in (genetic) toxicity assessment, and moreover, ultimately yield more precise metrics for HHRA and regulatory decision-making.

Importantly, use of historical data in this way does not solely rely on availability of endpoint data in ‘MegaStudy’ format (e.g., many dose groups and numerous replicates per group). In an analogous manner, more precise BMD estimates can be obtained through combined BMD analysis of many, less accurately defined (e.g., 3 doses plus control) *in vivo* datasets for shared endpoint and study design; obtained for instance, across different chemicals (20,35). Our companion paper (8) similarly demonstrated this approach across different types of ionizing radiation examined *in vitro*. Therefore, using historical data for a shared endpoint/study design to improve BMD precision for any newly-collected data obtained using a minimised-animal design (19) represents a promising strategy for appreciable animal reduction facilitated by the combined BMD-covariate approach (19,20,35).

5.7 Conclusions

In conjunction with our earlier companion paper (8), we have demonstrated the utility of the combined BMD-covariate approach for the quantitative analysis of genetic toxicity dose-response data across several *in vitro* and *in vivo* endpoints. Here, combined BMD approaches facilitated robust potency comparisons across wide-ranging *in vivo* covariates (e.g., study, sex, tissue, compound, etc.), more precise determinations of BMD and, by extension, an opportunity for more effective use of animal data for HHRA and regulatory decision-making.

More specifically, analysis across covariates identified shared dose-response model parameters that could be estimated from all included datasets during combined analyses, with the approach leading to improved BMD precision. Concomitant improvements in precision permit more effective potency rankings of prioritised substances, and could form the basis for assigning potency groupings to facilitating data gap filing by read across. Moreover, inclusion of historical data for an experiment with numerous doses can permit improved BMD precision for a dataset with fewer doses, and these improvements will be translated into more precise human exposure limit values (i.e., TDI, PDE, etc.) and/or MOEs. Thus, the analyses conducted herein indicate that the combined BMD-covariate approach permits effective and efficient use of animal data for regulatory evaluations, HHRA, and regulatory decision-making.

In summary, we offer the following concluding statements regarding the utility of the combined BMD-covariate approach for the analysis of *in vivo* genetic toxicity dose-response data:

1. Dose–response analysis using the combined BMD-covariate approach provides quantitative information on the potency of a compound that is more informative than simple dichotomous hazard identification.
2. Potency comparison requires careful consideration of BMD confidence intervals: plotting BMD confidence intervals provides an approach whereby both the magnitude of potential differences in BMDs and the uncertainty in the BMD estimates can be compared.
3. The precision of individual BMD estimates from genetic toxicity dose-response data can be improved by combined analysis with other datasets for the same endpoint/study design. This occurs when parameters in the fitted dose-response model are conserved across all included dose-response datasets, and thus can be estimated from all data in combination. Increased BMD precision affords an improved ability to rank potency values across covariates (e.g. study, compound, sex, tissue, etc.), and reduces the chance of Type II errors.
4. Improvements in the precision of BMD values derived from combined analysis of *in vivo* dose-response datasets allows more information to be obtained from the same number of animals, or vice-versa, the same information to be obtained using fewer animals.
5. Potency rankings determined using the combined BMD-covariate approach are an appropriate starting point for assigning substances to equipotent groupings. When considered in the context of available structural and/or physical-chemical properties, and/or MOA information, these groupings could permit reductions in animal use by facilitating data-gap filling via read across.

6. BMD comparisons across tissues provided robust potency rankings that permit the formulation of mechanistic hypotheses related to tissue-specific metabolic capacity, systemic distribution of parent compounds and metabolites, and the severity of the effect at the site of action.

5.8 References

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CHAPTER SIX

6 Chapter: Benchmark Dose Analyses of Multiple Genetic Toxicity Endpoints Permit Robust, Cross-Tissue Comparisons of MutaTMMouse Responses to Orally Delivered Benzo[a]pyrene

6.1 Preamble: Authors, Affiliations, and Style

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

6.2 Abstract

Genetic damage is a key event in tumorigenesis, and chemically induced genotoxic effects are a human health concern. Although genetic toxicity data have historically been interpreted using a qualitative screen-and-bin approach, there is increasing interest in quantitative analysis of genetic toxicity dose-response data. We demonstrate an emerging use of the benchmark dose (BMD)-approach for empirically ranking cross-tissue sensitivity. Using a model environmental carcinogen, we

quantitatively examined responses for four genetic damage endpoints over an extended dose range, and conducted cross-tissue sensitivity rankings using BMD₁₀₀ values and their 90% confidence intervals (CIs). MutaTMMouse were orally exposed to 11 doses of benzo[*a*]pyrene. DNA adduct frequency and *lacZ* mutant frequency (MF) were measured in up to 9 tissues, and *Pig-a* MF and micronuclei (MN) were assessed in immature (RETs) and mature red blood cells (RBCs). The cross-tissue BMD pattern for *lacZ* MF is similar to that observed for DNA adducts, and is consistent with an oral route-of-exposure and differences in tissue-specific metabolism and proliferation. The *lacZ* MF BMDs were significantly correlated with the tissue-matched adduct BMDs, demonstrating a consistent adduct conversion rate across tissues. The BMD CIs, for both the *Pig-a* and the MN endpoints, overlapped for RETs and RBCs, suggesting comparable utility of both cell populations for protracted exposures. Examination of endpoint-specific response maxima illustrates the difficulty of comparing BMD values for a fixed benchmark response across endpoints. Overall, the BMD-approach permitted robust comparisons of responses across tissues/endpoints, which is valuable to our mechanistic understanding of how benzo[*a*]pyrene induces genetic damage.

6.3 Introduction

Genetic damage is recognized as an enabler of cancer, and exposure to genotoxic substances is an important human health issue (Hanahan and Weinberg 2011). More specifically, exposures to genotoxicants can contribute to the accumulation of mutations in critical genes, such as tumour suppressor or proto-oncogenes (Fearon 1997; Hemminki et al. 2000; Wang et al. 2012; Kucab et al. 2016; Kucab et al. 2015), and this can lead to

sustained proliferative signalling, replicative immortality, and evasion of apoptosis and growth suppressors. Although genetic damage can occur and accrue spontaneously through endogenous damage and errors in replication, it is also induced by exposures to environmental genotoxins. Recent high-profile publications have re-emphasized the importance of extrinsic factors such as environmental exposures in determining cancer risk (Alexandrov et al. 2016; Wu et al. 2016).

The pervasive nature of environmental genotoxins, the recognition in the 1970s and early 1980s that humans are invariably exposed to environmental mutagens, and the established empirical and mechanistic links between genetic damage and carcinogenesis, initiated formal chemical screening programs in Canada, the United States, Japan, and Western Europe (MacGregor et al. 2015a). Importantly, chemical screening for genetic toxicity is now also motivated by recognition that cancer is not the only consequence of somatic mutations. Recent research has demonstrated links between *in utero* (geno)toxin exposures and neurodegenerative diseases (Modgil et al. 2014), reproductive defects (Fowler et al. 2008; Mocarelli et al. 2011), child development (Perera et al. 2015) and somatic mosaicism (Erickson 2010; Meier et al. 2016).

Traditional screening for chemically induced genetic toxicity involves high dose exposures followed by binary, qualitative evaluation of the results (i.e., genotoxic or not); little quantitative data analysis is conducted. This screen-and-bin approach is based on the assumption that it is not possible to identify a level below which effects are expected to be negligible (i.e., indistinguishable from the ever-present background); and moreover, that the relationships between exposure and genotoxic effect are linear to zero dose (Health Canada 1994; MacGregor et al. 2015a; Pottenger and Gollapudi 2010). This

assumption is increasingly being challenged, and it is now recognized that quantitative analyses of dose-related effects can be used to identify a dose below which the measured effect level is not significantly elevated (Gollapudi et al. 2013; Johnson et al. 2014; MacGregor et al. 2015b; Speit et al. 2000). However, due to the historical bias of examining effects at high doses, with few dose groups, determination of an accurate point-of-departure (PoD) (i.e., exposure level associated with a pre-defined level of effect) is complicated by the necessity to extrapolate below the tested doses.

The international genetic toxicology community recently examined the use of quantitative dose-response analyses for the determination of genetic toxicity PoD values, as well as their use to determine human exposure limits for regulatory decision-making (Gollapudi et al. 2013; Johnson et al. 2014). Although several methods can be used to analyse dose-response data and generate genotoxicity PoD metrics, the aforementioned works of Gollapudi *et al.* (2013), Johnson *et al.* (2014), and MacGregor *et al.* (2015a) expressed a distinct preference for the benchmark dose (BMD)-approach. The BMD method is a statistical approach for quantitative analysis of dose-response data, whereby a benchmark response (BMR) is selected as a pre-defined effect level (e.g., 10% greater than control) and the statistically determined BMD is the best estimation of the dose that will elicit this BMR. The approach is flexible with regard to study design (e.g., number of dose groups and number of animals) and critically, the 90% upper and lower confidence limits on the BMD (i.e., the BMDU and BMDL) enable statistically rigorous BMD comparisons (Crump 1984; MacGregor et al. 2015a; Slob 2002).

The BMD approach can be used for a variety of purposes, including sensitivity ranking, evaluation of mode-of-action (MOA) hypotheses, investigation of empirical

relationships between genetic toxicity endpoints, and determination of exposure limits. For example, we recently used BMD 90% confidence intervals (CIs) to conduct potency rankings across compounds and exposure regimes, and sensitivity rankings across tissues and cell types (Wills et al. 2016a; Wills et al. 2016b). The BMD approach for potency or sensitivity ranking is superior to comparisons based on the no or lowest observed genotoxic effect level (NOGEL or LOGEL), since the PoD derived (i.e., the BMD) is not restricted to the selected dose groups. Moreover, plotting BMD 90% CIs, and arranging them by mid-point, provides a visually intuitive way to compare BMD and evaluate trends across compounds, sex, cell type, tissue, exposure regime and other covariates.

The BMD approach has also proved useful for the demonstration of quantitative relationships between genotoxicity endpoints. Such demonstrations, which could investigate relationships between different endpoints or between tissues for a single endpoint, can be used to support the involvement of specific key events in the MOA to the adverse outcome (e.g., cancer). For example, Soeteman-Hernández *et al.* demonstrated correlations between *in vivo* genotoxic potency (i.e., BMD for induced mutant or micronucleus frequency) and carcinogenic potency (Hernández et al. 2011; Soeteman-Hernandez et al. 2015b). However, there is a paucity of data to investigate quantitative relationships along several well-understood key events, such as DNA adduct formation, mutations, and chromosomal damage that are triggered by genotoxicants in their carcinogenic MOAs.

Although it is well accepted that a sub-set of DNA adducts will contribute to mutation formation (Hemminki et al. 2000; Hemminki 1993), there is an acute need for research examining quantitative relationships between adduct frequency and mutation

frequency, especially at low doses (Hemminki et al. 2000; Sander et al. 2005). The lack of such analyses quantitatively linking *in vivo* genetic toxicity (e.g., frequency of DNA adducts) with *in vivo* mutagenicity, across numerous somatic tissues, is likely due to the difficulty of making all the required measurements. Somatic mutations *in vivo* are rare and they infrequently elicit phenotypic changes; thus, chemically induced changes in mutant frequency (MF) are notoriously difficult to detect; moreover, measurement of DNA damage and MF in the same animals constitutes a significant challenge. Although endogenous mutation detection systems do exist, they are either laborious, and hence, infrequently used (e.g., *Hprt* mutations), or restricted to haematopoietic tissues (e.g., *Pig-a*). Transgenic rodents (TGRs), which employ a bacterial target gene in a shuttle vector that can readily be recovered from genomic DNA, provide a convenient way to determine *in vivo* MF in any tissue (Lambert et al. 2009).

In this study, we used the prototypical genotoxic carcinogen benzo[*a*]pyrene (BaP), four well-characterized genetic toxicity endpoints, and the BMD approach, to scrutinise responses at low doses, rank potencies across tissues, and compare responses across endpoints. To permit simultaneous determination of several endpoints within the same animal, we employed the MutaTMMouse, a CD2F1 transgenic mouse containing a *lacZ* target in a lambda shuttle vector (Gossen et al. 1989). We used an extended range of BaP doses (i.e., 11 in total) in order to examine effects at doses below the region where significant increases in genotoxicity are observed. Although admittedly atypical, the study design employed herein affords improved BMD precision and a concomitant opportunity to robustly compare responses across tissues and endpoints; moreover, to

examine empirical relationships between responses of functionally related endpoints (e.g., induced DNA damage and mutations).

6.4 Materials & Methods

6.4.1 Animal exposures and tissue collection

We selected eleven doses of BaP (CAS # 50-32-8, purity: 99%; Sigma-Aldrich Canada, Oakville, ON, Canada), with the top doses based on previous work by our group (Lemieux et al. 2011). The selected doses, 0, 0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, and 50.00 mg BaP/kg body weight (BW)/day, were delivered in highly refined olive oil (Sigma-Aldrich). Adult male MutaTMMouse specimens (12-13 weeks old) were maintained as described previously (Long et al. 2016). There were 7 animals in each dose group, with 14 animals in the vehicle control group (84 animals total). Mice received BaP or olive oil by oral gavage at 0.005 ml/g body weight daily for 28 days. Two days after the final dose, blood was collected from the facial vein for MN analysis. A 3-day sampling time was employed for the transgene endpoint (OECD. 2013), and was also employed for the *Pig-a* endpoint. Mice were anesthetised with isoflurane gas and blood was collected via cardiac puncture for scoring *Pig-a* MF. Additionally at this time, blood was collected from 4 positive control mice that had been administered 80 mg ENU/kg BW *i.p.*, and 4 vehicle control mice that were administered phosphate buffer as single *i.p.* injection 3 weeks prior to blood collection. Mice were then euthanized by cervical dislocation. Mice were bred, maintained, and treated in accordance with the Canadian Council for Animal Care Guidelines and Health Canada's Animal Care Committee. Bone marrow, liver, lung, small intestine, and glandular stomach were

preserved as described previously (Long et al. 2016). Kidney, spleen, and bladder were flash frozen in liquid nitrogen. All tissues were stored at -80°C .

Preliminary data for bone marrow, liver, small intestine, glandular stomach, and lung for the *lacZ* MF endpoint only were previously published in Wills & Long (2016b); here the dataset is expanded with a further 2 tissues (i.e., kidney and spleen) for *lacZ*, and 3 additional endpoints.

6.4.2 Peripheral blood micronucleus assay

MicroFlow®-BASIC kits (Litron Laboratories, Rochester, NY) were used to prepare blood cells for enumeration of micronucleated reticulocytes (MN-RET) and normochromatic erythrocytes (MN-NCE) at Litron Laboratories, as previously described (Long et al. 2016). Frozen, coded samples were shipped on ice to Litron Laboratories for scoring by flow cytometry using a 3-colour labelling method as previously described (Dertinger et al. 2004).

6.4.3 Pig-a mutant scoring in peripheral blood

Following blood collection via cardiac puncture, 100 μL of blood was immediately transferred to a 0.5 mL K2-EDTA-coated microtainer (VWR, cat. no. 95057-299). Whole blood samples were placed in an ExactPak shipping container and shipped on ice overnight to Litron Laboratories. Flow cytometric scoring of *Pig-a* MF was conducted at Litron via MutaFlow® Kit reagents according to their published immunomagnetic enrichment method (Dertinger et al. 2011), with modifications for scoring in mouse (Labash et al. 2015). Based on these analyses, the number of *Pig-a* mutant cells per million were calculated for RETs as well as total red blood cells (RBCs).

6.4.4 DNA extraction

Bone marrow, glandular stomach, liver, small intestine, and lung were prepared for an overnight digestion in lysis buffer as described previously (Long et al. 2016). Spleen, kidney, and bladder were prepared for overnight lysis and genomic DNA extraction as follows: approximately $\frac{1}{4}$ of the spleen, $\frac{1}{2}$ of a kidney, or the entire bladder was defrosted on ice and minced into small pieces. The minced tissue was transferred to a tube containing 5 ml lysis buffer (1 mM Na₂EDTA, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% SDS (w/v)) and incubated overnight at 37°C with gentle shaking. Genomic DNA was isolated from lysed tissue using a phenol/chloroform extraction procedure described previously (Douglas et al. 1994; Vijg and Douglas 1996). Isolated DNA was dissolved in 50 - 100 μ l TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) and stored at 4°C until use.

6.4.5 ³²P-postlabelling for DNA adduct analysis

Bulky DNA adduct formation was analysed in DNA samples from liver, lung, bone marrow, glandular stomach, small intestine, spleen, kidney, and bladder via the nuclease P1 enrichment version of the thin-layer chromatography ³²P-postlabelling assay (Phillips and Arlt 2014). The procedure was performed as described previously (Krais et al. 2016; Wohak et al. 2016), and results are expressed as DNA adducts/10⁸ nucleotides.

6.4.6 Positive selection for lacZ mutants

The PGal (phenyl- β -D-galactoside) positive selection assay was carried out for the analysis of *lacZ* MF in DNA samples from spleen and kidney as previously described (Gossen et al. 1992; Lambert et al. 2005; Vijg and Douglas 1996). MF was calculated as the ratio of mutant plaque forming units (pfu) to total pfu. The *lacZ* MF could not be

scored in bladder as there was insufficient DNA remaining following DNA adduct analysis. The *lacZ* MF data for bone marrow, glandular stomach, small intestine, liver, and lung was recently published in Wills & Long (2016b), and these data were included in the analyses presented below.

6.4.7 Statistical analysis

Statistical analysis for determination of a treatment effect for all endpoints was carried out in SAS v.9.1 (SAS Institute, Cary, NC) by applying a Type 3 Chi-square analysis and employing a Poisson regression, in the same manner as described previously (Long et al. 2016). Post-hoc custom contrasts based on the asymptotic chi-square distribution of the likelihood ratio statistic were conducted to compare each dose group with the control.

6.4.8 Benchmark dose modelling

BMD analyses were conducted using the PROAST software (version 50.9 - <http://www.proast.nl>). Dose-response data were analysed using a family of nested exponential models (Slob 2002) recommended by the European Food Safety Authority for the assessment of continuous data (EFSA 2009). PROAST uses the likelihood ratio test to select the optimal model, with increasingly complex models using additional parameters only accepted if the difference in log-likelihood exceeds the critical value of $p < 0.05$. Dose-response datasets were processed individually (i.e. not using tissue as covariate) to avoid the possibility of correlation in tissues harvested from the same animals, and the concomitant generation of overly optimistic CIs (Wills et al. 2016b). The BMR selected for the current analyses (i.e., 100% or a two-fold increase in the response relative to control), was chosen as it is commonly used for the assessment of

genotoxicity data, and because it tended to lie within the range of experimental observation in the datasets herein as was thus ‘optimal’ for deriving CIs for sensitivity comparison (Wills et al. 2016a). The BMDL and BMDU values represent the lower and upper bounds of the two-sided 90% CI of the BMD respectively, with the difference between the BMDU and BMDL defining the uncertainty in the BMD estimate, and therefore its precision. As employed in previous work, CI plots arranged by the geometric midpoint of the BMDL-BMDU interval were utilised to permit robust sensitivity comparisons that account for estimation uncertainty (Bemis et al. 2016; Wills et al. 2016a).

6.5 Results

No overt signs of toxicity (i.e., body weight change, liver somatic index) were observed in any dose group, including controls. A significant level of induction of each genetic damage endpoint was observed in all tissues examined, although the responses occurred at different dose levels and the magnitude of the response varied across endpoints. Using PROAST, we employed the exponential model family to derive the BMD_{100} and accompanying 90% CIs (i.e. the BMDL and BMDU values). The BMD_{100} , BMDL, BMDU, along with the BMDU-BMDL ratio for each tissue and endpoint are summarised in **Supp. Table 6-I**, and BMD model fits for each analysis are presented in **Supp. Figure 6.1a-d**. The CIs (i.e. range between the BMDL to BMDU) bounding the BMD_{100} s were used for comparative evaluations of tissue sensitivities.

6.5.1 DNA adduct frequency

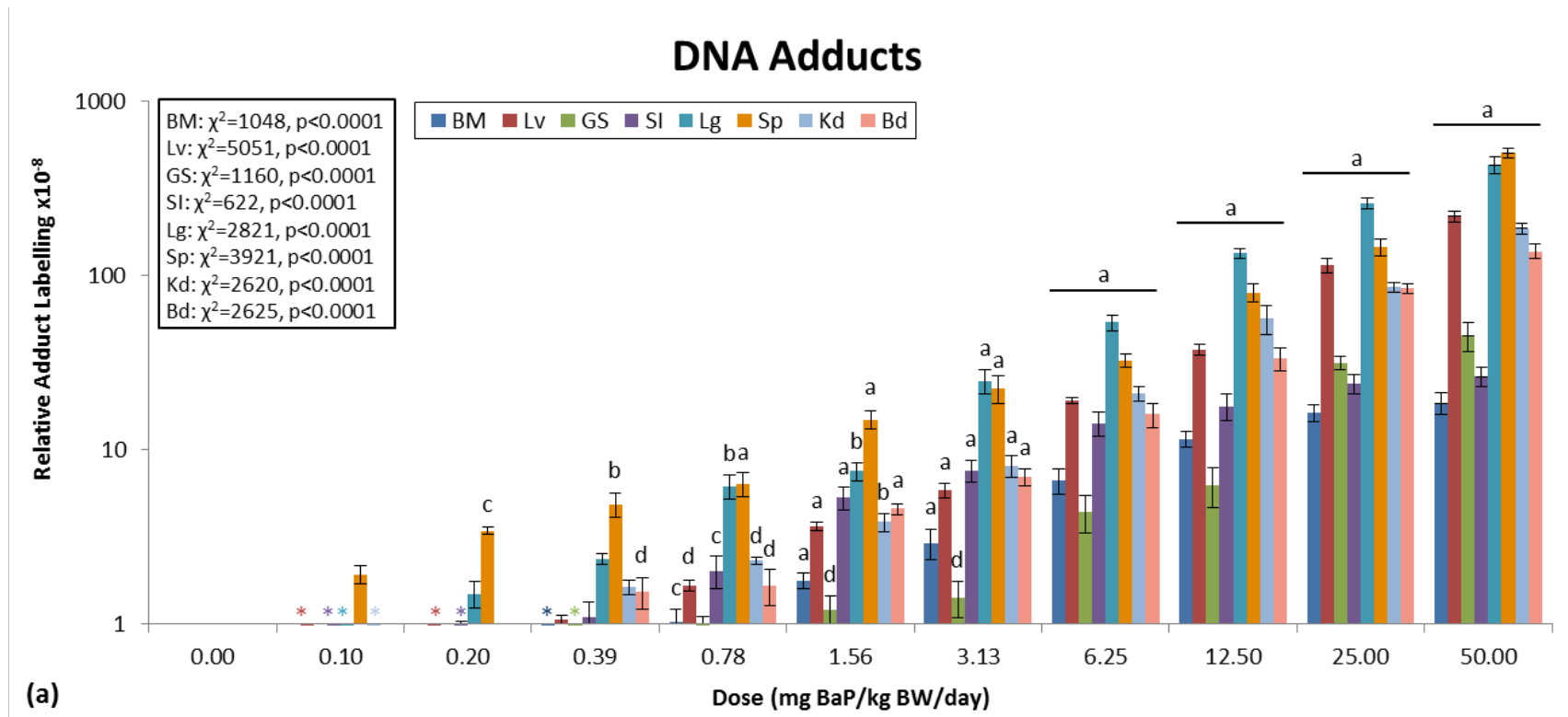
Using an external BaP-diol-epoxide-DNA standard (Phillips and Castegnaro 1999), the major DNA adduct detected was identified as dG- N^2 -BPDE (10-(deoxyguanosin- N^2 -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP), with BaP-DNA adducts being detected in all 7 tissues examined (**Supp. Figure 6.2**). The first dose in which we observed a significant increase in adduct frequency over control (i.e., the LOGEL) was 0.20 mg/kg BW/day for spleen, 0.39 mg/kg BW/day for bladder, 0.78 mg/kg BW/day for bone marrow, liver, small intestine, lung, and kidney, and 1.56 mg/kg BW/day for glandular stomach (**Figure 6.1a**; **Supp. Figure 6.3**). The highest fold change increase in DNA adduct levels over control was observed in spleen (506-fold), followed by lung (433-fold), liver (219-fold), kidney (187-fold), bladder (139-fold), glandular stomach (46.3-fold), small intestine (27.4-fold), and bone marrow (19.6-fold).

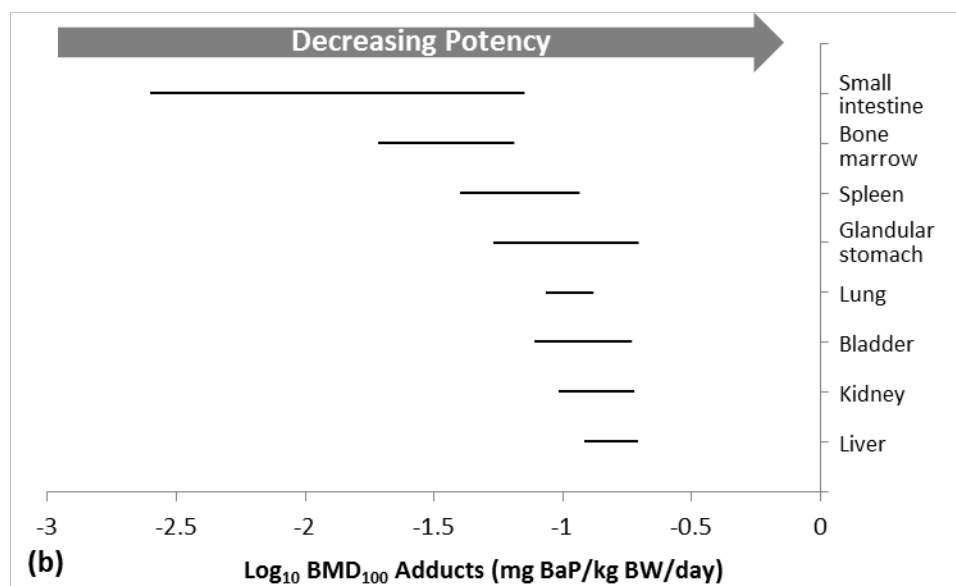
To visualize the difference in tissue sensitivities, the BMD CIs for each tissue were plotted using a log₁₀-scale (**Figure 6.1b**). In this way, tissues with non-overlapping BMD CIs can be sequentially ranked by sensitivity, with decreasing sensitivity shown from left to right (i.e. lowest to highest BMD). This method for illustration of sensitivity trends is superior to the common approach of simply listing the BMD/BMDL/BMDU values in a table (**Supp. Table 6-I**), as upon inspection of **Figure 6.1b** it is immediately apparent that the sensitivity order is: small intestine / bone marrow / spleen / glandular stomach / lung / bladder / kidney / liver. Due to the relatively large BMD CI for some tissues (e.g., 5 of 8 tissues had BMDU-BMDL ratios over 2, **Supp. Table 6-I**), there is some overlap in the BMD range of all adjacent tissues. However, it is still possible to obtain mechanistic information from these results; and moreover, conclude that the

sensitivity of small intestine and bone marrow are significantly higher than lung, bladder, kidney, and liver. It is also interesting to note that the sensitivity order obtained via BMD-modelling is not the same as the order of tissues according to the level of induced response (i.e., maximum fold-change over control), nor the order determined using lowest significant dose (i.e., the LOGEL).

Figure 6.1. (a) BaP dose-response data ($\pm$ standard error) for DNA adducts plotted using a $\log_{10}$ y-axis for better visualisation of the responses across all doses. Statistical results for the overall dose–response relationship are presented for each tissue. The level of significance for the custom contrast results for each dose vs. control are indicated as follows: a = $p < 0.0001$; b = $p < 0.001$; c = $p < 0.01$; d = $p < 0.05$. BM = Bone marrow; Lv = Liver; GS = Glandular stomach; SI = Small intestine; Lg = Lung; Sp = Spleen; Kd = Kidney; Bd = Bladder. *indicates where responses were below 1, and thus are obscured on a $\log_{10}$ scale. **Supp.**

Figure 6.3 shows dose-response data plotted on a linear scale with a restricted axis in order to visualise the low-dose responses. **(b)** BMD_{100} values and two-sided 90% CIs (i.e., the range between the BMDL and BMDU) determined using the exponential model for DNA adducts. BMDs can be considered different where CIs do not overlap.



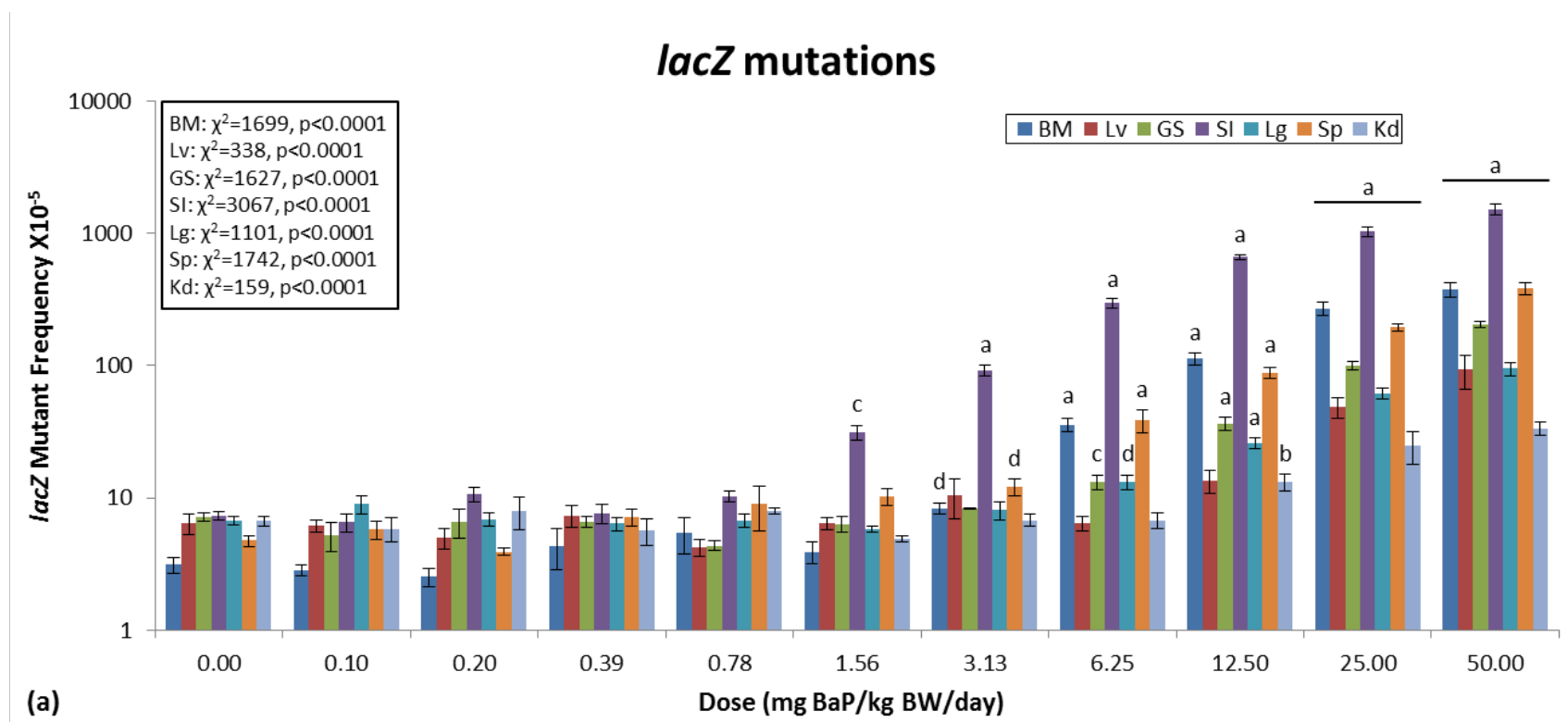


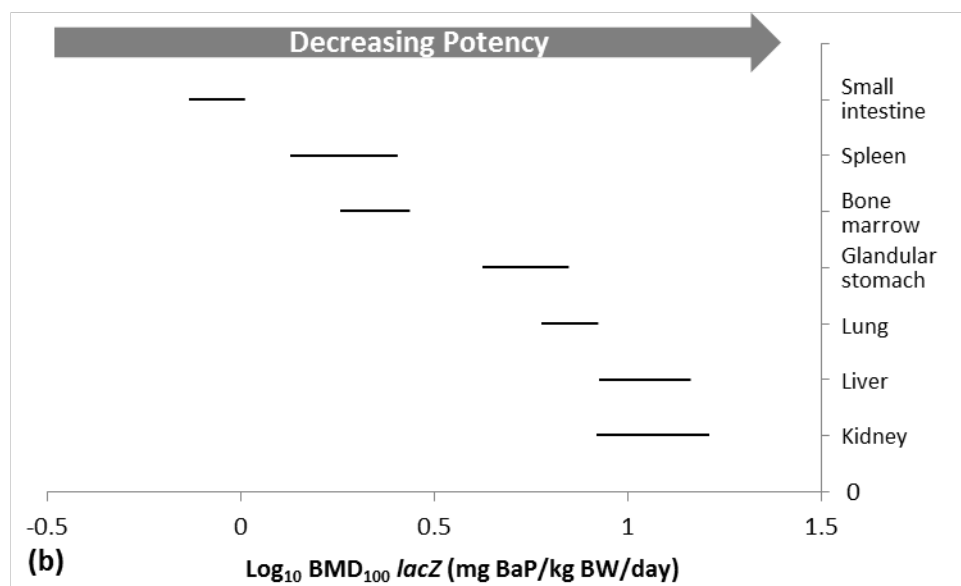
6.5.2 *lacZ* mutant frequency

A significant induction in *lacZ* MF was observed in all tissues examined, with LOGELs of 1.56 mg/kg BW/day for small intestine, 3.13 mg/kg BW/day for bone marrow and spleen, 6.25 mg/kg BW/day for lung and glandular stomach, 12.5 mg/kg BW/day for kidney, and 25 mg/kg BW/day for liver (**Figure 6.2a**). The highest fold change increase over control was observed in small intestine (208-fold), followed by bone marrow (120-fold), spleen (81.0-fold), glandular stomach (28.6-fold), liver (14.6-fold), lung (14.1-fold), and kidney (5.0-fold). As **Figure 6.2b** illustrates, there were considerable differences in BMDs across tissues, with the sensitivity order as follows: small intestine > spleen or bone marrow > glandular stomach or lung > liver or kidney. Despite minor differences, it is apparent that this sensitivity trend is similar to that observed for DNA adduct frequency. However, for the *lacZ* endpoint the BMDs were more precise (i.e., all BMDU-BMDL ratios below 2; **Supp. Table 6-I**), which resulted in fewer overlapping CIs. As a result, it is possible to distinguish *lacZ* sensitivity values between additional tissues, and in fact, to visualise three discrete groupings of tissues (i.e., small intestine > bone marrow / spleen > kidney / liver / lung / stomach). In this case the sensitivity order is fairly similar to the order according to induced response and LOGEL.

Figure 6.2. (a) BaP dose-response data ($\pm$ standard error) for *lacZ* mutations, plotted using a $\log_{10}$ y-axis for better visualisation of the responses across all doses. Statistical results for the overall dose-response relationship are presented for each tissue. The level of significance for the custom contrast results for each dose vs. control are indicated as follows: a = $p < 0.0001$; b = $p < 0.001$; c = $p < 0.01$; d = $p < 0.05$. BM = Bone marrow; Lv = Liver; GS = Glandular stomach; SI = Small intestine; Lg = Lung; Sp = Spleen; Kd = Kidney.

(b) BMD_{100} values and two-sided 90% CIs (i.e., the range between the BMDL and BMDU) determined using the exponential model for *lacZ* mutations. BMDs can be considered different where CIs do not overlap.

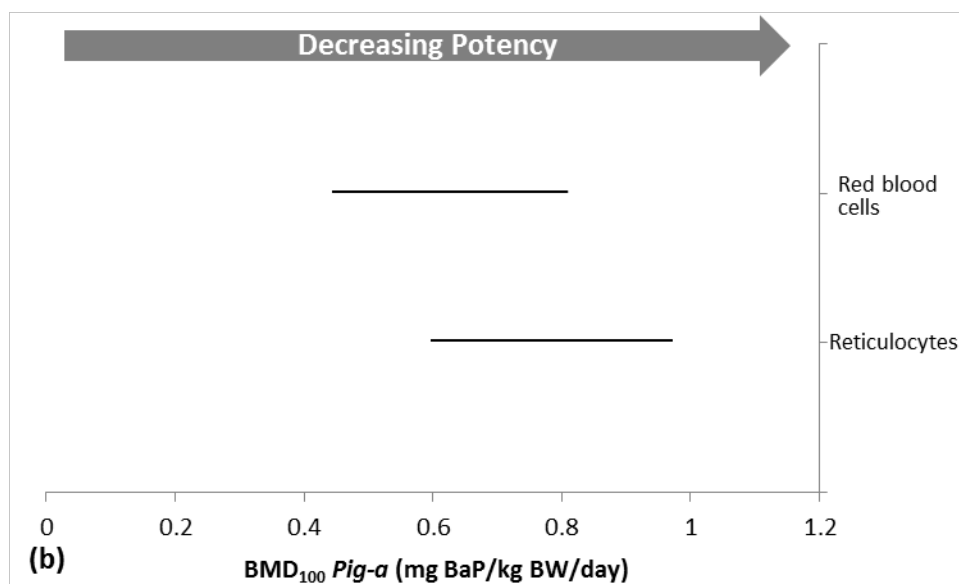
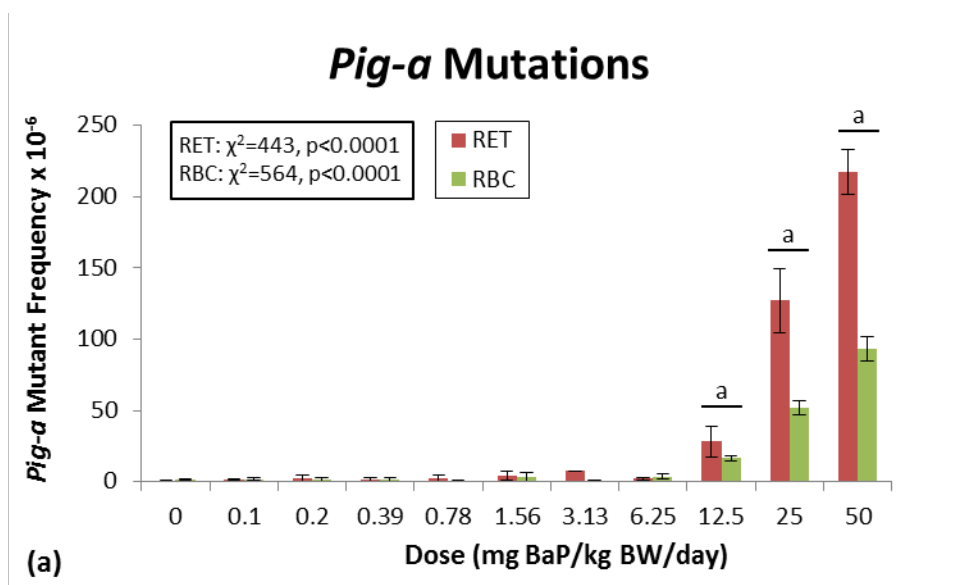




6.5.3 *Pig-a* mutant frequency in peripheral blood

Pig-a mutant phenotype frequency was examined in both RETs and RBCs. A significant positive increase was observed in both cell populations, with the first significant response (i.e., the LOGEL) appearing at 12.5 mg/kg BW/day in both RETs and RBCs (**Figure 6.3a**). The fold-increase over control observed for RBCs (69.7-fold) was only 1/5 as high as for RETs (385.5-fold). The BMD CIs for RETs and RBCs overlapped (**Figure 6.3b**), therefore a sensitivity order could not be established. This is interesting given the large difference in response magnitudes; however, the trend across these two cell populations is analogous to the observed cross-tissue trend in sensitivity to adduct formation (i.e., overlapping BMD ranks). In this case, issues related to the detection of the *Pig-a* MF resulted in reduced sample size that likely contributed to increases in the BMD CIs. In fact, the *Pig-a* BMDU-BMDL ratio is over 2 for both cell populations, whereas the *lacZ* BMDU-BMDL ratio is below 2 for all tissues examined.

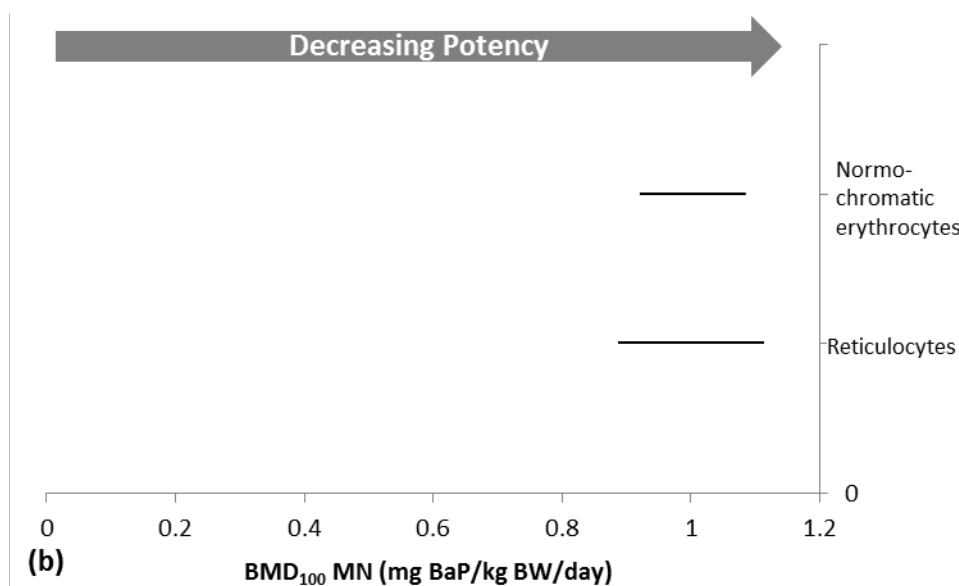
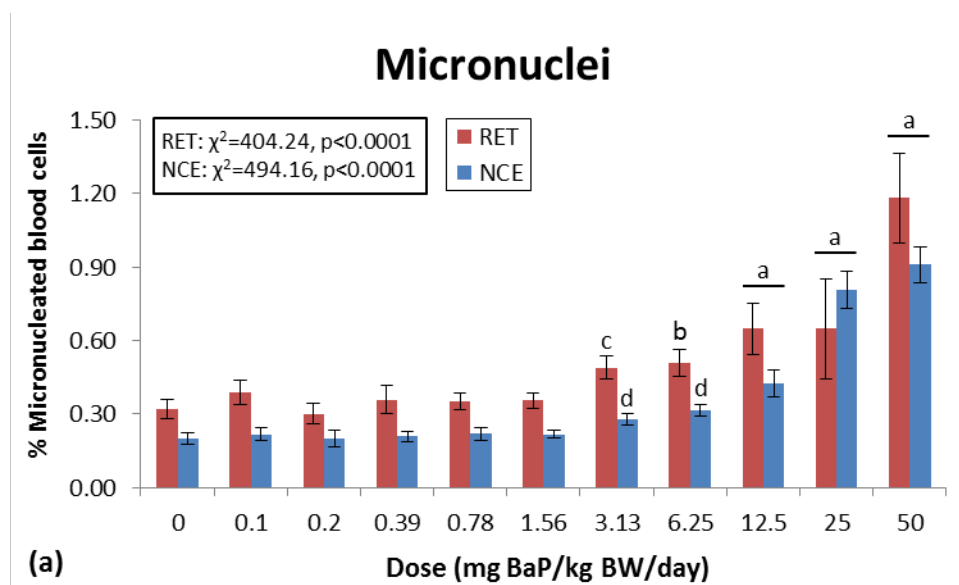
Figure 6.3. (a) BaP dose-response data ($\pm$ standard error) for *Pig-a* mutations. Statistical results for the overall dose–response relationship are presented for both cell populations. The level of significance for the custom contrast results for each dose vs. control are indicated as follows: a = $p < 0.0001$; b = $p < 0.001$; c = $p < 0.01$; d = $p < 0.05$. RET = reticulocyte; RBC = red blood cell. **(b)** BMD_{100} values and two-sided 90% CIs (i.e., the range between the BMDL and BMDU) for *Pig-a* mutations for both cell populations Where CIs overlap tissue responses cannot be considered different.



6.5.4 Percent micronuclei in red blood cells

The frequencies of micronucleated RETs and NCEs were examined in order to assess BaP-induced chromosomal damage. A significant increase in the percent micronucleated cells was observed in both RETs and NCEs at 3.13 mg/kg BW/day (**Figure 6.4a**). A slightly higher fold-change increase over control was observed in RETs (4.5-fold), in comparison with NCEs (3.7-fold). The ranges between the BMDL and BMDU overlapped (**Figure 6.4b**), therefore a sensitivity order could not be determined. The ratio of BMDU to BMDL was well below 2, indicating that BaP is similarly clastogenic in both cell populations.

Figure 6.4. (a) BaP dose-response data ($\pm$ standard error) for micronuclei. Statistical results for the overall dose-response relationship are presented for both cell populations. The level of significance for the custom contrast results for each dose vs. control are indicated as follows: a = $p < 0.0001$; b = $p < 0.001$; c = $p < 0.01$; d = $p < 0.05$. RET = reticulocyte; NCE = normochromatic erythrocyte. **(b)** BMD_{100} values and two-sided 90% CIs (i.e., the range between the BMDL and BMDU) for % micronuclei for both cell populations. Where CIs overlap tissue responses cannot be considered different.



6.5.5 Comparisons of endpoint-specific BMDs for hematopoietic tissues

Finally, we compared sensitivity across each endpoint for hematopoietic tissues. In order to do this, the 90% CIs for each hematopoietic tissue BMD₁₀₀ were plotted together for visual comparison across the endpoints examined (**Figure 6.5a**). The figure shows a progression of BMD₁₀₀s across endpoints from DNA adducts (i.e., lowest BMD), to *lacZ* and *Pig-a* mutants at doses approximately 25- to 90-fold higher, and finally, to chromosome damage at doses 2- to 4.5-fold higher than the mutant endpoints and 150- to 350-fold higher than for DNA adducts (**Supp. Table 6-I**). Thus, the observed pattern of BMDs in hematopoietic tissues seems consistent with the sequence of key events leading from genetic damage to cancer.

Although **Figure 6.5a** supports the contention that sequential genotoxic events precede tumor formation (Fukushima et al. 2016; MacGregor et al. 2015b) the manner in which the data are presented raises fundamental questions about the validity of such cross-endpoint comparisons. **Figure 6.5b** uses identical fixed y-axes to compare the modelled dose-responses for each endpoint in hematopoietic tissue (RBCs only for *Pig-a*, NCEs only for MN). By plotting the responses on the same y-axis scale, it becomes readily apparent that the inducible range in response varies considerably across endpoints (e.g. a small dynamic range in inducible response is particularly noticeable for the micronucleus endpoint). Additionally, model parameter ‘c’ displayed in the inset, which is the model-determined estimate of the maximum response, shows nearly a 10-fold reduction between the DNA adduct and MF endpoints, and an approximately 25-fold reduction between the MF and micronucleus endpoints (**Figure 6.5b**).

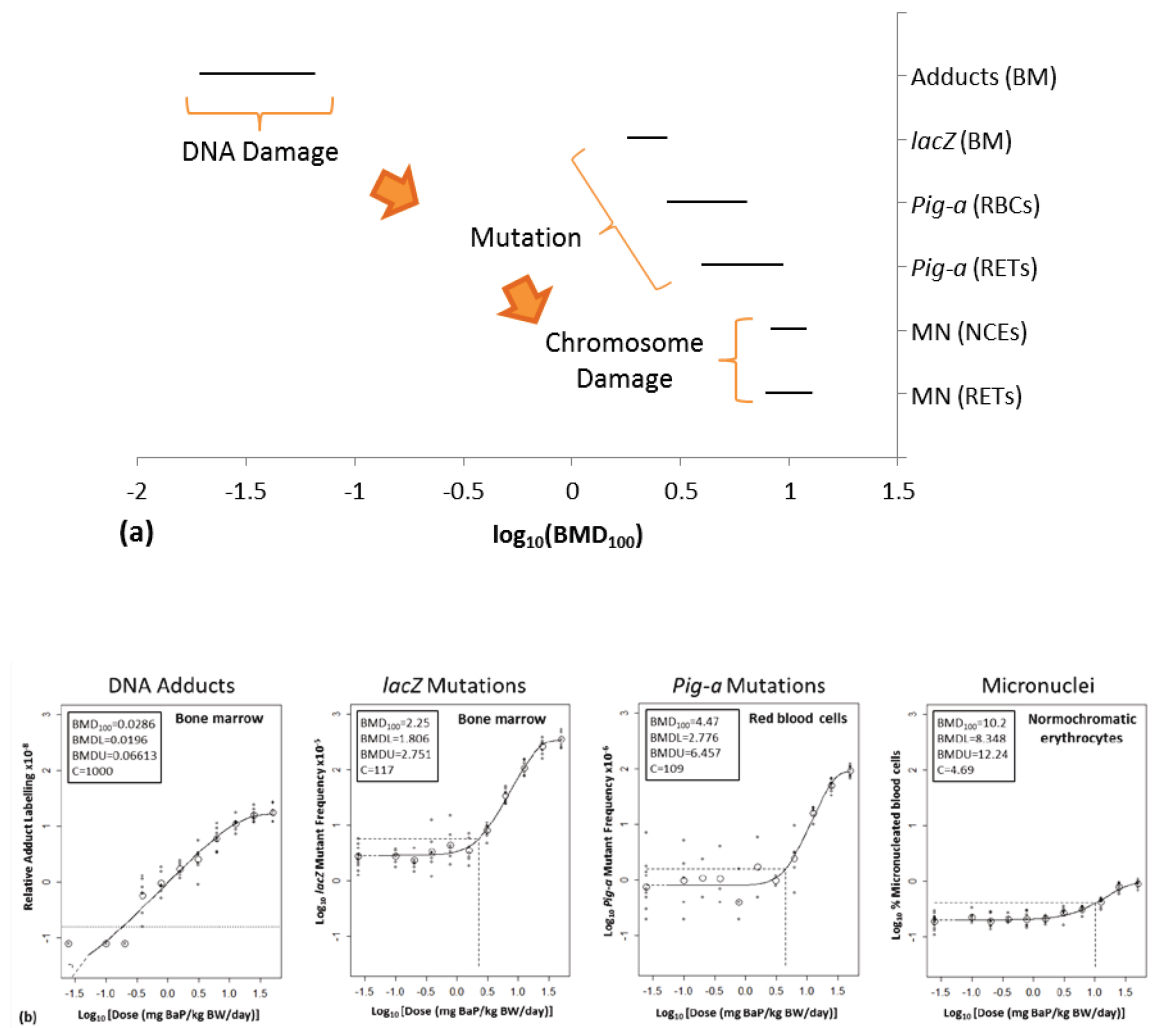


Figure 6.5. (a) BMD_{100} values and two-sided 90% confidence (i.e., the range between the BMDL and BMDU) for each endpoint in hematopoietic tissue. **(b)** Illustration of fitted functions showing variations in maximum response, indicated by model parameter 'c' (inset), across endpoints.

6.5.6 The empirical relationship between genotoxic sensitivity and mutagenic sensitivity

When visually comparing the dose-response data for DNA adduct frequency (**Figure 6.1a**) with that for *lacZ* MF (**Figure 6.2a**), there is no apparent empirical relationship between the tissue-specific induced damage levels. To examine the empirical relationship between these two endpoints, tissue-matched BMD₁₀₀ values, and their associated 90% CI, were plotted against each other. This analysis, which is shown in **Figure 6.6**, revealed a significant ($p = 0.002$) positive relationship between genotoxic sensitivity (i.e., induction of DNA adducts), and mutagenic sensitivity (i.e., induction of *lacZ* mutants) across 7 tissues. This relationship indicates that although there may be large differences in cross-tissue BMDs for a given endpoint, there appears to be a consistent cross-tissue rate for “conversion” of DNA adducts to mutations for this compound.

As cellular replication is a necessary factor for mutation fixation, we also wanted to investigate the influence of cellular proliferation on the conversion of DNA adducts to mutants. As part of our standard experimental protocol, we evaluated proliferation rate, as Ki-67 index, in liver, lung, and small intestine (**Supp. Methods**). The Ki-67 index plotted against the ratio of DNA adduct BMD to *lacZ* MF BMD for these three tissues (**Supp. Figure 6.4**), is positively correlated. Although this correlation is based on only three tissues, it supports the notion that cellular proliferation rate affects the tissue-specific conversion of BaP-induced DNA adducts to mutations.

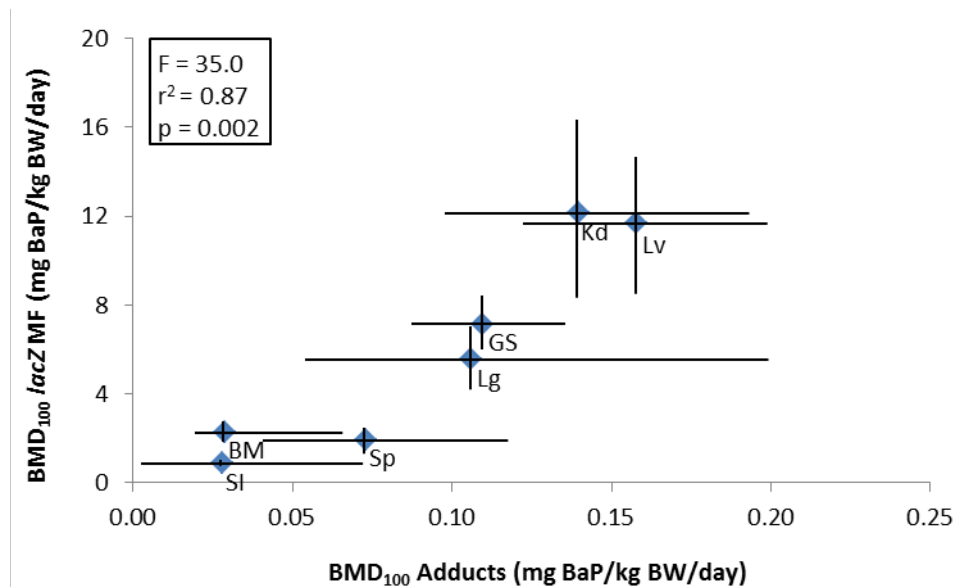


Figure 6.6. The relationship between the mutagenic sensitivity and genotoxic sensitivity to BaP (i.e., BMD₁₀₀ values and two-sided 90% CIs for the *lacZ* mutation endpoint vs. tissue-matched BMD₁₀₀ values and two-sided 90% CIs for the DNA adduct endpoint). Linear regression analysis results are inset. BM = Bone marrow; Lv = Liver; GS = Glandular stomach; SI = Small intestine; Lg = Lung; Sp = Spleen; Kd = Kidney.

6.6 Discussion

The quantitative assessment of genetic toxicity data is a rapidly advancing field, and the emerging application of the BMD-approach to derive PoDs has well-described advantages for regulatory decision-making and the protection of human health. Here, we present an advanced approach for BMD-modeling that allows for robust cross-tissue sensitivity ranking, thereby contributing important quantitative support for tissue-specific genotoxic effects. More specifically, this study used the BMD-approach to model BaP-induced *in vivo* genetic toxicity dose-response relationships across several tissues and an expanded dose range, and derived BMD₁₀₀ values and their associated 90% CIs for each tissue/endpoint combination. We subsequently employed the BMD CIs to conduct sensitivity ranking across 9 tissues for DNA adducts, 8 tissues for *lacZ* mutants, and 2 peripheral blood cell populations for both *Pig-a* MF and MN. As discussed below, examinations of tissue rankings for each endpoint, and empirical relationships between the endpoints, permitted general statements about the fundamental nature of the endpoints examined and the mechanistic relationships between the damage types.

It is well recognized that BaP primarily induces tumors via a genotoxic mode-of-action; specifically, via the conversion of bulky DNA adducts to point mutations that confer the hallmark properties of a cancerous cell (Hanahan and Weinberg 2011; IARC. 2010b). Following oral exposure, BaP has been shown to induce carcinomas in the oral cavity, forestomach, liver, and small intestine of male and female Wistar rats (Kroese et al. 2002), and kidney, mammary, and skin carcinomas in male Wistar rats (Kroese et al. 2002). Oral administration to various mouse strains resulted in tumours in lymphoid and haematopoietic tissues, as well as in lung, forestomach, liver, esophagus, and tongue

(IARC 2012). BaP also caused forestomach carcinomas, malignant lymphomas in lymphatic organs including the spleen, and bronchiolar-alveolar hyperplasia in the lung of male MutaTMMouse specimens orally exposed for only 5 days (Hakura et al. 1998). It is therefore not unexpected that we were able to detect significant increases in BaP-induced genotoxicity for all four endpoints in all tissues/cell populations examined, namely DNA adducts and *lacZ* mutants in bone marrow, glandular stomach, small intestine, liver, lung, kidney, spleen, and bladder (adducts only); as well as *Pig-a* MF and chromosome damage in peripheral blood cells. With respect to DNA damage, we were able to detect a significant increase in DNA adducts at 0.20 mg BaP/kg BW/day, the lowest oral dose used to date to investigate the *in vivo* genotoxicity of BaP. This robust genotoxic response demonstrates the utility of the MutaTMMouse TGR assay for simultaneously investigating several, complementary genetic toxicity endpoints across numerous tissues.

Ranking sensitivity across the measured tissues lends support for mechanistic understanding of the toxicological properties of the compound evaluated. In this study we observed that the *Pig-a* and MN BMD CIs overlapped for RETs and RBCs/NCEs, and therefore a sensitivity order could not be established between the two cell populations. This may simply be due to the accuracy of the measurement of these endpoints, with larger CIs being more likely to result in overlap. However, for the MN endpoint in particular, the BMDU-BMDL ratio is below 2 and is quite similar for both cell populations, therefore alternatively, this may indicate that RETs and NCEs are equally sensitive to MN induction by BaP. Therefore, that there may be comparable utility in examining either cell population for study designs that involve protracted exposures, such

as the one conducted here. Our novel approach to data analysis makes a direct comparison of our results with other publications using these endpoints difficult. However, in our recent paper we employed the BMD method to examine dose-response data from a *Pig-a* study examining the genotoxicity of *N*-ethyl-*N*-nitrosourea, and in that case, the BMD CIs for RBCs and RETs overlapped (Wills et al. 2016b), as they did in the current study. Conversely, the CIs for RBCs sampled at different time points were distinct (Wills et al. 2016b), which highlights that sampling time appears to be more important for RBCs than for RETs. Notably, for both the *Pig-a* and MN endpoints, far fewer RETs are interrogated than NCEs/RBCs, and this contributes to larger CIs. Additional use of the BMD approach will permit similar cross-cell comparisons for different species, experimental designs, and compounds.

The DNA adduct and *lacZ* MF sensitivity rankings resulted in very similar cross-tissue patterns; however, there was considerably more overlap in CI range across tissues for DNA adducts than for *lacZ* MF. This is likely a result of the fact that the *lacZ* MF data permit determination of more precise BMDs (as demonstrated by the smaller BMDU-BMDL ratio), allowing for greater sensitivity discrimination between tissues.

For both DNA adducts and *lacZ* MF, the cross-tissue pattern in responses is likely controlled by four factors – (1) tissue contact, (2) tissue-specific metabolism and the extent of systemic circulation of the parent compound and activated metabolites, (3) tissue-specific proliferation rate, and (4) tissue-specific differences in DNA repair capacity. As discussed in our recent manuscript (Wills et al. 2016b), the fact that small intestine was the most sensitive tissue for induction of *lacZ* mutants, and similarly, as seen here, for DNA adducts, is consistent with an oral route of exposure and the known

metabolic capacity of this tissue. BaP itself is not DNA-reactive. Its major genotoxic pathway via cytochrome P450 CYP1A1/1A2/1B1 metabolism, which is induced following aryl-hydrocarbon (AhR)-agonism (Shimada et al. 2002; Shimada and Fujii-Kuriyama 2004; Xue and Warshawsky 2005), leads to the conversion of BaP into the genotoxic metabolite BaP-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE exerts its mutagenic effect by covalently binding primarily to purines, causing a bulky DNA adduct, which if not repaired prior to DNA replication, can result in a mutation (IARC. 2014). Two alternate activation pathways for BaP are also recognized, namely the PAH-radical-cation pathway, which results in depurinating adducts, and the ortho-quinone pathway, which can generate covalent DNA adducts or oxidative DNA damage (Penning 2014). However, this study, which employed ³²P-postlabeling, only examined the frequency of BPDE-type adducts. Although ingested BaP would reach the stomach first, the small intestine is known to have much higher levels of *Cyp1a1* expression (Choudhary et al. 2003; Renaud et al. 2011), and therefore absorption by the small intestine would likely result in greater conversion to BPDE, a concomitant increase in damage in this tissue, and a higher sensitivity (i.e., lower BMD) in comparison with stomach (**Figures 6.1b & 6.2b**). Furthermore, BaP is known to undergo enterohepatic circulation (Miller and Ramos 2001; Ramesh et al. 2004), which causes cycling of BaP and its activated metabolites between the liver and the small intestine via the bile. This would permit re-exposure of the small intestine and liver to BPDE, leading to an increase in bulky adducts, and in turn, mutations. Taken together, the high level of tissue contact and effective metabolism in this tissue would certainly contribute to the high sensitivity in small intestine, as seen here.

As with the site-of-contact tissues, the spleen and bone marrow were quite sensitive to the induction of both DNA adducts and *lacZ* mutants. These are both distal tissues that require systemic circulation of BaP and/or activated metabolites for exposure to occur. BaP is well known to induce significant immunotoxicity in rodent models (Blanton et al. 1986; De Jong et al. 1999; Dean et al. 1983; Ginsberg et al. 1989), which appears to be at least partially mediated by cytotoxicity related to DNA adduct formation (Ginsberg et al. 1989). The lymphatic system, including the spleen, is also a target organ for tumors (lymphomas) in orally exposed MutaTMMouse (Hakura et al. 1998), and XPA-deficient mice (IARC. 2010a; Van Oostrom et al. 1999). *Cyp1b1* in the spleen and bone marrow appears to mediate the conversion of BaP to BPDE (Heidel et al. 1998; Uno et al. 2006) with several studies also suggesting the possibility that splenic exposure to BPDE is also occurring as a result of extra-splenic metabolism (Ginsberg et al. 1989; Ginsberg and Atherholt 1990). Specifically, BPDE in the blood can be sequestered by the serum (Ginsberg and Atherholt 1989) and transported to the spleen, where it is released and taken up by splenocytes (White et al. 1994). The results presented herein likely indicate that substantial levels of BaP are available for systemic circulation, and support the high sensitivity observed for both bone marrow and spleen.

The cross-tissue sensitivity ranking for the *lacZ* endpoint also highlights the likely role of tissue-specific proliferation, since the sensitivities for tissues with higher proliferative rates (i.e., small intestine, spleen, bone marrow, glandular stomach) were higher than tissues with slower rates of proliferation (i.e., lung, liver, kidney). For example, the sensitivity of the hepatic *lacZ* response is the lowest (i.e., highest BMD), and this is consistent with a relatively low proliferation rate in comparison with the other

tissues examined. In order for bulky adducts to result in sequence changes, DNA replication is a necessary process, therefore tissues that are not actively undergoing cellular proliferation would accrue fewer mutants in a given period of time, whereas a tissue with a higher mitotic index would be likely to accrue far more mutants. It is known that spleen (Muskhelishvili et al. 2003), bone marrow (White et al. Provisionally accepted), glandular stomach (Merritt et al. 1997; Ozkan et al. 2001; Radulescu et al. 2010; Snipes 1967), and small intestine (Muskhelishvili et al. 2003; White et al. Provisionally accepted) have mitotic indices that are at least an order of magnitude higher than those of slower proliferating tissues such as liver (White et al. Provisionally accepted), lung (Shami and Evans 1992), and kidney (Eldridge and Goldsworthy 1996). These higher replication rates would be expected to contribute to higher conversion of stable DNA adducts to mutations, higher mutant responses for a given dose, and a concomitantly lower BMD (i.e., lower sensitivity), which is consistent with our results. In fact, when we plotted the Ki-67 index for liver, lung, and small intestine against the ratio of the BMD₁₀₀ of DNA adducts to BMD₁₀₀ of *lacZ* mutations (**Supp. Figure 6.4**), we obtained a significant positive correlation. Taken together, the data presented here and in the literature highlight the importance of tissue-specific cellular proliferation in the conversion efficiency of stable DNA adducts to mutations.

The tissue-specific difference in DNA-repair capacity is also likely to contribute to the observed sensitivity pattern across tissues. Recent *in vitro* studies of potent alkylating agents have demonstrated that inactivation of O⁶-methylguanine DNA methyltransferase (MGMT) can shift the PoD for mutation and chromosome damage to the left (i.e., more potent)(Thomas et al. 2013; Zair et al. 2011), whereas murine

overexpression of the same repair pathway moves the PoD for tumor formation induced by alkylating agents significantly to the right (i.e., less potent) (Becker et al. 2014). Although differences in repair capacity across various tissues have not been fully explored, it is likely that tissue-specific differences in the presence and activity of certain DNA repair genes will affect the rate of conversion of bulky adducts to mutations. This is consistent with the aforementioned work of Thomas *et al.* (2015), which noted that the four key proteins involved in nucleotide excision repair (i.e., the pathway associated with BPDE removal) can be induced by genotoxic stress.

The direct comparison of BMDs across endpoints is an approach used to permit ranking of mechanistically sequential endpoints (Fukushima et al. 2016; Moffat et al. 2015; Thomas et al. 2007). The approach, which is borrowed from chemical risk assessors' practice of employing the most sensitive endpoint (i.e., lowest NOAEL) for regulatory decision-making, involves ordering endpoints that represent a series of plausible key events, and then comparing PoD metrics across these endpoints. When the BMD₁₀₀ values calculated here are compared across all hematopoietic tissue endpoints (**Figure 6.5a**), the observed BMD pattern is consistent with the sequence of key events leading from genetic damage to cancer. At first glance this is intuitive, as we expect DNA adducts to occur at a dose that is only limited by test article absorption, metabolism, and distribution, and tissue exposure to activated metabolites. Adducts may be repaired, however; and only when this response becomes overwhelmed, presumably at a higher dose, would we expect to see mutations and/or chromosome damage. Thus, the BMD for the next endpoint in the series (i.e., mutations) would be expected to be equal to or higher than that for adducts (Meek et al. 2014), as we found here with the BMD for *lacZ*

mutants in bone marrow, which preceded that for *Pig-a* MF. The lower BMD for *lacZ* mutants is likely a result of the fact that *lacZ* is a multi-copy silent transgene (Shwed et al. 2010), whereas *Pig-a* is an active, single copy endogenous gene (Phonethepswath et al. 2008). As such, *lacZ* transgene DNA is not subjected to transcription-coupled repair (Lambert et al. 2005), resulting in a higher MF for a given dose (i.e., lower BMD) in comparison with an endogenous gene like *Pig-a*. Finally, MN are the result of a relatively narrow range of damage types (i.e., double strand breaks or whole chromosome loss)(Fenech et al. 2011), in comparison with point mutations that can accrue via a range of processes (e.g., base-pair substitutions, frame-shifts, deletions)(Lambert et al. 2005). Therefore, it is logical that a higher BMD would be observed for the MN endpoint than for the mutant endpoints, as we see here.

Although the aforementioned approach, which orders PoD across a series of sequential endpoints, is mechanistically appealing, we contend that it is inappropriate to directly compare BMDs across endpoints with different dynamic ranges of inducible response (**Figure 6.5b**). As specified in Slob (2016) endpoints with very large inducible ranges (i.e., many fold changes between control response and maximum inducible response) can be *expected* to yield very low BMD values as it requires very little *effort* to move the response above background to the BMR. This is especially apparent when comparing an endpoint with a relatively high theoretical maximum and very low detection limit (e.g., DNA adducts) to a continuous endpoint such as MN frequency. For the MN endpoint, the maximum response generally observed is 10- to 20-fold above background (**Figure 6.5b**); in contrast, the observed maximum responses for the DNA adduct and *lacZ* mutant endpoints are 100- to 1000-fold above background. This

difference alone, regardless of the endpoints' sequential involvement in the determination of the adverse outcome, will contribute to a BMD series, for a set BMR, whereby adduct frequency is lowest, followed by mutation and chromosomal damage. The problem is further illustrated by consideration of the cancer endpoint, which is highly constrained with respect to maximum response, as the data are quantal (i.e., animals are tumor bearing or not). Therefore, at a BMR of 100, 100% of the animals are tumor bearing, and thus it is not possible to have an effect size $> 100\%$. However, for continuous endpoints, a BMR of 100 simply reflects a two-fold doubling of control, which is, in comparison a small, and relatively easily achieved effect size (Sander et al. 2005). A 10% increase in the incidence of cancer may be of concern, but, in contrast, a 10% increase in adduct frequency may not constitute a significant disease-related change given the relative *ease* with which the latter endpoint can be *moved* away from the background. The approach presented by Slob (2016) to correct for cross-endpoint differences in theoretical maximum, will be applied to this and other genetic toxicity datasets in a forthcoming manuscript.

Recent works by Soeteman-Hernández *et al.* employed the BMD approach to examine empirical relationships between several genetic toxicity and carcinogenicity endpoints (Hernández et al. 2011; Soeteman-Hernandez et al. 2015a; Soeteman-Hernandez et al. 2015b). However, there is a paucity of data examining quantitative relationships between DNA damage sensitivity (i.e., adduct sensitivity) and mutagenic sensitivity. In our study, when examining the relationship between DNA adducts and *lacZ* mutants, we were only able to see a significant cross-tissue relationship when the comparison was based on the BMD metric. This is the first demonstration of a

quantitative relationship between DNA adduct induction and mutation induction across several tissues, which suggests, at least for BaP, that there is a consistent conversion of adducts to mutations. DNA adduct frequency is a biomarker of exposure, whereas mutations and chromosomal damage are biomarkers of effect, and therefore DNA adduct data are currently used in more of a qualitative manner to establish exposure and genotoxic potential of a compound (Sander et al. 2005). Additional research will be required to demonstrate whether correlations between DNA adducts and mutations exist for other chemicals, but if this is confirmed, it may enable us to reliably use DNA adduct data in a more quantitative manner. This would be advantageous since DNA adduct frequency is much more readily assessed across tissues, and does not require the use of transgenic rodents.

Finally, it is important to note that this novel analysis approach need not be restricted to newly generated data such as those presented here. Analysis of published data, such as that previously presented by our group (Wills et al. 2016a; Wills et al. 2016b), can permit the determination of potency or sensitivity rankings across compounds, tissues, cell types, and/or exposure regimens. The results of such analyses can reduce the necessity of additional studies, thus contributing to an overall reduction in animal use without sacrificing the precision of metrics used for human health risk assessment.

6.7 Conclusions

We documented BaP-induced genetic damage across all endpoints and tissues examined, even at very low doses (e.g., 0.2 mg/kg BW/day for spleen adducts). By

ranking BMD CIs across tissues for a given endpoint, we observed that sensitivity varied significantly across tissues in regards to induced *lacZ* MF, and the differences were more pronounced in comparison with the DNA adduct endpoint. We were unable to observe cell population-specific sensitivity differences for *Pig-a* MF and MN frequency endpoints. We demonstrated that the cross-tissue BMD trend was similar for both DNA adducts and *lacZ* mutants, and that this trend is consistent with tissue-specific differences in metabolism, proliferation, and repair. Moreover, we showed quantitative evidence that the cross-tissue BMD_{100s} for DNA adduct induction are significantly correlated with the BMD_{100s} for *lacZ* mutant induction, illustrating that the *lacZ* MF in a given tissue is proportional to the level of genetic damage, and that this empirical dependency is consistent across the 7 tissues examined. Finally, our cross-endpoint comparisons of BMD₁₀₀ values raised questions regarding the validity of comparisons based on a fixed BMR value. Cross-endpoint comparisons of BMD metrics using a fixed BMR have become unfortunately common, but this work and the earlier work of Slob (2016) indicate that, for comparisons to be meaningful, BMR values must be scaled according to endpoint-specific theoretical maxima. Overall, the BMD-approach employed herein permitted robust comparisons of responses across tissues and endpoints, and the information obtained adds valuable information to our mechanistic understanding of how BaP induces an array of genetic damage across several tissues. BMD rankings within an endpoint, and empirical comparisons across endpoints, contribute to an improved understanding of tissue-specific, chemically induced genetic damage, and this knowledge can provide a foundation for the selection of tissues, endpoints and BMRs for use in human health risk assessments. To enhance current understanding regarding the tissue-

specific fixation of chemically induced genetic damage, future work should continue to examine the cross-tissue relationships between chemically induced mutations and/or chromosomal damage and induced DNA damage.

6.8 References

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CHAPTER SEVEN

7 Chapter: Quantitative relationships between mutagenic and genotoxic potency in MutaTMMouse tissues following oral exposures to polycyclic aromatic hydrocarbons

7.1 Preamble: Authors, Affiliations, and Style

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7.2 Abstract

Polycyclic aromatic hydrocarbons (PAHs) are mutagenic carcinogens that induce mutations via the formation and processing of bulky DNA adducts. However, there is a paucity of information regarding the quantitative, empirical relationship between DNA adduct formation and mutation establishment across tissues; likely because it is challenging to simultaneously make the required measurements. In this study we employed the transgenic MutaTMMouse to simultaneously assess the frequency of DNA

adducts and *lacZ* mutants across 5 tissues following oral exposure to 9 PAHs and 6 PAH mixtures. DNA adduct frequency was assessed in bone marrow, glandular stomach, small intestine, liver, and lung, and we previously determined *lacZ* mutant frequency in the same animals and tissues. The results revealed that despite cross-tissue and cross-agent variability in the frequency of induced DNA damage and mutations, the frequency of the former is inversely related to that of the latter. In large part this appears to be related to the experimental design, and the influence of repair and replication on adduct and mutant frequency. The benchmark dose (BMD)-approach was used to estimate BMD₁₀₀ values (i.e., the doubling dose) for each compound-tissue combination, and subsequently examine empirical relationships between mutagenic potency (i.e., *lacZ* BMD) and genotoxic potency (i.e., adduct BMD) across tissues. The results showed a significant linear relationship across all tissues, as well as for liver, glandular stomach, and lung when these tissues were examined independently. There was no significant difference between the tissue-specific slopes. The results demonstrate that the mutagenic potency of PAHs and PAH mixtures is empirically related to genotoxic potency; moreover, that there is cross-tissue congruence in the processing of PAH-induced damage. An improved quantitative understanding regarding the tissue-specific mutagenicity of stable adducts will improve the ability to interpret adduct frequency values in a risk assessment context.

7.3 Introduction

Polycyclic aromatic hydrocarbons (PAHs), which are produced by the incomplete combustion of organic matter, are a well-studied group of ubiquitous environmental carcinogens. PAHs are found in complex matrices such as tobacco smoke, air pollution,

coal tar, and diesel exhaust, all of which have been classified as Group 1 carcinogens (i.e., known human carcinogens) by the International Agency for Research on Cancer (IARC) (IARC. 2004; IARC. 2010; IARC. 2012; IARC. 2014; IARC. 2015).

Benzo[*a*]pyrene (BaP), also a Group 1 carcinogen (IARC. 2010), is the most well-studied PAH; consequently, it has been designated the prototypical carcinogenic PAH to which all others are compared. Several other PAHs have been classified by IARC as Group 2a (i.e., probable) or Group 2b (i.e., possible) human carcinogens (IARC. 2010).

Metabolites of carcinogenic PAHs readily react with DNA (i.e., they are genotoxic), and carcinogenic PAHs are thought to exert their effects via a genotoxic mode-of-action (IARC. 2010). The mechanistic relationship between tumour initiation/progression and genotoxicity is related to the ability of genetic damage, and concomitant mutations in selected genes, to confer cellular properties such as sustained proliferative signalling, ability to evade apoptosis, and replicative immortality, all of which are hallmarks of cancer (Hanahan and Weinberg 2011). When not properly repaired, DNA damage can lead to the establishment of mutations that are formed during the next round of DNA synthesis and replication. Both DNA damage and mutations are considered key events in the adverse outcome pathway leading from exposure to genotoxic carcinogens to tumour establishment, progression, and metastasis (Preston 2013).

In order for PAHs to react with DNA in the cells of a given tissue, they must (i) be metabolically transformed into DNA-reactive compounds, and (ii) reach the cells of the target tissue, not necessarily in that order. Oral exposure to PAHs is the most important route for humans, and most exposures are dietary (Phillips 1999). Following

ingestion, parent PAHs are absorbed by the gastro-intestinal (GI) tract, and subsequently transported to the liver. The majority of the parent compound is then detoxified via Phase I and Phase II metabolism; however, a subset can be metabolically transformed into highly reactive substances in the liver and GI tract (e.g., small intestine, stomach) (Choudhary et al. 2003). Reactive metabolites, such as PAH diol-epoxides, can subsequently react with deoxynucleosides, forming covalently-linked products known as bulky DNA adducts. From the liver, the parent compound or metabolites can either be returned to the intestine via enterohepatic circulation, resulting in intestinal re-exposure and/or excretion (Miller and Ramos 2001; Ramesh et al. 2004), or systemically circulated, permitting distribution to more distal tissues (e.g., bone marrow and lung).

The diol-epoxide pathway is considered to be the most mutagenic of the three metabolic pathways that are known to produce DNA-reactive PAH metabolites (IARC. 2014). In this pathway, cytochrome P450 1A1 (CYP1A1), CYP1B1, and epoxide hydrolase catalyze the production of the aforementioned PAH diol-epoxides that readily form stable, bulky DNA adducts (Parkinson 2001; Shimada 2006). Via arylhydrocarbon (AhR) receptor agonism, PAHs are able to induce the expression of CYP isozymes. In the case of BaP, the major reactive metabolite is BaP-7,8-dihydrodiol-9,10-epoxide (BPDE), which typically binds to the N² of deoxyguanosine (Hess et al. 1997; Jennette et al. 1977).

Stable bulky DNA adducts are important mutation precursors; however, not all DNA adducts will contribute to the establishment of permanent sequence changes (i.e., mutations). In order for a mutation to be established, the adduct must escape detection by the cell's repair machinery; and importantly, persist through the cell cycle. BPDE-DNA

adducts are primarily repaired via nucleotide excision repair (NER) (Braithwaite et al. 1998; Hess et al. 1997; Iyama and Wilson 2013). NER operates through both global genomic repair (GG)-NER, which scans the entire genome for damage, and transcription coupled repair (TC)-NER, which operates on the transcribed strand of actively transcribed regions (Iyama and Wilson 2013; Marteijn et al. 2014). GG-NER is constantly active and appears to be cell-cycle independent (Fagbemi et al. 2011; Iyama and Wilson 2013), with DNA damage checkpoints influencing cell cycle progression. By influencing cell cycle progression, these checkpoints can prevent damaged DNA from proceeding through critical cell cycle stages such as S-phase, where DNA synthesis takes place (Sancar et al. 2004). If a cell experiences considerable genetic damage it may undergo programmed cell death (i.e., apoptosis), preventing its ability to pass on mutations that may be deleterious.

PAH-DNA adducts that are not repaired can contribute to the establishment of mutations through a process known as translesion synthesis (TLS) (Bauer et al. 2007; Zang et al. 2006). During DNA synthesis the replication fork will stall when high-fidelity replicative polymerases encounter a bulky adduct (e.g., a BPDE adduct) (Jha et al. 2016). Subsequent polymerase switching at the stalled fork allows Y-Family polymerases to carryout TLS; that is, replicate past the damaged nucleotide (Liu et al. 2016; Prakash et al. 2005; Yang and Woodgate 2007). TLS is a process that permits cell survival, as inability to bypass a DNA adduct can lead to replication fork collapse and apoptosis (Roos and Kaina 2013). Importantly, TLS only relates to adduct bypass and not removal; therefore, until detected by NER, adducts can persist throughout the life of the cell,

potentially contributing to the establishment of a mutation during each subsequent round of replication.

Despite a significant body of knowledge related to the mechanisms underlying the establishment of PAH-induced mutations, there is little information regarding the quantitative relationship between adduct formation and mutation formation. The degree to which stable adducts are converted to mutations, which is sometimes referred to as *mutagenic efficiency* (Dosanjh et al. 1991; Jarabek et al. 2009; Verghis et al. 1997), is determined by repair efficacy, DNA replication fidelity, and cellular proliferation rate (i.e., mitotic index). Cellular replication is critically required for PAH adducts to generate permanent sequence changes (IARC. 2010), and consequently, the proportion of cells within a tissue that are undergoing replication will affect the frequency of mutation establishment. Organs such as glandular stomach, small intestine, and bone marrow have higher mitotic indices than organs such as liver and lung (White et al. 2017); therefore, under identical damage loads (i.e., adduct frequency), they can be expected to accrue more mutations for a given exposure regimen. Indeed, a recent *in vivo* study by White et al. (2017) showed that the mutagenic efficiency of total 3-NBA adducts is far lower in liver in comparison with bone marrow. Importantly, the rate of cellular proliferation and extrinsic genetic damage are both well recognized as key factors leading to the accumulation of mutations that contribute to tumour formation (Long et al. 2017b; Poirier 2004; White et al. 2017; Wu et al. 2016). Thus, a series of dynamic processes related to extrinsic damage formation, damage repair, and replication regulate the conversion of DNA adducts to mutations (i.e., the *mutagenic efficiency*).

DNA adduct frequency can be measured using a number of sensitive techniques such as ^{32}P -postlabelling (Phillips and Arlt 2007; Poirier 2016), and adduct frequency has been successfully employed to assess human exposures to DNA-damaging agents such as PAHs (reviewed in Poirier (2016)). However, despite the utility of tissue-specific adduct frequency as a human exposure metric, and the knowledge that DNA damage (i.e., adduct formation) is mechanistically linked to carcinogenesis (Hemminki et al. 2000; Hemminki 1993; Poirier 2004; Poirier 2016), there is little information regarding the quantitative link between tissue-specific adduct frequency and cancer risk. Therefore, although the formation of PAH-induced DNA adducts has been acknowledged as an important event along the continuum between PAH exposure and disease (i.e., cancer), the tissue-specific frequency of chemically induced adducts cannot be used as a quantitative indicator of cancer risk (Hemminki et al. 2000; Jarabek et al. 2009; Poirier 2016; Preston and Williams 2005; Sander et al. 2005; Swenberg et al. 2011). In large part, this is due to lack of knowledge regarding adduct fate, and the cross-tissue mutagenic efficiency of PAH-derived DNA adducts. In contrast, since many mutagens are carcinogens, and established mutations in critical genes have been definitively linked to carcinogenesis (Davies et al. 2002; Futreal et al. 1994; Li et al. 1997; Loeb et al. 2003; MacConaill et al. 2009; Pao et al. 2004; Parsons et al. 1995), induced mutant frequency can be used for regulatory decisions related to carcinogenic risk (Johnson et al. 2015; Johnson et al. 2014; MacGregor et al. 2015). Moreover, since a quantitative relationship between mutagenic potency and carcinogenic potency has been established (Hernández et al. 2011; Soeteman-Hernandez et al. 2015), it is becoming increasingly clear that cancer risk (i.e., carcinogenic potency) is positively correlated with increasing ability to induce mutations

(i.e., mutagenic potency). Improving the ability to interpret adduct frequency in a cancer risk context requires examination of the quantitative relationships between tissue-matched induction of DNA adducts and mutations (Fearon 1997; Hemminki et al. 2000; Preston and Williams 2005). Aside from the aforementioned work by White et al. (2017), and a recent study by Long et al. (2017b), this relationship has rarely been investigated.

Lack of information regarding the quantitative relationship between adduct frequency and mutant frequency is likely related to the difficulty of measuring both endpoints *in vivo* in the same tissue. However, transgenic rodents (TGR), such as the MutaTMMouse, the BigBlue[®] rat and mouse, the *lacZ* plasmid mouse, and the *gpt* delta rat and mouse, can readily be used to score both transgene mutant frequency and adduct frequency in almost any target tissue. Moreover, the same genomic DNA samples can be used to simultaneously assess both endpoints. In this study we used the MutaTMMouse TGR assay to simultaneously score the frequency of PAH-induced *lacZ* transgene mutants and DNA adducts across several target tissues.

The aforementioned MutaTMMouse 3-NBA study involved a single acute exposure, followed by assessment of DNA adduct frequency at 18 h after exposure, and assessments of *lacZ* mutant frequency at 3 and/or 28 days after exposure. Subsequently, mean tissue-specific mutation-to-adduct frequency ratio values were examined to assess the cross-tissue mutagenic efficiency of total adducts (White et al. 2017). More recently, we employed dose-response analysis and the benchmark dose (BMD) approach to investigate cross-tissue correlations between the ability of BaP to induce DNA adducts (i.e., genotoxic potency) and its ability to induce *lacZ* mutations (i.e., mutagenic potency), with both endpoints assessed in the same animals following a sub-chronic, oral

exposure to numerous doses (i.e., the 10 dose MegaBaP study). The results revealed a highly significant correlation between tissue-matched genotoxic and mutagenic potency BMD values (Long et al. 2017b). As a follow-up to those works, this work employs the BMD covariate approach, and dose-response data from smaller, sub-chronic, repeat-dose studies (i.e., 3 doses plus control), to determine genotoxic and mutagenic potency values across 5 tissues, 9 PAHs, and 6 PAH mixtures (i.e., BMD₁₀₀ or doubling dose values). Additionally, the work investigates empirical relationships between mutagenic potency (i.e., BMD₁₀₀ for *lacZ* mutant frequency) and genotoxic potency (i.e., BMD₁₀₀ for adduct frequency).

7.4 Materials & Methods

This study analysed new and previously published results to examine the quantitative relationships between PAH-induced DNA adducts and mutations. More specifically, we previously examined the frequency of MutaTMMouse *lacZ* mutants, across 5 tissues, following sub-chronic oral exposures to 9 PAHs and 6 PAH mixtures (Labib et al. 2012; Labib et al. 2015; Lemieux et al. 2011; Long et al. 2016a; Long et al. 2016b; Long et al. 2017a; Malik et al. 2013). We also employed ³²P-postlabelling to assess the frequency of DNA adducts in the same tissues. Although those studies examined both *lacZ* mutation and adduct data, and the qualitative correspondence between the endpoints, the adduct frequency data were not presented or analysed. With a few exceptions, those data are now presented and analysed herein. The frequency of DNA adducts induced by BaP was published in (Labib et al. 2012; Lemieux et al. 2011), and the frequency of hepatic DNA adducts induced by DBahA (dibenz[*a,h*]anthracene)

was published in (Malik et al. 2013). The frequency of DNA adducts in lung induced by DBahA, benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), chrysene (CHRY), benzo[*k*]fluoranthene (BkF), benzo[*g,h,i*]perylene (BghiP), and indeno[1,2,3-*c,d*]pyrene (INDENO) were published in Labib et al. (2015). Lastly, the frequency of DNA adducts induced by the organic extract of coal tar-based driveway sealcoat (all tissues) was presented in Long et al. (2016b). This is the first presentation of DNA adduct data related to MutaTMMouse exposures to organic extracts of two coal tar samples and three synthetic PAH mixtures, as well as the first quantitative presentation of DNA adduct frequencies induced by most of the individual PAHs in most tissues (i.e., with the exception of BaP). **Table 7-I** provides a summary of the data sources used for the analyses presented in the current work.

Table 7-I. Sources of *lacZ* mutant frequency and DNA adduct frequency data for 5 somatic tissues collected from Muta™Mouse following oral gavage exposures to 9 PAHs and 6 PAH mixtures. Test article doses were given daily for 28 days, with tissues collected 3 days after the final administration. GS: glandular stomach; SI: small intestine; BM: bone marrow.

Compound	<i>lacZ</i> Mutant Frequency					DNA Adduct Frequency				
	Tissue					Tissue				
	Liver	GS	SI	BM	Lung	Liver	GS	SI	BM	Lung
Benzo[a]pyrene	Lemieux et al. (2011)				Labib et al. (2012)	Lemieux et al. (2011)				Labib et al. (2012)
Dibenz[a,h]anthracene	Malik et al. (2013)	Long et al. (2016a)				This study*				Labib et al. (2015)
Benzo[a]anthracene	Long et al. (2016a)				Labib et al. (2015)	This study*				Labib et al. (2015)
Benzo[b]fluoranthene	Long et al. (2016a)				Labib et al. (2015)	This study*				Labib et al. (2015)
Chrysene	Long et al. (2016a)				Labib et al. (2015)	This study*				Labib et al. (2015)
Benzo[k]fluoranthene	Long et al. (2016a)				Labib et al. (2015)	This study*				Labib et al. (2015)
Benzo[g,h,i]perylene	Long et al. (2016a)				Labib et al. (2015)	This study*				Labib et al. (2015)
Indeno[1,2,3-cd]pyrene	Long et al. (2016a)				Labib et al. (2015)	This study*				Labib et al. (2015)
Dibenzo[a,l]pyrene	Long et al. (2016a)					This study*				
Coal tar-sealcoat	Long et al. (2016b)					Long et al. (2016b)				
Coal tar-1	Long et al. (2017a)					This study				
Coal tar-2	Long et al. (2017a)					This study				
Synthetic mixture-1	Long et al. (2017a)					This study				
Synthetic mixture-2	Long et al. (2017a)					This study				
Synthetic mixture-3	Long et al. (2017a)					This study				

* binary qualitative results (i.e., positive or negative) presented in Long et al. (2016a).

7.4.1 Animal treatment

The results of the animal studies were published in 3 different manuscripts (**Table 7-I**), with all experiments conducted in an identical manner under the same animal protocol (Long et al. 2016a; Long et al. 2016b; Long et al. 2017a). Briefly, adult male MutaTMMouse specimens were singly housed in a microVENT ventilated rack (Allentown Inc., Allentown NJ), maintained on a 12h light/12h dark schedule, and fed water and standard rodent chow (2014 Teklad Global standard rodent diet) *ad libitum*. The mice were randomly assigned to dose groups. Three doses of all PAHs and mixtures were prepared weekly, dissolved in highly refined olive oil (Sigma-Aldrich, Oakville, ON, Canada), and administered at 0.005 ml/g body weight. The compounds examined in this study are BaP, BaA, DBaA, BbF, BkF, CHRY, BghiP, INDENO, and dibenzo[*a,l*]pyrene (DBaP). In addition, we examined organic extracts of two coal tars (CT-1 and CT-2) and a coal tar-based driveway sealcoat (CT-Seal), as well as 3 synthetic PAH mixtures (Syn-1, Syn-2, and Syn-3). All mixtures are described in (Long et al. 2016b; Long et al. 2017a). Information on chemical sources, purity, and doses administered are presented in (Long et al. 2016a; Long et al. 2017a). Test articles and the vehicle control were administered daily for 28 days by oral gavage. There were 5 animals in each dose group and the vehicle control group. According to OECD test guideline #488, tissues were collected 3 days after administration of the final dose (OECD 2013). Animals were euthanized by cervical dislocation under isoflurane gas, followed by chest cavity opening. Bone marrow (BM), liver, glandular stomach (GS), small intestine (SI), and lung were processed as described previously (Long et al. 2016a) and stored at -80°C. Animals were bred, maintained, and treated in accordance with the Canadian Council for

Animal Care Guidelines, and Health Canada's Animal Care Committee approved the protocols.

7.4.2 Genomic DNA Isolation

Murine tissues were processed for isolation and purification of total genomic DNA as described previously (Long et al. 2016a). Genomic DNA was isolated and purified using a standard phenol/chloroform procedure described previously (Douglas et al. 1994; Vijg and Douglas 1996). Isolated DNA was dissolved in TE buffer (10 mM Tris pH 7.6, 1 mM EDTA) and stored at 4°C until use.

7.4.3 DNA adduct analysis

The nuclease P1 enrichment version of the thin-layer chromatography (TLC) ³²P-postlabelling assay (Phillips and Arlt 2014) was used to measure the frequency of DNA adduct formation in all DNA samples, as previously described (Krais et al. 2016; Wohak et al. 2016). For mixture exposure samples, total DNA adduct levels were measured in the diagonal radioactive zone (DRZ) area of the TLC plates. As specified in earlier studies, this technique yields values that effectively represent the total frequency of stable, P1 nuclease-resistant adducts induced by PAHs and other aromatic/hydrophobic PACs (polycyclic aromatic compounds) (Kim et al. 2011; Longhin et al. 2013; Molina et al. 2013; Siddens et al. 2012). Thus, the method provides an integrated measure of a complex mixture of adducts present in the postlabelling chromatograms. The results were expressed as DNA adducts/10⁸ nucleotides.

7.4.4 Data analysis

The DNA adduct frequency dose-response data were analysed in SAS v.9.1 (SAS Institute, Cary, NC) by Poisson regression. The data were fit to the model $\log(E(Y_i)) =$

$\log t_i + \beta x_i$, where $E(Y_i)$ is the expected value for the i th observation, β is the vector of regressions coefficients, x_i is a vector of covariates for the i th observation, and t_i is the offset variable used to account for differences in the observation count window. The offset was given a constant coefficient of 1.0 for each observation, and the log-linear relationships between mutants/adducts and test article concentration were specified by a natural log link function. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment.

BMD modeling of the DNA adduct and *lacZ* mutant frequency data was conducted using the PROAST software (www.proast.nl). Datasets were analysed using a nested family of exponential models recommend by the European Food Safety Authority (EFSA) (EFSA 2009). BMD analysis in PROAST uses the likelihood ratio test for selection of the optimal BMD model, with models of increasing complexity (i.e. containing additional parameters) only accepted where the difference in the log-likelihood ratio test exceeded the critical value at $p < 0.05$ (Slob 2002).

Datasets were combined by tissue/endpoint, and analysed using the factor discriminating the dose-response subgroupings (compound) as a covariate. During a combined analysis, the model parameters that require estimation for each subgroup, as well as those that can be considered constant across subgroups is determined. Here, the combined analyses typically assumed that model parameters ' c ' and ' d ' (i.e. maximum response and log-steepness after axis scaling, respectively) were constant across all subgroups, whereas the parameters ' a ', ' b ', and ' var ' (i.e., background response, potency and within-group variation, respectively) were tested for subgroup dependency. Where constant ' c ' and ' d ' shape parameters were utilised, the model fits to the data for each of

the subgroupings were used to visually assess the validity of this assumption of conserved shape.

The benchmark response (BMR) (or critical effect size) used in the presented analyses was 100% (i.e., the doubling dose or doubling relative to control). This value has previously been employed for genotoxicity data; it tends to lie within the range of experimental observations considered herein, and thus, is optimally suited for potency comparisons (Wills et al. 2016a). The BMDL and BMDU values represent the two-sided, lower and upper 90% confidence intervals of the BMD respectively, and thus the ratio BMDU/BMDL defines the precision of the BMD estimate. Confidence interval plots were used to visually compare differences in compound potency/tissue sensitivity, while taking estimation uncertainty into account. Dose-response relationships across subgroupings (e.g. different compounds) were concluded to be significantly different when their confidence intervals do not overlap (Wills et al. 2016a; Wills et al. 2016b).

Quantitative relationships between mutant frequency and adduct frequency were investigated by plotting the tissue-matched BMD₁₀₀ and associated CIs for *lacZ* MF (i.e., the mutagenic potency) versus the BMD₁₀₀ and associated CIs for DNA adducts (i.e., the genotoxic potency). The BMD₁₀₀ and associated CIs for both endpoints were measured in the same tissue in the same animal. Ordinary least-squares linear regression analysis was employed to investigate empirical relationships between genotoxic (i.e., adduct) potency and mutagenic (i.e., *lacZ* mutation) potency for all tissues. The slope of the linear regression may be viewed as a surrogate measure of the *mutagenic efficiency of total adducts* (i.e., the conversion of total bulky DNA adducts into *lacZ* mutants). For all

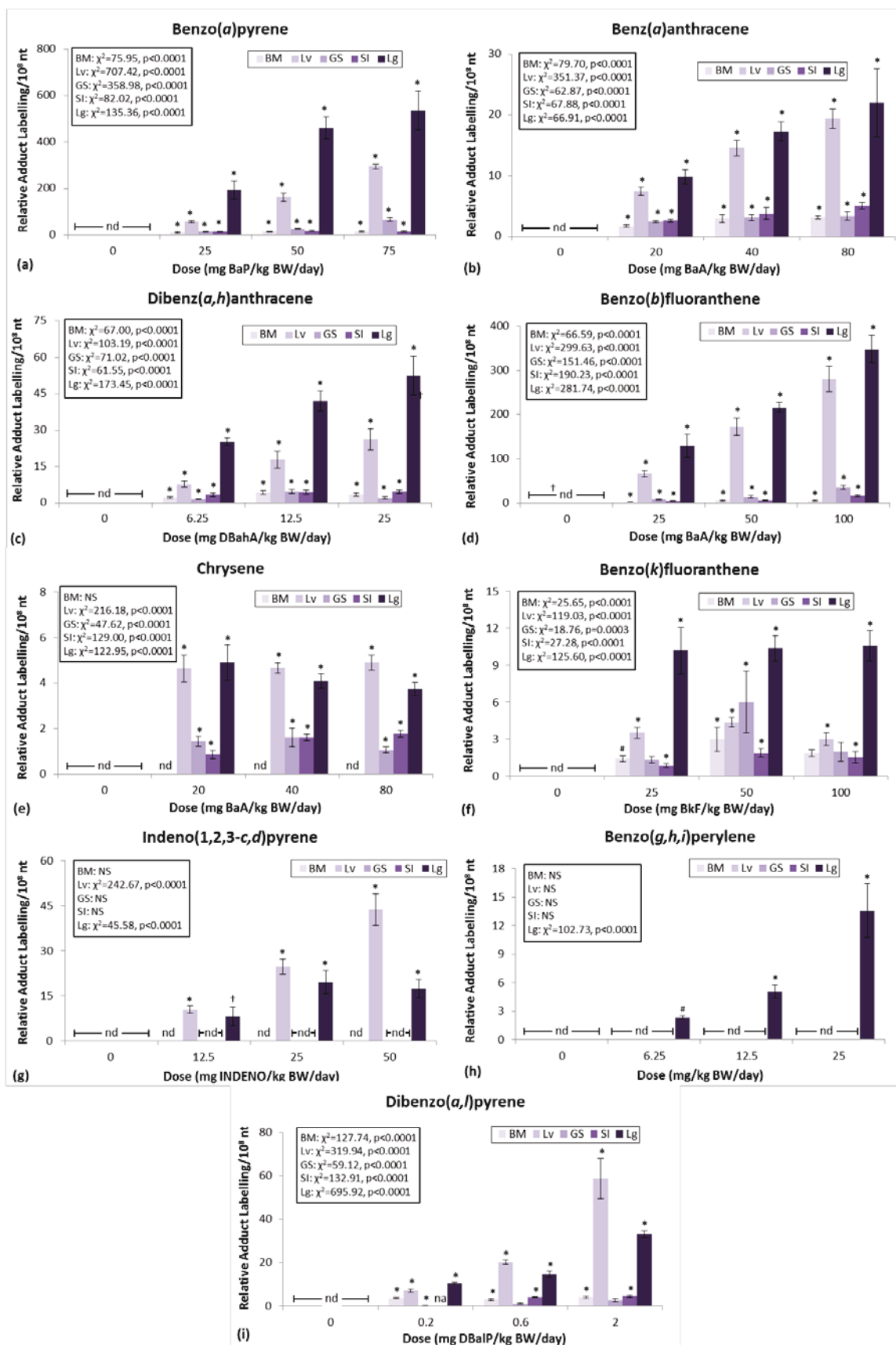
tissues, where a significant linear relationship was observed, the slopes were compared across tissues using ANOVA (analysis of variance).

7.5 Results

7.5.1 DNA adduct frequency

In our earlier study we described the qualitative correspondence (i.e., positive or negative) between the DNA adduct and *lacZ* mutation results for the individual PAHs examined herein (Long et al. 2016a). Here we present quantitative DNA adduct frequency data for most compounds and tissues, as well as new data for PAH mixtures (**Table 7-I**). The new and previously published data were used to examine the quantitative relationships between the induction of DNA adducts and the induction of *lacZ* mutants.

A statistically significant increase in the frequency of DNA adducts was observed for each test article in at least one tissue (i.e., liver, lung, GS, SI, BM) and dose group (**Figure 7.1; Supp. Figure 7.2**). More specifically, BaP, BaA, DBaA, BbF, BkF, DBaP, Syn-1, Syn-2, Syn-3, CT-1, CT-2, and CT-Seal induced a significant increase in adduct frequency across all tissues examined (**Figure 7.1 a-d, f, i-o**). CHRY induced a significant increase in adduct frequency in all tissues except BM (**Figure 7.1e**). INDENO only induced a significant increase in adduct frequency in liver and lung (**Figure 7.1g**), whereas BghiP induced a significant increase in adduct frequency in lung only (**Figure 7.1h**).



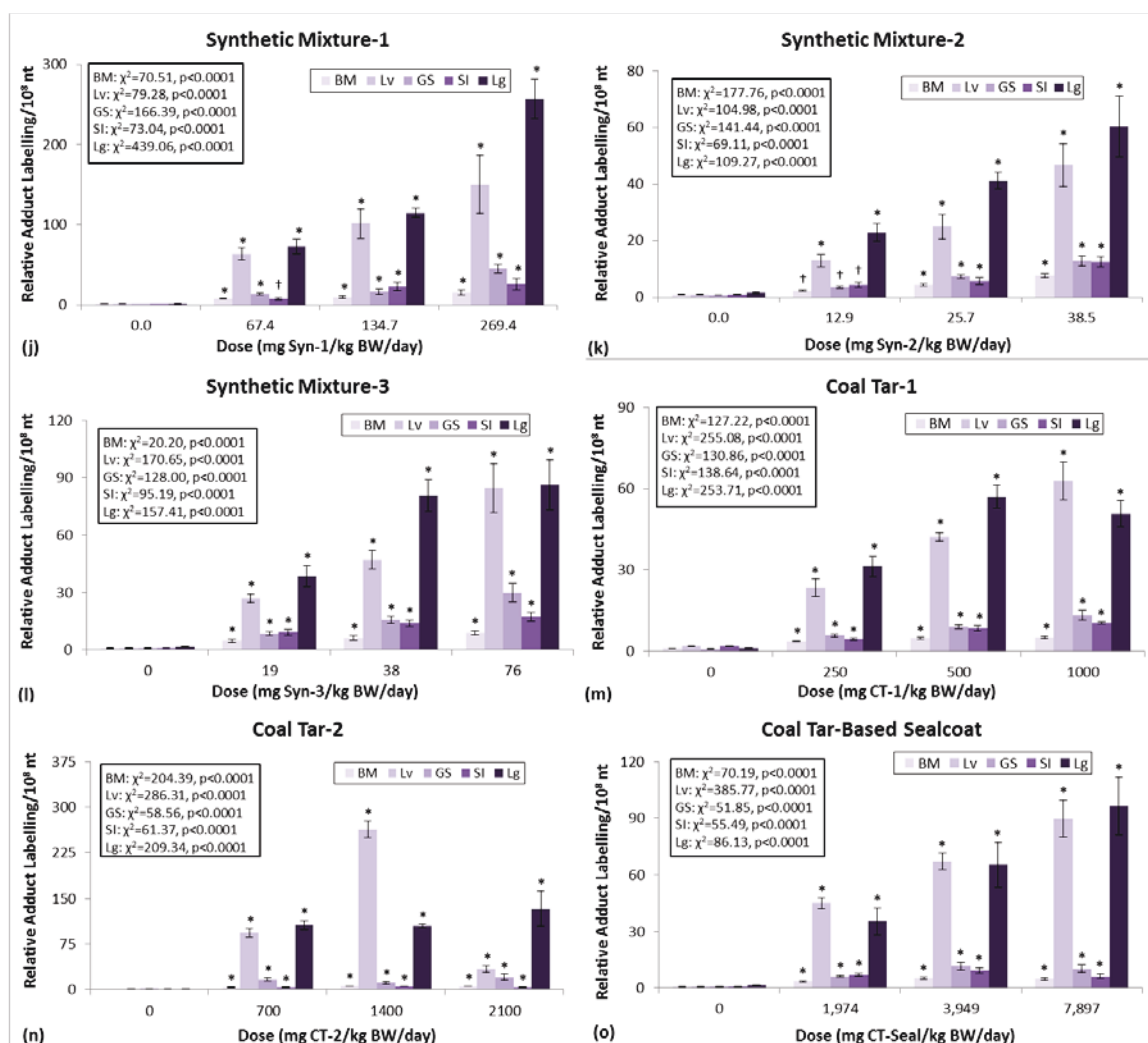


Figure 7.1. The frequency of PAH-induced DNA adducts in bone marrow (BM), liver (Lv), glandular stomach (GS), small intestine (SI), and lung (Lg). Relative Adduct Labelling (RAL) values per 10^8 nucleotides (nts) are displayed for each tissue and dose for (a) benzo[a]pyrene; (b) benz[a]anthracene; (c) dibenz[a,h]anthracene; (d) benzo[b]fluoranthene; (e) chrysene; (f) benzo[k]fluoranthene; (g) indeno[1,2,3-c,d]pyrene; (h) benzo[g,h,i]perylene; (i) dibenzo[a,l]pyrene; (j) synthetic mixture-1; (k) synthetic mixture-2; (l) synthetic mixture-3; (m) coal tar-1; (n) coal tar-2; (o) coal tar-based driveway sealcoat. Statistical results for the tissue-specific dose-response relationships are presented in the inlaid boxes. Results of the custom contrasts for each dose versus the concurrent controls are indicated as follows: *: $p<0.0001$; †: $p<0.001$; #: $p<0.01$. nd = not detected.

Across all compounds and mixtures, the highest frequency of DNA adducts was consistently observed in lung and liver, and the adduct levels in these tissues were notably higher than those observed in GS, BM, and SI. The adduct frequency in lung was higher than, or approximately equal to, that observed in liver for 9 of the 15 compounds, whereas adduct frequencies were higher in liver for 4 of the compounds. For BkF-exposed mice, the DNA adduct frequency in lung was substantially higher than in liver, which was approximately equal to the frequency of adducts observed in the other tissues examined. For BghiP-exposed mice, a significant increase in DNA adduct frequency was only observed in lung. For CT-2-exposed mice, it is interesting to note that the liver response is substantially higher at the mid dose (i.e., 108-fold over control) than the high dose (i.e., 14-fold over control), with the drop presumably related to excess cytotoxicity. This was not the case for CT-2-exposed lung, where the adduct frequency continued to increase with dose (i.e., 42-fold over control at the mid dose, 53-fold over control at the high dose). It was also notable that in BM and SI, the adduct levels frequently reached a plateau at the top two or three tested doses (i.e., 73% and 75% of tested compounds/mixtures that elicited a significant response, respectively). With a few exceptions, a dose-response plateau was not observed for the other tissues (i.e., plateau reached for 33% of compounds in liver and GS, and 36% of compounds in lung).

The *lacZ* mutant frequency data for the complex and synthetic mixtures are presented, with the results of the Chi-square analysis, in **Supp. Figure 7.1**. The results show statistically significant dose-related increases in induced *lacZ* mutant frequency across all tissues for BaP, DBaP, BbF, BkF, and all 6 PAH mixtures. BaA, DBaA, and INDENO induced a significant response in 3 or 4 tissues, whereas CHRY and BghiP

induced a significant response in only 2 tissues at a single dose. As noted, these data were originally presented in (Labib et al. 2012; Labib et al. 2015; Lemieux et al. 2011; Long et al. 2016a; Long et al. 2016b; Long et al. 2017a; Malik et al. 2013).

7.5.2 Correspondence between DNA adduct and *lacZ* mutant frequency data across tissues

When examining the levels of PAH-induced DNA adducts across tissues, the results reveal highest frequency values in different tissues than those where the highest mutant frequencies were observed. More specifically, the highest PAH-induced adduct frequencies are consistently observed in both liver and lung, with the observed adduct frequencies for BM, GS, and SI being routinely 1-2 orders of magnitude lower. This contrasts the *lacZ* mutant frequency results, where the overwhelmingly highest induction of *lacZ* mutants was observed in the SI. In fact, for many of the compounds with a robust response across all tissues, the adduct frequency results are inversely proportional to the mutant frequency results, with the mutant frequencies in SI and GS (e.g., BaA, BbF, BkF, syn mix-1, syn mix-2, syn mix-3) or BM (e.g., BaP, DBaP) often far surpassing those observed in liver and lung.

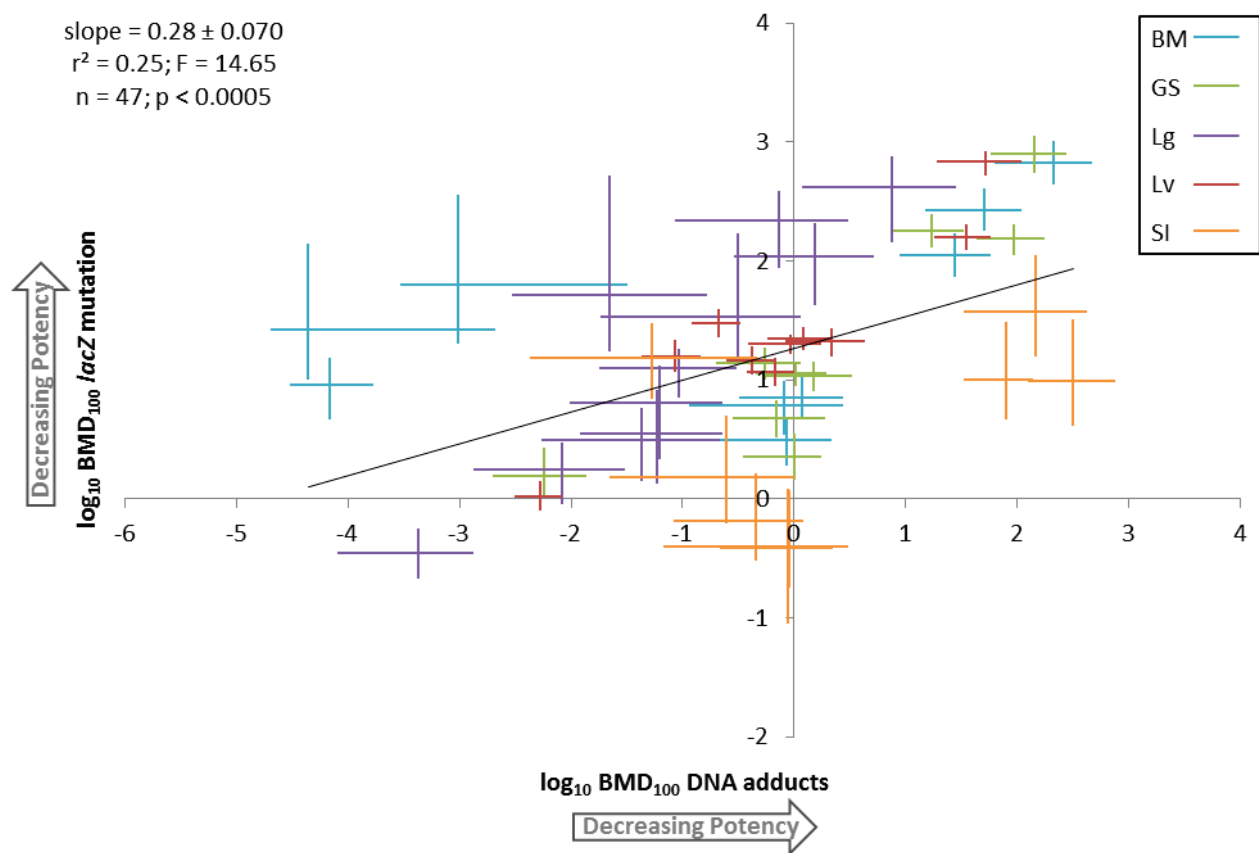


Figure 7.2. Relationship between the BMD_{100} values for the *lacZ* mutation endpoint and the tissue-matched DNA adduct endpoint. The length of the lines represents the 90% confidence limits of the BMD values. Tissues and compounds were included if the 90% confidence intervals were defined for both endpoints ($n = 47$). Linear regression analysis results are shown in the inset.

7.5.3 Quantitative relationships between mutagenic potency and genotoxic potency

The DNA adduct and *lacZ* mutant frequency dose-response data for all 9 PAHs and the 6 PAH mixtures were analysed to determine the BMD₁₀₀ and associated BMDL and BMDU values (i.e., the 90% CI) for each compound-tissue combination. In some cases, the BMDL or BMDU values could not be defined, and the results indicated an infinite CI. Compound-tissue combinations without defined CIs for both endpoints were not included in the subsequent analyses. To examine the empirical relationships between the ability to induce *lacZ* mutations and DNA adducts, tissue-matched BMD₁₀₀ values, and their associated 90% CIs, were plotted against each other (i.e., mutagenic potency vs. genotoxic potency). We first examined the relationship across all compounds and tissues (**Figure 7.2**), which includes 47 compound-tissue combinations that yielded defined CIs. The results revealed a significant positive relationship ($F = 14.65$, $p < 0.0005$) between the two endpoints across all tissues; however, the relatively low r^2 value (i.e., 0.25) indicates that variability in mutagenic potency is only partially explained by genotoxic potency.

To determine whether there are tissue-specific differences in the relationship between mutagenic potency and genotoxic potency, we plotted the BMD₁₀₀ and 90% CI values for the *lacZ* mutant endpoint versus DNA adduct endpoint for each tissue (**Figure 7.3a**). When plotted independently by tissue, the results show a significant positive relationship for three of the five tissues examined (**Figure 7.3a**). Specifically, we observed a significant positive relationship for GS ($F = 28.96$, $p = 0.001$) with an r^2 of 0.81, for liver ($F = 46.39$, $p < 0.0002$) with an r^2 of 0.85, and for lung ($F = 37.72$,

$p < 0.0002$) with an r^2 of 0.81 (**Figure 7.3a**). It is interesting to note that the DBalP value and the CT values anchor the regressions on the lower left and upper right areas of the plot, respectively, for all three tissues. A significant correlation between mutagenic potency and genotoxic potency was not observed for either BM or SI (**Supp. Figure 7.3**).

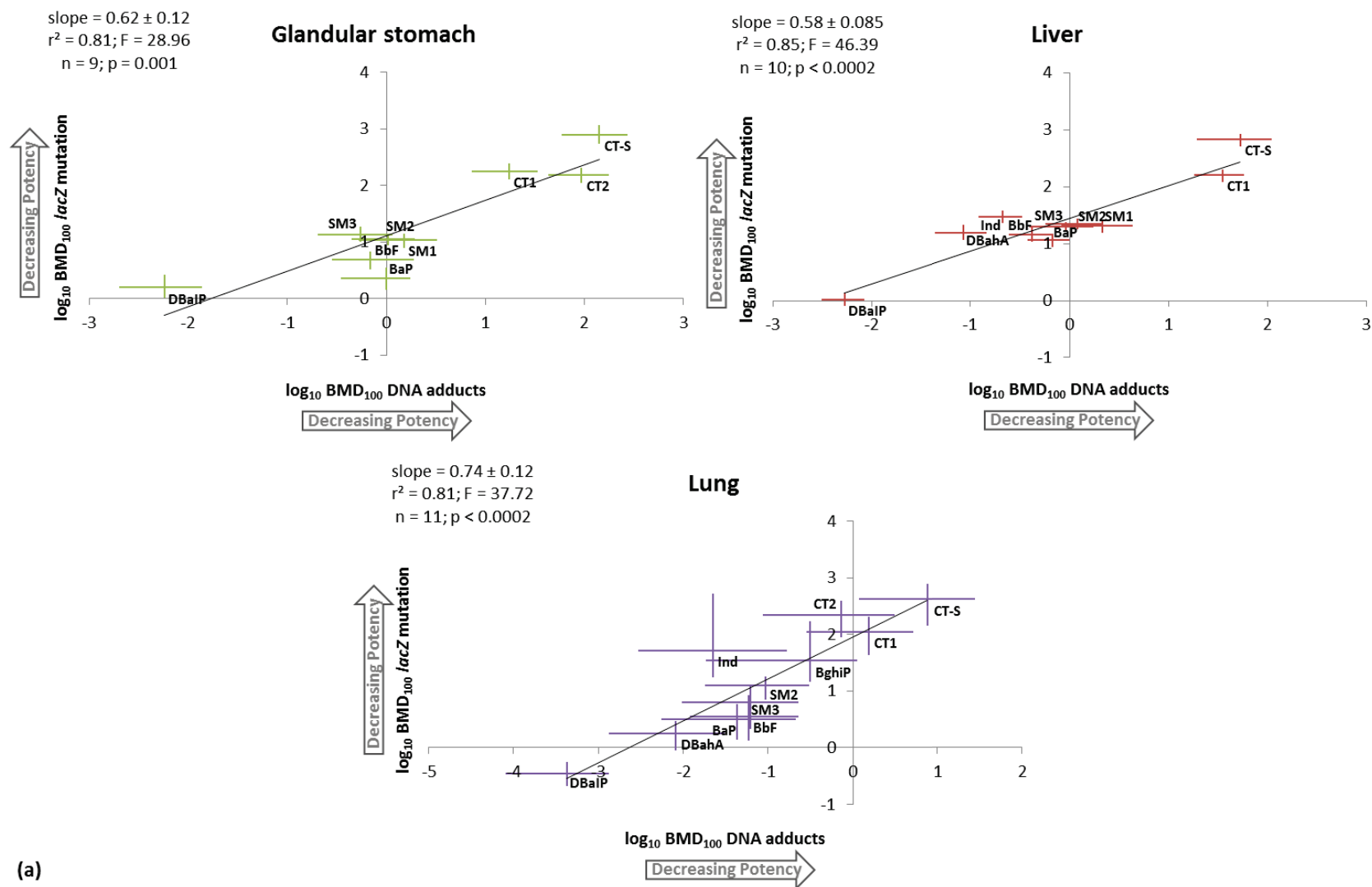
We subsequently re-plotted the data for the three aforementioned tissues (i.e., GS, liver, lung), leaving out the results for tissues where we did not observe a significant BMD correlation (i.e., BM, SI). This analysis also revealed a significant positive relationship between mutagenic and genotoxic potency (i.e., $F = 65.56$, $p < 0.0001$), with an r^2 of 0.70 (**Figure 7.3b**). Given the aforementioned 5-tissue relationship (**Figure 7.2**), and the relationships for GS, liver, and lung (**Figure 7.3a**), it is not surprising that a bivariate plot including only 3 tissues (i.e., without BM and SI) also shows a significant relationship between the endpoints. This relationship is clearly driving the 5-tissue relationship shown in **Figure 7.2**.

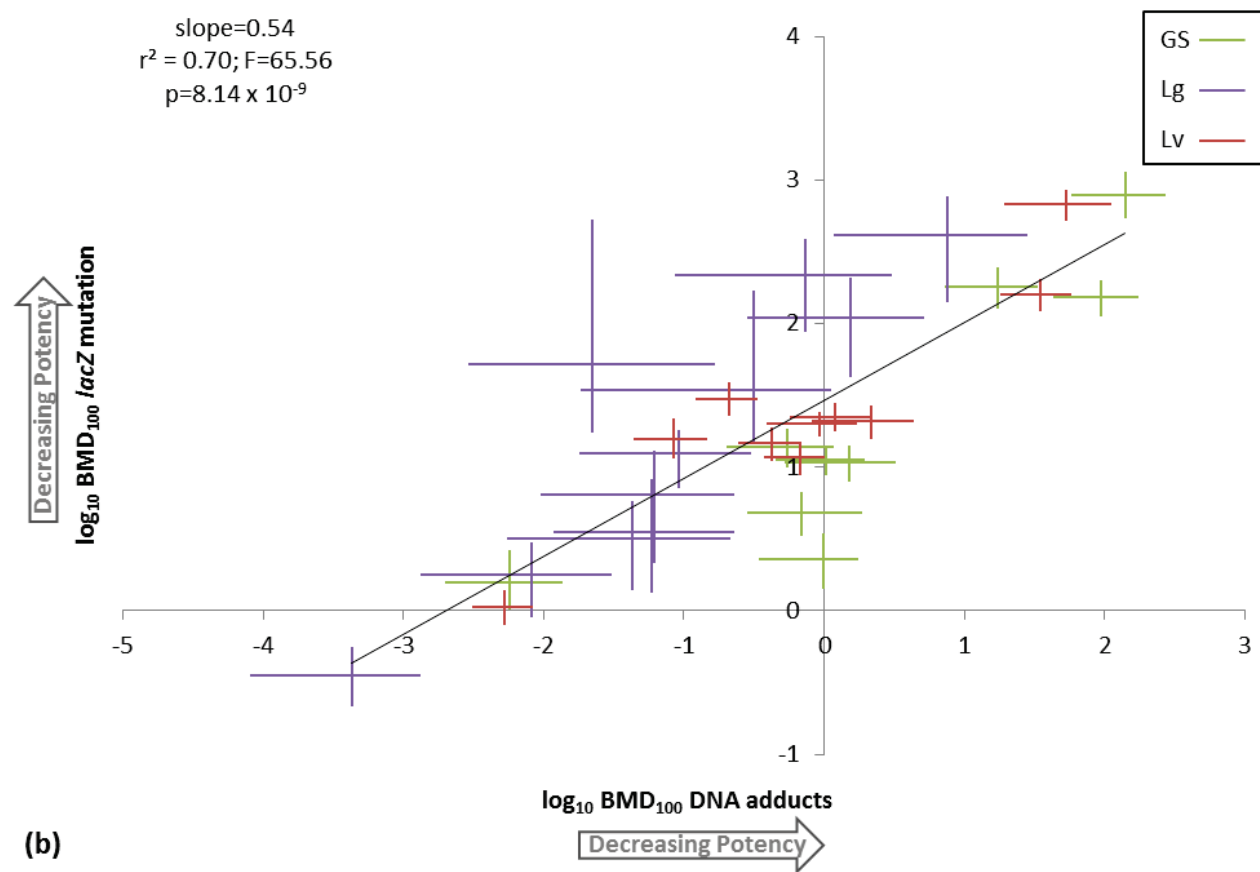
7.5.4 Comparisons of tissue-specific slopes of the mutation-adduct potency plots

We subsequently compared the slopes from the aforementioned bivariate plots that yielded significant empirical relationships (i.e., GS, liver, lung; **Figure 7.3a**). The slopes may be viewed as a surrogate for the *mutagenic efficiency of total adducts*, which has been defined as the ratio of mutant frequency to adduct frequency (White et al. 2017). The results did not reveal significant differences between the slope values across tissues.

Figure 7.3. (a) Relationship between the BMD₁₀₀ values for the *lacZ* mutation endpoint and matched DNA adduct endpoint for tissues that yielded a statistically significant relationship (i.e., p for F ratio <0.05). The length of the lines represents the 90% confidence limits of the BMD values. Data are plotted for glandular stomach, liver, and lung. For each tissue, test article BMD values were included if the 90% confidence intervals were defined for both endpoints. Linear regression analysis results are shown in the inset. The bone marrow and small intestine data did not reveal a significant relationship (see **Supp. Figure 7.3. a, b**).

(b) Relationship between the BMD₁₀₀ values for the *lacZ* mutation endpoint and the matched DNA adduct endpoint for tissues that showed a significant relationship (i.e., glandular stomach, liver, and lung). The length of the lines represents the 90% confidence limits of the BMD values. Tissues and compounds were included if the 90% confidence intervals were defined for both endpoints ($n = 47$). Linear regression analysis results are shown in the inset. BaP: benzo[*a*]pyrene; DBahA: dibenz[*a,h*]anthracene; BbF: benzo[*b*]fluoranthene; BkF: benzo[*k*]fluoranthene; Ind: indeno[1,2,3-*c,d*]pyrene; BghiP: benzo[*g,h,i*]perylene; DBaP: dibenzo[*a,l*]pyrene; SM1: synthetic mixture-1; SM2: synthetic mixture-2; SM3: synthetic mixture-3; CT1: coal tar-1; CT2: coal tar-2; CT-S: coal tar-based driveway sealcoat.





7.6 Discussion

The Muta™Mouse TGR assay is unique since it allows assessment of chemically-induced transgene mutant frequency in almost any tissue of interest (Lambert et al. 2009); accordingly, the assay is extremely useful for understanding tissue-specific genotoxic responses. The Muta™Mouse assay is known to be sensitive to a wide variety of mutagens, including PAHs (Lambert et al. 2009; Long et al. 2016a); moreover, many other genotoxicity assays can easily be integrated with the assay (Lemieux et al. 2011; Long et al. 2017b). The integrated assessment of multiple endpoints in various tissues maximizes the utility of each animal study, permitting examination of empirical and functional relationships between endpoints while reducing the number of animals required to investigate these relationships. Of particular interest is the relationship between DNA damage and mutation endpoints, since damage events precede the establishment of mutations, and this relationship has rarely been quantitatively investigated.

7.6.1 DNA adduct frequency

Although qualitative (i.e., binary) interpretations of some of the DNA adduct results have been presented previously, this is the first quantitative analysis of those data. As mentioned earlier, the DNA adduct frequency observed at a given post-exposure time point reflects the amount of a genotoxic compound or metabolite that has encountered the tissue of interest, the amount of a reactive metabolite or reactive metabolites that has/have interacted with DNA to form stable adducts, and the amount of adducts that have escaped cellular DNA repair. Differences in compound distribution, metabolism, repair, and adduct stability will therefore influence the adduct frequency observed at a

given sampling time (Braithwaite et al. 1998; Lagerqvist et al. 2011; Ramesh et al. 2004; Shimada 2006; Xue and Warshawsky 2005). Since we were able to observe DNA adducts in all tissues for some of the PAHs, it seems that there is sufficient systemic distribution of most compounds/metabolites, and/or sufficient metabolic capacity in the tissues examined. The exception is BM, for which we did not observe significant increases in adduct frequencies for CHRY, INDENO, and BghiP. It is not clear whether this is due to lack of BM exposure to systemically distributed metabolites, or a lack of tissue-specific metabolism, or both. BM is known to have high levels of CYP1B1, but minimal CYP1A1 (Heidel et al. 1998); and therefore, local ability to generate reactive metabolites may be limited. Another possibility is that adducts were induced in BM, but they were less stable and caused depurination prior to the sampling time (i.e., 3-days after exposure termination). Indeed, CHRY-induced DNA adducts have been observed to cause slow depurination over an observation period of 24 hours (Braithwaite et al. 1998). A more thorough (qualitative) discussion of the BM adduct results can be found in (Long et al. 2016a).

The observed tissue-specific adduct frequencies will be influenced by the post-exposure sampling time. For example, when examining adduct frequencies within a few hours of dosing termination, levels may not yet have reached a maximum due to ongoing distribution of compounds and/or metabolites to target tissues, and/or *in situ* metabolism of the parent compound. In contrast, since adducts will be eliminated through repair and apoptosis, and diluted by cellular proliferation, adduct frequencies can be expected to decline if the frequency is assessed at later post-exposure time-points (i.e., days after dosing termination). In our study, the tissues were collected 3 days following the last

dose, which is recommended as the most pragmatic time point for the transgene mutant frequency endpoint (OECD 2013). However, this sampling time may not be ideal for analysing DNA adduct frequency, especially in tissues with high proliferation rates. This assertion is consistent with an earlier study by our group that examined temporal trends in DNA adduct frequencies in MutaTMMouse BM following an acute oral exposure to DBaP (Chepelev et al. 2016). At 2 mg/kg BW/day, the high dose examined in the current sub-chronic study, the results showed that adduct frequency significantly declined with each successive post-exposure time point. More specifically, measured BM adduct frequencies (i.e., per 10⁸ nucleotides) at 4 h, 24 h, and 72 h were 14.7, 7.1, and 2.7 (Chepelev et al. 2016). These data permit estimation of the DBaP adduct half-life in BM, and the value obtained (i.e., 29 hours) indicates that the 3-day post exposure sampling time employed herein is nearly equivalent to 3 half-lives. Consequently, adduct levels at a 3 day sampling time can be expected to be 1/8 the level present at dose termination. In a separate study, we investigated adduct frequencies at 4 h, 16 h, 24 h, and 48 h following an acute oral MutaTMMouse exposure to BaP (unpublished). Those results revealed that although adduct frequencies 2-days post exposure indicated only a slight decline in lung (91% of adducts remaining), GS (83% of adducts remaining), and liver (65% of adducts remaining), the adduct frequencies in SI and BM decreased significantly (i.e., 43% and 37% of adducts remaining, respectively). Therefore, by only 2-days post exposure, the frequency of PAH adducts in some tissues can be reduced to less than 50% of the level present immediately after exposure termination. Sampling time is therefore an important determinant of adduct frequency in a given tissue, with the effects of sampling time being more pronounced in highly proliferative tissues such as SI and BM.

It is difficult to precisely delineate the causes of tissue-specific, post-exposure changes in adduct frequency across the PAHs examined; however, the current state of knowledge indicates that the aforementioned processes (i.e., metabolism, distribution, repair, proliferation) are critically involved. Since, the frequency of adducts at any given dose for any given compound-tissue combination is presumably driven by competition between adduct establishment and maintenance and the incorporation of new nucleotides during replication, variations in cellular proliferation rates across tissues can be expected to alter the adduct frequency observed at the sampling time (e.g., 3 days post-exposure). In a related study we examined the Ki-67 labeling index (LI) (i.e., a marker of cellular proliferation) in liver, lung, and SI of control MutaTMMouse specimens, and found that liver had the lowest LI (mean = 0.017 ± 0.0050), with lung having a 3-fold higher LI (mean = 0.056 ± 0.012), and SI being nearly 5-fold higher than lung and 15-fold higher than liver (mean LI = 0.26 ± 0.037) (Long et al. 2017b). That work did not examine LI for BM or GS; however, a recent publication by White et al. (2017) noted that the average of published MI (mitotic index) values for BM is 1.5-fold greater than that of SI. Neither study examined stomach; however, due to epithelial lining renewal, this tissue likely has an average MI that is similar to SI. In the present study, for a given test article, the adduct frequencies observed at 3-days post-exposure were consistently elevated in tissues with low proliferation rates (i.e., lung, liver), whereas the lowest adduct frequencies were observed in more proliferative tissues (i.e., SI, BM, GS). This observation is consistent with our contention that high proliferative capacity contributes to tissue-specific reductions in observed adduct frequency via dilution of the adducted nucleotides during replication. Therefore, optimal sampling times for tissue-specific adduct frequency

determination will vary according to proliferation rates. For tissues with high cellular proliferation rates (e.g., BM, SI, etc.), accurate assessment of PAH-induced adduct frequency likely requires tissue collection within a few hours of exposure termination. Timing would not be as critical for tissues with low proliferation rates (e.g., lung, liver).

It is also likely that tissue-specific differences in DNA repair are contributing to differences in adduct yield. As the vast majority of DNA repair studies have been conducted *in vitro*, there is little information on tissue-specific differences in repair capacity (Marteijn et al. 2014). However, it is recognized that GG-NER is likely to be most important for the viability and function of actively dividing cells (Iyama and Wilson 2013; Marteijn et al. 2014). GG-NER appears to be more important in highly replicative cells (Iyama and Wilson 2013), as it is thought that priority must be given to ensuring the integrity of the genome, whereas in non-dividing cells, priority must be given to transcriptional fidelity (i.e., TC-NER is prioritized) (Marteijn et al. 2014). In fact, in terminally differentiated cell types, there is reduced removal of UV-induced lesions throughout the genome, but TC-NER activity seems to be largely unaffected (Marteijn et al. 2014; Nospikel and Hanawalt 2002; van der Wees et al. 2007). This is congruent with the results of the current study, wherein we observed high adduct frequencies in slowly proliferating tissues, and low adduct frequencies in highly proliferative tissues. However, we also observed high mutant frequencies in the tissues with low adduct levels (and vice versa), so it is clear that repair is not solely responsible for the cross-tissue differences in adduct frequencies, and proliferation-induced dilution is likely the main contributor to the tissue-specific differences in DNA adduct frequency.

7.6.2 *lacZ* Mutant frequency

The measured frequency of *lacZ* mutants reflects the frequency of permanent sequence changes induced by exposures to the test article under investigation. This endpoint will be, at least in part, controlled by the aforementioned factors that regulate the formation of DNA adducts. However, mutation formation is also dependent on the fidelity of TLS, and implicitly dependent on tissue-specific replication rate (Hashimoto et al. 2012; IARC. 2010; Jha et al. 2016; Lagerqvist et al. 2011; Liu et al. 2014). Since DNA replication is required for the establishment of mutations (i.e., for lesions to be fixed into permanent sequence changes), proliferation rate can be considered a major determinant of chemically-induced mutant frequency in a given tissue (Lagerqvist et al. 2011; White et al. 2017). Therefore, as with DNA adducts, both the post-exposure sampling time and the tissue-specific proliferation rate can be expected to be important determinants of mutant yield. More specifically, for effective enumeration of chemically-induced *lacZ* mutants, longer sampling times are required for tissues with lower proliferation rates (Heddle et al. 2003; Thybaud et al. 2003). For this reason, although the OECD test guideline recommends a 28-day exposure followed by a 3-day sampling time, it also states that if slowly proliferating tissues are deemed to be of particular importance, a later sampling time should be considered (i.e., 28 days) (OECD 2013). Indeed, this fits with the cross-tissue trend in *lacZ* mutant frequencies observed in this study. Specifically, the highest mutant frequency was routinely observed in SI, with some compounds (i.e., BaP, DBaP) also inducing high frequencies in BM. Since SI has a high proliferation rate and is proximal to the exposure route, it would be expected to experience high exposures to reactive metabolites (Long et al. 2016a), and routinely exhibit high mutant frequencies

(Miller and Ramos 2001; Van Schooten et al. 1997). BM also has a high proliferation rate; however, as discussed, it is distal to the exposure route, and exposure to reactive metabolites may be limited by compound distribution and tissue-specific metabolic capacity. In contrast, we routinely observed the lowest PAH-induced mutant frequencies in liver, as well as lung for most PAHs; these are both tissues with low proliferation rates (Long et al. 2017b; White et al. 2017). It is important to note that in some cases we did observe robust responses in lung (i.e., for DBahA). In addition, for some compounds that induced positive responses in other tissues, we saw very low (i.e., for BbF, BkF, CT-1, CT-2, and CT-Seal) or no responses (i.e., for DBahA, INDENO, BghiP) in BM, highlighting that factors other than proliferation are important for determining the mutation burden of a tissue. A more thorough discussion regarding the likely causes of cross-tissue, compound-specific variability in mutant frequency is presented in Long et al. (2016a).

It is important to highlight that the detected *lacZ* mutations may arise from other forms of genetic damage (e.g., depurinating adducts) that do not produce stable DNA adducts detected using the ³²P-postlabelling assay (Phillips and Arlt 2007). Although formation of bulky adducts produced via the diol-epoxide pathway is largely considered the major mutagenic pathway of PAHs, two other metabolic pathways generate DNA reactive PAH derivatives (IARC. 2014). Thus, additional work is required to assess the relative ability of tissue-specific metabolic pathways to generate reactive PAH metabolites other than diol-epoxides; and moreover, the relative mutagenicity of these metabolites.

7.6.3 Comparisons between adduct and mutant frequencies across tissues

The results showed frequent contrasts between tissue-specific adduct frequencies and tissue-specific mutant frequencies. For example, tissues with high levels of adducts (i.e., liver, lung) were different from those with high levels of mutations (i.e., SI, GS, BM), and vice versa. This is presumably a result of the semi-conservative nature of DNA replication that would be expected to dilute adducts while concomitantly increasing the establishment of mutations via error-prone TLS and subsequent clonal expansion. For example, if one assumes that the initial adduct results in a mutation, and cells continue to proliferate after dose termination, then 20 successive rounds of replication would yield 2^{20} cells, half of which would be expected to contain a mutation via clonal expansion. In contrast, since DNA adducts will either be repaired or remain in the parent cell, post-exposure adduct frequency can be expected to be continually diluted by proliferation. Thus, we contend that differences in tissue-specific proliferation rates are a driving factor underlying lower adduct frequencies and concomitant higher mutant frequencies observed in tissues such as SI and BM; however, other factors such as sampling time, the level of tissue exposure and tissue-specific metabolism, the level of NER, and the fidelity of TLS, are also important.

Initially, we intended to employ the results of this study to examine the mutagenic efficiency of total adducts across compounds and tissues. Typically, mutagenic efficiency is assessed by examining the dose-specific ratios of mutant frequency and adduct frequency (White et al. (2017)). However, investigations of this nature require adduct frequency values that are determined shortly after exposure, with an extended follow-up for mutant frequency determination. Indeed, the study by White et al. (2017) employed a

sub-chronic sampling time for determination of mutant frequency (i.e., up to 28 days), and an acute sampling time for determination of adduct frequency (i.e., 18 h). In contrast, this study assessed both DNA adduct and *lacZ* mutant frequency at the sampling time recommended for the mutation endpoint (OECD 2013), and as mentioned above, kinetic differences between these two endpoints indicate that this sampling time is not ideal for the assessment of DNA adducts, especially in tissues with relatively higher proliferation rates. In fact, we did examine the relationships between *lacZ* mutant frequency and DNA adduct frequency in each tissue (data not shown). However, we did not find a correlation between these metrics, presumably due to the previously discussed differential effect of sampling time on the two endpoints. As an alternative tactic, the BMD-approach was employed to examine empirical relationships between genotoxic and mutagenic potency across compounds and tissues.

7.6.4 Comparisons of genotoxic and mutagenic potency using the BMD-approach

For DNA adducts, the results revealed a large range in genotoxic potency (i.e., BMD₁₀₀) across compounds. Potency differences across the PAHs and mixtures examined may be explained by differences in the ability to induce the expression of enzymes required to generate genotoxic metabolites, as well as differences in the stability of the adducts they produce and the manner in which the adducts are detected/repared. Since the PAHs examined herein are not equipotent AhR agonists (Machala et al. 2001; Ziccardi et al. 2002), variability in receptor agonism will influence the degree to which the exposure can augment enzyme expression, contributing to variations in the capacity to convert parent compounds to DNA-reactive metabolites. Moreover, variation in the structure and stability of PAH adducts is known to influence their ability to alter DNA

structure, and thus be detected by repair machinery (Lagerqvist et al. 2011). For example, some adducts such as those produced by BaP cause large double helix distortions, which allows them to be readily recognized by NER machinery. In contrast, DBaP adducts cause less distortion, thus increasing the ability to escape NER detection and effectively yield large dose-related increases in adduct frequency (i.e., lower BMD) (Buterin et al. 2000; Dreij et al. 2004; Dreij et al. 2005; Jankowiak et al. 1998; Lloyd and Hanawalt 2002; Melendez-Colon et al. 2000; Ruan et al. 2002; Scicchitano 2005; Suh et al. 1995). This is further supported by an *in vitro* study by Lagerqvist et al. (2011) that noted compound-specific differences in the DNA binding rate of BaP-, BPDE-, and DBaP-dihydrodiol epoxides; and moreover, the resulting adduct stability and NER detection efficacy. A separate study found that BaP adducts are much more stable than those produced by CHRY. The latter cause depurination resulting in AP sites that are thought to be more readily repaired; and moreover, would not be detected by ³²P-postlabelling (Christmann et al. 2016). All of these factors would be expected to contribute to the wide range in genotoxic potency across PAHs and PAH mixtures.

Across tissues, we might reasonably expect the aforementioned tissue-specific differences in metabolic profiles to affect the exposure of each tissue to reactive metabolites, resulting in different ranges in cross-tissue genotoxic potency. Interestingly, the BMDs actually overlapped fairly well across tissues, with lung and BM only having slightly lower BMDs than the remaining tissues. Both *Cyp1a1* and *Cyp1b1* are highly expressed in the mouse lung (Arlt et al. 2015; Choudhary et al. 2003; Renaud et al. 2011), and constitutive expression of *Cyp1b1* in BM has been demonstrated to be important in determining the toxicity of BaP in this tissue (Nebert et al. 2004; Nebert et al. 2013; Shi

et al. 2010; Uno et al. 2006). However, it is important to note that the BM adduct frequency responses often reached a plateau, with several doses exhibiting zero-order kinetics. Thus, the data are poorly suited to BMD estimation, and it is difficult to effectively compare BM potencies with those of other tissues. The lack of precision in the BMD₁₀₀ for BM is illustrated by the width of the CIs relative to those of other tissues (**Figure 7.2**). Further investigations regarding the cross-tissue differences in PAH genotoxic potency could assess frequency changes over refined dose ranges that are better suited to BMD modeling. Such data would permit more accurate and precise BMD determinations, and more robust cross-tissue comparisons.

7.6.5 Relationships between genotoxic and mutagenic potency

The plots of mutagenic potency versus genotoxic potency for individual tissues clearly show that DBaP is the most potent PAH for both endpoints, whereas the CTs are the least potent. These observations anchor the extremes of the regressions; consequently, they are likely influential observations. DBaP is a PAH with a Fjord-region, which confers increased stability of the diol-epoxide adduct (Glatt et al. 1991). Moreover, as mentioned above, the shape of the DBaP-diol-epoxide confers reduced detection by GG-NER (Buterin et al. 2000; Dreij et al. 2004; Dreij et al. 2005; Jankowiak et al. 1998; Lloyd and Hanawalt 2002; Melendez-Colon et al. 2000; Ruan et al. 2002; Scicchitano 2005; Suh et al. 1995). Stable, persistent adducts are considered the most mutagenic (Braithwaite et al. 1998), and the mutagenicity per adduct was found to be 4-times higher for the fjord-region diol-epoxide of DBaP, in comparison with BPDE (Lagerqvist et al. 2011). These published observations are consistent with the results of this study. In contrast, the reduced potency of the three CTs is likely related to their composition,

which would include a range of non-genotoxic PAHs (e.g., anthracene, naphthalene, etc.), as well as other PACs that are unlikely to generate stable bulky adducts (Long et al. 2016b; Long et al. 2017a). Thus, the complex CT samples are not expected to induce adducts and/or mutations at doses comparable to the other PAHs examined, all of which are mutagenic. It is interesting to note that the INDENO liver observation appears above the regression line, suggesting that the induced adducts are less mutagenic than the regression would predict. This observation is supported by the fact that INDENO does not have a classical bay region, is a non-alternant PAH, and there are no data to support a diol-epoxide mechanism for the formation of INDENO-induced DNA damage (IARC. 2010). Rather, bacterial data indicates that INDENO-1,2-oxide is the ultimate mutagenic metabolite (IARC. 2010).

The significant relationships between mutagenic and genotoxic potencies observed for lung, liver, and GS suggest that, within each tissue, there is similar processing of the PAH-induced adducts. As the measured adducts have already escaped repair by the time of tissue collection, TLS is likely the major contributor to their mutagenicity, with cross-compound similarity in TLS likely being responsible for the apparent similarity in tissue-specific conversion of adducts into mutations. That is, the results obtained suggest similarity in TLS processing of PAH adducts. Although the fidelity of Pol κ 's BPDE bypass is known to be determined by its compatibility with the active site (Jha et al. 2016), the ability of Y-class polymerases to similarly by-pass other PAH adducts is not known. Consequently, more work is required to determine the ability of Y-class polymerases to bypass PAH adducts; moreover, the efficacy of TLS across PAH adducts (i.e., error-prone versus error-free). Other Y- and B-family polymerases

have also been shown to have the ability to by-pass PAH adducts. For example, the Y-family polymerase Pol η and the B-Family polymerase Pol ζ can bypass BPDE adducts in an error-prone manner (Christmann et al. 2016; Hashimoto et al. 2012); however, it is unclear whether this is also the case for adducts induced by other PAHs. Furthermore, the bacterial Y-family polymerase Dpo4 can carryout TLS past all three N⁶-adenyl PAH adducts (i.e., two from BaA and one from BaP) in a largely error-free manner, with only slightly varying misincorporation frequencies (Zang et al. 2006). Thus, although there is already evidence to support the asserted similarity in the processing of the PAH-induced adducts into mutations, additional research will be required to delineate the fidelity of TLS across various types of PAH adducts. Although technically challenging, such research endeavours are necessary to determine whether different PAH adducts in different tissues are likely to induce mutations with similar efficiency.

For tissues where a significant relationship was observed between genotoxic and mutagenic potency (i.e., lung, liver, GS), the slopes were not significantly different. This suggests that cross-tissue processing of PAH-induced adducts may also be similar, a contention that is supported by published information pertaining to tissue-specific levels of TLS polymerases. For example, the gene encoding Pol κ (i.e., *Polk*) has been shown to be transcriptionally controlled by the aryl-hydrocarbon receptor (AhR), indicating that some PAHs will be able to induce its expression (Ogi et al. 2002). In addition, Pol κ appears to be expressed across many tissues including lung (Ogi et al. 2002; O-Wang et al. 2001), and a recent study observed PAH-induced expression of Pol κ in the lung of MutaTMMouse males (Labib et al. 2012; Labib et al. 2015). Nevertheless, it is not clear how the tissue-specific induction of *Polk* expression will affect the frequency of PAH-

induced damage and mutations across numerous tissues and agents. Unfortunately, information related to the cross-tissue expression and activity of TLS polymerases is very limited. More definitive understanding of the mechanisms underlying the cross-tissue similarities in the observed slopes of the mutation-adduct potency relationships will require more information regarding TLS processing of PAH-induced damage within and between tissues.

In addition to variations in the magnitude and fidelity of TLS across agents and tissues, differences in the nature and strength of the observed tissue-specific relationships between mutagenic potency and genotoxic potency may also be related to the effects of PAH-induced damage on the cell cycle. Cells can actively protect the integrity of the genome by inducing G1 arrest, a phenomenon mediated in part by tumour-suppressor proteins p53 and p21 (also known as CDKN1) (Sancar et al. 2004). G1 arrest, which is known to be induced by DNA damage, provides the opportunity to carryout DNA repair prior to entering S-phase, where damaged DNA can generate fixed mutations.

Interestingly, PAH diol-epoxides are known to induce “stealth properties”, that is, they have the ability to evade G1 arrest, thereby permitting large damage loads to accumulate in S-phase (Dipple et al. 1999; Khan and Anderson 2001). This stealth effect has been observed in lung cells following exposures to the diol-epoxides of BaP and benzo[g]chrysene, and the phenomenon is thought to be involved in PAH-induced lung cancer (IARC. 2010; Khan and Anderson 2001). The current study observed a strong cross-PAH correlation between lung mutagenic potency and lung genotoxic potency, and this relationship may indicate that the PAHs examined have the capacity to evade G1 arrest in response to PAH-induced damage; thus resulting in a higher proportion of the

adducts remaining at S-phase. This may include adducts that are easier to repair, which would not be expected to cause mutations in tissues where normal G1 arrest is manifested. Although there is no evidence in the literature to support this contention, the involvement of G1 arrest in the mutagenic efficiency of PAH adducts is worthy of investigation.

In contrast to the aforementioned relationships between mutagenic and genotoxic potency for GS, liver, and lung, the *lacZ* mutagenic potency of the tested agents in BM and SI does not appear to be dependent on genotoxic potency. Scrutiny of the bivariate plot containing data for all tissues (**Figure 7.2**) reveals that for SI and BM, the ranges in adduct BMDs are wide, and the confidence intervals are large. As noted earlier for BM, scrutiny of the adduct frequency dose-response data revealed that the responses frequently reached zero-order kinetics at the top two or three doses. Similar dose-response patterns were also observed for SI. In fact, for compounds with defined adduct BMDs, plateaued responses were observed for 67% of tested compounds in BM, and 50% of tested compounds in SI, but in contrast, only 0%, 11%, and 27% of tested compounds in liver, GS, and lung, respectively (e.g., observations on the far left and right of **Figure 7.1**, see also **Supp. Figure 7.3 a,b**). Unfortunately, BMD analyses of poorly defined dose-response relationships, such as those observed for PAH-induced adduct frequencies in BM and SI, provide imprecise and inaccurate doubling dose estimates (i.e., BMD₁₀₀). Such values are poorly suited to cross-endpoint potency comparisons, and further work would be required to appropriately define BMD estimates for both endpoints in BM and SI. Therefore, since the experimental design employed here did not permit accurate and precise determinations of BMDs for BM and SI, it is difficult to draw any

convincing conclusions regarding the relationship between PAH genotoxic and mutagenic potency for these tissues. The influence of a superior dose range on BMD precision is illustrated by the 10-dose MegaBaP study (Long et al. 2017b), which simultaneously examined both adduct and *lacZ* mutant frequency across 8 tissues (including SI and BM). Accordingly, we were able to observe a significant empirical relationship between mutagenic and genotoxic potency across all 8 tissues ($r^2 = 0.87$, $F = 35.0$, $p = 0.002$), even though the study still employed the same 3-day sampling time (Long et al. 2017b).

As noted earlier, the Muta™Mouse system permits assessment of multiple endpoints in a single study, contributing to reductions in animal use. Nevertheless, the results discussed above suggest that, in some cases, a wider dose range may be required for accurate and precise BMD estimation. However, generation of dose-response data that are well suited to BMD analyses does not necessarily require additional animals. Indeed, the use of more doses with fewer animals per dose has been highlighted as a means to reduce the need for additional animals while simultaneously providing dose-response data that is suitable for accurate and precise BMD determination (Slob 2014). Future dose-response studies should consider reducing the number of animals per dose group in favour of increasing the number of doses examined, thereby generating data that is better suited to accurate and precise BMD estimation.

In summary, this study investigated the frequency of adducts and *lacZ* mutants in numerous Muta™Mouse tissues following sub-chronic oral exposures to a wide range of PAHs and PAH mixtures. The results revealed that despite cross-tissue and cross-agent variability in the frequency of induced DNA damage and mutations, the frequency of the

former is inversely related to that of the latter. In large part this appears to be related to the experimental design (i.e., sampling time), and the influence of repair and replication on tissue-specific adduct and mutant frequency. The BMD approach was used to determine the genotoxic (adduct inducing) and mutagenic (mutant inducing) potencies; and moreover, investigate the relationships between the potencies across agents and tissues. The results obtained constitute the first demonstration of tissue-specific empirical relationships between mutagenic and genotoxic potency across numerous compounds. Although the results revealed significant relationships between genotoxic and mutagenic potencies for liver, lung, and GS, improved dose-response data would be required to definitively determine the applicability of these results for other tissues. These observed relationships suggest that, across tissues and compounds, PAH-induced adducts are being similarly converted into mutations. More specifically, the results suggest that TLS, an important driver of mutation establishment, is similarly processing PAH adducts across compounds and tissues. However, it is difficult to carefully scrutinise this assertion since there is very little published information regarding the cross-tissue levels and cross-compound efficacy of TLS polymerases. Thus, additional work should examine the mechanisms by which different PAH-induced lesions are bypassed by TLS polymerases, and whether the process is indeed consistent across tissues. An improved quantitative understanding regarding the tissue-specific mutagenicity of stable adducts will improve the ability to interpret adduct frequency in a risk assessment context, potentially allowing the use DNA adduct potency to predict mutagenic potency, an endpoint that is more relevant to human risk. This would constitute a considerable step forward since assessment of adduct frequency does not require the use of costly TGR systems.

7.7 References

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CHAPTER EIGHT

8 Chapter: General Conclusions

8.1 Summary of Study Outcomes and Evaluation of Thesis Hypotheses

The major goal of this thesis was to employ the transgenic MutaTMMouse to generate robust, *in vivo* multi-endpoint genetic toxicity data for PAHs (polycyclic aromatic hydrocarbons) and PAH mixtures; and moreover, examine patterns of genetic damage across tissues, endpoints, and compounds. More specifically, sub-chronic oral exposures to 9 individual PAHs and 6 PAH mixtures were carried out, and the frequency of genetic damage across 5 tissues (i.e., bone marrow, small intestine, glandular stomach, lung, liver) was assessed using 2 different endpoints (i.e., stable DNA adduct frequency, *lacZ* mutant frequency). The nature and magnitude of the responses were compared and contrasted across tissues, endpoints, and compounds; and moreover, novel dose-response analysis techniques were employed to quantitatively compare compound/mixture potency and/or tissue sensitivity in a statistically rigorous manner. Lastly, potency metrics (i.e., benchmark doses (BMDs)) were used to examine empirical relationships between genetic toxicity endpoints and/or tissue-specific effects.

The results presented in this thesis improve current understanding regarding the genetic toxicity of PAHs and PAH mixtures. More specifically, the results delineate (1) the cross-tissue similarities and differences in the genotoxicity and mutagenicity of PAHs, and by extension, the processes that determine tissue- and compound-specific effect levels, (2) the cross-tissue similarities and differences in the genotoxicity and mutagenicity of synthetic and complex PAH mixtures; moreover, the behaviour of priority PAHs in mixtures and the processes that determine tissue- and mixture-specific effect levels, (3) the cross-tissue sensitivity to various forms of genetic damage induced

by oral exposures to PAHs and PAH mixtures, and (4) the cross-tissue empirical relationships between the genotoxic potency and mutagenic potency of PAHs and PAH mixtures.

A more detailed chapter-by-chapter summary of the work presented in this thesis is provided below, along with a brief description of how the chapters contribute to the completion of the objectives outlined in **Chapter 1**.

Chapter 2

Hypothesis: Following an oral exposure, proximal tissues (e.g., small intestine, stomach, liver) will be more sensitive to PAH-induced genetic damage (i.e., yield a positive response for a known genotoxic carcinogen) in comparison with haematopoietic tissues.

Outcomes & Importance: This hypothesis was supported. All PAHs were positive for induction of *lacZ* mutations in at least one tissue (sensitivity = 100%), and for the 9 PAHs examined, one or more tissues proximal to the route of exposure (i.e., glandular stomach, liver, small intestine) were more sensitive than bone marrow. For example, the sensitivities of small intestine and bone marrow were 100% and 67%, respectively. In addition, all PAHs produced DNA adducts in at least one tissue. In contrast, only 5 PAHs yielded a positive response in the peripheral blood micronucleus (MN) assay (sensitivity = 56%). Interestingly, even though lung is a distal tissue for an oral exposure route, a marginally higher sensitivity was observed for this tissue (i.e., 89%) in comparison with glandular stomach or liver (i.e., both 78%). Moreover, lung was the only tissue for which a statistically significant increase in the frequency of exposure-induced DNA adducts was observed for all 9 compounds examined. The lung findings highlight the sensitivity of this tissue to PAH-induced genotoxic effects; moreover, its importance with respect to

cancer since, with the exception of BghiP, it is the only tissue in which tumours have been observed across all the mutagenic PAHs examined.

Genetic toxicity assays commonly used for regulatory evaluations assess effects in hematopoietic tissues only (e.g., reticulocytes). Therefore, the results presented herein indicate that routinely used assessment methods (e.g., MN in hematopoietic tissue) would not have detected the genotoxic hazards of 4 of the 9 tested PAHs. Moreover, 3 of the 9 PAHs would not be detected if the *lacZ* endpoint only examined bone marrow responses. Failure to identify genotoxic hazards could potentially result in unnecessary human health risk.

It is important to also note that this study is the largest sub-chronic *in vivo* genetic toxicity study of PAHs. More importantly, in many instances, the first evaluation of PAHs for some of the endpoints examined, and for some compounds, the first *in vivo* mutagenicity assessment with the first demonstration of a positive response for a specific endpoint and/or tissue. Thus, the results clearly demonstrate the utility of the TGR MutaTMMouse assay for mutagenicity assessment, especially for compounds that may not be systemically distributed, or for compounds where metabolic capacity in distal tissues (e.g., hematopoietic tissues) is insufficient to produce DNA-reactive (i.e., genotoxic) metabolites.

Chapter 3

Hypothesis: Oral murine exposure to an extract of a commercially-available coal tar-based driveway sealcoat will induce a statistically significant increase in genetic damage in multiple tissues.

Outcomes & Importance: This hypothesis was supported. Sub-chronic MutaTMMouse oral exposure to an extract of a commercially-available coal tar-based driveway sealcoat elicited statistically significant increases for all endpoints, in all tissues and cell types examined. More specifically, statistically significant increases in DNA adduct and *lacZ* mutant frequency in bone marrow, glandular stomach, small intestine, liver, and lung. Moreover, statistically significant increases in the frequency of micronucleated reticulocytes and normochromatic erythrocytes. The magnitude of the responses differed across tissues, with the highest frequency of adducts manifested in liver and lung, and the highest frequency of mutations occurring in small intestine. The results of this study constitute the first demonstration of *in vivo* mammalian genetic toxicity of coal tar-containing pavement sealcoat. Thus, the work provides *in vivo* evidence to support the contention that exposures to coal tar-based pavement sealcoats may increase the risk of adverse health effects in mammals, including humans.

Chapter 4

Hypothesis: The genetic toxicity of orally-delivered PAH mixtures will be equivalent to the sum of the incremental contributions from the targeted mixture components (i.e., the mixture components will act in an additive manner).

Outcomes & Importance: This hypothesis was not supported. The results consistently revealed more-than-additive mixture effects in select proximal tissues to the route of exposure (i.e., small intestine, liver), and less-than-additive mixture effects in distal tissues (i.e., bone marrow). Furthermore, the results suggest that the deviations from additivity (i.e., differences between the experimentally-observed mixture effects and predictions based on the sum of the contributions from the individual PAHs), are likely

caused by alterations of the processes that regulate tissue-specific metabolic capacity. It is important to emphasize that although the mutagenic responses deviated from additivity in both directions, and strictly speaking, this indicates that the hypothesis was not supported, the magnitudes of the deviations in either direction was typically within 5-fold, a surprisingly small range.

These results are significant since the assumption of dose additivity for complex PAH mixtures is an integral element of the approach employed for human health risk assessment (HHRA). More specifically, the results presented in **Chapter 4** demonstrated that additive predictions of mixture mutagenic potencies that are based on the concentrations and potencies of a small number of targeted PAHs are surprisingly close to potencies determined experimentally. These results were extended to determinations of benzo[*a*]pyrene (BaP)-equivalent concentrations in the complex samples investigated, and these values were compared to those calculated under the traditional assumption of additivity. Although the results should be interpreted with caution, they suggest that the risk assessment approach traditionally employed for PAH mixtures, which only examines the 8 priority PAHs that are mutagenic carcinogens, is surprisingly reasonable. Therefore, conclusions predicated on the results presented in **Chapter 4** support continued use of the additive assumption routinely employed for HHRA of PAH mixtures.

Chapter 5

Hypothesis: By simultaneously conducting dose-response analyses across experimental variables such as compound or sex, the BMD-covariate approach can increase the precision of genetic toxicity BMD estimates (i.e., reduce BMD confidence intervals);

resultant metrics and confidence limits permit potency ranking across *in vivo* covariates (e.g., compound, tissue, sampling time).

Outcomes & Importance: This hypothesis was supported. Across a number of datasets, the BMD-covariate approach did provide more precise BMD estimates (i.e., smaller confidence intervals). More specifically, using the BMD-covariate approach the results show that MN frequency dose-response data from multiple studies can be merged (i.e., no study effect). Subsequent analyses showed improved BMD precision, permitting attendant potency ranking of seven clastogens. Similarly, empirical comparisons of *Pig-a* mutant phenotype frequency data for males and females exposed to *N*-ethyl-*N*-nitrosourea (ENU) justified dataset merging across sex. This permitted more effective scrutiny regarding the effect of post-exposure sampling time on the mutagenicity of ENU in reticulocytes and total red blood cells. The BMD-covariate approach also revealed tissue-specific differences in the induction of *lacZ* transgene mutations in MutaTMMouse specimens exposed to BaP, with the results obtained permitting the formulation of mechanistic hypotheses regarding the observed potency ranking (e.g., low BMD for small intestine relative to liver). Lastly, the work illustrated how historical dose-response data for assessments that examined numerous doses (i.e., induced *lacZ* mutant frequency across 10 doses of BaP) can be used to improve the precision of BMDs derived from datasets with far fewer doses (i.e., *lacZ* mutant frequency across 3 doses of dibenz[*a,h*]anthracene). Collectively, the presented examples illustrate how innovative use of the BMD approach can optimise of the use of *in vivo* genetic toxicity dose-response data; optimised data use can improve the efficacy of experimental animal use without sacrificing point-of-departure (PoD) precision.

Chapter 6

Hypothesis: BMD analyses of Muta™Mouse genetic toxicity dose-response data related to sub-chronic, oral BaP exposure, and subsequent comparisons of BMD estimates across tissues and endpoints, permits statistical delineation of tissue sensitivities within an endpoint and empirical relationships between endpoints.

Outcomes & Importance: The hypothesis is supported since the BMD-approach revealed significant differences in sensitivity between tissues and/or groups of tissues, permitting empirical ranking of cross-tissue sensitivities to BaP-induced DNA adducts, *lacZ* mutations, MN, and *Pig-a* mutations. The cross-tissue patterns of BMD values for *lacZ* mutant frequency and DNA adduct frequency are similar, and consistent with an oral route of exposure and documented tissue-specific differences in metabolism and proliferation (i.e., small intestine, bone marrow, spleen most sensitive; liver, kidney least sensitive). In contrast, for MN frequency and *Pig-a* mutant phenotype, the results did not reveal any differences in the sensitivities of the two haematopoietic cell types examined. Additionally, the analyses revealed a significant empirical relationship between mutagenic potency (BMD₁₀₀ for *lacZ* mutants) and genotoxic potency (BMD₁₀₀ for DNA adducts) across 8 tissues, indicating consistent cross-tissue conversion of DNA adducts into mutations. Importantly, examination of endpoint-specific response maxima illustrated the difficulty of comparing BMD values across endpoints using a fixed benchmark response (BMR). Overall, the BMD-approach permitted robust comparisons of responses across tissues and endpoints, and these comparisons yielded valuable insight into the mechanisms underlying the *in vivo* induction of BaP-derived genetic damage.

Chapter 7

Hypothesis: The ability of PAHs and PAH mixtures to elicit permanent DNA sequence changes (i.e., mutations) is positively correlated with their ability to induce genetic damage (i.e., formation of bulky DNA adducts).

Outcomes & Importance: This hypothesis was partially supported. This study investigated the frequency of adducts and *lacZ* mutants in numerous MutaTMMouse tissues following sub-chronic oral exposures to 15 PAHs and PAH mixtures. The results revealed that despite cross-tissue and cross-agent variability in the frequency of induced DNA damage and mutations, the frequency of the former is inversely related to that of the latter. In large part this appears to be related to the experimental design, and the influence of repair and replication on tissue-specific adduct and mutant frequencies. However, we subsequently employed the BMD-approach to determine the genotoxic potencies (i.e., adduct BMD₁₀₀) and mutagenic potencies (i.e., mutation BMD₁₀₀) for each compound/tissue combination, and examined the empirical relationships between these metrics. A statistically significant quantitative relationship between mutagenic potency and genotoxic potency was observed when all tissues were analysed together; therefore the results demonstrate that the PAH potencies for these two endpoints are empirically related. When each tissue was examined independently, a significant relationship was observed for liver, glandular stomach, and lung, with no significant differences in the slopes between tissues. The latter results suggests that the measured adducts are being processed into mutations in a similar manner across tissues. Overall, the empirical analyses of cross-tissue and cross-compound relationships between mutagenic and genotoxic potency suggest that PAHs induce genetic damage in a consistent manner, with the results suggesting that translesion synthesis (TLS) is similarly processing PAH

adducts across compounds and tissues. A significant relationship was not observed in bone marrow and small intestine; however, the quality of the adduct frequency data yielded imprecise BMD estimates. Limitations attributable to the doses tested and the exposure regimen indicate that additional data will be required to definitively determine the validity and applicability of these results. This study constitutes the first demonstration of a quantitative relationship between mutagenic and genotoxic potency across several compounds, and moreover, in a number of tissues. An improved quantitative understanding regarding the tissue-specific mutagenicity of stable adducts will improve the ability to interpret adduct frequency in a risk assessment context. This would constitute a considerable step forward since assessment of adduct frequency does not require the use of costly TGR systems.

8.2 Overall Fulfillment of Thesis Objectives

The thesis introduction outlined 4 discreet objectives, and the results presented in **Chapters 2 - 7** permitted fulfillment of all objectives; the details are briefly outlined and summarised below. In some instances, the results presented in several chapters contribute to the fulfillment of an individual objective. Vice versa, the results presented in a single chapter may contribute to the fulfillment of multiple objectives.

Objective 1: To scrutinise the tissue-specific genotoxic responses of 9 individual PAHs.

Outcomes: This objective was fulfilled. In **Chapter 2**, the frequencies of exposure-induced DNA adducts and *lacZ* mutants were assessed across 5 tissues, and the frequency of MN was assessed in reticulocytes and normochromatic erythrocytes. The magnitude and potency of the responses, determined as the linear slopes of the dose-response

relationships, were compared and contrasted across tissues and endpoints in order to gain mechanistic insight regarding the manner in which PAHs induce genetic damage *in vivo*. In **Chapter 6**, four different genetic damage endpoints were examined following exposure to an extended range of BaP doses. Application of the BMD approach allowed precise comparisons of BaP sensitivity across tissues and cell types. Throughout this thesis, the results revealed patterns in the nature and magnitude of the *in vivo*, tissue-specific genotoxic responses to PAHs, and these patterns provided insight in to the physiologic mechanisms underlying the manifestation of genotoxic effects. For example, the results showed that tissues proximal to the oral exposure route are generally more sensitive to PAH-induced genetic damage and mutations than distal tissues, likely due to higher exposure and greater capacity for metabolic activation of the parent PAHs.

Objective 2: To assess the tissue-specific genetic toxicity of complex PAH mixtures, and moreover, scrutinise the assumption of component additivity that is routinely employed for regulatory HHRA of complex PAH mixtures.

Outcomes: This objective was fulfilled. The results presented in **Chapter 3** document DNA adduct and *lacZ* mutant frequencies across 5 tissues, as well as micronucleus (MN) frequency in reticulocytes and normochromatic erythrocytes, following MutaTMMouse exposure to an extract of a commercially available coal tar-based driveway sealcoat.

Chapter 4 investigated the frequency of *lacZ* mutants, across 5 tissues, following MutaTMMouse exposures to 2 coal tar extracts and 3 synthetic PAH mixtures. The assumption of component additivity was investigated by comparing mutagenic potencies of the complex and synthetic mixtures, expressed as the linear slope of the dose-response relationships, to additive potency estimates calculated as the product of the potencies and

mixture concentrations of the individual priority PAHs (i.e., the sum of the incremental contributions from each prioritised PAH). The analysis revealed more-than-additive mixture responses in proximal tissues (i.e., small intestine, liver), and less-than-additive mixture responses in bone marrow. However, the ratio of predicted (i.e., additive) potency to observed *in vivo* potency were largely less than 5-fold. In summary, the results indicate that, strictly speaking, PAHs in complex mixtures do not induce genotoxic effects in an additive manner, with both more-than-additive and less-than-additive responses being observed depending on the tissue examined. Nevertheless, observed and predicted mixture potencies are close enough to affirm that the risk assessment approach that is traditionally applied to PAH mixtures is reasonable. From a more mechanistic point of view, the differences that were observed, which range from 0.2-fold to 12.3-fold, suggest that differential physiologic alterations likely determine the precise nature and magnitude of the genetic damage elicited by orally-delivered PAH mixtures and individual priority PAHs. These differential alterations are likely related to metabolism, and concomitant balance between excretion and generation of DNA-reactive metabolites.

Objective 3: Employ recently-developed quantitative methods (i.e., BMD-combined covariate modelling) to analyse genetic toxicity dose-response data, thereby providing improved BMD precision that permits robust genotoxic potency ranking across experimental covariates.

Outcomes: This objective was fulfilled. **Chapter 5** employed the BMD-covariate approach to simultaneously analyse multiple genetic toxicity data sets, thus providing BMD estimates with smaller confidence intervals (i.e., increased precision) relative to estimates obtained via independent analyses of each data set (i.e., the traditional BMD-

approach). Smaller confidence intervals permitted robust differentiation between BMD values, and concomitant potency ranking across compounds, cell types, sampling time, and tissues. In **Chapter 6**, the traditional BMD-approach was employed to generate BMD₁₀₀ values and their associated 90% CIs; these values were used to rank tissue/cell type sensitivity within each endpoint. More specifically, novel applications of both the traditional and the recently-developed BMD methods permitted statistically rigorous comparisons of genotoxic sensitivity/potency (i.e., plotted BMD CIs compared across covariates, with non-overlapping CIs revealing statistical differences). These comparisons provided valuable mechanistic insights regarding the mechanisms underlying the manifestation of PAH-induced genetic damage.

Objective 4: Employ the BMD approach to examine empirical relationships between the ability of PAHs and PAH mixtures to induce the formation of bulky DNA adducts and *lacZ* mutations.

Outcomes: This objective was fulfilled. **Chapter 6** employed the BMD-approach to examine empirical relationships between the mutagenic potency (i.e., BMD₁₀₀ for *lacZ* mutants) and genotoxic potency (i.e., BMD₁₀₀ for DNA adducts) of BaP across 8 tissues; the results revealed a significant positive relationship. Moreover, in **Chapter 7**, the BMD approach was used to investigate empirical relationships between the ability of PAHs and PAH mixtures to induce DNA adducts (i.e., genotoxic potency) and mutations (i.e., mutagenic potency). The results revealed a significant relationship when all tissues were examined together, as well as for liver, glandular stomach, and small intestine when each tissue was examined independently. Although a few published studies have investigated empirical relationships between the mutagenic and genotoxic potency of individual

agents in a small number of tissues, the results presented in **Chapter 6** examined the relationships for a single compound across an unprecedented number of tissues (i.e., 8), whereas the results presented in **Chapter 7** examined the relationships across an unprecedented number of agents and tissues (i.e., 15 agents in 5 tissues). The empirical relationship between *in vivo* mutagenic and genotoxic potency of PAHs and PAH mixtures indicates that their mutagenicity is dependent on genotoxicity; at least in liver, lung, and glandular stomach. These results suggest that it may be possible, for a restricted series of compounds, to predict mutagenic potency via the dose-response assessment of DNA adduct frequency. Collectively, the studies presented in **Chapters 6 and 7** constitute the largest *in vivo* investigation to date regarding the empirical relationships between mutagenic and genotoxic potency.

8.3 Contributions to Scientific Knowledge

This thesis employed the transgenic MutaTMMouse to investigate the genetic toxicity of PAHs and PAH mixtures; numerous genetic toxicity endpoints were simultaneously assessed across multiple somatic tissues. The resulting data were used to address a number of specific questions, including those related to cross-tissue and cross-compound variability in the nature and magnitude of the induced genetic damage, the cross-tissue genetic toxicity of PAH mixtures and the additivity of PAHs in complex mixtures, and the utility of novel quantitative methods to interpret dose-response data, examine variability across covariates, and examine empirical relationships between genotoxic and mutagenic potency.

Most published *in vivo* toxicological data pertaining to PAHs relates to high dose studies of individual compounds. Moreover, beyond BaP, existing research has only examined genetic damage induced in a limited number of tissues across a limited number of compounds and endpoints; very few studies have examined *in vivo* induction of mutations. In contrast, every animal study conducted herein simultaneously examined the frequency of genetic damage across several tissues and endpoints, providing datasets to address applied questions such as (1) which tissues are most sensitive to PAH-induced genetic damage, and (2) what PAHs induce high frequencies of mutants in distal tissues such as bone marrow, and (3) is the mutagenic potency of PAHs dependent on their genotoxic potency. In addition, the results obtained permitted the formulation of several hypotheses regarding how cross-tissue variations in cellular processes related to metabolism, repair, and TLS contributes to tissue-, compound- and endpoint-specific differences in the observed levels of genetic damage. Moreover, in many instances, the data generated in this thesis provides the first demonstration of a positive response induced by specific PAHs and/or PAH mixtures for certain endpoint-tissue combinations. A more detailed summary of important contributions to scientific knowledge is provided below.

The thesis employed identical study designs to investigate cross-tissue genetic damage induced by sub-chronic oral exposures to 9 individual PAHs and 6 PAH mixtures. Prior to this work, the cross-tissue genotoxic effects associated with an oral exposure had not been delineated. The results of these studies demonstrated that PAH induction of *lacZ* mutants varies across tissues, with almost all PAHs inducing the highest frequency of *lacZ* mutants in small intestine, a tissue that is proximal to the route

of exposure. Moreover, the examined PAHs typically induced the lowest frequency of *lacZ* mutants in bone marrow and lung, both tissues that are distal from the exposure route. Interestingly, BaP and dibenzo[*a,l*]pyrene (DBaP) both elicited robust mutagenic responses in bone marrow, indicating that, in contrast to the other PAHs and PAH mixtures studied, there are unique factors controlling how these compounds are distributed and metabolised. BaP and DBaP are relatively poor AhR agonists in comparison with the other studied PAHs [Machala et al., 2001; Ziccardi et al., 2002]; therefore, they may not be capable of inducing sufficient P450-mediated metabolism in the gastrointestinal tract and liver to result in complete detoxication and excretion. Consequently, larger amounts of the parent compounds may be systemically distributed, with *in situ* metabolism in a distal tissue such as bone marrow locally generating substantial amounts of DNA-reactive metabolites. In contrast, the low or lack of bone marrow response observed with other PAHs suggests that they are more likely to be cleared (i.e., detoxified and excreted) prior to systemic distribution. In summary, existing knowledge related to the tissue specific metabolism of PAHs such as BaP and DBaP indicates that the distribution of PAH-induced genotoxic effects across tissues may be driven by difference in metabolism in tissues that are proximal and distal to the exposure route. It is difficult to precisely outline the specific isozymes and processes that govern the outcomes of the PAHs exposures conducted herein, and additional research is required to investigate the nature and magnitude of tissue-specific metabolic capacity.

Understanding tissue-specific differences in the induction of genetic damage is important since routine genetic toxicity hazard assessment frequently examines effects manifested in hematopoietic tissues only (i.e., blood, bone marrow). This study

demonstrated that the vast majority of PAHs examined induced positive mutagenicity responses in tissues other than bone marrow or blood (i.e., tissues proximal to the route of exposure). If the analyses had been restricted to hematopoietic tissues, the genotoxic hazards of several PAHs would have been missed. This could potentially result in misclassifications of compounds undergoing regulatory evaluation, and concomitantly unnecessary risk of adverse human effects. Going forward, it is clear that the results presented herein underscore the importance of appropriately assessing human hazard via analyses of effects manifested in numerous tissues. Wherever possible, the tissues should be selected on the basis of pre-existing ADME (absorption, distribution, metabolism, excretion). Although ADME investigations can be complex and laborious they are routinely carried out for pest-control and therapeutic products.

Coal tar-based driveway sealcoats, which are 10-35% coal tar by weight and are known to contain extremely high levels of PAHs, are readily available in Canada and the United States [Mahler et al., 2005]. Their use has been associated with high levels of PAHs in air [Van Metre et al., 2012], house dust [Mahler et al., 2010], and water runoff from sealed surfaces [Mahler et al., 2005]. In addition, they have been identified as the greatest contributor to elevated PAHs levels in the sediments of 40 US lakes [Van Metre and Mahler, 2010]. Thus, human exposure to PAHs from coal tar-based driveway sealcoat has been established, however, prior to the work presented herein there was no data demonstrating that coal tar-based sealcoats can induce genetic damage *in vivo* in experimental animals (i.e., rodents). This knowledge gap impaired the ability of regulatory agencies to predict human hazard, and evaluate the need to impose use restrictions. The thesis (i.e., **Chapter 3**) provides the first demonstration that a

commercially available coal tar-based driveway sealcoat can indeed induce *in vivo* genetic damage in a mammalian system. Moreover, the sealcoat induced genetic damage across all endpoints and tissues examined, with the highest frequency of mutants occurring in small intestine, and the highest frequency of DNA adducts occurring in lung. Thus, the study presented in **Chapter 3** supports the contention that exposures to coal tar-based driveway sealcoats will likely elicit adverse human health effects, and this contribution to original knowledge will permits regulatory authorities to formulate policies that appropriately reduce or eliminate human risks.

Component additivity is an assumption employed for risk assessment of PAH mixtures; however, there is a paucity of data to support the assumption. Most published PAH mixtures research is *in vitro* work, or involves analyses of simplistic (binary) mixtures [Lemieux et al., 2015a]. Using *in vivo*, cross-tissue genotoxicity assessment of individual PAHs and several complex and synthetic PAH mixtures, this thesis (i.e., **Chapter 4**) thoroughly scrutinised the assumption of component additivity. The results demonstrated that PAHs do not induce mutagenicity in a strictly additive manner, with more-than-additive effects consistently observed in tissues that are proximal to the exposure route, and less-than-additive effects consistently observed in distal tissues. Although the results did highlight mechanistically interesting differences between experimentally observed values and those calculated under an assumption of additivity, those differences can be reasonably regarded as small (i.e., less than an order of magnitude) relative to the magnitude of the uncertainties commonly associated with HHRA of materials contaminated with PAH mixtures (e.g., ingestion rates, potency equivalence factors, etc.). Indeed, regulatory authorities such as the Canadian Council of

Ministers of the Environment (CCME) assert that since animal studies have inherent uncertainties, “a difference of less than one order of magnitude would typically be considered quite minor for the purpose of acceptability of a model”[CCME., 2010]. Importantly, this study is the only comprehensive *in vivo* study to evaluate the assumption of dose-additivity for PAH mixtures. By demonstrating that cross-tissue deviations from additivity are within an acceptable range, the study provides, for the first time, a rigorous experimental evaluation of the assumption of dose-addition that is routinely employed for HHRA of PAH mixtures. Given that one third of the federally-owned contaminated sites in Canada contain complex mixtures of PAHs and related substances (i.e., approximately 3000 sites) this work fills a critically important knowledge gap.

It is well recognized that PAHs are mutagenic carcinogens that can induce mutations via the formation and processing of stable bulky DNA adducts. However, there is a paucity of information regarding the cross-tissue quantitative relationships between DNA adduct formation and mutation establishment. By employing the BMD approach, the work presented in **Chapters 6 and 7** provides the first demonstration that the genotoxic potency of PAHs (i.e., ability to induce adducts) is significantly related to mutagenic potency (i.e., ability to induce mutations). More specifically, for BaP, the work presented in **Chapter 6** defined an empirical relationship between these two endpoints across 8 somatic tissues. Additionally, across a number of PAHs and PAH mixtures, the work presented in **Chapter 7** described empirical relationships between these two metrics when all tissues were analysed together, and for liver, lung, and glandular stomach when these tissues were analysed independently. Moreover, there was

not a significant difference between the slopes of the tissue-specific relationships, suggesting that, for these tissues, the adducts are being processed into mutations in a similar manner. The works constitute the first demonstration of a significant quantitative relationship between genotoxic potency and mutagenic potency across a range of structurally related compounds. If this relationship is found to be consistent across other tissues, it may permit prediction of mutagenic potency from adduct (i.e., genotoxic) potency. Such an approach would be similar to that proposed by Hernandez et al.[2011] for the prediction of carcinogenic potency from mutagenic potency. However, convincing application of a predictive approach for the determination of mutagenic potency must be preceded by additional research to determine whether the results are applicable to other tissues, and/or more generally applicable to other compound types. Nevertheless, an improved quantitative understanding regarding the tissue-specific mutagenicity of stable adducts will improve the ability to interpret adduct frequency in a risk assessment context.

Although the studies conducted all involved murine exposures, and detailed interpretation of the results in a HHRA context is beyond the scope of the thesis, it is certainly important to at least consider the human relevance of the work. As repeatedly stated throughout the thesis, PAHs are produced via the incomplete combustion of organic material, and they are ubiquitously present in the environment. For the general population, dietary sources account for the largest fraction of human PAH exposures, although smoking and some occupations can make comparatively larger contributions to total exposure [Phillips, 1999; IARC., 2010]. Additionally, consumer products such as coal tar-based driveway sealcoats, which contain extremely high concentrations of PAHs,

can increase the potential for exposure. Indeed, the potential for human exposure to coal-tar based driveway sealcoats, which are readily available in Canada and the United States, has already been documented. **Chapter 6** demonstrated that sub-chronic exposures to even very low doses of BaP can significantly increase the frequency of DNA adducts and *lacZ* mutants in tissues such as spleen and small intestine, the most sensitive tissues respectively. Additionally, the no observed genotoxic effect level (NOGEL) values for these tissues can be used to estimate the human margin of exposure (MOE). Using the lowest NOGEL for BaP-induced *lacZ* mutations (i.e., 0.78 mg BaP/kg BW/day for small intestine), a conservative human body weight of 50 kg, and estimates of human dietary exposures to BaP (i.e., 0.04-2.8 µg/person/day) [IARC., 2010], the MutaTMMouse results provide an MOE of 13,900 – 975,000. Performing the same calculation using the small intestine BMDL_{1SD} (i.e., the lower confidence limit of the BMD associated with a response 1 standard deviation above control, 0.40 mg BaP/kg BW/day) provides a slightly lower MOE range of 7,214 – 505,500. The European Food Safety Authority (EFSA) has stated that if an MOE is based on a BMDL from an animal study, then a value of 10,000 or higher would indicate “low concern from a public health point of view” [EFSA, 2012]. In our case, using the BMDL_{1SD}, we obtained an MOE range that is marginally below this threshold for the upper limit of human dietary exposure. This type of calculation illustrates that the values generated by the MutaTMMouse studies conducted herein can be used to evaluate the mutagenic risks associated with typical human exposures to BaP. However, this calculation is compound specific and fails to acknowledge exposure to other mutagenic PAHs; moreover, other routes of exposure. By extension, it is tempting to use the experimentally-determined BaP

equivalent values for the PAH mixtures studied in **Chapter 4** to similarly calculate MOE values. However, such a calculation is prohibited by lack of information about exposure to the complex material examined (i.e., coal tar). Nevertheless, it is important to acknowledge that, going forward, quantitative dose-response analysis of MutaTMMouse *lacZ* mutant frequency data can be used to assess the risks of adverse human health effects. Improved human exposure data for individual PAHs as well as complex PAH mixtures in complex environmental matrices (e.g., soil, settled house dust) would facilitate such calculations.

Despite the utility of the MOE calculations for human health risk assessment, it is important to note that the BaP exposure values only include dietary intake via, for example, consumption of grilled/barbequed foods (up to 4 ng BaP/g of cooked meat) [Kazerouni et al., 2001] and coffee (0.25-5 ng BaP/cup) [De Kruijf et al., 1987; Tfouni et al., 2013; Orecchio et al., 2009]. Other activities and habits, such as tobacco smoking (3-28 ng BaP/cigarette) [Kaiserman and Rickert, 1992] and use of coal tar based-driveway sealcoats (32 mg BaP in a 15 L pail) [Long et al., 2016] can significantly augment PAH exposures. In addition, some occupational settings can also elevate total daily PAH exposures. These include settings involving charcoal production, road paving, rubber manufacturing, coke and refined metals production, cooking, and firefighting [IARC, 2010; IARC, 2012; IARC, 2010]. In summary, the BaP MOE based on dietary exposure alone suggests cause for concern at the highest exposure levels; however, humans are also exposed to other genotoxic PAHs, and PAHs from a variety of sources. Thus, a risk assessment that acknowledges exposures to all mutagenic PAHs from all sources would be required to reliably assess the risks posed by PAHs from all sources and exposure

routes. Despite the fact that it is not possible to assess the mutagenic risks posed by all PAHs from all sources, the MOE for BaP alone, and the results presented in the thesis, indicate that PAH exposures should be avoided wherever possible. Exposure reduction would presumably involve avoidance of PAH contaminated environments (e.g., campfires, pavement sealed with coal tar-based products), avoidance of tobacco smoke, and avoidance of grilled or char-broiled foods. Where avoidance of PAH contaminated environments is not possible (e.g., occupational settings), every effort should be made to properly use PPE (personal protective equipment) such as gloves, a face masks for fumes and dust, or, in extreme environments, a self-contained breathing apparatus (SCBA).

8.4 Future Directions

The studies presented in this thesis collectively generated a vast amount of data regarding the *in vivo* genetic toxicity of PAHs and PAH mixtures. More specifically dose-response data across tissues, agents, and endpoints that permit investigations regarding the sensitivity of different assays and endpoints, the tissue-specific differences in the genotoxicity of PAHs and PAH mixtures, the effects of PAH mixtures relative to what is expected under an assumption of component additivity, the utility of the BMD approach to rank potency values across experimental covariates, and the empirical relationships between endpoints that are key events in the mode-of-action of genotoxic carcinogens. In addition to what was already stated above, there are numerous avenues for follow-up research.

It is clear that the nature and magnitude of the PAH and PAH-mixture effects across tissues and endpoints are dependent on several cellular processes. More

specifically, toxicokinetic processes that regulate the exposure of target tissues to DNA-reactive metabolites, damage removal processes such as NER, and damage by-pass processes such as TLS. However, with the possible exception of BaP and DBaP, a thorough review of the literature revealed little information regarding how these processes determine the nature and magnitude of cross-tissue *in vivo* genotoxic effects elicited by oral exposures to the PAHs and PAH-mixtures investigated. BaP is a well-studied PAH, and there are numerous published works outlining how metabolism, distribution to target tissues, nucleotide excision repair, and translesion synthesis contribute to its genotoxicity. For most other PAHs, a few studies have investigated the influence of some of these processes on genotoxicity; however, for the most part, they have not examined how all of these factors contribute to the nature and magnitude of genotoxic effects. Thus, there is a need for follow-up mechanistic studies that assess the involvement of toxicokinetics, repair, and lesion by-pass in determining the tissue-specific effects of orally delivered PAHs. For example, future studies could examine tissue-specific, temporal changes in gene expression, protein production, and enzyme activity related to these processes (e.g., activity of P450 isozymes, expression of repair and cell cycle control genes). In addition, rodent knock-out models (e.g., *Polk* knockout) could be used to assess the involvement of specific genes/proteins in determining the outcome of oral PAH exposures.

For BaP, the available information clearly indicates that metabolism plays a critical role in determining the exposures of both distal and proximal tissues, with effects on distal tissues being largely dependent on the ability to metabolise and excrete orally delivered PAHs [Nebert et al., 2013]. This is likely the case for most of the PAHs

examined; however, the paucity of data hampers the ability to make definitive statements. Additionally, the cross-compound complexity of PAH metabolism, the lack of oral *in vivo* studies (i.e., many are dermal), and the lack of cross-tissue information on PAH metabolism and damage processing hampers definitive, robust interpretation of the results presented herein. For example, as noted in **Chapter 1**, several metabolic pathways have been described for PAHs [Penning, 2014; Penning et al., 1996; IARC., 2010], and even within a single pathway there are contributions from a number of different enzymes and/or P450 isozymes (e.g., CYP1A1, CYP1B1, CYP1A2, epoxide hydrolase) [Nebert et al., 2013; Shimada, 2006; Shimada and Fujii-Kuriyama, 2004; Xue and Warshawsky, 2005]. However, most published murine studies only examined the expression and contributions of a handful of genes/enzymes in a few tissues; cross-tissue and/or cross-compound differences in basal gene expression, induced gene expression, and enzyme activity levels are rarely reported. The follow-up research avenues outlined above could also examine cross-tissue differences in basal and induced levels of metabolic enzymes; these research avenues could determine the mechanistic causes underlying the observed cross-compound differences between effects manifested in proximal (e.g., glandular stomach, small intestine, liver) and distal (e.g., bone marrow, lung) tissues.

The rate and type of PAH lesion processing are also determinants of PAH-induced genetic damage. Current knowledge indicates that the major mutagenic metabolite of BaP is benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), and BPDE-DNA adducts are generally thought to be correctly repaired by via nucleotide excision repair (NER). DBaP has been demonstrated to induce mutagenicity not only as a result of highly persistent bulky adducts, but also due to depurinating adducts, with substitution

mutations resulting from incorrect repair of abasic sites [Chakravarti et al., 2000]. Additionally, chrysene seems to produce unstable adducts that slowly depurinate; however, the resulting abasic sites are thought to be correctly and rapidly repaired by base excision repair [Braithwaite et al., 1998]. Unfortunately, little repair data exists for other forms of PAH-induced DNA damage. Moreover, the vast majority of the existing repair research has been conducted *in vitro* [Marteijn et al., 2014], and *in vivo* studies on this topic are only beginning to be published. Although *in vivo* DNA repair studies are challenging, improved understanding of the mechanisms underlying cross-tissue and cross-compound differences in the repair of PAH lesions will require *in vivo* tracking of adduct fate. Moreover, adduct fate could be examined in wild type animals as well as NER (e.g., *Xpa*) and/or BER knockouts.

Information related to cross-tissue and cross-compound TLS across PAH adducts is markedly more limited than information on metabolism and repair. For some PAHs, even the structure of the mutagenic adducts remain to be elucidated (e.g., BkF, INDENO, BghiP) [IARC., 2010], and, with the exception of BaP, the role of TLS in the processing of PAH-induced damage is not at all clear. In addition, although the literature documents error-free polymerase κ bypass of dG-BPDE lesions, as well as error-prone bypass of the same lesions by polymerase η , the tissue-specific level and efficacy of lesion by-pass has not been investigated. Since incorrect lesion bypass is thought to be the main mechanism of PAH-induced mutagenesis, and different Y-Family and B-family polymerases [Liu et al., 2014; Jha et al., 2016; Yang and Woodgate, 2007; Christmann et al., 2016; Hashimoto et al., 2012] are known to bypass dG-BPDE adducts with different fidelities, follow up work could address the nature and efficacy of lesion bypass for other PAHs.

This type of research, which could also make use of knockout models, could investigate the cross-tissue, cross compound and cross-adduct efficacy of TLS. Although this type of work would be challenging and time-consuming, it could dramatically enhance the mechanistic understanding of PAH lesion processing *in vivo*.

Evaluations of hypotheses regarding the processes that determine the nature and magnitude of PAH-induced genotoxic effects *in vivo* (e.g., metabolism, repair and lesion bypass) could employ knockouts in TGR animals. Such knockouts, which could restrict the aforementioned processes that drive mutation establishment (e.g., metabolism, repair, TLS), would combine the utility of TGRs for *in vivo* mutation detection with the utility of targeted deficiencies. For example, evaluations of tissue-specific, induced mutant frequencies in TGRs lacking functional genes known or suspected to be involved in PAH metabolism, or genes known to be involved in repair and/or TLS, would afford the aforementioned improvements in mechanistic understanding. There are several published examples illustrating how TGR knockouts can be used to investigate the mechanisms underlying the induction of spontaneous and induced genetic damage [Van Oostrom et al., 1999; Wang et al., 2012; Huamani et al., 2004; Stancel et al., 2009]. For example, BigBlue® mice (i.e., *lacI* and *cII* mutation targets on λ -LIZ shuttle vector) have been crossed with *Polk*^{-/-} mice and *Polh*^{-/-} mice, producing two different TGR knockout strains used to investigate the involvements of Y-Family polymerases in the formation of spontaneous mutations. Interestingly, the authors concluded that *Polk*^{-/-} mice have a spontaneous mutator phenotype [Stancel et al., 2009]. Another study crossed *lacZ* plasmid mice with *Xpa*^{-/-} and *p53*^{+/-} mice, creating both single and double knockouts that permitted investigations regarding the contribution of nucleotide excision repair and cell

cycle arrest on the frequency of BaP-induced mutations [Van Oostrom et al., 1999]. By creating double knockouts for *Nat1* and *Nat2*, another group investigated the mutagenic impact of alterations in the metabolism and bioactivation of 4-aminobiphenyl in the MutaTMMouse [Wang et al., 2012]. Finally, spontaneous mutagenesis in *lacI* mice heterozygous for AP endonuclease (i.e., *lacI*⁺, *Apex*^{+/-}) was also investigated [Huamani et al., 2004]. Collectively, these studies show that genetic manipulation of TGR rodents; more specifically, manipulation of processes related to metabolism, repair, TLS, and cell cycle control, can be used to determine the mechanisms underlying compound- and tissue-specific induction of *in vivo* mutations. Nevertheless, it should be noted that TGR knockout models must employ the TGR rodent strains (e.g., BALB/C x DBA2 for MutaTMMouse, C57BL6/J for *gpt* delta mouse), and this could restrict the ability to construct useful TGR knockout models. Such restrictions would not apply to the aforementioned use of knock-outs to study PAH-induced DNA damage. With respect to the MutaTMMouse results presented herein, good starting points would be investigation regarding the impact of single and multiple knockouts of *Cyp1b1*, *Cyp1a1*, *Xpa*, and *Polk* on the tissue-specific frequencies of PAH-induced DNA adducts; moreover, the impact on the tissue-specific spectrum of *lacZ* mutants observed following sub-chronic oral exposures to individual PAHs and PAH mixtures.

It is important to note that evaluations of hypotheses related to tissue-specific metabolism and processing of PAH-induced damage (i.e., **Chapters 2, 5, and 6**) can also be addressed using the wild-type MutaTMMouse. Indeed follow-up experiments are already partly completed. More specifically, follow-up MutaTMMouse studies are examining tissue-specific temporal changes (i.e., 4 h, 12 h, 24 h, and 28 h post-exposure) in adduct

frequency and targeted gene expression following oral BaP exposure. Additional MutaTMMouse studies, which are also underway, are examining the cross-tissue levels of DNA adducts, the expression of genes related to metabolism, DNA damage and DNA repair, and the activity of several P450 isozymes following single acute oral exposures to the 9 PAHs examined in this thesis. Assessments of cross-tissue changes in PAH-induced expression of *Cyp1* genes will permit evaluation of hypotheses regarding the involvement of compound-specific induction of metabolism in the GI tract in determining the differential elicitation of genotoxic effects in proximal and distal tissues. Moreover, examination of cross-tissue differences in the expression of NER and TLS genes will provide *in vivo* results to interpret the observed cross-compound and cross-tissue relationships between mutagenic and genotoxic potency.

Chapter 4 investigated whether oral exposures to complex PAH mixtures elicit mutant frequency levels that are comparable to what would be expected under an assumption of component additivity. Although dietary sources make up the largest fraction of PAH exposure for the general population, inhalation and dermal exposures can also be significant. With respect to the former, tobacco smoking and participation in specific occupations can dramatically augment daily exposure levels [IARC., 2010; Phillips, 1999]. Moreover, the general population is also exposed to airborne PAHs in polluted (urban) air, diesel exhaust, environmental tobacco smoke, biomass combustion emissions (e.g., wood, straw, dung, etc.) associated with indoor cooking and heating, and other combustion-derived aerosols [IARC., 2010]. Thus, a natural follow up to the mixtures study presented in **Chapter 4** would entail investigations of mixture effects manifested following exposures via inhalation, intratracheal instillation, or dermal

contact. However, it should be noted that experimental evaluation of the additivity assumption for inhalation exposure would not be trivial. Inhalation studies are operationally challenging and laborious, and the required studies of the 8 targeted PAHs examined herein, in addition to PAH mixtures, would take approximately 5 years to complete. In light of the anticipated cost, complexity and time commitment, such studies are not planned. Nevertheless, a single *in vivo* intratracheal instillation MutaTMMouse study will be carried out during the summer of 2017. The study will examine tissue-specific mutagenic effects induced by pulmonary exposure to a complex PAH mixture from contaminated urban air. MutaTMMouse will be exposed via intratracheal instillation, and dose-related changes in the frequency of DNA damage and *lacZ* mutants will be assessed in the same tissues examined in the oral studies. Although this study will not address the question of component additivity, the work, which if successful will be the first demonstration of *in vivo* mutations induced by exposure to a complex PAH mixture from urban air particulate matter, could be followed up with suitably-matched assessments of individual PAHs [IARC., 2015].

There are numerous other avenues for meaningful follow-up on the PAH mixtures work presented in **Chapters 3 and 4**. For example, additional work could also examine PAH mixtures that are not derived from coal tar (e.g., extracts of particulate matter from diesel exhaust, cigarette smoke, or wood smoke). The PAH composition of such mixtures would almost certainly differ from those investigated in this thesis. Numerous studies have shown that the profiles of PAHs in complex PAH mixtures are source dependent [Yunker et al., 2002]; the proportional representation of specific PAHs (e.g., high molecular weight PAHs) will almost certainly influence mixture genotoxicity and the

suitability of the component additivity paradigm employed for HHRA. Examination of more simplistic synthetic mixtures may also prove useful for investigating the mechanisms underlying the observed more-than-additive and less-than-additive mutagenic effects in selected tissues. Finally, as we observed considerable differences in the potency of individual PAHs and mixtures across tissues, resulting in different levels of experimentally-derived BaP-equivalent levels across tissues, the development of tissue-specific PEFs could be undertaken in order to conduct cross-tissue HHRA via the PEF-method described in **Chapter 4**. Interestingly, the USEPA is currently in the process of developing tissue-specific PEFs, also known as RPFs (relative potency factors), for tissue specific cancer risk assessments of PAH mixtures. However, the draft document, which was first posted online in 2010, has not been finalised. Finally, future studies could also investigate the contributions of other compounds in complex PAH mixtures (e.g., non-priority or uncharacterised PAHs, more polar PACs) to the observed *in vivo* mutagenicity. This could be investigated by preparing fractions of extracts from PAH-contaminated matrices such as coal tar, and examining the cross-tissue effects induced by sub-chronic oral exposures to each fraction. The contributions from non-targeted PAHs and polar PACs are not currently considered in HHRA of PAH contaminated material (e.g., soil) [Lemieux et al., 2015a; Lemieux et al., 2015b]; therefore, this is an important data gap that should be scrutinised.

The studies in **Chapters 6 and 7** presented quantitative relationships between the mutagenic and genotoxic potencies of PAHs. Significant cross-compound relationships were identified for liver, lung, and glandular stomach; however, significant relationships between these two endpoints were not observed for bone marrow and small intestine.

Scrutiny of the adduct frequency dose-response data for bone marrow and small intestine revealed that the responses frequently reached a plateau at the top two or three doses examined. As a result, the results failed to show dose-dependent increases in response, and concomitantly, the dose-response analyses yielded poor potency estimates (i.e., BMD₁₀₀ values). Although the MutaTMMouse system permits assessment of multiple endpoints in a single study, thereby contributing to reductions in animal use, the results obtained suggest that a wider dose range may be required to permit accurate and precise BMD determination. Nevertheless, the use of more doses with fewer animals per dose can reduce the need for additional animals, and this animal use strategy has been recommended for accurate and precise BMD determination [Slob, 2014]. The influence of additional doses on BMD precision is illustrated by the 11-dose MegaBaP study (**Chapter 6**) that simultaneously examined both adduct and *lacZ* mutant frequency across 8 tissues (including small intestine and bone marrow). Additional investigations are necessary to determine whether superior data would permit detection of significant relationships between mutagenic and genotoxic potency for other tissues. Such studies would require determination of DNA adduct frequency induced by lower PAH doses, and re-estimation of the BMD₁₀₀ and associated confidence intervals. Moreover, investigations of quantitative relationships between mutagenic and genotoxic potency should be expanded to other compound types. An improved quantitative understanding regarding the tissue-specific mutagenicity of stable adducts will improve the ability to interpret adduct frequency data in a risk assessment context, potentially allowing the use DNA adduct potency to predict mutagenic potency, an endpoint that is more relevant to human risk.

All of the studies presented in this thesis employed the transgenic MutaTMMouse to assess the frequency of *in vivo lacZ* mutants in selected tissues. This technology allowed scoring of the frequency of chemically-induced mutations; and moreover, straight-forward investigations of cross-tissue patterns of induced mutant frequencies. This type of analysis is simply not possible with other *in vivo* genetic toxicity assays, which are largely restricted to hematopoietic tissues. Analyses of TGR results such as those presented herein readily permit investigations of differences in response across, as mentioned, compounds and tissues, as well as routes of exposure and transgenic loci. In addition to the use of TGR dose-response data for mechanistic investigations related to cross-tissue and cross-compound differences in chemically-induced mutations, the analyses also permit scrutiny of TGR assay regulatory utility. The results presented in **Chapter 2** demonstrated that accurate assessment of mutagenic hazard often requires analyses of more than one tissue, and the role of tissue selection and exposure regimen on accurate detection of mutagenic hazard is worthy of further investigation.

Although it is unlikely that TGR assays, which have proven to be extremely useful for regulatory evaluations of chemicals, will be replaced anytime soon, it is interesting to note that novel next generation sequencing (NGS) approaches for genetic toxicity assessment are being developed. In theory, NGS-based approaches could score any locus; however, sequencing fidelity does not currently permit accurate enumeration of rare mutations. Nevertheless, NGS technology has been recently adopted for high-throughput sequencing of *lacZ* mutations, and related determinations of mutation spectra [Beal et al., 2015]. Although analyses of mutation spectra, which have been conducted since the 1980's [Cariello et al., 1994; Adams and Skopek, 1987], can be used to examine

mutagenic mechanism, until recently the required sequencing of mutant loci was time consuming and expensive. Consequently, studies of MutaTMMouse transgene mutation spectra commonly involved Sanger sequencing the short (294 bp) *cII* gene [Jacobsen et al., 2011]. The NGS approach has all but replaced the need to use Sanger sequencing to examine induced and spontaneous mutation. Despite the current restriction of NGS technologies to mutation spectra determination, it must be acknowledged that the current TGR technologies for *in vivo* mutation enumeration will eventually be superseded. Future work should focus on refining technologies for high fidelity, single-cell NGS analysis that permits accurate enumeration of induced somatic and germ cell mutations in experimental animals and/or humans. This would eliminate the need for expensive TGRs. Moreover, an NGS approach could be used for human biomonitoring of induced mutations, and follow-up molecular epidemiological analyses to identify lifestyles, diet, habits, and occupations that increase the risk of mutagenic effect in easily monitored tissues (e.g., buccal swabs).

It is interesting to note that analyses of mutation spectra are now being used to link specific exposures with the spectrum of mutations in human tumours. Although this type of analysis is not new, prior to the advent of NGS technology it was accomplished via Sanger sequencing of exons in single loci [DeMarini et al., 2001]. More recently, NGS technology has been used to rapidly examine mutation spectra in human tumours, derive mutational signatures across the entire genome, and investigate the mutational etiology of human cancers [Alexandrov et al., 2013]. More specifically, Alexandrov and colleagues have used mutation spectrum analyses to examine base-pair substitutions, along with the local sequence context of the mutations (i.e., one nucleotide on either

side), to identify, compare, and contrast mutation signatures across human cancers. To date, many of the signatures have been associated with endogenous biological processes such as aging, inherited or acquired mutations in genes controlling the production and processing of DNA damage (e.g., BRCA1/2, Polymerase ϵ), and exogenous exposures such as ultraviolet light, aflatoxin, aristolochic acid, and BaP [Alexandrov et al., 2013]. Additionally, Alexandrov and colleagues recently identified mutational signatures that are specifically associated with tobacco smoking [Alexandrov et al., 2016]. Collectively, these works are rapidly advancing our understanding of the biological processes and exogenous exposures that generate the mutations linked to human carcinogenesis. Similar spectral analysis of mutations in experimental animals exposed to various genotoxic agents could permit the identification of mutational signatures that can subsequently be linked to those observed in specific types of human tumours.

One noteworthy disadvantage of the TGR systems employed herein is the time required for sample processing and scoring. Numerous toxicity assessment programs (e.g., the US Environmental Protection Agency's ToxCast™ program) now employ automation that permits rapid, high-throughput sample processing; however, the method employed to tabulate mutant and titre plaques is not readily amenable to automation. Thus, the TGR assay systems, as they currently exist, are incongruent with emerging regulatory programs that have become increasingly focused on high throughput assays that are amenable to semi- or complete automation [National Research Council, 2007]. However, advances in image analysis may provide an avenue for improvements of TGR systems. Recently, a group at the Massachusetts Institute of Technology developed a transgenic mouse that permits visual identification of homologous recombination (HR)

events via a fluorescent signal. The mouse, termed the **Rosa26-GFP Direct Repeat-GFP** (i.e., RaDR) mouse [Sukup-Jackson et al., 2014], contains two truncated EGFP expression cassettes. When HR occurs, the combined cassettes permit production of a functional protein and digital image analysis can be used to score HR events in tissue sections. This type of scoring eliminates the need for DNA extraction (1.5 days per 20-30 samples), the need to adequately dissolve high molecular weight DNA in buffer (2 days), and the need to conduct a mutant frequency enumeration assay (e.g., PGal positive selection assay, 2 days per 20-30 samples). Although the RaDR mouse can only detect HR events, the principles employed to construct the RaDR system could be used to design a TGR mouse that elicits a mutation-induced signal that can be assessed using image analysis. Such a system could make use of automated (fluorescence) microscopy and high-content, high-throughput image processing that permits rapid, accurate *in situ* scoring of mutant signals in tissue sections, or even individual cells within a tissue section [Kriston-Vizi and Flotow, 2017].

8.5 Concluding remarks

Overall, this thesis demonstrated that several PAHs are genotoxic and mutagenic across many tissues, and the nature and the magnitude of the effects in individual tissues is determined by a complex series of dynamic processes that regulate metabolism and damage processing (i.e., repair and TLS). Complex PAH mixtures can induce very high levels of genetic damage and mutations, and the magnitude of the tissue-specific responses are surprisingly close to those expected from a small number of mixture components. Use of the BMD approach for dose-response analysis of genetic toxicity

data permits the determination of potency metrics that can be used to assess cross-tissue and cross-compound relationships between genotoxicity and mutagenicity; and moreover, the impact of experimental covariates on dose-response functions. Given that mutations have been associated with several human diseases, including cancer and various genetic disorders, such exposures are clearly undesirable. Complete elimination of exposure is not possible; however, avoiding or reducing contact with combustion-derived materials can minimize exposures, and by extension, the associated risk of adverse health effects.

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APPENDIX A

Appendix A : In Vitro mammalian mutagenicity of complex polycyclic aromatic hydrocarbon mixtures in contaminated soils

A.1 Preamble: Authors, Affiliations, and Style

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Style: This chapter was prepared according to the style guide for *Environmental Science and Technology*.

A.2 Abstract

This study employed an *in vitro* version of the *lacZ* transgenic rodent mutation assay to assess the mutagenicity of non-polar neutral and semi-polar aromatic soil fractions from ten PAH-contaminated sites, and evaluated the assumption of dose additivity that is routinely employed to calculate the risk posed by PAH mixtures. Significant mutagenic activity was detected in all non-polar neutral fractions, and 8 of 10 semi-polar aromatic fractions (non-polar > semi-polar). Mutagenic activity of synthetic PAH mixtures that mimic the PAH content of the soils (i.e., 5-PAH or 16-PAH mix) were greater than that of the PAH-containing soil fractions, with 5-PAH mix > 16-PAH-mix. Predictions of mutagenic activity, calculated as the sum of the contributions from the mutagenic mixture

components, were all within two-fold of the observed activity of the non-polar neutral fractions, with one exception. Observed differences in mutagenic activity are likely the result of dynamic metabolic processes, involving a complex interplay of AhR agonism and saturation of metabolic machinery by competitive inhibition of mixture components. The presence of hitherto unidentified polar compounds present in PAH-contaminated soils may also contribute to overall hazard; however, these compounds are generally not included in current contaminated site risk assessment protocols.

A.3 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants formed via the incomplete combustion of organic material. Many PAHs have been classified as either known human carcinogens (e.g., benzo[*a*]pyrene, BaP), probable carcinogens (e.g., dibenz[*a,h*]anthracene), or possible carcinogens (e.g., benz[*a*]anthracene, benzo[*b*]fluoranthene) ¹. Carcinogenic PAHs generally act via a mutagenic mode of action; they are first converted into electrophilic metabolites (e.g., benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide) that can react with DNA resulting in the formation of bulky adducts, which in turn can contribute to the formation of permanent sequence changes – i.e., mutations. Many PAHs are also aryl hydrocarbon receptor (AhR) agonists that are capable of inducing their own metabolism via regulation of CYP enzymes controlled by AhR-mediated pathways ².

PAHs are almost always found in the environment as components of complex mixtures that contain hundreds, or even thousands, of substances, and many PAH-containing mixtures have also been classified as known human carcinogens (e.g., coal tar, tobacco

smoke) or probable carcinogens (e.g., coal tar creosote) ^{1,3}. Many former industrial sites contain soil that is contaminated with complex mixtures of PAHs and related substances. For example, industrial sites that were involved in the manufacturing of coal-tar creosote for wood preservation are often highly contaminated with a variety of PAHs and other polycyclic aromatic compounds (PACs) (e.g., O- and S- heterocyclics) ⁴. Industrial sites involved in the production of manufactured gas (also known as coal gas or town gas) and/or coke have also been shown to be contaminated with mixtures of PAHs and related compounds due to the production and improper disposal of coal tar ^{5,6}.

Hazard assessment of contaminated soils containing complex mixtures of PAHs and related compounds is not a simple task. The presence of unidentified compounds in the mixtures is a well-known stumbling block that hinders accurate assessment of hazard and risk. Moreover, there is a paucity of reliable information regarding the interactions of selected PAHs that have been prioritized for assessment and control. In recent years, some studies have employed methods such as bioassay-directed chemical fractionation to isolate and identify hazardous compounds, such as PACs, that are present at contaminated sites such as former manufactured gas plants (MGPs) sites (for example, see Brooks et al, 1998⁷). In principle, these substances can be toxicologically assessed and subsequently included in determinations of hazard and/or risk. Other studies have investigated the validity of some of the assumptions and methods routinely used for the assessment of hazard and risk posed by PAHs present at these sites ⁸⁻¹⁰ (e.g., dose additivity, potency equivalency factors, etc.). Numerous mechanistic studies have also been carried out in an effort to understand the genotoxicity of PAHs in mixtures (for example, see ¹⁰⁻¹⁹).

The methodology routinely employed to assess the excess lifetime cancer risks posed by complex PAH-contaminated materials (e.g., soil) rarely involves biological assessment of the actual whole mixture. Moreover, although some groups have recommended the use of sufficiently similar “surrogate” mixtures to assess the risks posed by PAH mixtures, suitable data are rarely available²⁰⁻²³. Rather, cancer risk assessment methods for mixtures, which are currently advocated by governmental agencies such as the U.S. Environmental Protection Agency (U.S. EPA) and Health Canada (HC), calculate total risk as the incremental sum of the contributions from a targeted set of chemicals that are assumed to have the same mode of action^{21, 24}. For example, assessments for PAH-contaminated material typically calculate risk as the incremental sum of the contributions from a subset of PAHs highlighted by the U.S. EPA as priority PAHs²⁵. The contributions of each monitored PAH to the total hazard of the mixture are generally inferred by employing the comparative potency of each compound relative to BaP, and an assumption of simple additivity is used to calculate the total concentration of BaP equivalents in the mixture. Although there is debate about the value of the Potency Equivalency Factors (PEFs, also called Relative Potency Factors and Toxic Equivalency Factors) required to calculate total BaP equivalents for a PAH mixture, governmental agencies in several countries (e.g., USA, Canada, Sweden, the Netherlands, the UK), as well as the WHO Internal Programme on Chemical Safety (IPCS), advocate the use of the PEF concept²⁶. For a more detailed discussion of complex mixture risk assessment methods, the reader is referred to our companion paper²⁷.

Previous work in our laboratory used the Salmonella reverse mutation assay (i.e., the Ames test) to scrutinise the aforementioned approach for the assessment of excess

lifetime cancer risk attributable to mutagenic PAHs in complex mixtures. More specifically, an additive, chemical-specific approach was employed to predict the mutagenic activity of complex PAH mixtures in ten contaminated soils, and these predictions were compared to the observed mutagenic activity. We demonstrated that the sums of the contributions from the individual priority PAHs detected in the soils, as well as the mutagenic activity of synthetic mixtures of priority PAHs that constituted sufficiently similar mixtures, were both greater than the observed activities of the corresponding complex PAH mixtures obtained from the contaminated material (i.e., organic fractions of the contaminated soils) ⁸. These results suggested that risk assessment methods currently employed for carcinogenic PAHs with a mutagenic mode of action, which express total risk as the sum of the incremental contributions from a small number of targeted substances, may be conservative (i.e., overestimate actual risk). However, the Salmonella test system employs an exogenous metabolic activation mixture with extraordinarily high cytochrome P4501A1 activity, and is therefore exceptionally sensitive to some priority PAHs (e.g., BaP). Furthermore, when tested in isolation, mutagenic PAHs have exclusive access to the enzymes required for metabolic activation. Thus, the resulting individual potency values can contribute to a sum that greatly exceeds the observed activity of the mixture, and the conclusions of our earlier study may not be relevant to the determination of risk for mammalian cells. In our previous study, we demonstrated that polar compounds also contribute to the overall mutagenic activity of soils contaminated with complex mixtures of PAHs and PACs. Such compounds are therefore expected to contribute to the overall hazard and risk of a contaminated site;

however, their contribution is not addressed by current contaminated site risk assessment protocols.

The aim of the current study was to expand on our earlier work, and, employing an *in vitro* mammalian cell system for assessment of mutagenic activity, continue to evaluate the aforementioned assumption of dose additivity that is routinely employed to calculate the risk posed by PAH mixtures. We contend that a test system based on mammalian cells can provide a more realistic assessment of potential human health hazard, and a more robust evaluation of the assumptions routinely employed for risk assessment of PAH mixtures. More specifically, we measured the induction of mutations in a transgenic murine cell line following exposure to non-polar neutral (i.e., PAH-containing) or semi-polar aromatic fractions derived from ten PAH-contaminated soils. We then compared the observed levels of mutagenic activity to those of sufficiently similar synthetic PAH mixtures, and moreover, to predicted activities calculated as the sums of the contributions from the individual priority PAHs detected in each of the samples. Assessment of mutagenic activity employed the MutaTMMouse FE1 cell line, an *in vitro* version of the *lacZ* transgenic rodent mutation assay²⁸. The FE1 cell line, which is a cytogenetically stable lung epithelial cell line derived from an adult male, is metabolically competent and can readily convert a variety of PAHs and PACs, including nitroarenes and aromatic amines, into DNA-reactive mutagens. FE1 cells are known to express CYP1A1, CYP1A2, and CYP1B1 and their activities are sufficient to metabolize PAHs to DNA-reactive metabolites^{29, 30}.

A.4 Materials and Methods

Chemicals

Safety warning – several PAHs are known or suspected human carcinogens; they should be handled with extreme care.

All chemicals used for the extraction, fractionation and chemical analysis of the soil samples were analytical grade ($\geq 99\%$) and obtained from EMD Chemicals (Gibbstown, NJ). All reagents, cell culture media, and media supplements used for the mutagenicity assessments were obtained from Gibco-Invitrogen (Burlington, ON, Canada), unless otherwise indicated. PAHs were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

Soil pre-treatment, extraction, fractionation and chemical analysis

Ten soil samples, obtained from five PAH-contaminated industrial sites in Sweden, were examined in this study. The sites included three wood preservation facilities (Holmsund, Forsmo and Hässleholm), one MGP site (Husarviken) and one coke oven site (Luleå). All facilities, with the exception of the coke production facility in Luleå, were no longer in operation at the time of sampling. Detailed site information can be found in Lemieux et al. (2008) ⁸.

The soils were previously characterized by our research group, and a complete description of the soil handling and extraction procedures is outlined in Lemieux et al. (2008) ⁸. These procedures have been extensively validated and show acceptable recoveries and reproducibility ³¹⁻³³. Briefly, the soil samples were air-dried and sieved (2

mm), and organic substances extracted via pressurized liquid extraction using an ASE 200 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA). The extracts were then fractionated on open silica columns (10% w/w deactivated) into three fractions: (i) aliphatics, (ii) non-polar neutral compounds, and (iii) semi-polar aromatic compounds using hexane, followed by hexane:dichloromethane (3:1), and dichloromethane, respectively. Prior validation of the fractionation protocol confirmed that homocyclic, unsubstituted PAHs and alkyl-PAHs, including the U.S. EPA priority PAHs, are contained in the second fraction, and semi-polar PACs, including oxy-PAHs and nitrogen heterocyclic PACs, are contained in the third fraction³². The first fraction was discarded, and the subsequent two fractions were evaporated to ~1 ml using a gentle stream of ultra-pure nitrogen. 500 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) was then added, and the samples reduced under nitrogen to a final volume of 500 µl. All samples were stored at 4°C until mutagenicity testing. Characterization of the PAHs in the soils was carried out using gas chromatography-mass spectrometry as described previously^{8, 32}.

PAHs and synthetic mixtures of priority PAHs

Stock solutions of individual priority PAHs and synthetic PAH mixtures were prepared in DMSO. Two types of synthetic PAH mixtures were prepared, each containing PAHs in matched proportions to the PAH profiles of the non-polar neutral soil fractions. The first type of mixture included all 16 U.S. EPA priority PAHs (i.e., naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene, benzo[*a*]anthracene, benzo[*k*]fluoranthene, chrysene, BaP, benzo[*b*]fluoranthene,

indeno[1,2,3-*c,d*]pyrene, dibenz[*a,h*]anthracene and benz[*g,h,i*]perylene). The second type of mixture included only the 5 PAHs that were previously observed to induce mutations at the transgenic *lacZ* locus in FE1 cells (i.e., benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene and dibenz[*a,h*]anthracene). The mixtures are hereinafter simply referred to as the 16-PAH and 5-PAH mixtures, respectively. All PAHs and PAH mixtures were stored in amber glass vials at -20°C until use.

Mutagenicity of Soil fractions, PAHs and Synthetic PAH Mixtures

FE1 cell line

A pulmonary epithelial cell line, denoted FE1, derived from the transgenic MutaTM Mouse, was used to assess the mutagenic activity of the soil fractions, PAHs, and synthetic PAH mixtures. This cell line has been characterized, previously used for mutagenicity assessment, and is described in detail in White et al (2003)²⁹ and Berndt-Weiss et al (2009)³⁰. Note that FE1 cells also contain a functional P53 gene (i.e., are p53-competent) (unpublished results). FE1 cells were maintained in DMEM/F12 (1:1) supplemented with 2% v/v foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 ng/ml murine epidermal growth factor (Roche Diagnostics, Laval, QC, Canada). Treatment medium, prepared without FBS, was used to maintain the cells during all chemical exposures. All incubations were carried out at 37°C, 95% humidity and 5% CO₂.

Exposure to PAHs, Synthetic Mixtures and Soil Fractions

FE1 cells were initially exposed to each of the 16 U.S. EPA priority PAHs. PAHs that induced a significant increase in *lacZ* mutant frequency at a concentration of 10 µg/ml or less were selected for further testing, at multiple concentrations (i.e., 4 or more), in duplicate. These included benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene and dibenz[*a,h*]anthracene. The cells were also exposed to each of the ten non-polar neutral soil fractions, each of the ten semi-polar aromatic soil fractions, and each of the ten synthetic 5-PAH and 16-PAH mixtures. In each case, FE1 cells were exposed to at least four concentrations, in duplicate.

Preliminary experiments on PAHs included assays conducted with and without an exogenous metabolic activation mixture containing Aroclor-1254 induced rat liver S9 from male Sprague Dawley rats (Molecular Toxicology Inc., Boone, NC). However, no significant difference in mutant frequency was observed upon the addition of an exogenous metabolic activation mixture (data not shown), and all subsequent exposures were carried out without S9. A positive control (0.1 µg BaP/ml) and a solvent (DMSO) control were run concurrently during each exposure, in duplicate.

Approximately 3×10^5 cells were seeded into 100-mm polystyrene culture dishes and grown overnight (~16 h) to approximately 20% confluence. The following morning, the medium on each plate was replaced with 5 ml of serum-free medium containing 50 µl of the appropriate dilution of the desired test article (i.e., soil fraction, individual PAH, 5-PAH- or 16-PAH- mixture). All chemical dilutions were freshly prepared in DMSO on the morning of the exposure. FE1 cells were incubated in the treatment medium for 6 h,

washed with Dulbecco's phosphate buffered saline (DPBS), and incubated for 72 h in serum-containing medium to permit mutation fixation.

Following the mutation fixation period, the medium was removed and cells incubated overnight in 3 ml of lysis buffer (10 mM Tris pH 7.6 (Caledon Laboratories Ltd, Georgetown, Canada), 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich Canada Ltd., Oakville, Canada), 150 mM NaCl, 1% (w/v) sodium dodecyl sulfate (SDS) and 1mg/ml proteinase K (≥ 20 units/mg)). Total genomic DNA was then isolated from each plate using standard phenol-chloroform extraction, followed by precipitation in ethanol, as previously described²⁹. DNA was stored at 4°C until mutation scoring via the phenyl- β -D-galactoside (P-Gal) positive selection assay.

Detection of lacZ mutations

The frequency of mutant *lacZ* loci in genomic DNA isolated from exposed FE1 cells was determined using the P-Gal positive selection assay as described elsewhere³⁴. Briefly, *lacZ* transgenes were rescued from total genomic DNA and packaged into λ phage particles using the Transpack™ lambda packaging system (Agilent Technologies, Mississauga, ON, Canada). Phage particles were mixed with the host bacterium (*Escherichia coli lacZ⁻, galE⁻, recA⁻, pAA119 with galT and galK*), plated on minimal medium containing the selective agent (i.e., 0.3% w/v P-Gal), incubated overnight at 37°C, and scored for *lacZ* mutants (i.e., plaques). The total number of plaque-forming units (pfu) (i.e., number of λ vectors containing the *lacZ* transgene rescued from genomic DNA) was measured on concurrent titre plates without P-Gal. Mutant frequency (MF) was calculated as the ratio of mutant plaques to total pfu. Induced MF, calculated as the

observed MF minus the experiment-specific spontaneous MF, was used in calculating all mutagenic potency values. This permits the elimination of inter-day variations in observed spontaneous mutant frequencies.

Data Analysis

All data and statistical analyses were carried out in Microsoft Excel 2010. For each PAH, soil fraction, and PAH mixture, concentration response curves were constructed by plotting induced MF versus concentration. Mutagenic potency was calculated for each sample using ordinary least-squares linear regression on the linear portion of the concentration-response function. Only mutagenic potencies with $p < 0.05$ (i.e., slope significantly greater than zero) were considered significant.

The mutagenic potencies of the individual PAHs were used to calculate a predicted mutagenic potency for each non-polar neutral soil fraction according to **Equation 1**⁸:

$$[1] \quad \text{Total Predicted Mutagenic Activity} = \sum_{i=1}^n \text{Observed Activity of PAH}_i \times \text{PAH}_i \text{ Concentration in Soil Fraction}$$

for priority PAHs 1 through n .

Where total predicted mutagenic activity is expressed as induced mutants $\times 10^{-5}$ /mg eq. dry soil/ml, the observed activity of each PAH is expressed as induced mutants $\times 10^{-5}$ /μg PAH/ml, and the concentration of PAHs in the soil fraction is expressed as μg PAH/mg eq. dry soil.

The mutagenic potencies of the synthetic PAH mixtures were compared to those of the corresponding non-polar neutral fractions using the two-tailed Student *t*-test ($p \leq 0.05$) with the appropriate multiple test correction (i.e., Bonferroni)⁸. Statistical comparisons of the mutagenic activities calculated using equation [1] and the corresponding observed activities of the soil fractions were not carried out due to the excessive variance associated with the predicted values. The high variance can largely be attributed to the variance associated with predicted contributions of each PAH to the total mutagenic activity of the mixture. Since it was not possible to include the detected concentrations of each PAH in the range of tested doses, the variance associated with each of the ‘new predictions’ is expected to be large³⁵.

A.5 Results

PAH Composition of Soils

The results of the chemical analyses have been published elsewhere. For complete details the reader is referred to Lemieux et al (2008)⁸. Briefly, the soil samples were found to contain several PAHs and alkyl PAHs, including the 16 U.S. EPA priority PAHs. Total priority PAH levels ranged from 70 – 9300 µg PAH/g dry soil. The **Supporting Information** includes a table that provides a summary of the PAH levels for the soils investigated.

Observed Mutagenicity of Soil Fractions

Mutant frequencies of positive (BaP) and negative (DMSO) controls, run concurrently during each exposure, were well within established historical limits. More specifically,

the mean mutant frequencies ($\times 10^{-5}$) were $578 \pm 29 \times 10^{-5}$ and $44 \pm 2.2 \times 10^{-5}$ mutants for positive and negative controls, respectively.

The mutagenic activity of the non-polar neutral and semi-polar aromatic fractions of the ten PAH-contaminated soils were evaluated using the *in vitro lacZ* transgenic mutation assay in Muta™Mouse FE1 cells. Linear concentration-responses functions were analysed, and the slope of the initial linear portion of the curve (i.e., the mutagenic potency) used as a measure of mutagenic activity for each fraction of each soil. The **Supporting Information** includes a figure that depicts a typical concentration-response plot for a non-polar neutral soil fraction and its corresponding semi-polar aromatic fraction. **Table A-I** summarizes the mutagenic potency values for each fraction of each of the 10 soils. The non-polar neutral fractions from all 10 soils elicited significant positive responses, and the semi-polar aromatic fraction from 8 of the 10 soils elicited significant positive responses. The mutagenic potencies of the non-polar neutral fractions ranged from 16 to 463×10^{-5} mutants/mg soil eq./ml, and those of the semi-polar aromatic fractions ranged from not detectable to 217×10^{-5} mutants/mg soil eq./ml.

Table A-I. Mutagenic potencies of non-polar neutral and semi-polar aromatic soil fractions measured using the *lacZ* transgene mutation assay in Muta™Mouse FE1 cells. Mutagenic potency is expressed as induced mutant frequency ($\times 10^{-5}$)/mg soil eq./ml $\pm$ standard error. In all cases the slope values are significant at $p < 0.01$.

	Mutagenic Potency (induced mutant frequency ($\times 10^{-5}$)/mg soil eq./ml)	
Soil	Non-polar neutral soil fraction	Semi-polar aromatic soil fraction
Holmsund1	16 $\pm$ 3	60.5 $\pm$ 17.5
Holmsund2	463.1 $\pm$ 50.1	3.9 $\pm$ 0.4
Holmsund3	174.6 $\pm$ 19.1	NM ^a
Luleå	29.9 $\pm$ 5.6	5.4 $\pm$ 1.3
Husarviken1	30.1 $\pm$ 6.1	3.8 $\pm$ 1
Husarviken2	49.7 $\pm$ 13	18.3 $\pm$ 4.3
Forsmo1	287.4 $\pm$ 4.3	216.6 $\pm$ 32.3
Forsmo2	397.7 $\pm$ 87.6	NM
Hässleholm1	221.2 $\pm$ 36.9	1.6 $\pm$ 0.4
Hässleholm2	102.1 $\pm$ 13.7	15.7 $\pm$ 4.5

^a NM = not mutagenic

The mutagenic potencies of the non-polar neutral fractions were generally greater, and often far greater, than those of the corresponding semi-polar aromatic fractions (e.g., 463.1×10^{-5} versus 3.9×10^{-5} mutants/mg soil eq./ml for Holmsund-2). The only exceptions were Holmsund-1, where the observed potency of the semi-polar aromatic fraction (i.e., 60.5×10^{-5} mutants/mg soil eq./ml) was greater than the corresponding non-polar neutral fraction (i.e., 16×10^{-5} mutants/mg soil eq./ml), and Forsmo-1, where the potencies of the two soil fractions were not significantly different at $p < 0.05$. Moreover, the mutagenic activity of the non-polar neutral soil fractions was found to be significantly correlated with both total and priority PAH concentration (i.e., $r^2 = 0.47$ for both, $p < 0.03$). The mutagenic activity of the semi-polar aromatic fractions was not correlated with PAH concentration.

Mutagenic Activities of Synthetic PAH Mixtures and Predicted Activities Based on Expected Contributions from Priority PAHs

The observed mutagenic potencies of the non-polar neutral fractions were compared to (i) the observed activity of the synthetic priority PAH mixtures, prepared using the results shown in **Suppl. Table A-I**, and (ii) the predicted activity calculated using equation [1]. Prediction of potency for the PAH-containing fractions, according to equation [1], required the mutagenic activity of each of the 16 U.S. EPA priority PAHs. As noted, only five priority PAHs (i.e., benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene and dibenz[*a,h*]anthracene) induced significant positive responses in the *lacZ* mutation assay in MutaTMMouse FE1 cells (data not shown). **Figure A.1** shows a comparison between the observed mutagenic potencies of the non-polar neutral fractions, and the predicted

potencies calculated using Equation [1]. The comparison shows that the predicted potencies are all within two-fold of the observed potencies, except for Holmsund-1, where the prediction was 21-fold higher than the observed potency. For 6 of the 10 soils studied, the predicted potencies were less than the corresponding observed values, while for 4 of the 10 soils the predicted potencies were greater than the observed values.

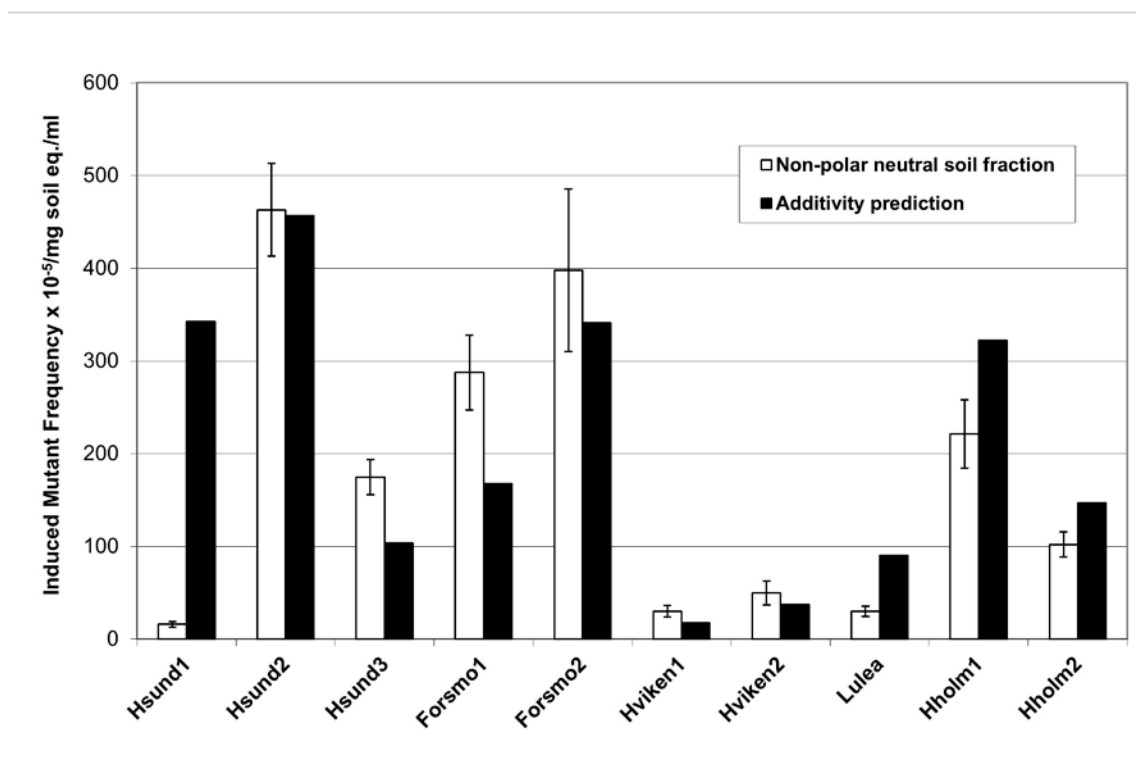


Figure A.1. Comparison of observed mutagenic potencies for the non-polar neutral soil fractions (□) to predictions of their activity calculated using equation [1] (■). All mutagenic potencies were measured using the *lacZ* transgene mutation assay in MutaTMMouse FE1 cells. Error bars represent standard error. Standard error values for predictions of mutagenic activity based on PAH additivity are not presented (see Materials and Methods for explanation). (Hsund = Holmsund; Hviken = Husarviken; Hholm = Hässleholm).

The observed potency values for the non-polar neutral fractions were also compared to the potencies of the synthetic PAH mixtures (i.e., sufficiently similar mixtures). Both the 5-PAH mixture, which contained only PAHs that elicited a positive response in the MutaTMMouse FE1 assay, and the 16-PAH mixture, which contained all 16 U.S. EPA priority PAHs, were compared to the PAH-containing fractions. Each of the synthetic PAH mixtures induced a significant response; however, the 5-PAH mixtures consistently elicited a greater response than the 16-PAH mixtures. **Figure A.2** shows a comparison of the mutagenic activities for both types of synthetic mixtures, and the corresponding non-polar neutral fractions. The 5-PAH mixture consistently yielded mutagenic activities that were significantly greater than, or not significantly different from, the corresponding soil fractions. Only a single simplified mixture yielded a response less than the corresponding non-polar neutral fraction (i.e., Forsmo-1). Similar results were obtained for the 16-PAH mixtures. Again, 9 of 10 mixtures elicited responses that were greater than, or not significantly different from, the corresponding soil fractions, and only one mixture yielded a response significantly less than its corresponding non-polar neutral fraction (i.e., Forsmo-1)

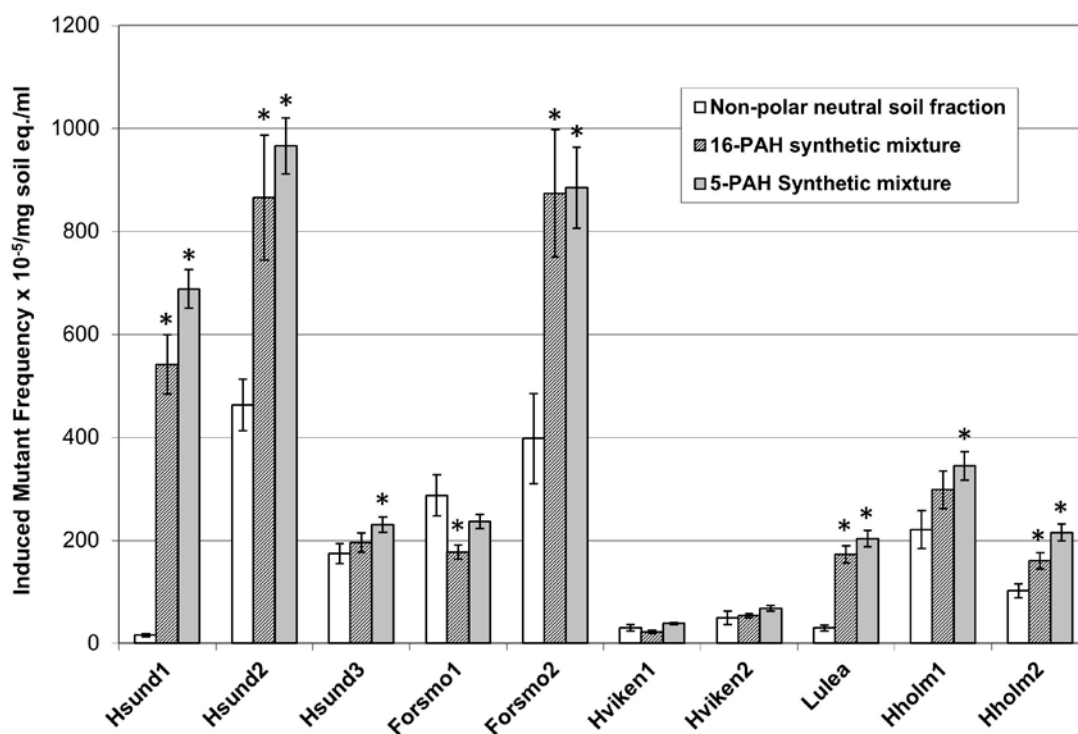


Figure A.2. Comparison of observed mutagenic potencies for the non-polar neutral soil fractions (□) to the activity of synthetic PAH mixtures composed of either 16 priority PAHs (▨) or 5 mutagenic PAHs (▩). All mutagenic potencies were measured using the *lacZ* transgene mutation assay in MutaTMMouse FE1 cells. Error bars represent standard error, and asterisks' (*) indicate where the mutagenic potency of the simplified, synthetic mixture was significantly different from the non-polar neutral fraction ($p < 0.05$). (Hsund = Holmsund; Hviken = Husarviken; Hholm = Hässleholm).

A.6 Discussion

Observed mutagenicity of soil fractions

To our knowledge, no studies have used cultured mammalian cells to evaluate the mutagenicity of soil extracts from a PAH-contaminated site; however, several studies have used cultured mammalian cells to assess the mutagenicity of PAH-containing extracts and/or fractions from other complex environmental samples such as urban air particulate matter^{36, 37}, diesel and gasoline exhaust particles³⁸, and coal oil³⁹. This study evaluated the mutagenicity of organic fractions from ten PAH-contaminated soils, and the results clearly show that both the non-polar neutral and semi-polar aromatic fractions from coal-tar and creosote contaminated soils induce a significant response in the *in vitro* *lacZ* transgenic mutagenicity assay in MutaTMMouse FE1 cells. The results follow those of our previous study, where we showed that these same soil fractions were mutagenic in a bacterial test system (i.e., the Salmonella reverse mutation assay)⁸. Other studies of PAH-contaminated soils have also noted potent activity in soil extracts, and PAH-containing extract fractions using the Salmonella assay. For example, de Souza Pohren et al. (2012) found significant Salmonella mutagenic activity in organic, PAH-containing extracts of soil from a wood preservation site⁴⁰, and Hughes et al. (1998) detected significant Salmonella mutagenicity in several soil fractions from a creosote-contaminated Superfund site⁴¹.

Here, and in our previous study⁸, the mutagenic activities of the PAH-containing soil fractions exceeded those of the corresponding semi-polar aromatic soil fractions, indicating that the mutagenic activity associated with extractable organics is dominated

by PAHs and similar non-polar neutral compounds (e.g., alkyl-PAHs). This assertion is supported by the significant positive correlation between the mutagenic activity of the non-polar neutral fractions and the PAH content of the soils examined. Moreover, several of the detected unsubstituted, homocyclic PAHs (e.g., BaP, benzo[*b*]fluoranthene) are recognized mutagens⁴²⁻⁴⁵. O- and S- heterocyclic compounds may also contribute to the mutagenicity of this fraction since earlier validation of the fractionation protocol showed that they can elute into the non-polar neutral fraction³².

The fact that the semi-polar aromatic soil fractions elicited significant positive responses, albeit weaker compared with those of the corresponding non-polar neutral fractions, should not be disregarded. Mutagenic activity in this soil fraction indicates that the soils contain hitherto unidentified mutagens that are more polar than priority PAHs, yet the hazards posed by these substances cannot currently be included in a routine risk assessment. The identities of mutagenic polar PACs present at PAH-contaminated sites remain largely unknown; however, insight into the types of compounds that might be expected in this fraction has been provided by other studies of PAH-contaminated sites (e.g.,^{8, 32, 46}). For example, such compounds may include oxygenated PAHs (i.e., oxy-PAHs), nitroarenes, or aromatic amines, including N-heterocyclic compounds, some of which have been shown to be mutagenic in bacteria⁴⁷, and mammalian cells^{44, 48}.

Indeed, recent studies of soils from wood preservation and MGP sites have attempted, sometimes unsuccessfully, to identify hitherto unknown polar mutagens³². For example, Park et al. (2008) detected significant mutagenic activity in polar soil fractions from former MGP and wood preservation sites, but they could not identify the putative mutagens responsible for the activity⁴⁹. Similar studies of contaminated sediments from

industrial sites in Germany noted that polar PACs such as nitroarenes, azaarenes and keto-PAHs contributed to the observed mutagenic activity; however, a multitude of other PACs, including hydroxy compounds, lactones and quinones were also tentatively identified in mutagenic polar fractions⁵⁰⁻⁵². The contributions of the latter substances could not be quantified due to a lack of chemical standards. Despite the obvious analytical challenge of positively identifying mutagenic polar PACs in contaminated soils, several of the researchers mentioned above have commented on the necessity, and moreover, have established the required analytical methodology^{33, 53-55}. The importance of investigating polar PACs in contaminated soils, particularly oxy-PAHs, has been highlighted by Lundstedt et al. 2007⁴⁶, and the identification and hazard assessment of polar PACs in contaminated soils in general is among the stated objectives of the Polar PAC Network (<http://www.mcnio.com/projects/the-polar-pac-network.html>).

Comparisons of the Complex Soil Fractions, Synthetic PAH Mixtures and the Sum of the Expected Contributions from Priority PAHs

Overall, the results show that predictions of mutagenicity based on the sum of the activity for each of the targeted, mutagenic PAHs are similar to the observed activities of the PAH-containing fractions, with the noteworthy exception of Holmsund-1 (**Figure A.1**). In fact, most predictions are within 2-fold of the measured activity. Nevertheless, a combination of factors, which are addressed in more detail below, can account for differences that were observed. These include alterations in metabolic capacity, as well as contributions from unidentified mixture components.

Instances where the predicted mutagenic activities are less than those observed for the PAH-containing fractions (e.g., Holmsund-3, Forsmo-1) are likely attributable to the presence of unidentified mutagens in the complex soil fractions. Predicted activity values are based solely on the contributions from priority PAHs and, as already noted, other compounds present in the soils may be contributing to the mutagenicity of the non-polar neutral soil fraction (e.g., alkyl PAHs, O- and S-heterocyclics). Information regarding the structure and mutagenic activity of such compounds is rarely available. Although these substances will contribute to the hazards posed by non-polar neutral pollutants at PAH-contaminated sites, the significance of that hazard cannot be ascertained until the putative mutagens are identified.

Another likely driving force behind the greater-than-additive effects of the complex PAH-containing fractions is the induction of enhanced AhR-mediated metabolism of the mixture components. Conversion of mutagenic PAHs to DNA-reactive metabolites is known to involve cytochrome P450 isozymes such as CYP1A1, CYP1A2 and CYP1B1, all of which are known to be inducible in FE1 cells, and the production of these isozymes is known to be controlled by AhR agonism. Since PAHs are AhR agonists, and are thus capable of inducing the enzymes required for their own metabolism², it is not unreasonable to expect that dynamic modifications in metabolic capacity plays an important role in determining the mutagenicity of a PAH mixture. For example, Mahadevan et al showed that exposure to a complex PAH-containing mixture (i.e., coal tar extract) increased the levels of CYP1A1 and CYP1B1 proteins in human MCF-7 cells; however, the same study showed that DNA adduct levels observed following co-treatment with coal tar extract and either BaP or DBaP (dibenzo[*a,l*]pyrene) were lower

than those observed for BaP or DBaP alone¹⁵. A similar study of urban air particulate matter showed that in vitro exposure can increase the expression of *CYP1A1* and *CYP1B1* genes, and co-treatment with BaP and urban dust augments gene expression relative to BaP alone; again, the level of DNA adducts for the co-treatment was lower than that for BaP alone¹³. Related studies by Courter et al. showed increased EROD (ethoxyresorufin-*O*-deethylase) activity and *CYP1B1* expression following in vitro co-exposure to diesel exhaust particulate matter extract and DBaP, compared to DBaP alone, and a slight increase in skin tumorigenicity following co-treatment with BaP and diesel exhaust particulate extract, compared to BaP alone^{17, 18}. Interestingly, several studies have also noted that in vitro exposures of human cells to complex PAH-containing mixtures such as cigarette smoke condensate⁵⁶ and diesel exhaust particulate matter extract¹⁷, or co-exposure to diesel particulate extract and either BaP or DBaP, can augment the expression of aldo-keto reductase (AKR) genes (e.g., *AKR1C1*). AKRs catalyse the conversion of PAH dihydrodiols to *o*-quinones that are known to be capable of inducing abasic sites and oxidative DNA lesions⁵⁷. In addition, non-mutagenic PAHs such as anthracene have been shown to enhance the mutagenic activity of BaP⁵⁸. Thus, mutagenic and non-mutagenic PAHs in the soil fractions investigated is likely contributing to the augmentation of the metabolic machinery in FE1 cells (e.g., P450 isozymes, AKRs, etc); this would enhance the metabolism of mutagenic PAHs to reactive metabolites, and thus augment the overall mutagenic activity of the mixture.

Conversely, instances where the predicted mutagenic activities calculated using equation [1] were greater than those observed for the PAH-containing fractions may result from competitive inhibition or saturation of enzymes involved in the metabolism and

activation of PAHs. Such phenomena would contribute to a reduction in the mutagenic activity of a complex PAH-containing mixture relative to the theoretical maximum calculated as the sum of the contributions of each of the mutagenic priority PAHs. It seems likely that both mutagenic and non-mutagenic PAHs in the complex PAH-containing fraction compete for access to CYP isozymes. In fact, many non-mutagenic PAHs, such as phenanthrene and naphthalene are known P450 substrates^{59, 60}, and several PAHs have been shown to inhibit the activity of human CYP1A1, 1A2 and 1B1⁶¹. Moreover, some studies have shown that the presence of non-mutagenic PAHs in a mixture with mutagenic PAHs, such as BaP, reduces mutagenic activity. In contrast to inhibition of mutagenic activity by co-exposure with non-mutagenic PAHs, *in vitro* exposures to mutagenic PAHs in isolation permit the mutagen to have exclusive access to the metabolic machinery required to convert the substance to a DNA-reactive metabolite. The resulting potency of each individual PAH can be viewed as a maximum, and this value may contribute to over-estimation of mixture activity according to equation [1].

This notion of ‘metabolic insufficiency’ for mutagenic PAHs in complex mixtures is supported by several studies. Courter et al. showed that the tumorigenicity of BaP is significantly delayed by co-treatment with a PAH-containing complex mixture (i.e., urban particulate matter extract), and that this effect is likely associated with dose-related, non-competitive inhibition of CYP1A1 and CYP1B1 activity¹⁶. Related studies noted that *in vitro* co-treatment with diesel particulate extract and BaP elicited significantly decreased levels of DNA adducts, compared with BaP alone; co-treatment of Sencar mice with DBaP and diesel particulate extract markedly reduced tumorigenicity, compared with DBaP alone^{17, 18}. The latter observation is thought to be related to the stronger

inhibitory effect of PAH-containing complex mixtures on CYP1B1 activity, relative to CYP1A1^{14, 18}. The aforementioned Mahadevan et al. studies showed that a complex PAH-containing mixture derived from coal tar can decrease the levels of DNA adducts formed by BaP or DBaP¹⁵. Follow-up work by the same group demonstrated competitive inhibition of human CYP enzymes in V79 cells, and moreover, that this competitive inhibition resulted in a reductions of BaP- and DBaP-induced DNA adduct formation; the inhibitory effects of the complex mixture on CYP1B1 activity was found to be much stronger in comparison with CYP1A1¹⁴. Similarly, a study by Binkova & Sram showed that environmental PAH mixtures containing BaP (i.e., extractable organic matter from respirable air particles) induced lower levels of DNA adducts in human embryonic lung fibroblast cells compared to BaP alone¹⁹.

The comparisons between the mutagenic activity of the PAH-containing non-polar neutral fraction and the synthetic PAH mixtures showed that the activity of the 5-PAH and 16-PAH mixtures is the same or higher than their corresponding complex mixtures. More specifically, the overall trend shows that the complex PAH-containing fractions have the lowest activity, followed by the 16-PAH mixture, and then the 5-PAH mixture. In other words, increased mixture complexity is associated with decreased mutagenic activity. This pattern is consistent with the aforementioned hypothesis of ‘metabolic insufficiency’, i.e., mutagenic PAHs in a more simplified mixture can be more effectively metabolized than mutagenic PAHs in a complex fraction. Comparison of the 5-PAH and 16-PAH mixtures revealed that the more simplified mixture containing only PAHs that elicit a significant positive response in the FE1 MutaTMMouse assay (i.e., benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, BaP, chrysene and dibenz[*a,h*]anthracene) is

always more mutagenic, thus providing additional support for the contention that competition with non-mutagenic PAHs for the limited metabolic machinery likely limits the mutagenic activity of a PAH mixture *in vitro*.

The results of this work provide only circumstantial evidence for competitive inhibition and/or metabolic saturation of CYPs by non-mutagenic PAHs in a complex mixture, and additional research would be required to confirm the hypothesis of ‘metabolic insufficiency’. For example, follow-up experiments could involve augmentation of enzymatic capacity *in vitro* exposure via the addition of exogenous microsomes isolated from FE1 cells or MutaTMMouse tissue. In theory, the addition of microsomal enzymes should boost the mutagenic activity of the non-polar neutral soil fractions, or the 16-PAH mixtures, to the level observed for the 5-PAH mixture. Measurements of AhR agonism, and the induction of various CYP isozymes, following exposure to single compounds and PAH mixtures would also contribute to improved understanding regarding the metabolic alterations induced by PAHs and PAH mixtures. Regardless, critical examination of the ‘metabolic insufficiency’ hypothesis is a profitable area for follow-up research since improved understanding of the toxicological behaviour of PAHs in complex mixtures may impact contaminated site risk assessment.

The results of this study indicate that the mutagenic activity of PAHs in complex mixtures, and by extension, potential carcinogenic hazard, is influenced not only by the levels of known mutagens in the mixture, but also by the presence of non-mutagenic PAHs and related compounds. Moreover, the dynamic metabolic processes that catalyze the conversion of PAHs to oxidised metabolites, some of which are DNA reactive, are

controlled by a complex, dynamic interplay of AhR agonism and stimulation of P450 isozyme production, and competitive inhibition of P450 isozymes by both mutagenic and non-mutagenic PAHs in the mixtures. In addition, soils contaminated with complex mixtures of PAHs and related compounds, such as those examined in this study, also contain more polar mutagens that may be contributing to overall hazard. These are currently not included in routine contaminated site risk assessment protocols. It is important to emphasise that whereas most of the aforementioned studies of complex PAH mixtures investigated changes in DNA damage frequency and/or metabolic capacity, the current study examined induced mutant frequency, which is determined by the complex dynamic interplay between metabolism and DNA damage processing. Several studies have investigated factors that influence the formation, persistence and mutagenicity of DNA damage induced by individual PAHs and PAH-containing complex mixtures^{17, 62-64}.

In our companion paper²⁷, we describe a novel bioassay-based approach to calculate levels of BaP equivalents for use in the assessment of excess lifetime cancer risk, and compare the results obtained to those generated using the standard risk assessment paradigm based on a small number of targeted PAHs and an assumption of additivity.

Follow-up work, which is currently underway, is employing sub-chronic (i.e., 28-day), repeat-dose oral MutaTMMouse exposures, and subsequent quantification of *lacZ* mutant frequency in multiple tissues (e.g., stomach, small intestine, liver, bone marrow), to extend investigations into the mutagenic activity of PAH mixtures and the degree to which observed responses are consistent with those expected based on the concentrations and activities of priority PAHs. Tissue-specific alterations in metabolic capacity and

DNA damage processing are also being examined. This work will ultimately contribute to an improved understanding regarding the mutagenic and carcinogenic hazards posed by PAHs in complex mixtures.

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APPENDIX B

Appendix B : Cancer risk assessment of polycyclic aromatic hydrocarbon contaminated soils determined using bioassay-derived levels of benzo[*a*]pyrene equivalents

B.1 Preamble: Authors, Affiliations, and Style

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Style: This chapter was prepared according to the style guide for *Environmental Science and Technology*.

B.2 Abstract

Here we evaluate the excess lifetime cancer risk (ELCR) posed by ten PAH-contaminated soils using (i) the currently advocated, targeted chemical-specific approach that assumes dose additivity for carcinogenic PAHs, and (ii) a bioassay-based approach that employs the *in vitro* mutagenic activity of the soil fractions to determine levels of benzo[*a*]pyrene equivalents and, by extension, ELCR. Mutagenic activity results are presented in our companion paper¹. The results show that ELCR values for the PAH-containing fractions, determined using the chemical-specific approach, are generally greater (i.e., 8 out of 10)

than those calculated using the bioassay-based approach; most are less than 5-fold greater. Only two bioassay-derived ELCR estimates are greater than their corresponding chemical-specific values; differences are less than 10%. The bioassay-based approach, which permits estimation of ELCR without *a priori* knowledge of mixture composition, proved to be a useful tool to evaluate the chemical-specific approach. The results suggest that ELCR estimates for complex PAH mixtures determined using a targeted, chemical-specific approach are reasonable, albeit conservative. Calculated risk estimates still depend on contentious PEFs and cancer slope factors. Follow-up *in vivo* mutagenicity assessments will be required to validate the results and their relevance for human health risk assessment of PAH-contaminated soils.

B.3 Introduction

Industries involved in the production of manufactured gas (also known as coal gas or town gas), coking operations, and wood preservation facilities generate or use coal tar and/or coal-tar creosote. Improper disposal and release of coal tar and creosote has resulted in an abundance of contaminated land at or nearby these industrial sites. The contaminated areas contain complex mixtures of hundreds of chemicals, including a range of polycyclic aromatic hydrocarbons (PAHs) and related polycyclic aromatic compounds (PACs) (e.g., oxygenated PAHs, and O-, N- and S- heterocyclic compounds)². Several PAHs are known or suspected human carcinogens³, thus human health risk assessment (HHRA) of PAH-contaminated sites generally includes an assessment of excess lifetime cancer risk (ELCR) for a given level of exposure. The results of these risk assessments drive custodial decisions and risk management activities, including access restriction, prioritization for remediation, and determination of the

suitability of the sites for subsequent agricultural, residential, commercial or industrial use. The primary route of exposure for PAHs at contaminated sites is generally assumed to be via non-dietary ingestion of PAHs adsorbed to soil particles⁴.

Complex mixture risk assessment is by no means a simple task. Some researchers and governmental agencies argue that insufficient knowledge regarding the interaction(s) of PAHs in mixtures, coupled with the presence of hitherto unidentified hazardous compounds in the mixtures; necessitate the development and implementation of HHRA methodologies that involve biological (i.e., hazard) assessment of the whole mixture. However, difficulties related to toxicity assessment of complex materials such as contaminated soil have led to the development of HHRA methodologies that focus on a small number of targeted substances that have been highlighted by governmental regulatory agencies for concern and control.

In 2000, the United States Environmental Protection Agency (U.S. EPA) published a guidance document for conducting HHRA of chemical mixtures⁵, and in 2010, Health Canada published an analogous “Guidance on Human Health Detailed Quantitative Risk Assessment for Chemicals (DQRA)”⁶. These documents provide guidance for HHRA of contaminated sites, including federally-owned contaminated sites in Canada, most of which contain complex mixtures of toxic substances. Both agencies currently advocate and employ similar strategies for estimating the ELCR attributable to complex PAH mixtures, including (i) evaluation of the risk attributable to the actual mixture of concern (i.e., the PAH-contaminated soil), (ii) evaluation of the risk attributable to a *sufficiently similar* mixture, or (iii) evaluation of the risk attributable to a small number of targeted

PAHs in the mixture (e.g., the 16 PAHs highlighted by the U.S. EPA⁷), and application of an assumption of additivity to calculate total risk as the sum of the incremental contributions from each targeted PAH⁵⁻⁶. Methods currently advocated by Health Canada and the USEPA focus on only 7-8 PAHs that have been highlighted as known, probable, or possible human carcinogens (i.e., benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, BaP, dibenz[*a,h*]anthracene, benzo[*ghi*]perylene, indeno[1,2,3-*c,d*]pyrene^{3-4, 8}). Analogous approaches are recommended by several other countries (e.g., Sweden, the Netherlands, the UK), as well as the World Health Organization's International Programme on Chemical Safety (WHO/IPCS)⁹. Some jurisdictions advocate, where possible, inclusion of a broader range of PAHs and PACs in HHRAs and concomitant regulatory decisions¹⁰⁻¹².

Although contaminated site risk assessment, and any subsequent regulatory decisions, should be based on the risk attributable to the actual mixture of concern, assessment of the actual PAH-contaminated material, or even a sufficiently similar mixture of targeted PAHs in the material, is rarely practical. Thus, the aforementioned targeted, chemical-specific approach (iii, above) is most often employed; the incremental contributions of each known (i.e., targeted) carcinogen in the mixture are assumed to be additive, and the total risk is equal to the sum of the incremental risks. Such an approach does not require any direct measurements of hazard for the actual material being evaluated (i.e., the PAH-contaminated soil), but rather, employs chemical analyses to determine the concentrations of the targeted PAHs, applies potency equivalency factors (PEFs) to convert the concentrations of each of the targeted PAHs in the mixture to an equivalent amount of the reference carcinogen benzo[*a*]pyrene (i.e., BaP equivalents), and calculates the total

quantity of BaP equivalents in the material as the sum of the contributions from each targeted, carcinogenic PAH. The oral cancer slope factor for BaP can then be employed to calculate the total estimated ELCR posed by the contaminated material at a given site. The choice of PEFs for each of the targeted PAHs, the cancer slope factor, and the assumptions regarding the frequency and duration of exposure, all of which may vary across jurisdictions, all influence the magnitude of the calculated ELCR.

In an earlier study¹³, we employed the Salmonella reverse mutation assay (i.e., Ames test) to assess the mutagenic activity of organic extracts of PAH contaminated soils, and moreover, used the calculated mutagenic potencies for each soil, and a novel bioassay-based approach (i.e., the mutagenic potency ratio or MPR method), to derive an estimate of ELCR. This bioassay-based approach to HHRA does not assume additivity of targeted PAHs, but rather estimates risk using bioassay-derived levels of BaP-equivalents, which are calculated using the measured mutagenic potency of the mixture and its ratio to BaP potency^b. A comparison of risk estimates derived using the bioassay-based approach to those derived using the targeted, chemical-specific approach suggested that current risk assessment methods may be underestimating the risks posed by the PAH-containing fraction of contaminated soils. Moreover, mutagenic activity assessment of semi-polar aromatic fractions of soil organic extracts suggested that more polar compounds, which remain largely unidentified and are not ordinarily included in the risk assessment process, may pose additional risk. Although interesting and pertinent to regulatory evaluations and decision-making, these results were based on measurements obtained using the Salmonella reverse mutation assay. The assay system permits reductive bacterial

^bFormerly called the Mutagenic Potency Ratio (MPR) approach

metabolism and employs an exogenous metabolic activation system derived from the livers of Aroclor-induced rats for oxidative metabolism. As such, it is renowned for its sensitivity to some PACs. Thus, our evaluation of targeted, chemical-specific HHRA methods for PAH-contaminated soils based solely on *Salmonella* mutagenic potency may not be generally applicable to the determination of risk for mammalian systems.

We have now assessed the mutagenic activity of organic extracts from the same PAH-contaminated soils using a transgenic mammalian cell line (i.e., the MutaTMMouse FE1 cell line) that has an endogenous capacity to convert PAHs such as BaP to DNA-reactive metabolites¹⁴⁻¹⁵, and the reader is referred to the companion paper¹ for details of the mutagenic activity results. The current study continues our evaluation of the assumption of additivity routinely used for risk assessment of complex PAH mixtures. Specifically, we (i) employ mutagenic activity data from the *lacZ* transgene mutation assay in MutaTMMouse FE1 cells, and the aforementioned bioassay-based approach, to derive estimates of ELCR for non-dietary ingestion of PAH in contaminated soils, and (ii) compare the bioassay-based risk estimates to those calculated using the targeted, chemical-specific approach currently advocated by the U.S. EPA and Health Canada.

B.4 Materials and Methods

Soils

Ten soil samples obtained from PAH-contaminated sites in Sweden were analysed (**Table B-I**). These sites include three wood preservation sites (Holmsund, Forsmo and Hässleholm), one manufactured gas plant site (Husarviken) and one coke oven site

(Luleå). Detailed site information and the results of chemical analyses can be found in Lemieux et al. (2008)¹³.

Table B-I. Characteristics and location of contaminated sites investigated (reproduced with permission from Lemieux et al., 2008¹³, SETAC Press)

Location	Industry	Period of operation	Known pollutants	Sampling depth	Soil Type	LOI ^a (%)
Holmsund-1	Wood preservation	1943-1983	Creosote, CCA ^b , zinc	20-30 cm	Sandy Till	9.8
Holmsund-2	Wood preservation	1943-1983	Creosote, CCA, zinc	10-20 cm	Sandy Till	6.9
Holmsund-3	Wood preservation	1943-1983	Creosote, CCA, zinc	10-20 cm	Sandy Till	2.7
Luleå	Coke production	^c	PAH, arsenic	Top soil	Sediment	13
Forsmo-1	Wood preservation	1933-1950	Creosote, CCA	2-18 cm	Fine sand	2.6
Forsmo-2	Wood preservation	1933-1950	Creosote, CCA	0-10 cm	Fine sand	13
Hässleholm-1	Wood preservation	1946-1965	Creosote, CCA, zinc	40 cm	Coarse sand	6.8
Hässleholm-2	Wood preservation	1946-1965	Creosote, CCA, zinc	40-60 cm	Coarse sand	2.2
Husarviken-1	Gas work	1893-1972	Coal tar, heavy metals, cyanide	^d	Sand	19
Husarviken-2	Gas work	1893-1972	Coal tar, heavy metals, cyanide	^d	Sand	12

^a loss on ignition (measure of total organic content), determined by heating samples at 130°C overnight, and then at 550°C for 2h.

^bChromated copper arsenate

^cFacility still in operation at the time of sampling, start unknown

^dnot known

Organic pollutants were extracted from each of the ten contaminated soils using accelerated solvent extraction on an ASE200 (Dionex, Sunnyvale, CA), and the extracts were fractionated into non-polar neutral and semi-polar aromatic soil fractions on open silica gel as described in the companion paper¹ and in Lemieux et al. (2008)¹³. Prior validation of the fractionation protocol confirmed that homocyclic, unsubstituted PAHs (including the U.S. EPA priority PAHs), alkyl-PAHs, and O- and S- heterocyclic compounds are contained in the non-polar neutral soil fraction, whereas, the semi-polar aromatic soil fraction contains oxygenated PAHs, nitroarenes and aromatic amines, including N-heterocyclic compounds¹⁶. Chemical analyses of selected PAHs and PACs were carried out by gas chromatography-mass spectrometry as previously described^{13, 16}.

Cancer risk assessment

The ELCR for an adult exposed to each of ten PAH-contaminated soils was evaluated using two methods: (1) the targeted, chemical-specific approach currently advocated by regulatory agencies such as Health Canada and the U.S. EPA^{5, 6, 10} and, (2) a bioassay-based approach, originally described in Lemieux et al. (2008)¹³. Note that in our previous publication this approach is referred to as the mutagenic potency ratio (MPR) approach.

Targeted, chemical-specific approach

The targeted, chemical-specific approach employs the soil concentrations of a targeted group of known PAHs, a soil ingestion rate, exposure factors, body weight, an oral slope factor for BaP, and PEFs to calculate risk according to **Equation 1**. The targeted PAHs included are those currently identified by the U.S. EPA as probable human carcinogens (i.e., B2 carcinogens benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene,

benzo[*k*]fluoranthene, BaP, dibenz[*a,h*]anthracene and indeno[1,2,3-*c,d*]pyrene)⁸. This approach assumes dose additivity to determine the total ELCR for a given soil. This method only allows for risk estimations that relate to selected components of the non-polar neutral fractions of organic soil extracts (i.e., compounds for which PEFs have been assigned). Contributions related to components of the semi-polar aromatic soil fractions are rarely incorporated into risk estimation since the chemical composition of the fraction remains largely unknown, and, with few exceptions, PEFs for PACs have not been determined^{11-12, 17}.

$$[1] \quad \text{Total Excess Lifetime Cancer Risk} = \left(\sum_{i=1}^n \left(\frac{C_i \times IR \times EF \times 1000}{BW} \right) \times PEF_i \right) \times SF$$

for PAHs 1 through n.

Where C_i refers to the soil concentration of each targeted PAH ($\mu\text{g PAH/g soil}$) (available in companion work¹), and IR refers to the soil ingestion rate (mg soil/day). Adults were assumed to consume 20 mg soil per day during the exposure period¹⁰. The exposure factor (EF) was calculated according to Health Canada recommendations¹⁰ (i.e., 5 days/week, 48 weeks/year, 35 years of exposure, life expectancy of 75 years), and a body weight (BW) of 70.7 kg was assumed¹⁰. The cancer slope factor (SF) for BaP employed for these analyses was 2.3 per mg BaP/kg/day¹⁸. The PEFs or potency equivalency factors are from CCME (2010)⁴. Upper and lower limits of risk estimates were calculated using highest and lowest PEF values, respectively, from the scientific

literature^{4, 19-27}. Excess lifetime cancer risk is expressed as the number of expected cases in excess of 1 in a million (i.e., 10^{-6}).

Bioassay-based approach

The bioassay-based approach, which we described previously¹³, employs the experimentally determined mutagenic potencies of each of the soil fractions, as well as the mutagenic potency of BaP to derive risk estimates according to **Equation 2**.

Mutagenic potencies are defined as the slope of the linear portion of the concentration-response functions for the induction of *lacZ* transgene mutations in FE1 cells, and are reported in the companion paper¹. Rather than using the concentrations of targeted PAHs, and the associated PEFs, this method determines the equivalent concentration of BaP required to elicit the observed response for a selected fraction of a given soil sample, and uses this concentration of BaP equivalents and the soil ingestion rate to determine the daily dose of BaP equivalents. Since the method employs a bioassay response for the complex soil fractions to provide a BaP equivalent dose, PEFs and an assumption of additivity are not required.

$$[2] \quad \text{Total Excess Lifetime Cancer Risk} = \left[\left(\frac{Activity_{\text{soil}}}{Activity_{\text{BaP}}} \times IR \times EF \right) \times BW^{-1} \right] \times SF$$

Where $Activity_{\text{soil}}$ is the mutagenic potency of the non-polar neutral (i.e., PAH-containing) or semi-polar aromatic soil fractions measured using the *in vitro* transgenic mutation assay in FE1 cells (mutant frequency $\times 10^{-5}$ /dry mg equivalent soil eq./ml) and

$Activity_{BaP}$ is the mutagenic potency of BaP as measured using the same assay (mutant frequency $\times 10^{-5}/\text{mg BaP/ml}$). IR refers to the soil ingestion rate (mg soil/day). Adults were assumed to consume 20 mg soil per day during the exposure period¹⁰. The exposure factor (EF) was calculated according to Health Canada recommendations¹⁰ (i.e., (i.e., 5 days/week, 48 weeks/year, 35 years of exposure, life expectancy of 75 years), and a body weight (BW) of 70.7 kg was assumed¹⁸. For the bioassay-based risk calculations, the aforementioned Health Canada cancer slope factor for BaP was used (i.e., 2.3 per mg BaP/kg/day)¹⁸. 95% confidence intervals of bioassay-derived risk estimates were determined using propagated error values calculated from the standard errors of the mutagenic potencies for BaP and the soil fractions examined, and the appropriate critical values of the t distribution (two-sided, $p=0.05$)²⁸.

B.5 Results

Total PAH levels (i.e., ΣPAH_{24}) ranged from 72 – 9256 $\mu\text{g PAH/g dry soil}$; with priority PAH levels from 60 to 8823 $\mu\text{g/g}$. The levels of carcinogenic PAHs ranged from 29 to 1707 $\mu\text{g PAH/g dry soil}$, which accounted for 18 – 60% and 19 to 67% of the total and priority PAHs, respectively. Detailed chemical characterization of PAHs and PACs in the soils evaluated in this study can be found in Lemieux et al. (2008)¹³ and the companion paper¹. As noted, the targeted, chemical-specific approach for risk assessment employs the concentrations of targeted PAHs, PEF values, and a BaP cancer slope factor to calculate estimates of cancer risk according to Equation 1. Using this method, the calculated risk (per million) ranged from 1.4 for Husarviken-1, the least contaminated soil sample, to 46.6 for Holmsund-2, the most contaminated soil sample. In contrast, the bioassay-based approach calculates risk using bioassay response data, and, as such, can

be employed for both of the soil fractions investigated (i.e., non-polar neutral and semi-polar aromatic soil fractions). Mutagenic potency values for the non-polar neutral soil fraction (i.e., the PAH-containing fraction) ranged from 16 to 463×10^{-5} mutant frequency/mg soil equivalent/ml, whereas those of the semi-polar aromatic fractions ranged from not detected (i.e., Forsmo-2 and Holmsund-3) to 217×10^{-5} mutant frequency/mg soil eq./ml (i.e., Forsmo-1). Details regarding the mutagenic potency of the soil fractions can be found in the companion paper¹. The mutagenic potency of BaP was 3989 ± 257 mutant frequency/ μ g BaP/ml, and the bioassay-based ELCR values for the non-polar neutral (i.e., PAH-containing) fractions (per million) ranged from 0.8 for Holmsund-1 to 23.2 for Holmsund-2.

Figure B.1 compares the bioassay-based risk estimates for the non-polar neutral fractions with those derived using the targeted, chemical-specific approach. The figure shows that the targeted, chemical-specific approach yields risk estimates that are often higher (i.e., for 8 of 10 soils) than those calculated using the bioassay-based approach. The chemical-specific ELCR values range from 1.2-fold greater than the corresponding bioassay-derived values for Holmsund-3 and Husarviken-2, to 42.6-fold greater for Holmsund-1. With the exception of Holmsund-1, all chemical-specific ELCR values are less than 5-fold greater than their corresponding bioassay-derived values, with the geometric mean ratio of chemical-specific ELCR to bioassay-derived ELCR equal to 2.9. More detailed scrutiny of the data used to generate **Figure B.1** indicates that although the chemical-specific ELCR values generally exceed the corresponding bioassay-derived values, chemical-specific ELCR values calculated using the aforementioned CCME PEF values⁴ exceed the upper 95% confidence limit of the corresponding bioassay-derived values for

only 5 soils (i.e., Holmsund-1, Holmsund-2, Luleå, Häsleholm-1, Häsleholm-2).

Moreover, for 4 of these 5 soils (i.e., Holmsund-1, Luleå, Häsleholm-1, Häsleholm-2), even the lower limit of the calculated chemical-specific ELCR values (i.e., determined using the lowest published PEF values) exceeds the upper 95% confidence limit for the bioassay-derived value. In contrast, only 2 of the ten soils investigated (i.e., Forsmo-1, Husarviken-1) yielded bioassay-based ELCR values that are greater than their corresponding chemical-specific values; moreover, the increases are only 1.5 and 5.4%, respectively. Thus, the chemical-specific ELCR estimates are generally greater than their corresponding bioassay-derived values, and often significantly greater; whereas, for the few instances where the bioassay-derived value is greater, the differences are small (i.e., <10%).

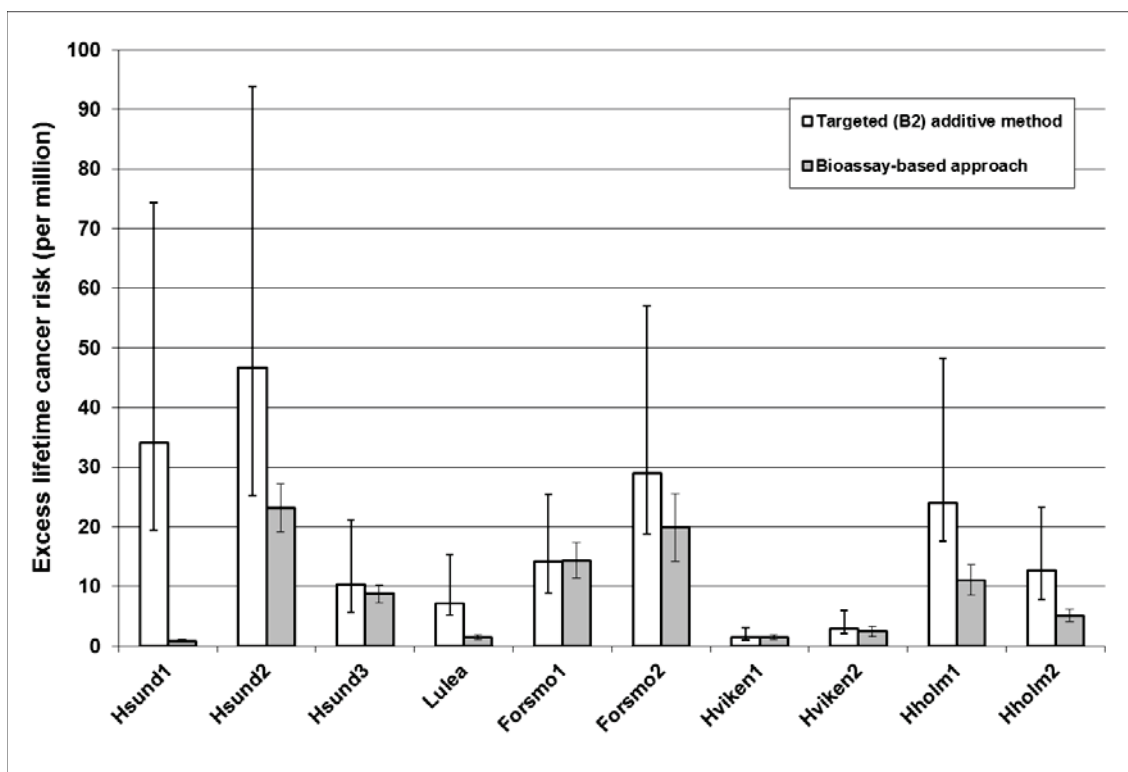


Figure B.1. Excess lifetime cancer risk associated with non-dietary ingestion of PAH-contaminated soils by a typical adult. Risk was calculated using the targeted, chemical-specific approach (i.e., B2 additive method), as well as the bioassay-based approach described in the text (non-polar neutral fraction only). Error bars associated with B2 additive values represent minimums and maximums calculated using the lowest and highest PEF values for B2 PAHs, respectively, from the scientific literature^{4, 19-27}. The error bars for the bioassay-based values represent the composite standard error calculated from the standard errors of the mutagenic potencies for BaP and the non-polar neutral soil fractions. All risk calculations employed the slope factor for BaP recommended by Health Canada¹⁶. Hsund=Holmsund, Hviken=Husarviken, Hholm=Hälsleholm

In principle, the bioassay-based approach can be employed for any soil fraction that induces a response in the bioassay employed to assess mutagenic activity. However, since the identities of the components in the semi-polar aromatic fraction are not known, and there is no evidence that the putative mutagens in this fraction are mutagenic carcinogens, the use of BaP as a reference compound cannot be systematically defended. Nevertheless, since eight of the ten semi-polar aromatic fractions yielded a significant mutagenic response in the FE1 assay¹, and, as noted, several PACs are mutagenic carcinogens, it is reasonable to assert that components in the fraction can contribute to total ELCR. Guarded calculations of BaP equivalents for the semi-polar aromatic fractions provides ELCR values greater than 10^{-6} for only two sites (Holmsund-1, Forsmo-1). The values associated with this fraction surpass that of the non-polar neutral fraction for only one site (i.e., Holmsund-1).

B.6 Discussion

This study compares estimates of ELCR calculated using a targeted, chemical-specific approach that focuses on a small number of PAHs with those based on a bioassay-based approach that employs a biological response to complex soil fractions (i.e., FE1 mutagenic potency for the non-polar neutral and semi-polar aromatic fractions). This bioassay-based approach, first described in Lemieux et al. (2008)¹³, uses the mutagenic potency of complex soil extracts/fractions (i.e., ability to induce *lacZ* transgene mutations in MutaTM Mouse FE1 cells) to calculate BaP-equivalent dose, and thus does not rely on chemical-specific PEF values or an assumption of additivity.

Our companion paper¹ showed that all ten non-polar neutral fractions derived from the sites examined, and 8 of the 10 corresponding polar aromatic fractions induced a

significant response in the *lacZ* transgene mutation assay in FE1 cells exposed *in vitro*. This bioassay response, and an assumption of an empirical relationship between carcinogenic potency and mutagenic potency (discussed below), permitted the use of the bioassay-derived method for estimation of ELCR values for the non-polar neutral fractions. Estimates were then compared with ELCR values calculated using the targeted, chemical-specific approach based on the additive responses of seven targeted PAHs (i.e., the U.S. EPA B2 PAHs). The results (**Figure B.1**) show that, if the PEFs and slope factor recommended by Health Canada^{4, 18} (2.3 per mg BaP/kg/day) are employed, then the traditional additive ELCR estimates are generally greater than those derived using the bioassay-based approach (i.e., 8 out of 10 soils). Moreover, even when the bioassay-derived ELCR values were greater, the differences are small (i.e., <10%). This suggests that, despite the fact that the complex soil fractions contain a mixture of known and hitherto unknown PAHs and related compounds, risk estimates based on a small subset of PAHs may yield conservative estimates of ELCR. However, it is important to reiterate that the relationship between the bioassay-derived estimates and the traditional additive estimates will depend on the PEF values employed to determine the concentration of BaP equivalents. Use of the lowest and highest PEF values presented in the scientific literature provided a means for more rigorous comparisons of traditional additive and bioassay-derived ELCR values. The results obtained indicate that for half of the eight soils that yielded higher chemical-specific ELCR values, even ELCR estimates based on the lowest available PEF values were above the upper 95% upper confidence limit of the bioassay-derived value. Thus, the traditional additive ELCR values are generally greater, and for 40% of the soils examined, they are significantly greater.

The choice of cancer slope factor used for the calculation of ELCR can also significantly affect the outcome of the risk assessment and subsequent risk management decisions, and the effect of slope factor choice will affect both the chemical-specific and bioassay-derived ELCR estimates. Cancer slope factors routinely used by various jurisdictions vary by approximately 20-fold (**Table B-II**), and this variability can be attributed to differences in the data used to derive the value (e.g., epidemiological, animal bioassay), and the model used for dose/species extrapolation (e.g., differences in allometric scaling methods)²⁹. Unfortunately, the studies used to derive cancer slope factors are often older studies that may contain critical weaknesses. This study employed the oral slope factor for BaP recommended by Health Canada (i.e., 2.3 per mg/kg-day); however, this and several other BaP slope factors are based on the 1967 Neal & Rigdon study³⁰, which does not contain sufficient information on exposure duration. Consequently, some jurisdictions (e.g., The Netherlands) are considering methods that do not rely on dose-response analysis and derivation of cancer slope factors. For example, Kroese et al. (2001) discusses the *virtually safe dose* approach, where linear extrapolation from a point-of-departure such as the LOAEL (i.e., the lowest dose level associated with significant tumour response) is used to determine an acceptable dose³¹. Although interesting, this approach yields acceptable substance-specific dose levels, and as such, it cannot be applied to mixtures.

Table B-II. Summary of BaP cancer slope factor values employed by different regulatory agencies for assessment of excess lifetime cancer risk. All values derived from dose-response data for gastric tumors in mice or rats. For details, see New Zealand Ministry for the Environment (2011)³² and California Environmental Protection Agency (1997)³³.

Jurisdiction	Cancer Slope factor (per mg/kg bw/day)	Key Study	Reference
USA, New Zealand	7.3	(30, 34)	(32, 35)
Canada	2.3	(30)	(18)
WHO Drinking water	0.46	(36)	(37, 38)
California	9.03	(30)	(33)

The bioassay-based approach employs bioassay-derived potency data for a complex soil component (i.e., non-polar neutral compounds); knowledge of the mixture composition is not required. Thus, in principle, the bioassay-based approach can evaluate the potential health risks posed by chemicals that are not typically included in the targeted, chemical-specific approach used by many governmental organizations. For the soils studied here, both non-polar neutral and semi-polar aromatic soil fractions were mutagenic in the *Salmonella* reverse mutation assay¹³ and in FE1 cells¹, and the bioassay-based approach can provide an estimate of ELCR posed by mutagenic compounds found in both fractions. However, lack of information regarding the identity, physical-chemical properties, and carcinogenicity of mutagenic components in the semi-polar aromatic fraction impedes convincing use of BaP as a reference compound, and by extension, realistic estimation of human ELCR. Nevertheless, it is useful to reemphasize that the components of the semi-polar aromatic soil fraction would not be monitored in a conventional risk assessment, despite the fact that this fraction could be expected to contain PACs such as oxygenated PAHs, nitroarenes and aromatic amines, including N-heterocyclic compounds³⁹ and nitro-PAH derivatives, some of which are known mutagens and possible human carcinogens (e.g., 3-nitrobenzanthrone⁴⁰⁻⁴²).

The bioassay-based approach assumes that BaP is an appropriate reference compound for conversion of mutagenic activity values for complex PAH-containing mixtures to chemical equivalents that can be employed for HHRA. Our companion work noted a significant empirical relationship between the mutagenic potency of the non-polar neutral fractions, and by extension, the calculated level of BaP equivalents, and the concentrations of both total measured and priority PAH. The lack of a correlation

between bioassay-derived level BaP equivalents for the semi-polar aromatic fraction and PAH level (not shown) confirms that, although interesting, estimation of ELCR for the semi-polar fraction cannot be mechanistically defended, and must be interpreted with caution.

The bioassay-based approach also assumes a correlation between mutagenic and carcinogenic potency (i.e., an assumption that the ratio of mutagenic potency values for BaP and soil extracts is equivalent to the ratio of the corresponding carcinogenic potency values). In practice, validating this assumption is not practical since it would require evaluations of the carcinogenicity of each soil extract in a two-year rodent cancer study. Nevertheless, analysis of the empirical relationship between the relative mutagenic potency of five targeted PAHs (i.e., relative to BaP), measured using the *lacZ* mutation assay in MutaTM Mouse FE1 cells, and the corresponding relative rodent carcinogenic potency values, revealed a highly significant relationship ($r^2 = 0.98$, F ratio = 77.0, $p < 0.004$). In addition, the results published by Hernández et al. (2011) support the contention that mutagenic potency is indeed empirically related to carcinogenic potency when a mutagenic mode of action has been documented. In that study, the authors revealed a strong correlation between mutagenic and carcinogenic potency for 18 carcinogens with a mutagenic mode of action (including BaP)⁴³. More specifically, the authors examined the correlation between benchmark dose (BMD₁₀) values derived from the *in vivo* micronucleus assay and *in vivo* transgenic rodent mutation assay, and those derived from murine carcinogenicity studies.

The traditional (i.e., targeted, chemical-specific) approach to PAH risk assessment inherently assumes that the cancer risk of all PAHs present in a mixture are additive. The

convention of assuming risk additivity for PAHs is largely accepted because it is understood that carcinogenic PAHs generally act via a common mode of action (i.e., the formation of bulky DNA adducts that contribute to the establishment of mutations and cancer initiation)^{5, 10}. Several studies support the contention that PAHs in mixtures can exhibit additive effects^{13, 44}; however, there is also evidence to support sub-additive phenomena, particularly at higher concentrations or doses^{1, 45-46} where competition for enzymatic catalysis may be expected. Supra-additive or synergistic effects are also possible, and these are most likely due to metabolic augmentation (e.g., upregulation of CYP isozymes by mutagenic and non-mutagenic PAHs⁴⁷⁻⁴⁸). For a more comprehensive discussion regarding factors that control the generation of activated metabolites, and their influence on the (geno)toxicity and carcinogenicity of PAH mixtures, the reader is referred to our companion paper. Without comprehensive information about the chemical composition of a mixture, and of the interactions of all components, neither of which are feasible, the assumption of response additivity for a subset of targeted PAHs in a complex mixture is a pragmatic approach for routine assessment of ELCR. Interestingly, although the targeted, chemical-specific approach only focuses on a small subset of PAHs that might be expected in a contaminated soil, the ELCR estimates presented here are generally higher, and largely within an order of magnitude, of those determined using a bioassay-based approach. The chemical-specific approach yielded a value more than an order of magnitude above the corresponding bioassay-derived value for only one site (i.e., Holmsund-1), and the geometric mean ratio of chemical-specific to bioassay-derived ELCR suggests that the former are generally about 3-fold greater than the latter. Moreover, for the two soils where the bioassay-derived ELCR values are greater than

their corresponding chemical-specific values, the difference is very small (i.e., 1.5 and 5.4%). It is therefore reasonable to contend that ELCR estimates calculated using the targeted, chemical-specific approach are pragmatic, realistic, and somewhat conservative. Thus, despite the fact that they only focus on a small subset of PAHs, they can be used for sound custodial decisions (i.e., access restriction, site remediation and reclamation).

The convention of assuming response additivity for PAHs in mixtures is exemplified by the widespread use of PEFs for complex PAH mixture risk assessment. Nevertheless, PEFs, or the related toxic equivalency factors (TEFs), which are broadly used for the risk assessment of polychlorinated dioxin-, dibenzofuran-, biphenyl-containing mixtures⁴⁹, do have their limitations. First, PEFs are usually only available for a small subset of carcinogenic PAHs, restricting mixture risk assessments to effects attributable to those PAHs. Indeed, it is now recognized that some carcinogenic PAHs (e.g., dibenz(*a,l*)pyrene, DBa/P) are more mutagenic and carcinogenic than BaP⁵⁰⁻⁵³, and even a small amount of these potent substances in a PAH-contaminated soil could contribute to a substantial increase in estimated cancer risk. However, potent PAHs such as DBa/P are not included in the U.S. EPA's priority PAH list and as such, have not traditionally been monitored at contaminated sites or incorporated into risk calculations. Some jurisdictions (e.g., California Environmental Protection Agency, Minnesota Department of Health, Health Canada) are now recommending, where possible, the use of PEFs for other PAHs and PACs, including DBa/P and potent alkylated PAHs, thus expanding the scope of ELCR assessments for PAH-contaminated sites^{10-12, 17}. Nevertheless, it should be noted that it may not be practical or desirable to derive and apply PEF values for an extensive list of components identified in complex PAH-containing mixtures. The analyses

conducted herein indicates that ELCR values based on only a small subset of PAHs is already conservative; addition of risks attributable to additional PAHs such as those mentioned above will only serve to enlarge the gap between chemical-specific and bioassay-derived values. Moreover, determining the identity of the toxicologically-relevant components, and routine quantification of these components in complex matrices undergoing regulatory evaluation, constitutes a significant analytical challenge.

Derivation of PEFs for an extended series of compounds necessitates the use of inadequate bioassay results, adoption of several assumptions, and extrapolation to the suspected target tissue(s) in humans. For example, PEFs are often derived from the results of dermal (skin painting), and or intrapulmonary installation studies⁵⁴; however, they are routinely employed for HHRA calculations that assume oral exposure (i.e., ingestion as the primary route for contaminated soils). This raises the concern that toxicokinetic differences may affect the suitability of the PEFs for contaminated site risk assessment. Moreover, differences in the PEF values for PAHs recommended by different governmental agencies (for review see Delistraty, 1997⁵⁵ and WHO, 1998⁵⁶) only serve to further hamper a generalised interpretation of HHRA for PAH mixtures. Interestingly, the current Health Canada guidance document for quantitative risk assessment of federal contaminated sites lists 44 PEF values for PAHs and alkylated PAHs^{6, 17}; however, few site assessments quantify an extensive range of PAHs and related compounds (e.g., alkylated PAHs). The aforementioned recommendations of the MN Department of Health includes PEFs for an extended list of only 19 PAHs, indicating that monitoring of additional PAHs that could constitute a carcinogenic hazard is problematic due to analytical challenges and/or toxicological uncertainty. The U.S.

EPA's IRIS (Integrated Risk Information System) Program is currently engaged in an extensive review of the RPF (relative potency factor) approach for PAH mixtures (see http://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=194584); however, the results have yet to be released.

It is important to note that only the non-dietary ingestion route was evaluated in the current study, since it is considered the primary route of exposure for soil-bound PAHs and other PACs at contaminated sites. In an actual risk assessment, other routes of exposure would also be considered (e.g., inhalation of airborne soil particles); however, such an evaluation is beyond the scope of the current study. Moreover, the analyses only assessed risk for a typical adult, and other receptors with different exposure factors (e.g., construction workers) would also be considered in a more comprehensive assessment⁶. Assumptions include exposure levels (e.g., soil ingestion rates) for various receptors, the degree to which contaminants are absorbed by the human gastrointestinal tract (i.e., bioaccessibility), and the assumption that the risks posed by mixture components are additive⁶. Moreover, different international jurisdictions base regulatory decisions on different thresholds for acceptable risk. Even provincial jurisdictions within Canada differ in their HHRA methods and the levels of acceptable cancer risk⁵⁷. It should also be noted that the current study only evaluated ELCR attributable to targeted PAHs or mutagens in the organic fractions examined. It is not unreasonable to assert that other components in the contaminated soils could contribute to carcinogenic risk. Although it would prove interesting to conduct an analogous *in vivo* study that investigated the effects of unaltered soils ingested by experimental animals, rodents would not be physiologically appropriate for studies to be interpreted in an HHRA context. Porcine

models have been offered as physiologically-relevant alternatives (Bode et al 2010); however, the costs of porcine studies are generally prohibitive⁵⁸.

In contrast to the results of this study, our previous analyses showed that bioassay-based risk estimates obtained using *Salmonella* reverse mutation assay results for the non-polar neutral fractions were much *higher* than estimates obtained using the targeted, chemical-specific approach¹³. In light of the current results, and the aforementioned sensitivity of the *Salmonella*-based mutagenicity assessment system, it is reasonable to contend that the earlier results may not be generally applicable to mammalian systems. Oversensitivity of the bacterial test system, at least for the complex soil fractions examined here and in our earlier study, can likely be attributed to the inherent ability of *Salmonella* to metabolically activate selected PACs (e.g., nitroarenes and aromatic amines), as well as the extraordinarily high P450-mediated enzymatic capacity of the exogenous activation system commonly employed (i.e., CYP1A1, CYP1A2, CYP2B1 activities). The enhanced sensitivity will contribute to an inflated measure of mutagenic potency relative to BaP, a concomitant higher level of BaP equivalents, and ultimately, a higher estimate of risk. The bioassay-based risk estimates derived here, which are based on results from metabolically-competent mammalian cells, are indeed 1-2 orders of magnitude lower than those calculated using the *Salmonella* assay results. We contend that the current risk estimates represent a more realistic evaluation of human health risk compared to those derived in our previous study.

The results of the current study show that for complex mixtures of chemicals present at PAH-contaminated sites, estimates of ELCR determined using existing risk assessment strategies (i.e., a targeted chemical-specific approach) are generally greater than those

determined using a bioassay-based approach. Although the assumption of dose additivity inherently assumed by the targeted chemical-specific approach may be simplistic in light of the known toxicokinetic and toxicodynamic complexities for different groups of substances, different dose levels, and different routes of exposure, for the soils evaluated in this study, it does appear to provide risk estimates that are conservative relative to those derived using an approach based on the biological activity of the complex mixture of PAHs in the soil matrix. It is important to note that it is not our intention to suggest that the bioassay-based approach supplant the traditional, chemical-specific additive approach that relies on well-recognised, accurate methods for chemical analyses. Rather, the bioassay-based approach employed herein, which does not require *a priori* knowledge regarding the identity and levels of the putative soil toxicants or assumptions regarding the toxicological behaviour of these compounds in a mixture, provided a convenient means to evaluate the chemical-specific approach. Although it has proved to be a useful tool, and indeed, has revealed that chemical-specific assessments based on only seven B2 PAHs is likely conservative, routine application of a bioassay-based approach for evaluation of complex PAH-contaminated matrices (e.g., soil) would likely be impractical.

In this work we have employed a mammalian cell bioassay-based approach to critically evaluate the HHRA method for PAH-contaminated matrices based on additivity of a small number of components, and shown that the targeted chemical-specific approach provides higher risk estimates that are generally well within an order of magnitude of those determined using a bioassay-based approach. Moreover, consistent with our

previous work¹³, preliminary calculations suggest that additional hazard and risk may be attributable to more polar compounds, such as oxygenated PAHs and nitroaromatic compounds that are known to be present in PAH-contaminated soils. Lundstedt et al. (2007) recommend monitoring of oxygenated PAHs at contaminated sites such as those studied here⁵⁹; however, incorporation of these compounds into a risk assessment would require compound-specific PEF values, and these are generally not available. Although the current work employed a metabolically-competent mammalian cell line, rigorous evaluation of the additive, chemical-specific approach for HHRA of complex PAH-containing mixtures will require *in vivo* analyses in selected tissues following oral exposure. Indeed, follow-up investigations, which are already underway, are employing *in vivo* mutagenicity assessment in selected MutaTMMouse tissues to extend the current evaluation of the bioassay-based approach. Additional assessments of non-priority PAHs, which are also underway, will contribute to a comprehensive, objective evaluation of cancer risk assessment methods that are routinely applied to mixtures of PAHs and related compounds.

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APPENDIX C

Appendix C : Supplementary Material

C.1 Chapter 1

(none)

C.2 Chapter 2

(none)

C.3 Chapter 3

(none)

C.4 Chapter 4

Supp. Table 4-I. Mutagenic potencies of individual PAHs determined using the MutaTMMouse transgenic rodent assay. Mutagenic potency, which was calculated for each compound-tissue-combination, is the slope of the linear portion of the dose-response function. Original data published in Long et al (2016)²⁰.

Compound	Mutagenic Potency (<i>lacZ</i> Mutant Frequency x 10 ⁻⁵ /mg PAH/kg body weight/day)				
	Bone marrow	Liver	Glandular Stomach	Small Intestine	Lung
Benzo(<i>a</i>)pyrene	8.69	2.90	4.66	18.8	1.96
Benz(<i>a</i>)anthracene	0.0264	0.0322	0.184	0.0523	NS
Dibenz(<i>a,h</i>)anthracene	NS	0.577	0.390	16.2	2.60
Benzo(<i>b</i>)fluoranthene	0.242	2.08	3.09	7.89	0.335
Chrysene	NS	NS	NS	0.357 ^a	0.125 ^a
Benzo(<i>k</i>)fluoranthene	0.0228	0.058	0.161 ^a	0.561 ^a	0.134
Indeno(1,2,3- <i>c,d</i>)pyrene	0.0244	0.263	0.118	3.27	0.0736
Benzo(<i>g,h,i</i>)perylene	NS	NS	NS	0.181	0.218
^a indicates where data were truncated prior to the determination of potency (i.e., fewer than 4 dose groups included in the analysis)					

Supp. Table 4-II. The Canadian Council of Ministers of the Environment (CCME) potency equivalency factors (PEFs) ¹⁸employed to calculate BaP-equivalent concentrations via the PEF-method (i.e., PEF-M). A literature review was conducted to identify the lowest and highest published PEFs for each compound. These values were used to calculate the lower and upper bounds of the PEF-M-derived BaP-equivalent values, respectively.

Compound	CCME PEF	Lowest PEF	Highest PEF
Benz[<i>a</i>]anthracene	0.1	0.0005 ³⁸	0.145 ³¹
Chrysene	0.01	0.001 ^{14, 30}	0.1 ³²
Benzo[<i>b</i>]fluoranthene	0.1	0.06 ³⁵	0.62 ⁴¹
Benzo[<i>k</i>]fluoranthene	0.1	0.03 ³⁷	0.1 ^{32, 33, 35, 40, 41}
Benzo[<i>a</i>]pyrene	1	1	1
Dibenz[<i>a,h</i>]anthracene	1	0.69 ²⁹	5 ³¹
Indeno[1,2,3- <i>c,d</i>]pyrene	0.1	0.017 ³⁰	0.278 ⁴²
Benzo[<i>g,h,i</i>]perylene	0.01	0.01 ⁴⁰	0.022 ³¹

Supp. Table 4-III. The concentrations of selected polycyclic aromatic hydrocarbons (PAHs) in coal tar-1, coal tar-2, and the coal tar-based driveway sealcoat. In order to remove the effects of contributions from other mixture components, the results of the chemical analyses were used to prepare synthetic mixtures containing only the CCME carcinogenic PAHs. The proportional concentration of each CCME PAH in the synthetic mixture is presented below.

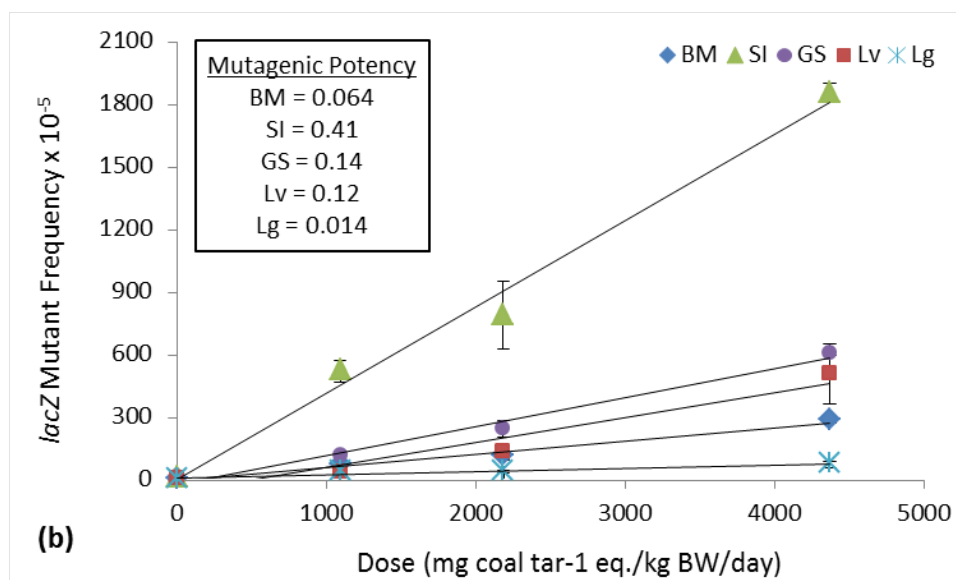
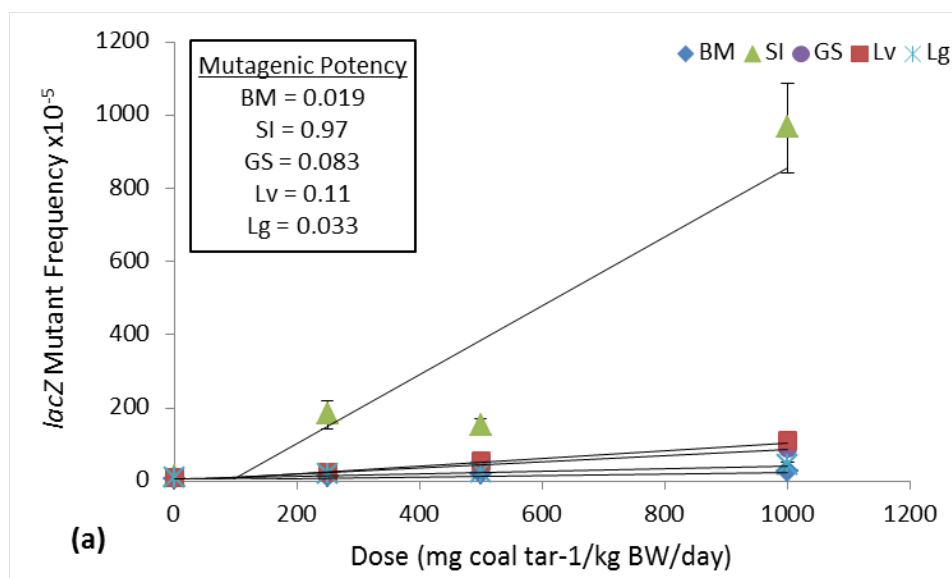
Polycyclic Aromatic Hydrocarbons	CAS #	Coal tar-1 extract (mg PAH/kg coal tar)	Synthetic Mixture-1 (%)	Coal tar-2 extract (mg PAH/kg coal tar)	Synthetic Mixture-2 (%)	Coal tar-Sealcoat (mg PAH/kg sealcoat)	Synthetic Mixture-3 (%)
Acenaphthene	83-32-9	747.4		2,530.2		2,432.8	
Acenaphthylene	208-96-8	6,681.0		8,576.8		BDL	
Anthracene	120-12-7	8,108.8		9,401.5		1,581.6	
Benzo[a]anthracene [†]	53-55-3	11,546.3	18.7%	5,080.1	27.7%	1,325.2	13.8%
Benzo[a]pyrene [†]	50-32-8	9,589.6	15.5%	2,982.1	16.3%	1,905.6	19.8%
Benzo[b]fluoranthene [†]	205-99-2	12,216.1	19.8%	2,662.1	14.5%	1,407.5	14.6%
Benzo[g,h,i]perylene [†]	191-24-2	5,200.2	8.4%	768.6	4.2%	957.6	10.0%
Benzo[k]fluoranthene [†]	207-08-9	6,293.2	10.2%	1,603.2	8.7%	783.5	8.1%
Biphenyl	92-52-4	2,333.9		2,074.9		87.1	
Chrysene [†]	218-01-9	10,982.2	17.8%	4,189.4	22.8%	2,408.6	25.0%
Dibenzo[a,h]anthracene [†]	53-70-3	1,089.4	1.8%	134.6	0.7%	150.9	1.6%
Fluoranthene	206-44-0	28,980.3		12,799.2		8,705.9	
Fluorene	86-73-7	8,408.5		12,139.5		1,866.9	
Indeno[1,2,3-cd]pyrene [†]	193-39-5	4,773.6	7.7%	930.3	5.1%	682.0	7.1%
1-Methylnaphthalene	90-12-0	2,466.1		14,514.6		201.2	
2-Methylnaphthalene	91-57-6	5,623.3		27,643.7		351.1	
Naphthalene	91-20-3	86,024.1		43,873.7		725.5	
Phenanthrene	85-01-8	42,130.7		29,722.0		11,849.7	
Pyrene	129-00-0	23,568.5		10,853.0		6,819.6	
TOTAL		276,763.2	100.0%	192,479.5	100.0%	44,242.3	100.0%
Total CCME carcinogenic PAHs		61,690.6	100.0%	18,350.4	100.0%	9,621.0	100.0%

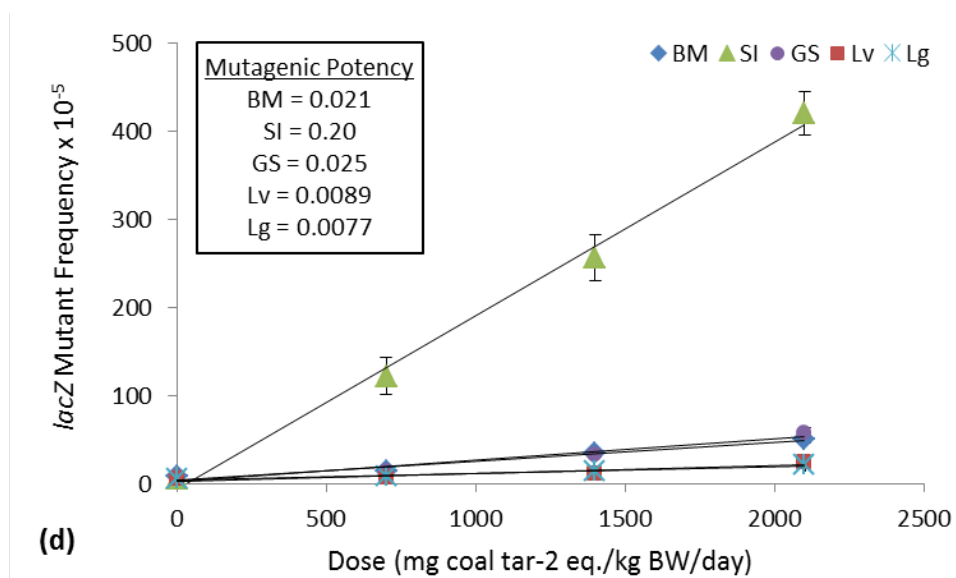
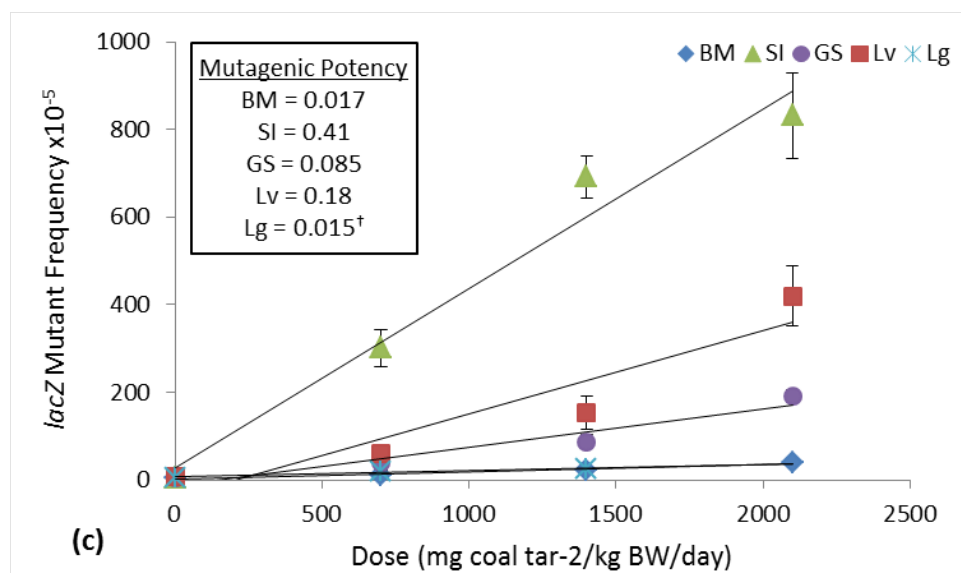
Supp. Table 4-IV. Qualitative summary of the MutaTMMouse *lacZ* mutation assay results for bone marrow, small intestine, glandular stomach, liver, and lung. Data for the CT-based sealcoat was previously published in Long *et al.* (2016)²⁰.

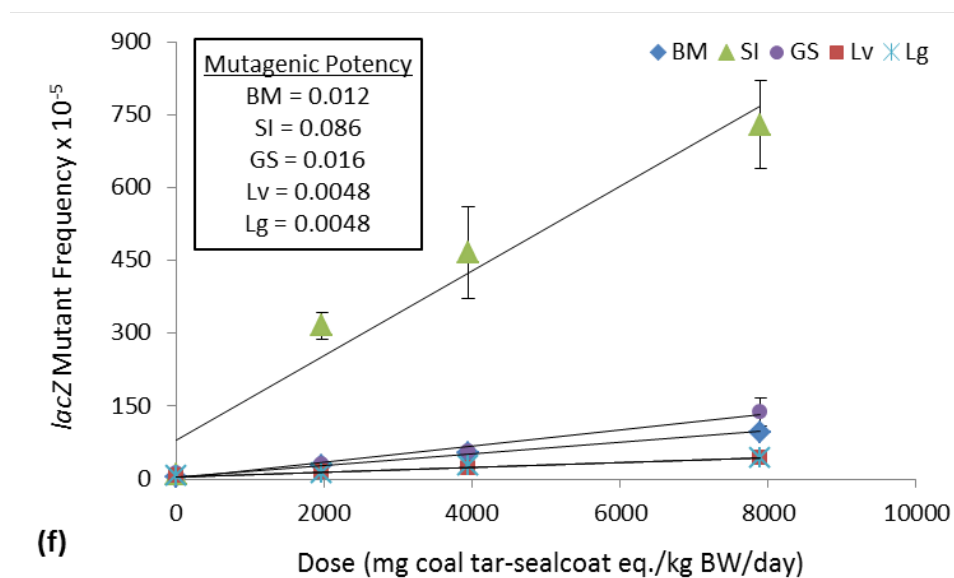
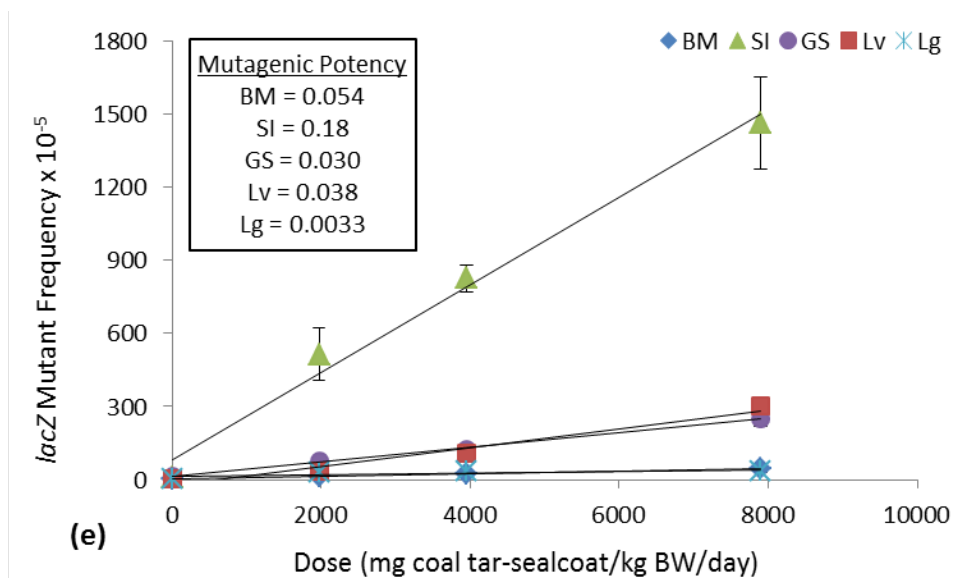
Compound	<i>lacZ</i> mutations				
	Bone Marrow	Small Intestine	Glandular Stomach	Liver	Lung
Coal Tar-1	++	+++	+++	+++	++
Synthetic Mixture-1	+++	+++	+++	+++	+++
Coal Tar-2	+++	+++	+++	+++	++
Synthetic Mixture-2	++	+++	++	+	+
Coal Tar-Sealcoat	+++	+++	+++	+++	++
Synthetic Mixture-3	+++	+++	++	++	++

+++ : Dose-response, greater than 10X control (CTR), ++ : Dose-response, 5-10X CTR, + : Dose-response, 1.5-5X CTRL

Supp. Figure 4.1. Tissue-specific MutaTMMouse *lacZ* mutagenicity results for the 6 mixtures examined in this study. Dose-response plots show mutant frequency $\times 10^{-5} \pm$ standard error for (a) coal tar-1, (b) synthetic PAH mixture-1, (c) coal tar-2, (d) synthetic PAH mixture-2, (e) coal tar-based driveway sealcoat and (f) synthetic PAH mixture-3. Each plot is the result of an independent animal exposure. Responses are shown for bone marrow (BM), small intestine (SI), glandular stomach (GS), liver (Lv), and lung (Lg), and MutaTMMouse *lacZ* mutagenic potency values for each tissue are inlaid. Potency is defined as the slope of the linear portion of the dose-response function (mutant frequency $\times 10^{-5}/\text{mg/kg BW/day}$). [†] indicates where data were truncated prior to potency determination (i.e., instances where potency was determined using fewer than 4 dose groups).



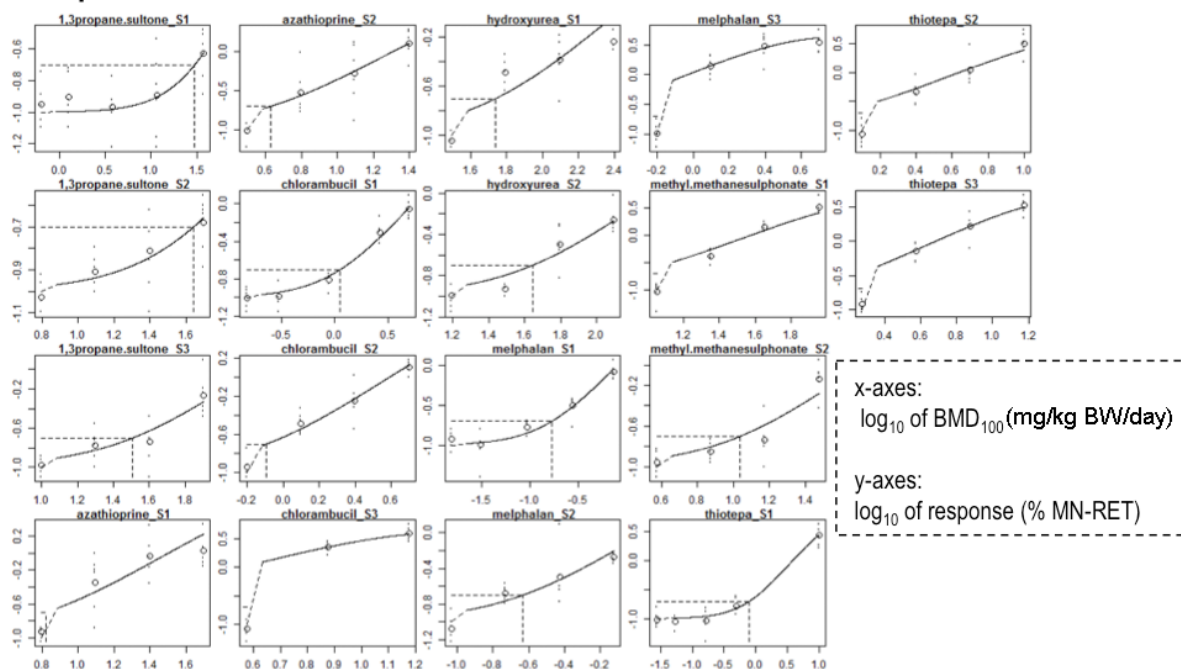




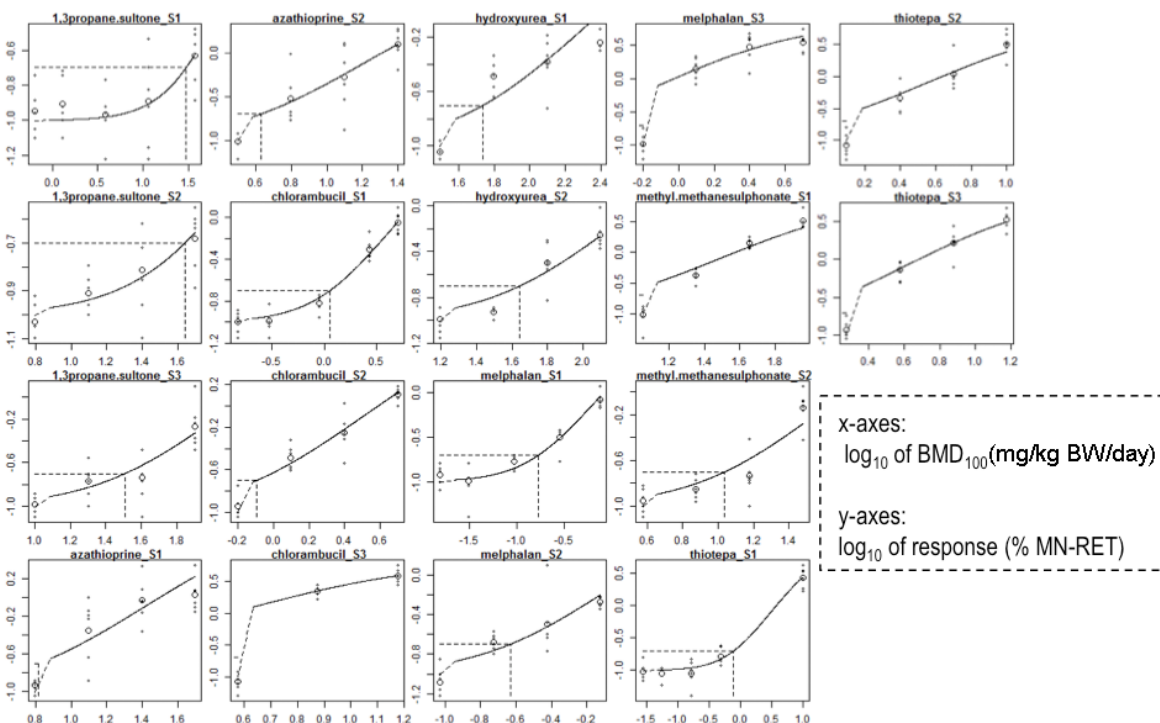
C.5 Chapter 5

Supp. Figure 5.1. BMD model fits of *in vivo* micronucleus data to examine study (S1, S2, S3) reproducibility across seven clastogenic compounds (21-23). The curves reflect the fitted four-parameter exponential or Hill model with covariate (study) dependent parameters a , b and within-group variance, and with ‘shape’ parameters c and d assumed to be equal in all subgroups. Horizontal and vertical dashed lines represent the BMR of 100% and BMD_{100} , respectively. Note that the control group (i.e., dose zero) is situated at minus infinity on a $\log_{10}$ -scale and a ‘placeholder’ on the X axes is required to permit visualisation.

Exponential model fits

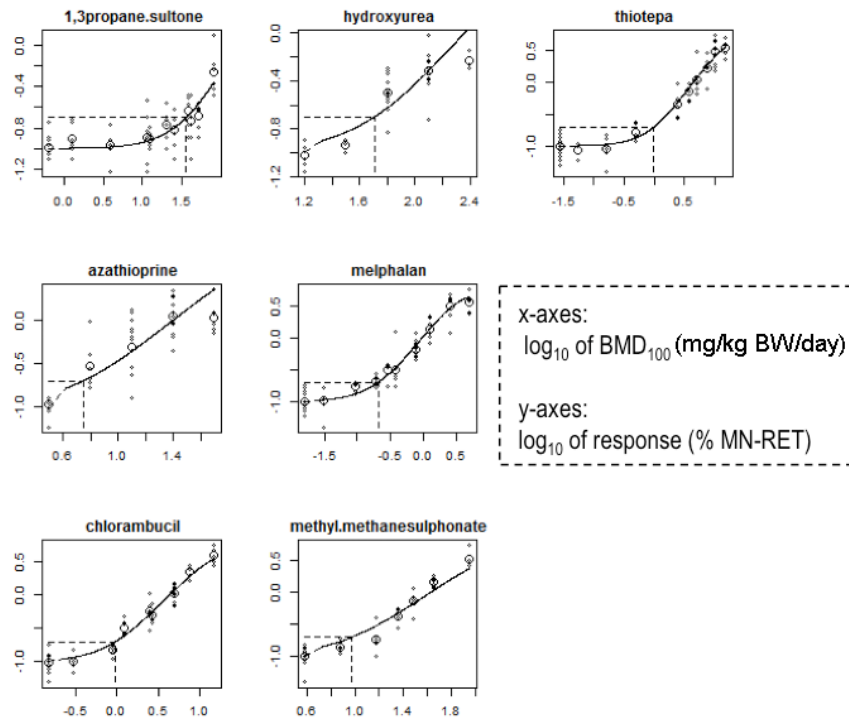


Hill model fits

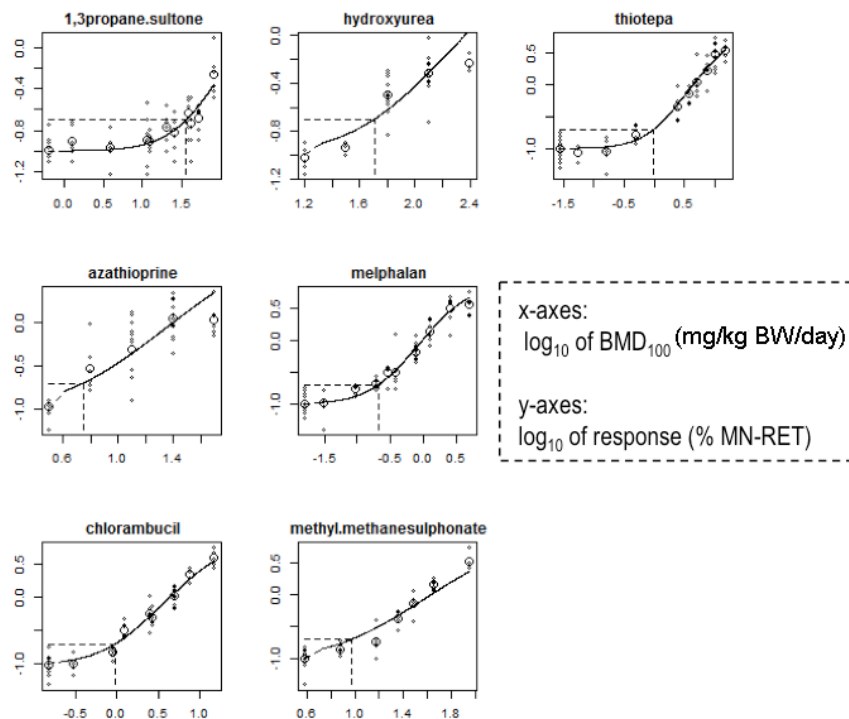


Supp. Figure 5.2. BMD model fits of *in vivo* micronucleus data to examine the potency of seven clastogenic compounds. Small points are individual observations, larger circles are geometric means (21-23). The curves reflect the fitted four-parameter exponential or Hill model with covariate (compound) dependent parameters a , b and within-group variance, and with ‘shape’ parameters c and d assumed to be equal in all subgroups. Horizontal and vertical dashed lines represent BMR of 100% and BMD_{100} , respectively. Note that the control group (i.e., dose zero) is situated at minus infinity on a $\log_{10}$ -scale and a ‘placeholder’ on the X axes is required to permit visualisation.

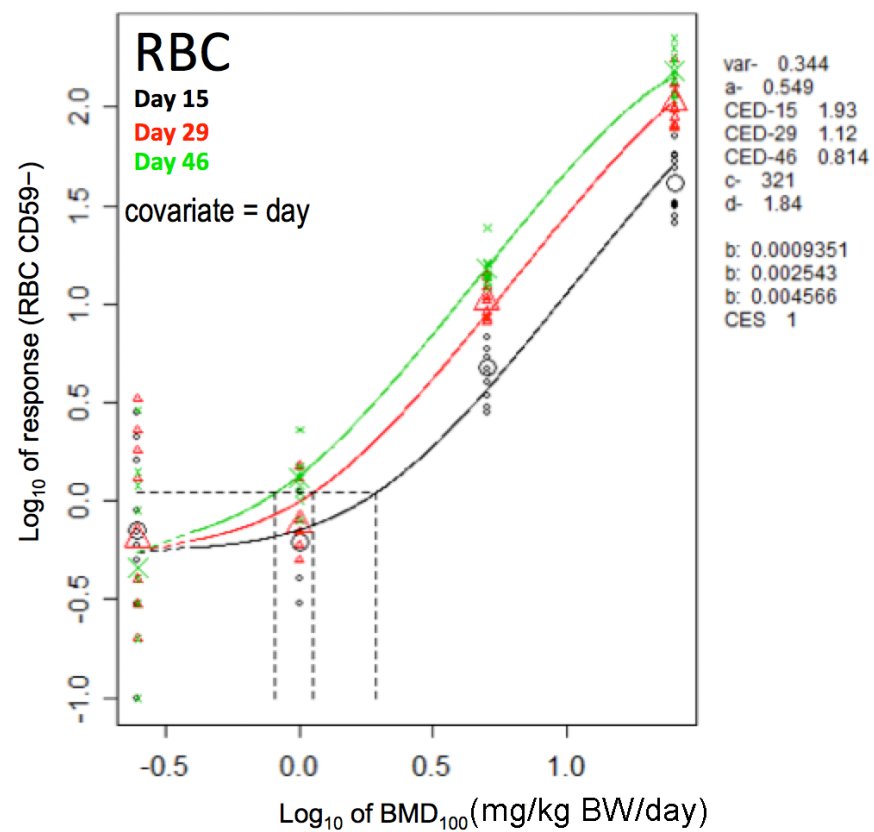
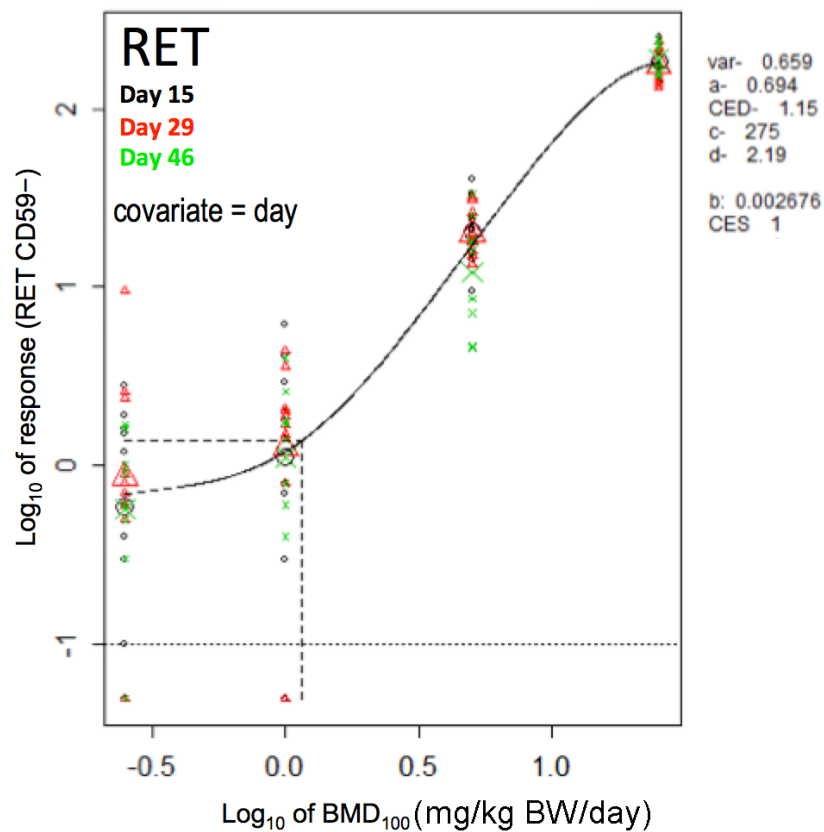
Exponential model fits



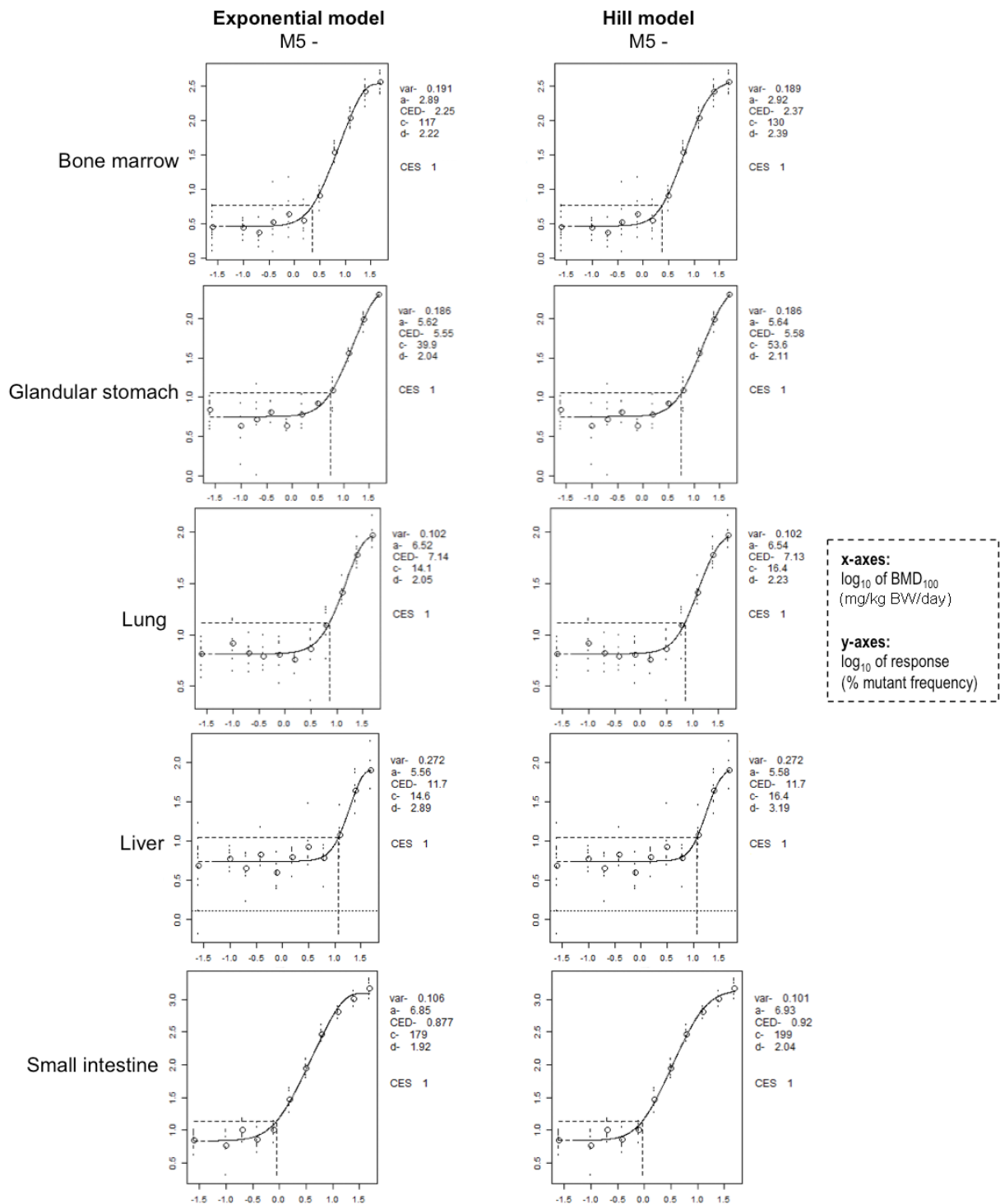
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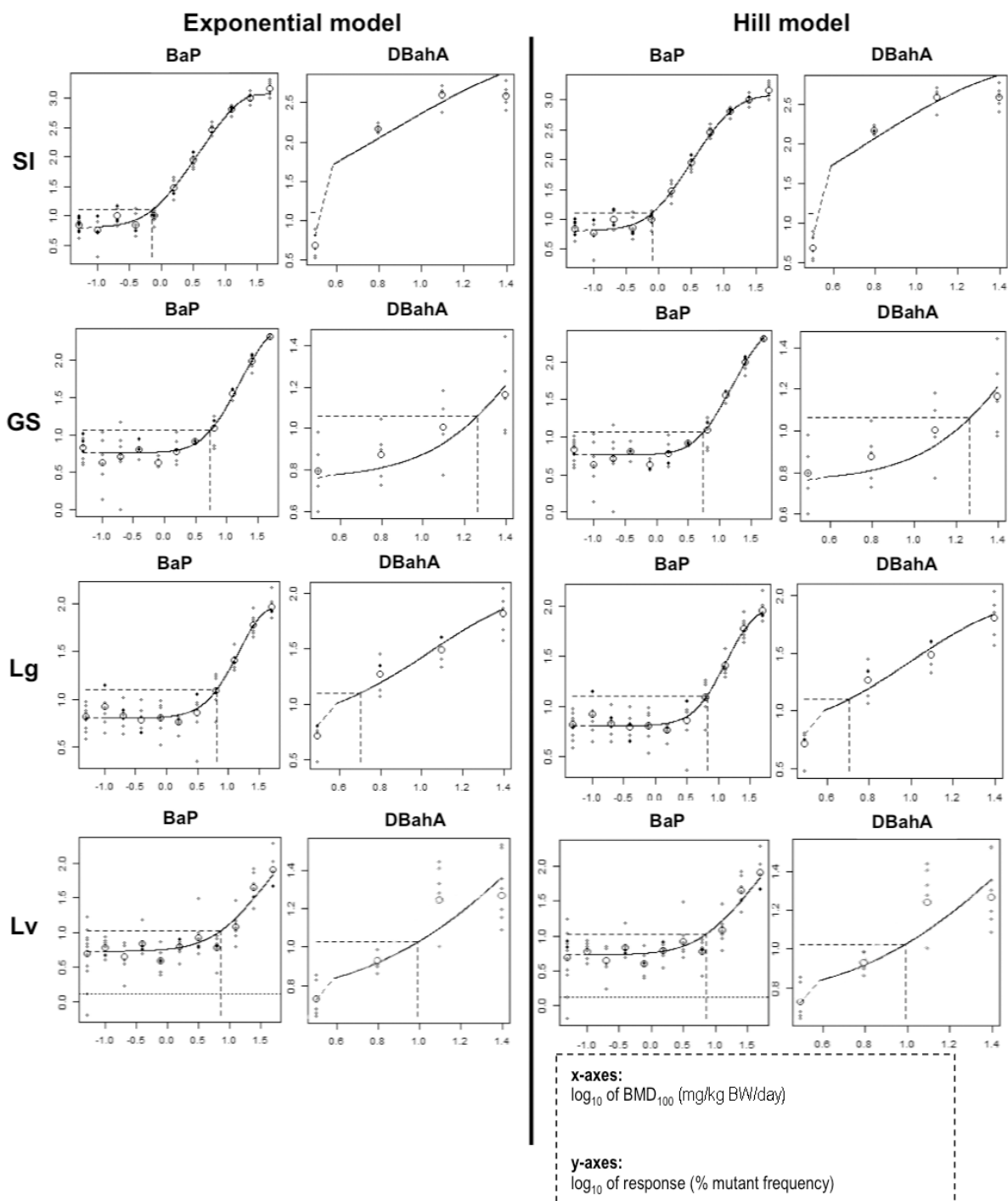
Supp. Figure 5.3. *Pig-a* mutant phenotype frequency for male and female rats exposed to ENU via oral gavage at 24-h intervals for three consecutive days prior to mutant frequency enumeration on days 15, 29 and 46 (24). Using ‘day’ as a covariate and pooling the data across sexes, the combined BMD-covariate analyses shows that sampling time is less important for mutant reticulocyte (RET CD59-) frequency (left panel) (i.e., the data were adequately described using a single exponential model curve with constant parameters for within-group variation (V), background (parameter a), potency (b), max-response (c) and log-steepness (d)). In contrast, mutant red blood cell (RBC CD59-) frequency showed sampling time dependency, requiring potency (b) estimation for each subgroup (right panel). Horizontal and vertical dashed lines represent BMR of 100% and BMD_{100} , respectively. Note that the control group (i.e., dose zero) is situated at minus infinity on a $\log_{10}$ -scale and a ‘placeholder’ on the X axes is required to permit visualisation. The horizontal dotted line in the left (RET) panel represents the response limit of quantification used in the statistical calculations.



Supp. Figure 5.4. Induced *lacZ* mutant frequency in response to benzo[*a*]pyrene (BaP) in the MutaTMMouse transgenic rodent assay (small points are individual observations; larger circles are geometric means). The curves reflect the fitted four-parameter exponential or Hill model with the values used for parameters *a* (response at dose zero), *b* (potency), *c* (maximum response) and *d* (log-steepness) shown to the right of each plot. Horizontal and vertical dashed lines represent a BMR of 100% and BMD₁₀₀, respectively. Note that the control group (dose zero) is situated at minus infinity on the log₁₀-scale and a 'placeholder' on the X axes is required to permit visualisation. Horizontal dotted lines (in the plots for liver) represent the response limit of quantification used in the statistical calculations.



Supp. Figure 5.5. Induced *lacZ* mutant frequency in response to benzo[*a*]pyrene (BaP) or dibenz[*a,h*]anthracene (DBaA) (28) exposure across several tissues (small intestine (SI), glandular stomach (GS), lung (Lg) and liver (Lv)) in the MutaTMMouse transgenic rodent assay (small points are individual observations; larger circles are geometric means). During analysis, the DBaA and BaP data were combined for each tissue and analysed using compound as a covariate. The curves reflect the fitted four-parameter exponential or Hill model with parameters *a* (response at dose zero) and *b* (potency) estimated for each subgroup, and with parameter *c* (maximum response) and *d* (log-steepness) assumed to be equal in all subgroups. Horizontal and vertical dashed lines represent a BMR of 100% and BMD₁₀₀, respectively. Note that the control group (dose zero) is situated at minus infinity on the log₁₀-scale and a ‘placeholder’ on the X axes is required to permit visualisation. Horizontal dotted lines (in the plots for liver) represent the response limit of quantification used in the statistical calculations.



C.6 Chapter 6

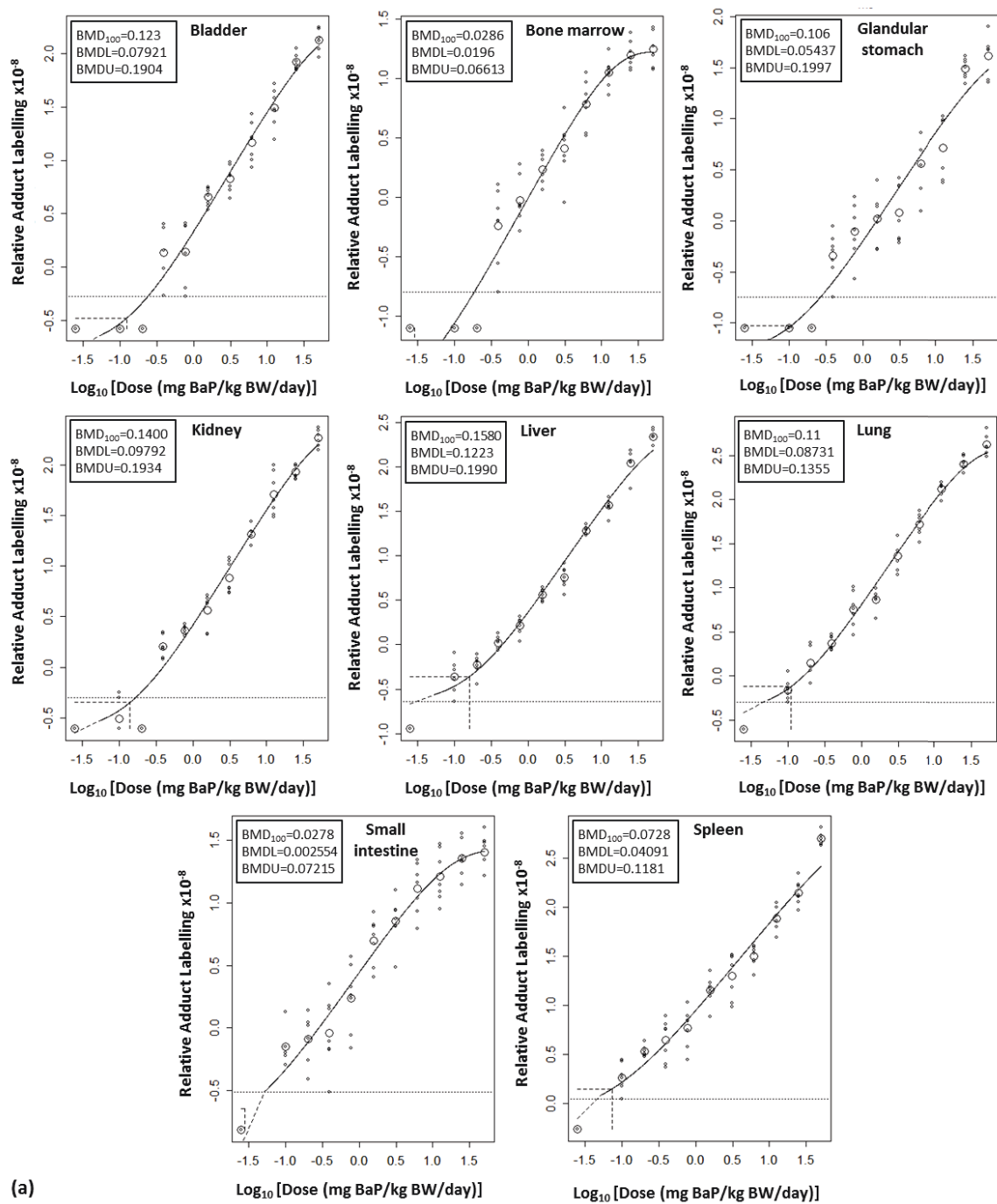
Supplementary Methods:

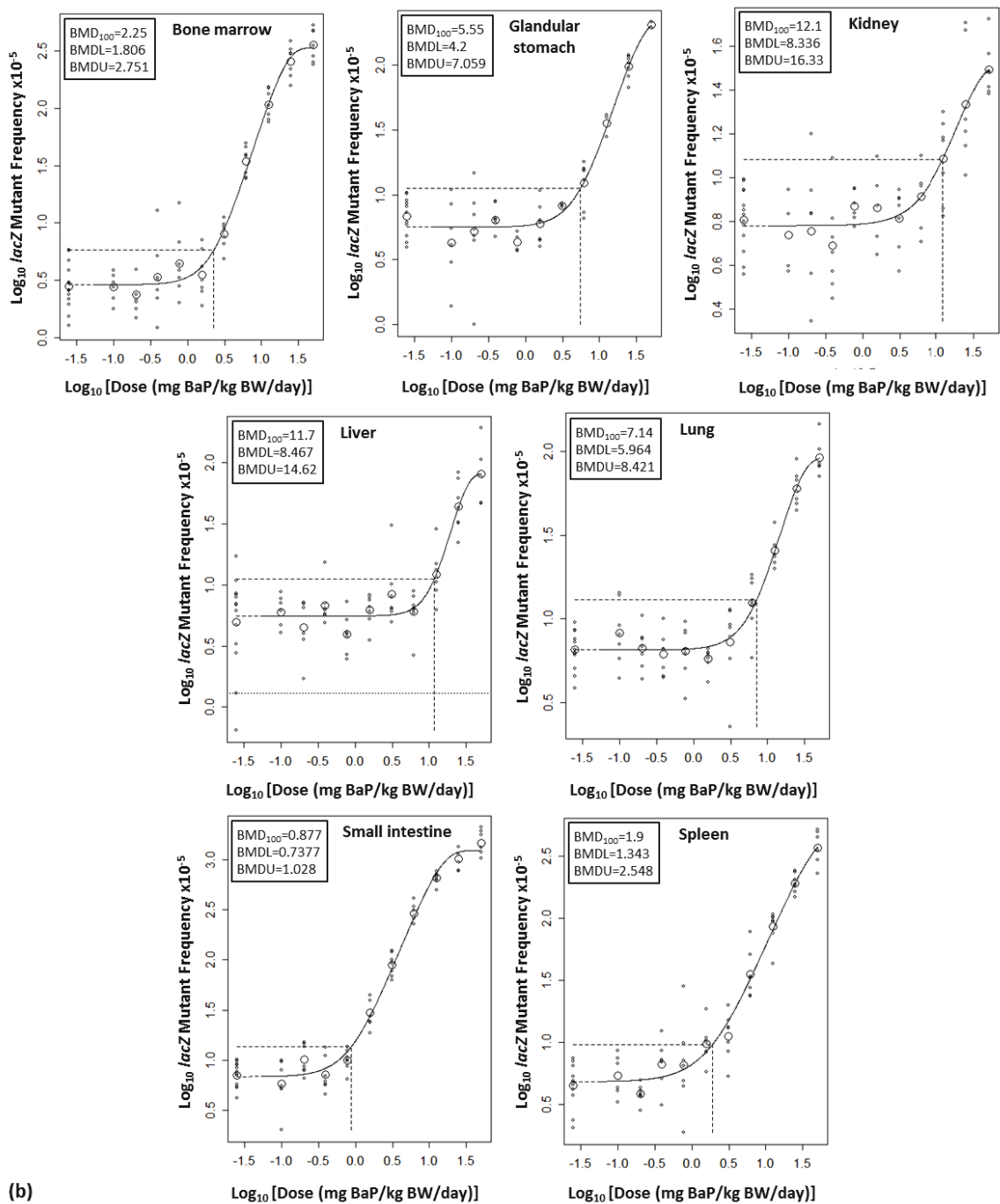
The lung and heart were excised attached, and the heart and right lobe of the lung were clamped off and removed. The left lung lobe was then perfused with 10% neutral buffered formalin (Surgipath, Winnipeg, MB) via the trachea until inflated. The duodenum was excised, flushed with phosphate buffered saline, followed by 10% NBF. The caudate lobe of the liver was excised and all tissues were immediately preserved in 10% NBF for immunohistochemical analysis. Formalin-fixed tissue samples were embedded in paraffin the day following necropsy and the resulting formalin-fixed paraffin-embedded tissues were sectioned to 4- μ m-wide slices. Tissue slices were mounted on slides and dried overnight. The slides were analysed for cell proliferation (ki67) using ki67 antibody (clone TEC-3) (DAKO, Burlington, ON). All slides were visualised on a Leica DM 4500 B microscope equipped with an Olympus DP 70 camera. Image acquisition and data analysis were conducted using Visiopharm Integrator System (v.3.2.8.0, Hoersholm, Denmark). Ki67 index (i.e., proliferation index) was calculated as the frequency of ki67-positive stained cells.

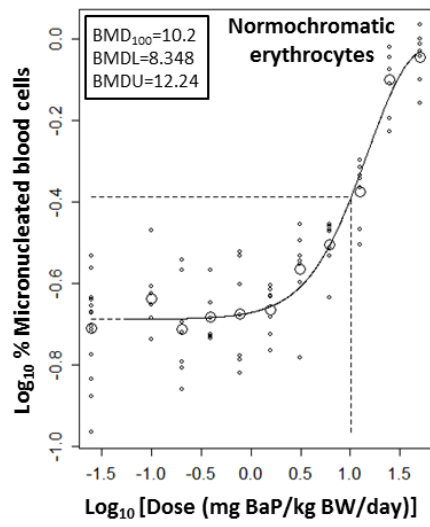
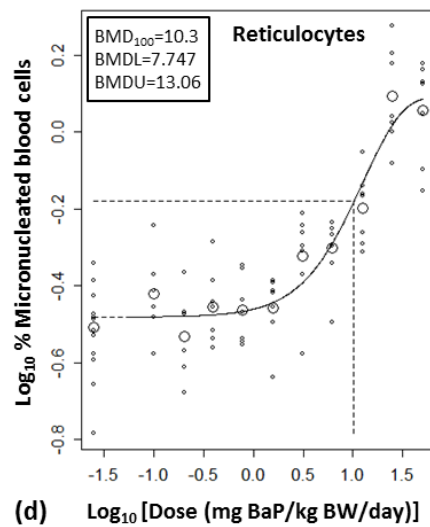
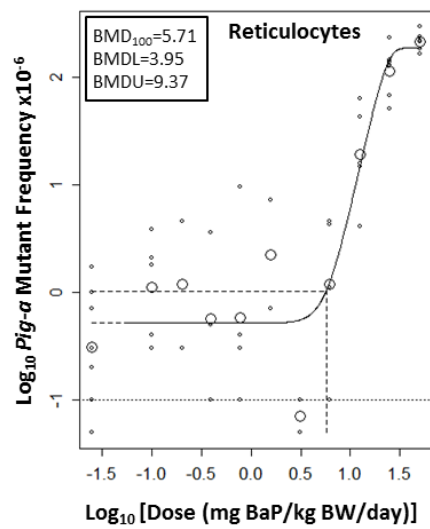
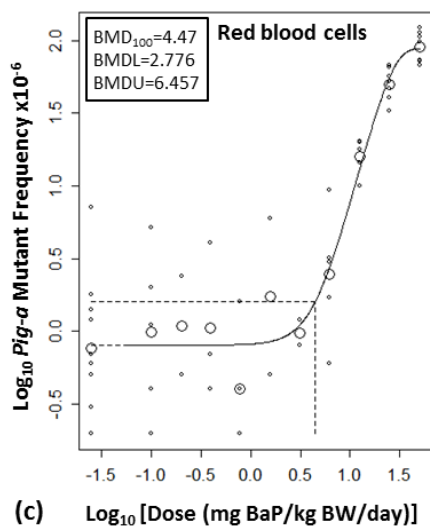
Supp. Table 6-I. BMD₁₀₀ values and two-sided 90% confidence intervals (i.e., the BMDL & BMDU) for each endpoint and tissue/cell population. BMDs were determined (in mg BaP/kg BW/day) using the 4-parameter exponential model in PROAST. BM = Bone marrow; Lv = Liver; GS = Glandular stomach; SI = Small intestine; Lg = Lung; Sp = Spleen; Kd = Kidney, RET = reticulocyte; RBC = red blood cell; NCE = normochromatic erythrocyte.

Tissue	BMD ₁₀₀	BMDL	BMDU	BMDU-BMDL Ratio
Adducts				
SI	0.0278	0.00255	0.0722	28.3
BM	0.0286	0.0196	0.0661	3.37
Sp	0.0728	0.0409	0.118	2.89
GS	0.106	0.0544	0.200	3.68
Lg	0.110	0.0873	0.136	1.56
Bd	0.123	0.0792	0.190	2.40
Kd	0.140	0.0979	0.193	1.97
Lv	0.158	0.122	0.199	1.63
<i>lacZ</i>				
SI	0.878	0.738	1.03	1.39
Sp	1.904	1.34	2.55	1.90
BM	2.25	1.81	2.75	1.52
GS	5.55	4.20	7.06	1.68
Lg	7.14	5.96	8.42	1.41
Lv	11.7	8.47	14.6	1.73
Kd	12.1	8.34	16.3	1.96
MN				
NCEs	10.2	8.35	12.2	1.47
RETs	10.3	7.75	13.1	1.69
<i>Pig-a</i>				
RBCs	4.47	2.78	6.46	2.33
RETs	5.71	3.95	9.37	2.37

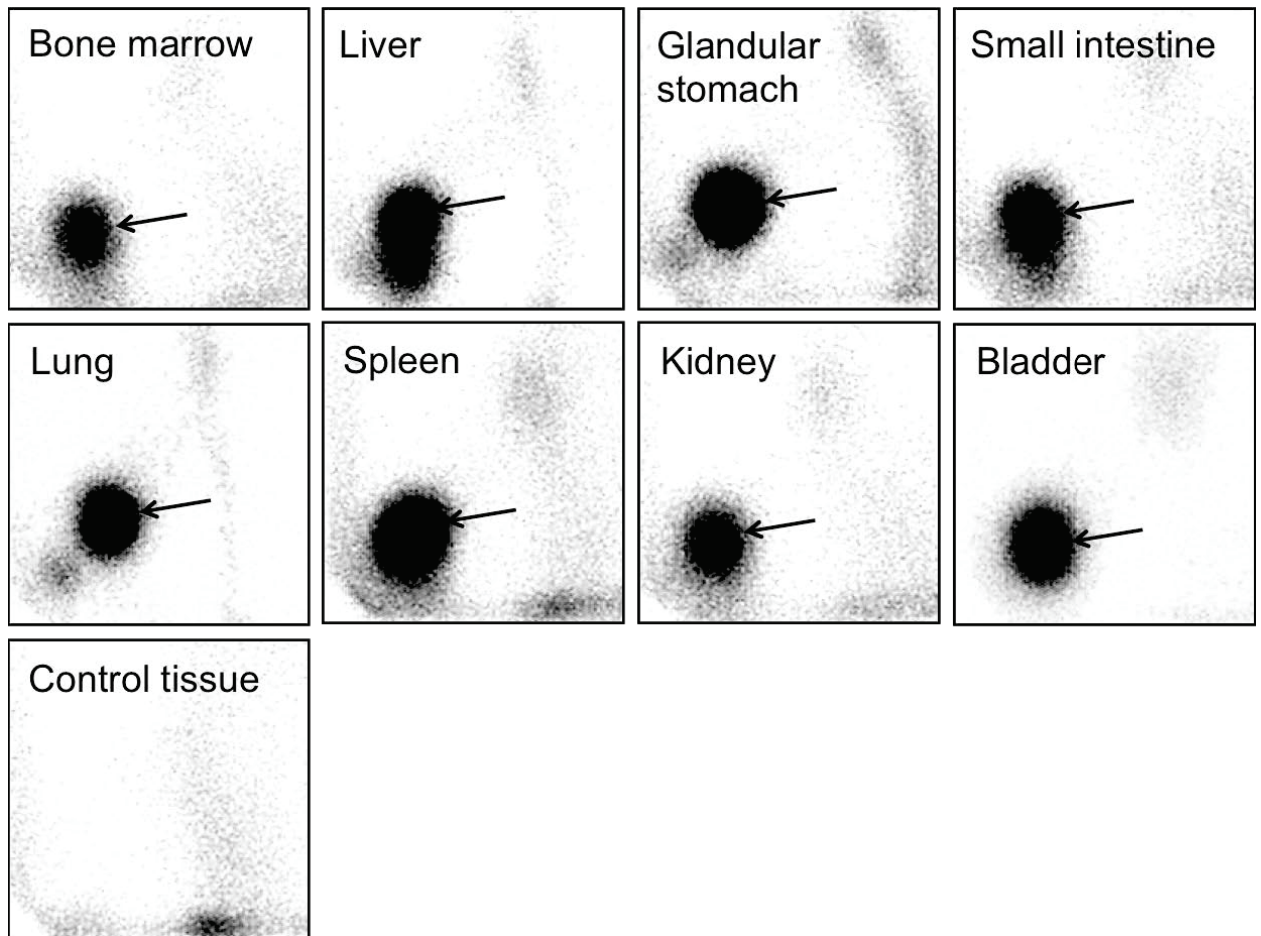
Supp. Figure 6.1. PROAST model fits for **(a)** DNA adducts, **(b)** *lacZ* mutations, **(c)** *Pig-a* mutations, and **(d)** micronucleus frequency data. Small points are individual observations, larger circles are geometric means. The curves reflect the fitted four-parameter exponential model. Horizontal and vertical dashed lines represent BMR of 100% and BMD_{100} , respectively. The BMD_{100} , $BMDL$, and $BMDU$ are inset. Note that the control group (i.e., dose zero) is situated at minus infinity on a $\log_{10}$ -scale and a ‘placeholder’ on the X-axis is required to permit visualization. The horizontal dotted line represents the response limit of quantification used in the statistical calculations.



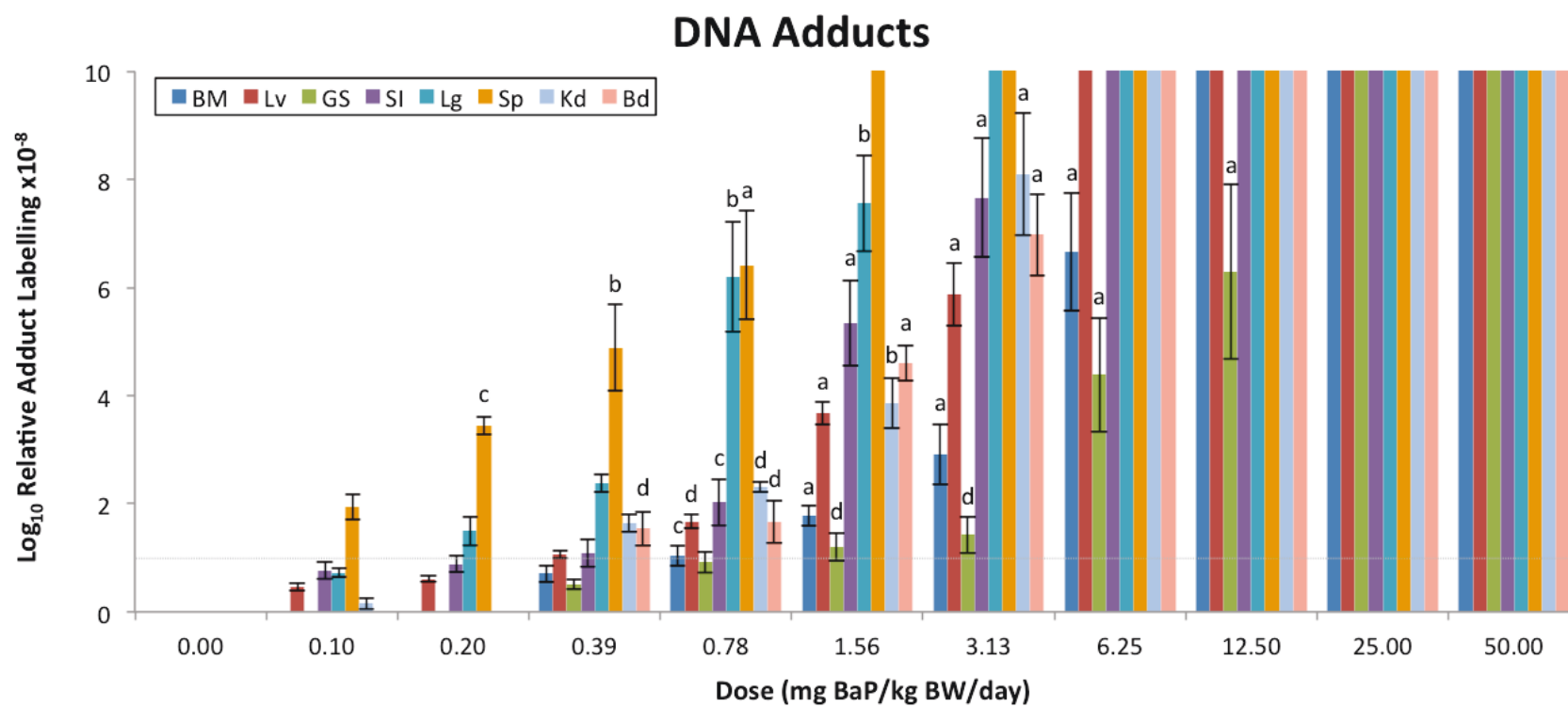


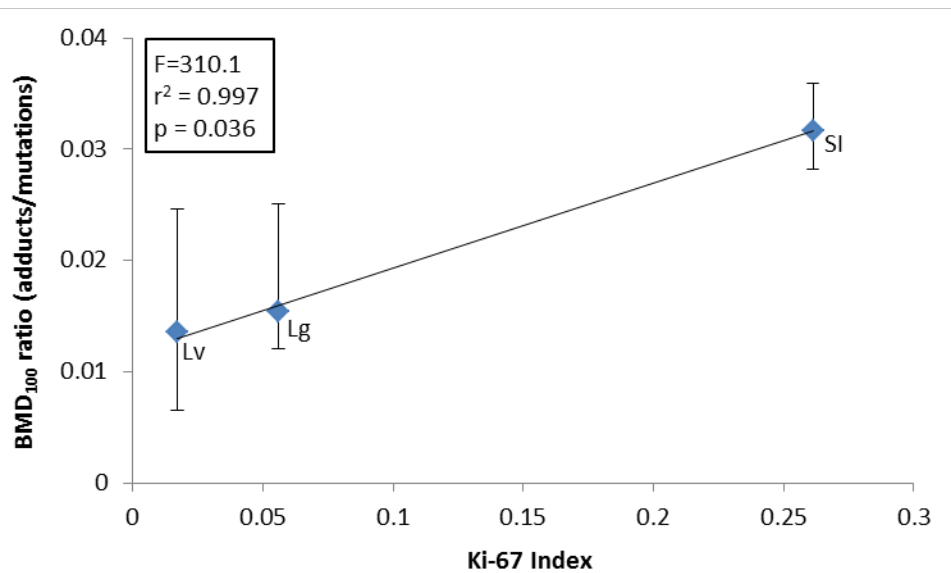


Supp. Figure 6.2. Autoradiographic profiles of DNA adducts, measured by thin-layer chromatography ^{32}P -postlabelling, in various tissues exposed to 50 mg/kg BW/day BaP. The adduct profiles shown are representative of the cross-tissue pattern observed throughout the dose range examined. No BaP-DNA adducts were detected in control tissues. Solvent conditions for the separation of BaP-derived DNA adducts were as follows: D1, 1.0 M sodium phosphate, pH 6.0; D3, 3.5 M lithium-formate, 8.5 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. The origins, at the bottom left-hand corners, were cut off before imaging. The arrow indicates the adduct spot of 10-(deoxyguanosin- N^2 -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP (dG- N^2 -BPDE) used for quantitation.



Supp. Figure 6.3. Benzo[*a*]pyrene dose-response data ($\pm$ standard error) for DNA adducts with the results plotted on a restricted linear y-axis in order to visualize responses at low doses that were obscured when plotted on a $\log_{10}$ y-axis (as in **Figure 6.1a**). Statistical results for the overall dose–response relationship are presented for each tissue. The level of significance for the custom contrast results for each dose vs. control are indicated as follows: a = $p < 0.0001$; b = $p < 0.001$; c = $p < 0.01$; d = $p < 0.05$. BM = Bone marrow; Lv = Liver; GS = Glandular stomach; SI = Small intestine; Lg = Lung; Sp = Spleen; Kd = Kidney; Bd = Bladder.

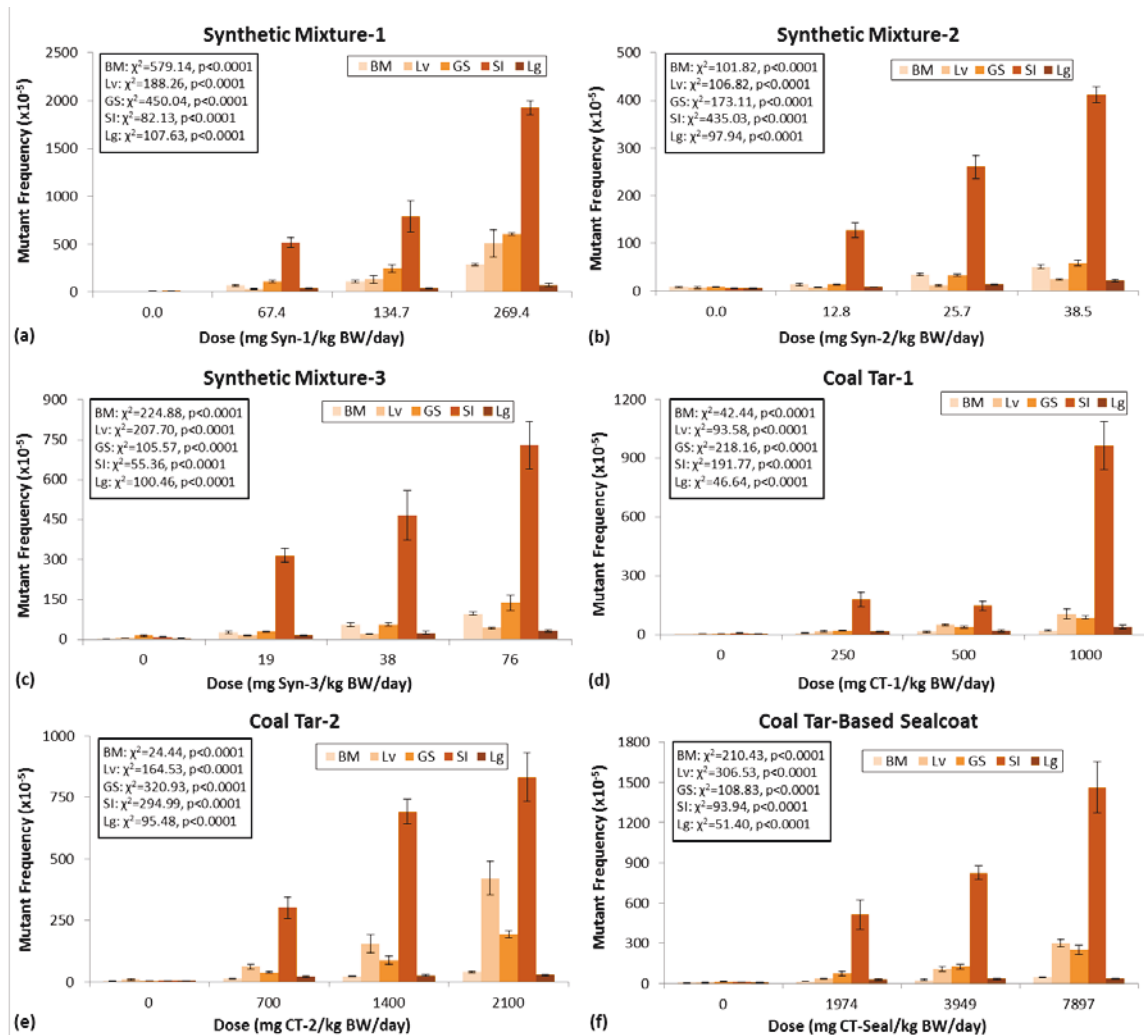


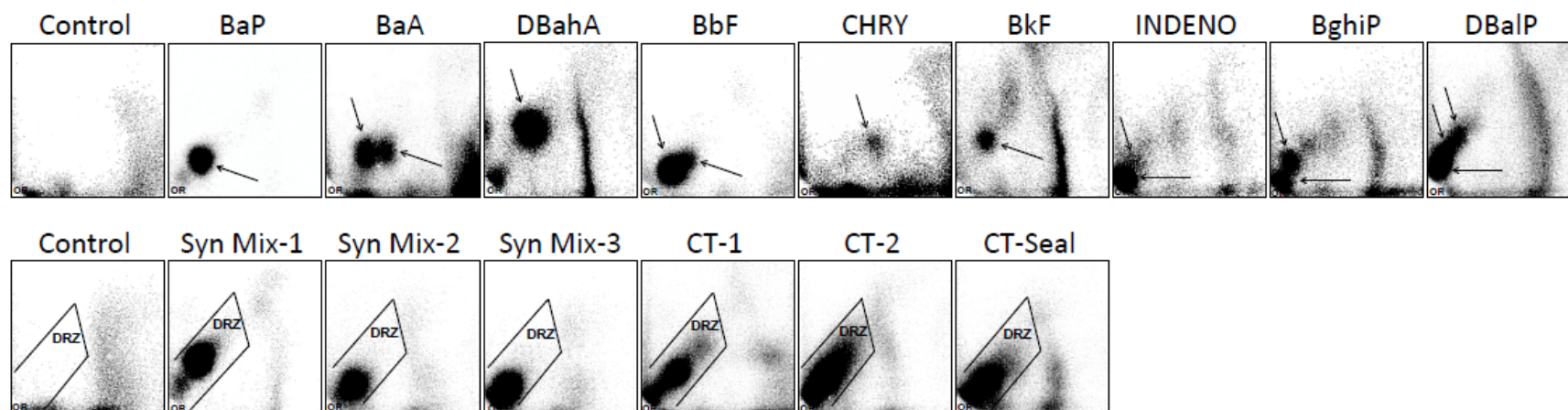


Supp. Figure 6.4. BMD₁₀₀ ratio of DNA adducts to *lacZ* mutations for liver (Lv), lung (Lg), and small intestine (SI) versus the Ki-67 index. Ki-67 index was determined via comparative immunohistochemical analyses of FFPE tissues slices from control and exposed animals.

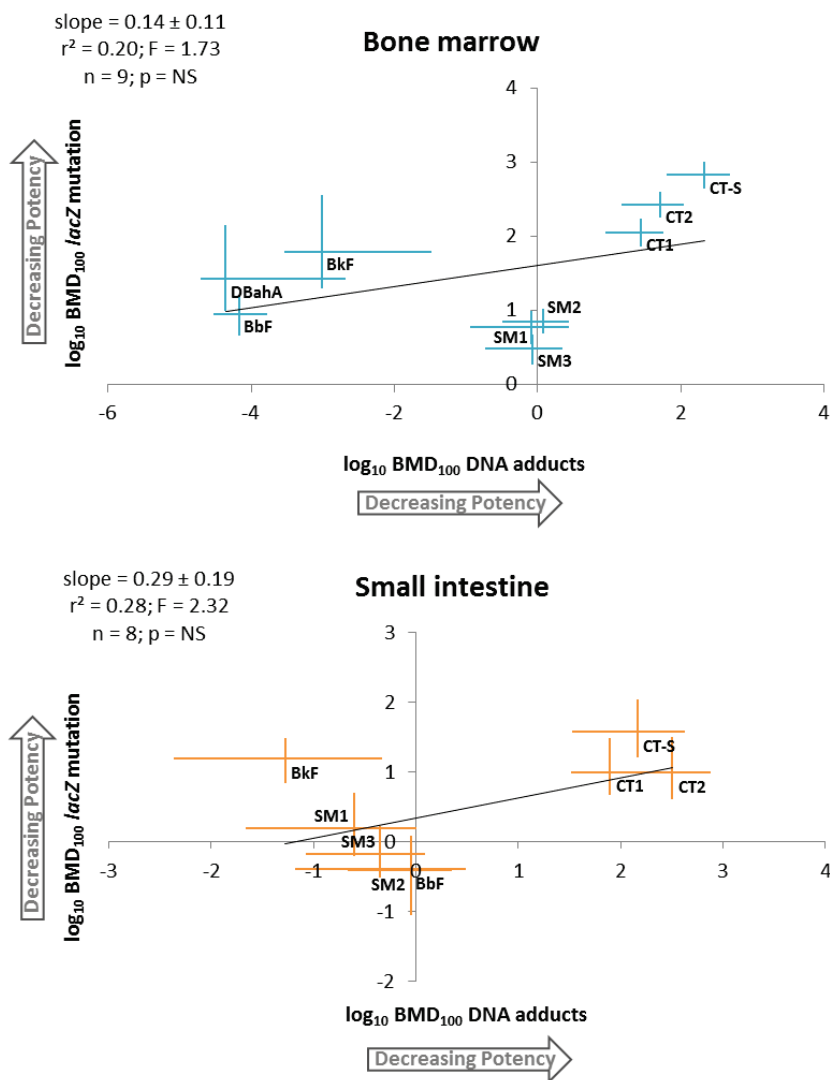
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Supp. Figure 7.1. The *lacZ* mutant frequency in bone marrow (BM), liver (Lv), glandular stomach (GS), small intestine (SI), and lung (Lg) induced by exposures to synthetic PAH mixtures and complex organic extracts of coal tar or coal tar-based driveway sealcoat. Mutant frequencies $\pm$ standard error are displayed for each tissue and dose for **(a)** synthetic mixture-1; **(b)** synthetic mixture-2; **(c)** synthetic mixture-3; **(d)** coal tar-1; **(e)** coal tar-2; **(f)** coal tar-based driveway sealcoat. Statistical results for the tissue-specific dose-response relationships are presented in the inlaid boxes. Custom contrasts for each dose versus the concurrent control revealed significant ($p < 0.0001$) increases in mutant frequency for all compounds and doses in each tissue, with the exception of the low dose of Synthetic mixture-2 in liver, GS, and lung.





Supp. Figure 7.2. Representative autoradiographic profiles of DNA adducts, measured by ^{32}P -postlabelling, in lungs from control MutaTMMouse animals (A & K) or MutaTMMouse specimens subchronically exposed to individual PAHs (B-J), synthetic PAH mixtures (L-N), coal tar extracts (O-P), or an extract of a commercially available coal tar-based driveway sealcoat (Q). These profiles in the lung DNA are representative of adduct profiles for other tissues including liver, glandular stomach, small intestine, and bone marrow. For the individual PAHs, the arrow(s) indicate the major adduct spot(s) used to calculate DNA adduct levels. For the synthetic and complex mixtures, the diagonal radioactive zone (DRZ) was used to calculate DNA adduct levels. Solvent conditions for the separation of PAH-derived DNA adducts using thin-layer chromatography were as follows: D1, 1.0 M sodium phosphate, pH 6.0; D3, 3.5 M lithium-formate, 8.5 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea. The origins (OR), at the bottom left corner, were cut off before imaging. BaP: benzo[*a*]pyrene; BaA: benz[*a*]anthracene; DBaA: dibenz[*a,h*]anthracene; BbF: benzo[*b*]fluoranthene; CHRY: chrysene; BkF: benzo[*k*]fluoranthene; INDENO: indeno[1,2,3-*c,d*]pyrene; BghiP: benzo[*g,h,i*]perylene; DBaP: dibenzo[*a,l*]pyrene; Syn-1: synthetic mixture-1; Syn-2: synthetic mixture-2; Syn-3: synthetic mixture-3; CT-1: coal tar-1; CT-2: coal tar-2; CT-Seal: coal tar-based driveway sealcoat.



Supp. Figure 7.3. Relationship between the BMD₁₀₀ values for the *lacZ* mutation endpoint and the matched DNA adduct endpoint for bone marrow and small intestine. The length of the lines represents the 90% confidence limits of the BMD values. For each tissue, compounds were included if the 90% confidence intervals were defined for both endpoints. Linear regression analysis results are shown in the inset. BaP: benzo[*a*]pyrene; DBaHA: dibenz[*a,h*]anthracene; BbF: benzo[*b*]fluoranthene; BkF: benzo[*k*]fluoranthene; Ind: indeno[1,2,3-*c,d*]pyrene; BghiP: benzo[*g,h,i*]perylene; DBaIP: dibenzo[*a,l*]pyrene; SM1: synthetic mixture-1; SM2: synthetic mixture-2; SM3: synthetic mixture-3; CT1: coal tar-1; CT2: coal tar-2; CT-S: coal tar-based driveway sealcoat.