

Utility of toxicogenomics tools for the toxicological assessment
of polycyclic aromatic hydrocarbons and complex polycyclic
aromatic hydrocarbon mixtures

by

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Abstract

Human exposures to polycyclic aromatic hydrocarbons (PAHs) occur as components of complex mixtures. Evaluations of health risks posed by complex mixtures containing PAHs rely on the toxicological knowledge of prioritized PAH mixture components, assuming that these PAHs share a common mode of action and that the sum of the contributions of these PAHs equals the toxic potency of the mixture (i.e., additivity). Traditional toxicity testing methods emphasizing apical endpoints have had limited success at evaluating the validity of these assumptions. Toxicogenomic tools that are able to rapidly generate toxicologically-relevant and mechanistic information have gained acceptance in the regulatory arena for individual chemicals; however, the applicability of these tools for chemical mixtures has been inadequately addressed. This thesis used toxicogenomic tools to (1) improve the understanding of mechanisms underlying the adverse, toxicological responses induced by individual PAHs and (2) evaluate the contention that transcriptional profiles and pathway information can be used to critically examine interactions between individual PAHs in PAH-containing mixtures, and the assumption of additivity. Microarrays were used to profile gene expression changes (transcriptomes) in forestomach, liver, and lung tissues (targets of PAH exposure) from mice orally exposed to three doses of eight individual PAHs, two defined PAH mixtures, and one complex PAH-containing mixture (coal tar). The results revealed that each PAH induced transcriptional changes that were significantly associated with several pathways implicated in carcinogenesis. However, despite a uniform ability to induce DNA damage (i.e., DNA adducts), mutations, and increases in enzyme activity, the pathways differ across PAHs and tissues. A novel strategy that employs single-PAH transcriptome data to models of additivity revealed that the assumption of additivity in PAH mixtures is valid at the pathway level; however, the independent action model of additivity yielded better estimates compared to concentration addition (used in human health risk

assessment of PAH mixtures) or generalized concentration addition. Additionally, predicted and observed coal tar-induced transcriptional benchmark doses were comparable to those derived from previously published coal tar-induced murine lung tumour incidence data. Overall, this thesis demonstrates the utility of toxicogenomic data to expand the current understanding regarding the toxic potential of individual PAHs and PAH-containing complex mixtures.

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Table of Contents (brief)

Chapter		Page
1	Introduction: Review of Pertinent Literature	1
2	Subchronic Oral Exposure to Benzo(a)pyrene leads to Distinct Transcriptomic Changes in the Lungs that are Related to Carcinogenesis	41
3	Toxicogenomics Outcomes Predictive of Forestomach Carcinogenesis Following Exposure to Benzo(a)pyrene: Relevance to Human Cancer Risk	71
4	Comparative Transcriptomic Analyses to Scrutinize the Assumption that Genotoxic PAHs Exert Effects via a Common Mode of Action	108
5	A Framework for the Use of Single Chemical Transcriptomics Data in Predicting the Hazards Associated with Complex Mixtures of Polycyclic Aromatic Hydrocarbons	155
6	General Conclusions	195
7	References	211
Appendices		
I	Comparison of Toxicogenomics and Traditional Approaches to Inform Mode of Action and Points of Departure in Human Health Risk Assessment of Benzo(a)pyrene in Drinking Water	234
II	Nano-risk Science: Application of Toxicogenomics in an Adverse Outcome Pathway Framework for Risk Assessment of Multi-Walled Carbon Nanotubes	278
III	Integrating Toxicogenomics into Human Health Risk Assessment: Lessons Learned from the Benzo[a]pyrene Case Study	296
IV	Transcriptomic Analysis Reveals Novel Mechanistic Insight into Murine Biological Responses to Multi-Walled Carbon Nanotubes in Lungs and Cultured Lung Epithelial Cells	306
V	Supplemental Material	332

Table of Contents (full)

ABSTRACT	II
ACKNOWLEDGEMENTS	IV
TABLE OF CONTENTS (BRIEF)	VII
TABLE OF CONTENTS (FULL)	VIII
LIST OF ABBREVIATIONS	XIV
LIST OF TABLES.....	XVII
LIST OF FIGURES.....	XVIII
LIST OF APPENDICES	XX
LIST OF SUPPLEMENTARY FILES	XXI
PREFACE	XXIII
STATEMENT OF CONTRIBUTIONS.....	XXIV
1 CHAPTER: GENERAL INTRODUCTION	1
1.1 TOXICITY TESTING AND HUMAN HEALTH RISK ASSESSMENT	1
1.1.1 <i>The human health risk assessment paradigm</i>	1
1.1.2 <i>Risk assessment of chemical mixtures</i>	3
1.1.2.1 Additivity	4
1.1.3 <i>The current landscape of toxicity testing</i>	6
1.1.4 <i>Toxicogenomics</i>	7
1.1.4.1 Toxicogenomics in risk assessment	9
1.1.4.2 Mixtures toxicology from a toxicogenomics perspective.....	10
1.2 THE TOXICOLOGY AND RISK ASSESSMENT OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)	11
1.2.1 <i>Identity, use, and sources of PAHs</i>	12
1.2.2 <i>Carcinogenicity associated with PAHs</i>	14
1.2.2.1 Mechanisms of PAH-induced carcinogenesis	16

1.2.3	<i>Risk assessment of PAHs</i>	21
1.2.4	<i>Risk assessment of PAH-containing mixtures</i>	22
1.2.4.1	The PEF approach for risk assessment of PAH mixtures	23
1.3	RATIONALE FOR THIS THESIS	24
1.4	TABLES	29
1.5	FIGURES	35
2	CHAPTER: SUBCHRONIC ORAL EXPOSURE TO BENZO(A)PYRENE LEADS TO DISTINCT TRANSCRIPTOMIC CHANGES IN THE LUNGS THAT ARE RELATED TO CARCINOGENESIS	41
2.1	ABSTRACT	41
2.2	INTRODUCTION	42
2.3	MATERIALS AND METHODS	44
2.3.1	<i>Animal Treatment</i>	44
2.3.2	<i>Tissue Selection</i>	45
2.3.3	<i>Tissue DNA Extraction</i>	45
2.3.4	<i>DNA Adduct Analysis</i>	46
2.3.5	<i>LacZ Mutant Frequency (Positive Selection)</i>	46
2.3.6	<i>RNA Extraction and Purification</i>	47
2.3.7	<i>Microarray Hybridization and Analysis</i>	47
2.3.8	<i>qRT-PCR Array Validation</i>	48
2.3.9	<i>Bioinformatics</i>	49
2.4	RESULTS	50
2.4.1	<i>BaP-induced DNA adduct formation in lung and liver tissue</i>	50
2.4.2	<i>Mutagenic activity of BaP in the lung tissue</i>	51
2.4.3	<i>General overview of pulmonary gene expression profiles</i>	51
2.4.4	<i>RT-PCR validation of microarray results</i>	54
2.5	DISCUSSION	55
2.6	TABLES AND FIGURES	61

3 CHAPTER: TOXICOGENOMIC OUTCOMES PREDICTIVE OF FORESTOMACH

CARCINOGENESIS FOLLOWING EXPOSURE TO BENZO(A)PYRENE: RELEVANCE TO HUMAN

CANCER RISK	71
3.1 ABSTRACT	71
3.2 INTRODUCTION	72
3.3 METHODS	75
3.3.1 <i>Animal treatment</i>	75
3.3.2 <i>Tissue RNA extraction and purification</i>	76
3.3.3 <i>Microarray hybridization and analysis</i>	77
3.3.4 <i>Bioinformatics and pathway analysis</i>	78
3.3.5 <i>Mining of public databases for human disease correlation</i>	79
3.3.6 <i>Quantitative Real Time (qRT)-PCR array validation</i>	80
3.3.7 <i>Western blot analysis</i>	80
3.4 RESULTS	81
3.4.1 <i>General overview of forestomach gene expression profiles</i>	81
3.4.2 <i>Biological function and pathway analysis of significantly differentially expressed genes</i>	82
3.4.3 <i>Disease prediction</i>	84
3.4.4 <i>qRT-PCR Validation of Representative Genes</i>	85
3.4.5 <i>Protein Validation</i>	85
3.5 DISCUSSION	86
3.5.1 <i>BaP-induced DNA damage response in the forestomach</i>	87
3.5.2 <i>BaP activates antigen processing and presentation pathway</i>	89
3.5.3 <i>MHC class I-mediated detoxification of BaP</i>	91
3.5.4 <i>Human relevance</i>	93
3.5.5 <i>Conclusion</i>	97
3.6 TABLES AND FIGURES	97

4 CHAPTER: COMPARATIVE TRANSCRIPTOMIC ANALYSES TO SCRUTINISE THE

ASSUMPTION THAT GENOTOXIC PAHS EXERT EFFECTS VIA A COMMON MODE OF ACTION 108

4.1	ABSTRACT	108
4.2	INTRODUCTION	109
4.3	METHODS	112
4.3.1	<i>Materials and dose selection</i>	112
4.3.2	<i>MutaTMMouse exposures</i>	112
4.3.3	<i>Tissue RNA extraction and purification</i>	113
4.3.4	<i>Microarray hybridization and analysis</i>	113
4.3.5	<i>Bioinformatics and pathway analysis</i>	114
4.3.6	<i>DNA extraction from lung tissue</i>	116
4.3.7	<i>LacZ mutant frequency in lung tissue</i>	116
4.3.8	<i>DNA adduct analysis in lung tissue</i>	116
4.3.9	<i>Enzymatic analysis of lung Cyp1a activity</i>	116
4.3.10	<i>Benchmark dose (BMD) calculations</i>	116
4.4	RESULTS	117
4.4.1	<i>General overview of the microarray results</i>	117
4.4.1.1	Forestomach transcriptional response	118
4.4.1.2	Hepatic transcriptional response	121
4.4.1.3	Pulmonary transcriptional response	124
4.4.2	<i>Apical responses to PAHs in the lungs</i>	127
4.4.2.1	Pulmonary DNA adduct formation	127
4.4.2.2	Pulmonary <i>lacZ</i> mutant frequency	128
4.4.2.3	EROD enzymatic activity analysis	128
4.4.3	<i>BMD analysis</i>	128
4.5	DISCUSSION	130
4.5.1	<i>Conclusions</i>	137
4.6	TABLES AND FIGURES	139
5	CHAPTER : A FRAMEWORK FOR THE USE OF SINGLE CHEMICAL TRANSCRIPTOMICS DATA IN PREDICTING THE HAZARDS ASSOCIATED WITH COMPLEX MIXTURES OF POLYCYCLIC AROMATIC HYDROCARBONS.....	155

5.1	ABSTRACT	155
5.2	INTRODUCTION	156
5.3	METHODS	159
5.3.1	<i>Chemicals</i>	159
5.3.2	<i>Preparation of PAH mixtures and animal exposures</i>	160
5.3.3	<i>Tissue RNA extraction and purification</i>	162
5.3.4	<i>Microarray hybridization and analysis</i>	162
5.3.5	<i>Bioinformatics and pathway analysis</i>	163
5.3.6	<i>Transcriptional benchmark dose (BMD) analysis</i>	164
5.3.7	<i>Application of the component-based reference models to evaluate additivity</i>	165
5.3.8	<i>BMD analysis of lung tumour incidence data</i>	169
5.4	RESULTS	170
5.4.1	<i>General overview of microarray results</i>	171
5.4.2	<i>Prediction of mixture pathway perturbations</i>	172
5.4.3	<i>BMD modeling of published cancer data</i>	177
5.5	DISCUSSION	177
5.5.1	<i>Pathway perturbations induced by PAHs or PAH-containing mixtures</i>	179
5.5.2	<i>Predicting pathway perturbations for mixtures of PAHs</i>	181
5.5.3	<i>Quantitative comparisons of coal tar-induced mouse pulmonary tumours with transcriptomic-based pathway perturbations using predictive models of additivity</i>	185
5.5.4	<i>Conclusions</i>	186
5.6	TABLES AND FIGURES	187
6	CHAPTER: GENERAL CONCLUSIONS	195
6.1	SUMMARY OF STUDY OUTCOMES	195
6.2	UTILITY OF TOXICOGENOMICS IN CONTEMPORARY HUMAN HEALTH RISK ASSESSMENT	204
6.3	CONCLUDING REMARKS	209
7	REFERENCES	211

APPENDICES.....	234
APPENDIX I. COMPARISON OF TOXICOGENOMICS AND TRADITIONAL APPROACHES TO INFORM MODE OF ACTION AND POINTS OF DEPARTURE IN HUMAN HEALTH RISK ASSESSMENT OF BENZO[A]PYRENE IN DRINKING WATER.	234
APPENDIX II NANO-RISK SCIENCE: APPLICATION OF TOXICOGENOMICS IN AN ADVERSE OUTCOME PATHWAY FRAMEWORK FOR RISK ASSESSMENT OF MULTI-WALLED CARBON NANOTUBES.	278
APPENDIX III INTEGRATING TOXICOGENOMICS INTO HUMAN HEALTH RISK ASSESSMENT: LESSONS LEARNED FROM THE BENZO[A]PYRENE CASE STUDY.	296
APPENDIX IV TRANSCRIPTOMIC ANALYSIS REVEALS NOVEL MECHANISTIC INSIGHT INTO MURINE BIOLOGICAL RESPONSES TO MULTI-WALLED CARBON NANOTUBES IN LUNGS AND CULTURED LUNG EPITHELIAL CELLS.	306
APPENDIX V SUPPLEMENTAL MATERIALS.	332

List of Abbreviations

AHR	Aryl Hydrocarbon Receptor
ANT	Anthracene
BaA	Benz[<i>a</i>]anthracene
BaP	Benzo[<i>a</i>]pyrene
BbF	Benzo[<i>b</i>]fluoranthene
BghiP	Benzo[<i>ghi</i>]perylene
BkF	Benzo[<i>k</i>]fluoranthene
BMD	Benchmark Dose
BMDL	Benchmark Dose Lower Confidence Limit
BMDS	Benchmark Dose Software
BMR	Benchmark Response
BPDE	BaP Diol Epoxide
CA	Concentration Addition
CCME	Canadian Council of Ministers of the Environment
CEPA	Canadian Environmental Protection Act
CHR	Chrysene
CSE	Cigarette Smoke Extract
CT	Coal Tar
CYP	Cytochrome P450
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DBahA	Dibenz[<i>ah</i>]anthracene
DBaP	Dibenzo[<i>al</i>]pyrene
DE	Diesel Exhaust
DEG	Differentially Expressed Gene
EROD	EthoxyResorufin-O-Deethylase
FDR	False Discovery Rate
FLA	Fluoranthene
FLU	Fluorene
GCA	Generalized concentration addition
GEO	Gene Expression Omnibus

GSH	Glutathione
GST	Glutathione S Transferase
HAH	Halogenated Aromatic Hydrocarbons
HPA	Health Protection Agency (UK)
IA	Independent Action
IARC	International Agency for Research on Cancer
IP	Indeno[123,cd]pyrene
IPA	Ingenuity Pathway Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LOAEL	Lowest Observable Adverse Effect Level
LOWESS	Locally Weighted Scatterplot Smoothing
MOA	Mode of Action
mRNA	Messenger RNA
MWCNT	Multi-Walled Carbon Nanotubes
NCBI	National Center for Biotechnology Information
NOAEL	No Observable Adverse Effect Level
NRC	National Research Council (US)
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
PEF	Potency Equivalence Factor
POD	Point of Departure
PYR	Pyrene
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
QSAR	Quantitative Structure Activity Relationship
RDRC	Resources Development Research Centre
REACH	Registration, Evaluation, Authorization, and Restriction
RIVM	National Institute for Public Health and the Environment (Netherlands)
ROS	Reactive Oxygen Species
RPF	Relative Potency Factor

TCDD	2,3,7,8-tetrahydrodibenzo-p-dioxin
TEF	Toxicity Equivalence Factor
TUE	Toxic Unit Extrapolation
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration

List of Tables

<i>Table 1-1 Summary table of toxicogenomic studies investigating mixtures</i>	29
<i>Table 1-2 Physico-chemical properties of select PAHs</i>	31
<i>Table 1-3 Effect of binary and ternary mixtures of PAHs on DNA adduct formation, enzyme activity, and enzyme gene expression in vivo and in vitro</i>	32
<i>Table 2-1. Agilent probes with FDR adjusted P value ≤ 0.05 common to all three dose groups as measured by microarrays on lung tissue</i>	62
<i>Table 2-2 p53 regulated genes that were significantly differentially expressed with microarrays and real-time PCR in lungs</i>	63
<i>Table 2-3 Genes validated using real-time PCR arrays divided based on functional association in lungs.</i>	64
<i>Table 2-4 Cancer pathway (KEGG) associated genes significantly differentially expressed in microarrays in the lung and not in the liver.</i>	66
<i>Table 2-5 p53 regulated genes identified in liver by Malik et al. (2012) were cross-validated in the lung using real-time PCR arrays.</i>	67
<i>Table 3-1 Meta-analysis in NextBio of significant genes in BaP-treated mouse forestomach and human profiles in which esophageal, gastric, intestinal, or colon cancer was a phenotype.</i>	98
<i>Table 3-2 Genes validated by real-time PCR in the forestomach of BaP treated mice divided by function.</i>	100
<i>Table 4-1 Total numbers of differentially expressed genes in forestomach, liver, and lung tissues.</i>	139
<i>Table 5-1 Predicted and observed benchmark dose (BMD50) values for selected pathways perturbed by each mixture.</i>	188

List of Figures

Figure 1-1 Low-dose extrapolation approaches	35
Figure 1-2 Schematic describing the predictive reference models of additivity	36
Figure 1-3 Structure of PAHs	37
Figure 1-4 Rodent carcinogenicity of PAHs and PAH-containing mixtures, by route of exposure	38
Figure 1-5 Schematic showing mammalian metabolism of BaP	39
Figure 1-6 Formation of Bay region and DNA-reactive BaP metabolites.	40
Figure 2-1 (a) DNA adduct formation and (b) lacZ mutant frequency in the lungs and livers from Muta TM Mouse subchronically exposed to BaP.	68
Figure 2-2 Functional analysis of differentially expressed genes using IPA for (a) lung and (b) liver.	69
Figure 2-3 Molecular relational network of genes modulated by BaP in lung tissues enriched for cancer function using IPA for all three doses with p53 at the central node.	70
Figure 3-1. Step-wise organization of the most significant gene ontology processes perturbed in BaP-exposed forestomach showing dose-dependent transition in the response from antigen processing and presentation to epithelial cell differentiation.	103
Figure 3-2. Upstream regulator analysis.	104
Figure 3-3. Activation of the immunoproteasome in BaP exposed forestomach.	105
Figure 3-4. Molecular relational network of genes modulated by BaP in the forestomach using MetaCore for all three doses.	106
Figure 4-1 Forestomach data analyses part I.	140
Figure 4-2 Forestomach data analyses part II.	142
Figure 4-3 Liver data analyses part I.	144
Figure 4-4 Liver data analysis part II.	146
Figure 4-5 Lung data analysis part I.	148
Figure 4-6 Lung data analysis part II.	150
Figure 4-7 Apical endpoints and BMD analyses.	152
Figure 4-8 Activation or suppression of cell signaling pathways in support of pulmonary carcinogenic transformation.	154
Figure 5-1 Workflow to select pathways for mathematical modeling and mixture prediction analyses	189
Figure 5-2 General overview of microarray results data analysis.	191

<i>Figure 5-3 Comparison of cancer-related pathways for individual PAH and PAH-mixtures</i>	<i>192</i>
<i>Figure 5-4 Comparisons of predicted and observed mixture effects for (A) a mixture of 4 PAHs and (B) a mixture of 8 PAHs.....</i>	<i>193</i>
<i>Figure 5-5 Comparison of predicted and observed effects of a PAH-containing coal tar extract</i>	<i>194</i>

List of Appendices

<i>Appendix 1 Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo(a)pyrene in drinkwater.</i>	<i>234</i>
<i>Appendix 2 Nano-risk science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes.</i>	<i>278</i>
<i>Appendix 3 Integrating toxicogenomics into human health risk assessment: lessons learned from the benzo[a]pyrene case study.</i>	<i>296</i>
<i>Appendix 4 Transcriptomic analysis reveals novel mechanistic insight into murine biological responses to multi-walled carbon nanotubes in lungs and cultured lung epithelial cells.</i>	<i>306</i>
<i>Appendix 5 Supplemental Materials.</i>	<i>332</i>

List of Supplementary Files

<i>Supplementary Table 2. 1 Significant gene list for BaP exposed lungs</i>	332
<i>Supplementary Table 2. 2 Gene ontology analysis of BaP exposed lungs</i>	332
<i>Supplementary Table 2. 3 KEGG pathway analysis of BaP exposed lungs</i>	332
<i>Supplementary Table 2. 4 PCR validation of BaP exposed lung microarray data</i>	332
<i>Supplementary Table 2. 5 Relaxed gene list from BaP exposed lungs</i>	332
<i>Supplementary Table 3. 1 The 59 curated studies in NextBio used in meta-analysis.</i>	332
<i>Supplementary Table 3.2 Significant probe list</i>	333
<i>Supplementary Table 3. 3 Upstream regulator analysis.</i>	333
<i>Supplementary Table 3. 4 NextBio disease prediction.</i>	333
<i>Supplementary Table 3. 5 Genes validated using real-time PCR arrays in the forestomach of BaP-treated mice.</i>	333
<i>Supplementary Table 4. 1 Collapsed functional groupings for each pathway and process.</i>	333
<i>Supplementary Table 4. 2 Significant probe and gene lists for each pathway and process.</i>	333
<i>Supplementary Table 5. 1 Analytical assessment of purified coal tar.</i>	333
<i>Supplementary Table 5. 2 Gene lists for PAH-containing mixtures in lung tissues.</i>	333
<i>Supplementary Table 5. 3 List of median and mean gene expression changes for each pathway in lungs from mice exposed to the 4PAH-Mix, 8PAH-Mix, CT-Mix, and the individual PAHs.</i>	333
<i>Supplementary Figure 2. 1 Hierarchical cluster analysis of BaP exposed lungs.</i>	334
<i>Supplementary Figure 3. 1 VENN diagram of significantly differentially expressed genes.</i>	335
<i>Supplementary Figure 3. 2 Hierarchical cluster analysis.</i>	336
<i>Supplementary Figure 3. 3 Pathway analysis of differentially expressed genes</i>	337

<i>Supplementary Figure 4. 1 IPA upstream regulator analyses.....</i>	<i>338</i>
<i>Supplementary Figure 4. 2 Sample autoradiographs ³²P-post-labeling of PAHs in lung.</i>	<i>339</i>
<i>Supplementary Figure 5. 1 Hierarchical cluster analyses.</i>	<i>340</i>
<i>Supplementary Figure 5. 2 Impact of no statistical filtering on mixture predictions using mathematical models of additivity</i>	<i>341</i>
<i>Supplementary File 4. 1 Detailed methodology</i>	<i>342</i>

Preface

This thesis is written as a series of articles as outlined in the guidelines provided by the Faculty of Graduate and Postdoctoral Studies. The thesis begins with an introductory chapter (Chapter 1) that reviews pertinent literature necessary to understanding the research questions and results reported in the following chapters and finishes with a synthesis and conclusion (Chapter 6) that provides a global summary of work achieved.

This thesis contains four studies that have been prepared for publication in peer-reviewed journals. Chapters 2, 3, and 4 are already published and chapter 5 will be submitted shortly. While other authors contributed to each study, I was responsible for the majority of the experimental work, data analysis, and manuscript writing. An outline of the specific contributions of each author is included below.

In addition to the main chapters of my thesis, I was involved in several related projects during the course of my graduate studies that resulted in 1st authorship on one manuscript and 2nd and 3rd authorship on three articles published in peer-reviewed journals. These manuscripts are included in the appendices to this thesis and are discussed where appropriate in Chapter 6.

Statement of Contributions

Chapter 2 Sub-chronic oral exposure to benzo(a)pyrene leads to distinct transcriptomic changes in the lungs that are related to carcinogenesis.

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Chapter 3 Toxicogenomic outcomes predictive of forestomach carcinogenesis following exposure to benzo(a)pyrene: relevance to human cancer risk.

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Chapter 4 Comparative transcriptomic analyses to scrutinize the assumption that genotoxic PAHs exert effects via a common mode of action.

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Chapter 5 A framework for the use of single chemical transcriptomics data in predicting the hazards associated with complex mixtures of polycyclic aromatic hydrocarbons. TBD

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Appendix I Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo[a]pyrene in drinking water.

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Appendix II Nano-risk Science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes.

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Appendix III Integrating toxicogenomics into human health risk assessment: lessons learned from the benzo[a]pyrene case study.

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***Appendix IV* Transcriptomic analysis reveals novel mechanistic insight into murine biological responses to multi-walled carbon nanotubes in lungs and cultured lung epithelial cells.**

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1 Chapter: General Introduction

Exposure to chemical contaminants in the environment is ubiquitous; they are found in food water, air, and consumer products, throughout the workplace, home, and communities. In Canada alone there are approximately 23,000 chemicals that are either manufactured in or imported into Canada (Canadian Environmental Protection Act (CEPA), 1999). In the United States (US) and European Union there are over 75,000 and 140,000 chemicals listed, respectively. Humans exposed to these chemicals encounter them as components of chemical mixtures, which can contain hundreds or thousands of known and unknown chemicals. Moreover, depending on the medium, location, and source, the chemical composition of the mixture can vary in terms of the type of chemicals and their respective concentrations (Goodsaid and Frueh, 2007). Thus, the landscape of environmental chemicals to which humans are exposed is complex and large.

The potential for chemicals to cause adverse health effects has been recognized for centuries. Ibn Wahshiyya, a 10th century alchemist first documented it in his book of poisons and their antidotes (Hamarneh, 2008). Modern toxicology has attributed numerous human health effects to exposures to environmental chemicals, including mortality, reproductive and developmental effects, neurotoxic effects, immunologic effects, respiratory effects, cardiovascular effect, nephric effects, hepatic effects, and cancer. Moreover, environmental contaminants are regularly detected in the blood and urine of humans (Centers for Disease Control and Prevention, 2009; Canadian Health Measures Survey, 2015), which raises concern for the potential health risks posed by exposures to environmental chemicals.

1.1 Toxicity testing and human health risk assessment

1.1.1 The human health risk assessment paradigm

The presence of chemicals in the environment does not necessarily imply that adverse human health effects will be manifested; rather, adverse effects are dependent on exposure and dose.

Regulatory agencies worldwide, including Health Canada and the US Environmental Protection Agency (USEPA), are faced with the task of characterizing the nature and probability of adverse health effects (i.e., toxicological risk) posed by chemicals in the environment. The human health risk assessment process involves four phases: hazard assessment, dose-response assessment, exposure assessment, and risk characterization (USEPA, 2005).

Hazard assessment. This phase of the risk assessment process examines whether the chemical has the potential to cause harm to humans, and if so, under what circumstances. Additionally, this phase evaluates the scientific information to understand how each type of toxicity occurs (i.e., mode of action; the sequence of events starting with the interaction between the agent and biological molecule, resulting in the effect). The outcomes of the hazard assessment are the adverse health effect of concern and mode of action that are used as the basis for the dose-response assessment (below).

Dose-response assessment. This phase of the risk assessment process examines the relationship between the exposure dose and the observed response with the goal of identifying a dose that is deemed to be acceptable for human exposure. This phase involves the (i) selection of a point of departure (POD), which is used as a starting point for quantitative risk assessment, and (ii) extrapolation of the POD to a dose level that is unlikely to cause adverse effects in humans. The POD can be a NOAEL or no observable adverse effect level (i.e., the highest level at which there is no increase in the frequency or severity of the adverse effect), a LOAEL or lowest observable adverse effect level (i.e., the lowest level at which there is an increase in the frequency or severity of an adverse effect), or a BMD or benchmark dose (i.e., a mathematically-derived dose level associated with a predetermined effect level). The extrapolation can proceed via either a non-linear or linear low-dose extrapolation approach that depends on the chemical's

toxic mode of action (a detailed description of these two approaches is presented in Figure 1-1).

The outcome of the dose-response assessment determines the dose at which the risk occurs.

Exposure assessment. This phase of the risk assessment process examines what is known about the frequency and levels of human exposures. The outcome of the exposure assessment determines the relevant routes of exposure, the circumstances under which exposure occurs, and the level of human exposure, all of which are used in informing risk.

Risk characterization. In the final step of the risk assessment, the assessments from the first three phases are integrated to estimate the likelihood of adverse health effects to vulnerable populations (e.g., children, construction workers, pregnant women) or the general population. Based on this risk characterization, and considering technical, economical, and political factors, regulatory decisions are made that permit effective management of chemicals in commerce (i.e., risk management).

1.1.2 Risk assessment of chemical mixtures

Applications of the risk assessment process to complex chemical mixtures requires additional considerations. Risk assessors rely on two general approaches: (a) a whole mixture approach that considers the hazard of the actual mixture of interest or a sufficiently similar mixture, and (b) a component-based approach that considers the hazards of the known mixture components.

The preferred approach involves hazard (toxicity) assessment of the actual mixture (USEPA, 2000); however, due to difficulties in collecting and processing mixtures, and lack of standards, the whole mixture approach is not practical for routine risk assessment of complex mixtures. Similarly, comparing each newly identified mixture to a sufficiently similar mixture is not practical because of the relative differences in mixture compositions and the influence of individual mixture components on mixture toxicity.

The component-based approach has been deemed to be the most appropriate approach for routine assessments of complex mixtures (USEPA, 2010). The approach assumes that the total risk and hazard associated with a mixture is the sum of the contributions of the known mixture components (i.e., contributions of mixture components are additive), which is known as the assumption of additivity. Deviations from what would be expected under the assumption of additivity are termed interactions. When the combined effect of the individual mixture components is larger than expected, the outcome is assumed to be driven by a synergistic interaction. In contrast, when the combined effect is smaller than expected, the outcome is assumed to be driven by an antagonistic interaction. The component-based approach relies on available toxicological data for the individual mixture components. This approach is employed by most regulatory agencies, including Health Canada (CCME, 2010), the USEPA (2010), and the European Commission (2001).

1.1.2.1 Additivity

The assumption of additivity is tested using two mathematical models: the independent action model or IA (i.e., Bliss additivity, response addition, or effect addition) or the concentration addition model or CA (i.e., Loewe additivity or dose addition) (Figure 1-2).

IA was the first method described for assessing the toxicity of mixtures (Bliss, 1939); the approach is used for mixtures of chemicals with dissimilar modes of action. In such cases, the response/effect level of the mixture can be estimated from the sum of the responses or effect levels of the mixture components. Several studies have shown that the IA model is useful for estimating the response/effect levels of mixtures of dissimilarly acting chemicals (Backhaus et al., 2004; Faust et al., 2003).

CA is applied for mixtures of chemicals with similar modes of action that differ only in their potencies. In CA, the dose/concentration of the mixture that induces a particular response/effect

level can be estimated from the sum of the doses/concentrations of the mixture components after scaling for difference in relative toxicity. Mathematically, CA is only valid if (a) the dose-response curves of the mixture components are parallel and if (b) the maximal effect level (i.e., the maximum effect a compound can induce) of each component is equal. Due to the latter criterion of CA, the maximal effect level of the weakest inducer (i.e., the chemical that induces the lowest effect level compared to other mixture components) is the maximal effect that can be calculated for the mixture. Therefore, the CA model may be of limited value for chemicals that act through a receptor. For example, in a mixture of full receptor agonists and partial receptor agonists, the model can only estimate a mixture dose up to the maximal effect level of the partial agonist (i.e. the weakest inducer). Despite these limitations, CA is the most frequently used model of additivity and has been highlighted as a reasonable default assumption for groups of chemicals able to induce the same toxic response (Boobis et al., 2011).

Several researchers have proposed strategies for overcoming the problems faced when dealing with mixtures containing chemicals with different maximal effect levels. Examples include the toxic unit extrapolation approach or TUE (Scholze et al., 2014) and the generalized concentration addition approach or GCA (Howard and Webster, 2009). The TUE model addresses the issue of maximal effect levels by extrapolating the maximal effect level of the weakest inducer to a higher maximal effect level. This model accurately predicted the estrogenicity of full and partial estrogen receptor agonists (Scholze et al., 2014). The GCA model addresses the problem by considering the maximal effect level of every mixture component; it has been used to accurately predict the joint effect of a mixture of full aryl hydrocarbon receptor (AHR) agonists and partial AHR agonists including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,3',4,4'-Pentachlorobiphenyl (PCB105); 3,3',4,4',5-

pentachloro-biphenyl (PCB126); and 2,3,7,8-tetra-chloro-dibenzofuran (TCDF) (Howard et al., 2010). These newer models still require further testing and validation; nevertheless, they presently provide a means for overcoming the limitations that hamper the estimation of mixture effects based on CA.

1.1.3 The current landscape of toxicity testing

Toxicity testing serves to generate information on a chemical's toxicological properties and potency, which is integral to the evaluation of human health risk. Toxicity testing methods often involve studies of whole animals as surrogates of human exposure with different testing methods designed to evaluate numerous outcomes, including cancer. The testing methods may focus on a particular life stage (e.g., pregnancy, infancy, adulthood), sex, or condition of exposure (e.g., oral, inhalation, dermal) over a wide range of exposure scenarios (e.g., short term vs. chronic; episodic vs. continuous). The results from these studies can provide valuable information for the identification of hazards posed by a chemical, and often, for describing dose-response relationships; however, limitations exist. For example, these studies are designed to evaluate specific outcomes and endpoints, such as tumour formation, which can require lots of time before any discernable effects are observed. Studies are conducted on large numbers of animals in order to minimize variance, bias, and the potential for false positive and false negative results, which is both cost- and animal-intensive. Additionally, testing methods require exposure doses that exceed environmental exposures by several orders of magnitude to improve test sensitivity, which can affect test specificity and the applicability to human exposures. There is a growing recognition that because traditional toxicity testing methods are time-consuming, resource intensive, and use high exposure doses, the current approach cannot adequately assess the human health risk of the large number of existing and newly identified chemicals (NRC, 2007a).

In recent years toxicology has undergone a paradigm shift towards more mechanistic testing strategies that was promulgated by a seminal report from the US National Research Council (NRC) entitled “Toxicity Testing in the 21st Century: A Vision and a Strategy” (NRC, 2007b). This report proposes that toxicologists exploit recent advances in technology, including toxicogenomics, bioinformatics, and computational toxicology, to move towards a toxicity testing paradigm that relies on high-throughput, systems biology approaches (Krewski et al., 2010). Importantly, this report underscored the value of identifying mechanism-related toxicity pathways in an attempt to infer the long-term adverse manifestations of effects recorded shortly after the exposure. This type of mechanistic data can be invaluable in improving the confidence in selecting critical endpoints for follow-up testing, and in enhancing the understanding of the mechanisms underlying eventual adverse outcomes; and moreover, the relevance for human risk. In an effort to minimize uncertainties, recent work has contributed to an increased emphasis on mechanism-based approaches for improvement of human health risk assessment (Adeleye et al., 2015; Bourdon-Lacombe et al., 2015; European Commission, 2015, USEPA, 2015a; USEPA, 2015b; OECD, 2016).

1.1.4 Toxicogenomics

Toxicogenomics is a field of toxicological sciences that employs molecular biology tools to study the global toxic response in organisms at the level of DNA sequence (genomics), gene expression (transcriptomics), protein expression (proteomics), and metabolite levels (metabolomics). The value of these technologies for pure and applied toxicology has been recognized since 1999, when the term “toxicogenomics” was first coined (Nuwaysir et al., 1999). In addition to providing a rapid overview of the global response of a cell population or a target

tissue to a toxicant exposure, toxicogenomics can help elucidate the underlying mechanisms of toxicity, and thus potentially aid in predicting the development of adverse outcomes.

Although there are several technologies and endpoints that are encompassed by toxicogenomics, each has its use in terms of understanding a chemical's toxicity. Transcriptional profiling, which measures changes in messenger RNA that occur following exposure, is considered one of the most well-developed techniques applied in toxicogenomics. This is due to the availability of mature technologies (microarrays, quantitative real-time PCR, RNA sequencing), well developed bioinformatics and pathway analysis software, and robust statistical analysis methodologies; several studies have demonstrated the reproducibility of transcriptomic measurements within and across platforms and laboratories (Chen et al., 2007; Larkin et al., 2005; Li et al., 2014; Webster et al., 2015a; Webster et al., 2015b; Yauk and Berndt, 2007). Thus, the majority of toxicogenomics studies published use transcriptomic technologies.

Initial cellular responses to chemical stressors are often manifested as altered gene expression, which ultimately causes increases or decreases in specific proteins required to carry out important tasks related to the maintenance of homeostasis. Gene expression changes occur relatively rapidly, within hours or days after the exposure (Nuwaysir et al., 1999), and therefore, they can be useful in the identification of early changes that potentially predict long-term toxic or pathological effects (Daston et al., 2008; NRC, 2007a). Effects at the level of gene expression are also known to be induced at concentrations well below those required to elicit pathophysiological changes (Hamadeh et al., 2002). Thus, a carefully designed transcriptomic study can detect chemically-induced toxic effects at very low doses, and thus in principle can improve our ability to evaluate the risks of low-dose, environmentally relevant exposures (NRC, 2007a).

Numerous studies have demonstrated the validity of this technology for providing mechanistic information regarding the adverse effects induced by chemical exposures. These studies have applied multiple approaches to effectively employ and interpret toxicogenomics data, including the use of bioinformatics and pathway analysis tools to identify pathophysiologic changes that lead to toxic, adverse outcomes (Bourdon et al., 2012; Halappanavar et al., 2011a; Halappanavar et al., 2011b; Husain et al., 2013; Husain et al., 2015; Euling et al., 2013; Jackson et al., 2014; Labib et al., 2012; Labib et al., 2013; Labib et al., 2015; Poulson et al., 2013), to identify gene signatures predictive of adverse outcomes such as liver tumours, lung tumours, and renal toxicity (Ellinger-Ziegelbauer et al., 2008; Fielden et al., 2005; 2007; 2011; Nie et al., 2006; Thomas et al., 2009; Uehara et al., 2011), to mine existing gene expression repositories for signatures that predict hazards (e.g., meta-analyses) (Bourdon et al., 2013; Jackson et al., 2014; Labib et al., 2013; Nikota et al., 2016), to employ clustering methods to differentiate between chemicals with different modes of action (Ellinger-Ziegelbauer et al., 2009; Halappanavar et al., 2015; Thomas et al., 2012), and lastly, to examine gene expression profiles to determine modes of action (Budinsky et al., 2014; Corton et al., 2014; Elcombe et al., 2014; Moffat et al., 2015; Rouquié et al., 2014; Tinwell et al., 2014). Thus, the application and utility of toxicogenomics to understanding a chemical's underlying molecular mechanisms and potential toxicity is well established.

1.1.4.1 Toxicogenomics in risk assessment

A framework to include toxicogenomics in the human health risk assessment process is very much desired, and the strategies are currently being implemented; both the USEPA and USFDA are actively developing guidelines for its use in regulatory assessments (Cote et al., 2016; Dix et al., 2006; European Commission, 2015; Goodsaid and Frueh, 2007; USEPA, 2004; USEPA, 2014a). Recent advances in computational analysis of toxicogenomic data have allowed for high-

content dose-response modeling combined with functional annotation of toxicogenomics data to derive transcriptional BMD values for single genes or biological pathways associated with mechanistically-linked adverse outcomes hazards (Chepelev et al., 2014; Jackson et al., 2014; Labib et al., 2016; Moffat et al., 2015; Thomas et al., 2011; Thomas et al., 2013b; Webster et al., 2015a), and several researchers have proposed the use of these metrics to efficiently establish PODs for data-poor chemicals (Chepelev et al., 2015; Moffat et al., 2015; NRC, 2007a; NRC, 2007b; Thomas et al., 2013b) and nanomaterials (Labib et al., 2016). The successful application of toxicogenomics data for risk assessment requires a large database of gene expression signatures for known chemical toxicants, a thorough understanding regarding the meaning of gene expression changes and their relationship to observed phenotypes, and lastly, a demonstrated ability to quantitatively employ gene expression data to estimate a chemical's toxicological potency (Bourdon-Lacombe et al., 2015). The case studies that are necessary to demonstrate these requirements are currently being developed.

1.1.4.2 Mixtures toxicology from a toxicogenomics perspective

Whereas toxicogenomics has been widely implemented for the toxicological evaluation of single chemicals, the utility of these tools for the study of complex mixtures is still being defined. Studies that consider toxicogenomic changes following exposures to whole complex mixtures primarily focus on applying bioinformatics and pathway analysis tools to identify changes that can lead to toxic outcomes (Halappanavar et al., 2009; John et al., 2005; Maertens et al., 2013; Sen et al., 2007; Vaccari et al., 2015; Yauk et al., 2012); however, the application of other approaches to generate mechanistic information such as that outlined above (e.g. gene signatures, meta-analyses, mode of action determination) have not been well explored. Additionally, while these studies provide valuable mechanistic information about specific mixtures, they do not

provide information about the identity of the mixture components that elicit the adverse effects, nor do they address the concept of additivity.

Several studies have investigated toxicogenomic changes, including gene expression changes, observed following exposures to complex mixtures or simple, defined mixtures, alongside individual mixture components, and employed comparative analyses to scrutinize the assumption of additivity (Table 1-1). These studies have applied a combination of bioinformatics and pathway analysis tools to identify molecular changes (e.g., gene expression changes) leading to toxic outcomes, and clustering methods to identify differences or similarities between mixture components and the mixtures themselves. Several studies investigated the assumption of additivity via qualitative and quantitative comparison of the gene expression responses; however, only three studies applied mathematical models of additivity to test the additivity assumption (Kopec et al., 2011; Staal et al., 2007; Zucchi et al., 2014). These studies employed statistical models of additivity to interpret toxicogenomics data (i.e., expression changes of single genes); however, they did not use the seminal models of additivity described above (i.e., CA and IA) (section 1.1.2.1). Despite these efforts, a precise methodology for using toxicogenomic data to scrutinize the assumption of additivity has not been defined.

1.2 The toxicology and risk assessment of polycyclic aromatic hydrocarbons (PAHs)

PAHs and PAH-containing mixtures are ubiquitously found in the environment and are regularly prioritized for human health risk assessment. The following pages contain a brief overview of the toxicological properties of PAHs, and where available, PAH-containing mixtures. Most published studies examined benzo[*a*]pyrene (BaP), a PAH frequently detected in PAH-contaminated samples, as a point of reference for PAH-related toxicity. Thus, the review will focus primarily on information pertaining to BaP, with information on additional PAHs added where available.

1.2.1 Identity, use, and sources of PAHs

PAHs are a group of organic aromatics containing two or more fused rings formed during the incomplete combustion of organic materials. As a class of chemicals, over 1500 PAHs have been identified with diverse structural features. In this thesis, the term PAH specifically refers to unsubstituted, homocyclic PAHs (Figure 1-3); however, PAHs belong to the class of polycyclic aromatic compounds that also include N-, S-, and O-substituted PAHs, as well as heterocyclic compounds containing N, S, or O atoms within the ring structure (Achten and Andersson, 2015). Overall, the PAHs have limited water solubility and as the number of rings and molecular weight increases, the compounds become increasingly less water soluble (Table 1-2) (Achten and Andersson, 2015). Even so, even the low molecular weight PAH naphthalene, with a log octanol-water partition coefficient (i.e., $\text{Log}_{10}K_{ow}$) of 3.36 would partition into octanol (a surrogate of biological lipids) at a ratio of 2991 parts for every part partitioned into water. Lower molecular weight PAHs also tend to be more volatile (higher vapor pressure) and readily partition into air from pure water (higher Henry's law constant).

Multiple PAHs are produced during the combustion of organic material (Wang et al., 2007). Natural sources include forest fires and volcanic eruptions that contribute to background levels of PAHs in the atmosphere. Anthropogenic activities, including internal combustion vehicle operation, furnace operation for heating, coke production, cigarette smoking, and agricultural burning are primary sources of atmospheric PAHs. Because of the PAHs' physico-chemical properties, these emissions lead to the deposition and accumulation of PAHs in soil, on vegetation, and in aquatic sediments (Mackay and Paterson, 1991). For example, 82% of emitted BaP is estimated to partition to soil, 17% to sediment, 1% to water, and less than 1% to air (Hattemer-Frey and Travis, 1991).

Human PAH exposures occur as components of highly complex and dynamic mixtures with delivery via multiple routes, including oral ingestion (e.g., contaminated seafood, charbroiled meats, house dust, and drinking water), dermal contact (e.g., road paving and roofing materials in an occupational setting), and inhalation (e.g., polluted urban air, cigarette smoke, and vehicular exhaust). 82-97% of human exposure to BaP and PAHs is estimated to be via oral exposure through food consumption (Hattemer-Frey and Travis, 1991; Phillips, 1999), as PAHs occur in numerous food items such as fruits, vegetables, cereals and grains, eggs, and meat. Although exposure via inhalation is typically very low, occupational exposures and cigarette smoking significantly increase exposure and concomitant risk.

Due to their ubiquity in the environment, PAHs and PAH-containing mixtures are consistently found on priority lists of compounds selected for toxicity testing, risk assessment, and risk management (CEPA, 1994; CCME, 2010; USEPA, 2014b). These priority lists, such as the USEPA list of 129 priority pollutants (Keith and Telliard, 1979), commonly include 16 PAHs that have become widely known as the priority PAHs. These compounds, which are thought to effectively represent this group of compounds, include acenaphthene (ACN), acenaphthylene (ACE), anthracene (ANT), benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF), benzo[*ghi*]pyrene (BghiP), benzo[*k*]fluoranthene (BkF), chrysene (CHR), dibenz[*a,h*]anthraene (DBahA), fluoranthene (FLA), fluorene (FLU), indeno[123,*cd*]pyrene (IP), naphthalene (NAT), phenanthrene (PHE), and pyrene (PYR). It has recently been suggested that the list of priority PAHs be expanded to include higher molecular weight PAHs, substituted PAHs, alkylated PACs, as well as N-, S-, and O-heterocyclic compounds (Andersson and Achten, 2015). How these particular unsubstituted PAHs were selected as “priority” PAHs was mired in mystery until a recent publication by Lawrence Keith,

formerly of the USEPA (Keith, 2015). Shortly after the USEPA's inception, several environmental advocacy organizations took the USEPA to court, claiming that it was inadequately enforcing its regulatory responsibilities because it was only regulating water quality parameters and was not regulating specific pollutants. This resulted in a consent decree (i.e., binding judicial ruling) requiring that the USEPA rapidly produce a list of toxic pollutants and standardised analytical methods to measure them. The aforementioned list of 129 pollutants included 16 PAHs that were selected because analytical standards were available, the compounds were suspected carcinogens, or the compounds were known to be found in coal tar (a complex mixture containing PAHs). BghiP was selected as a representative of six-membered ring PAHs. This list became US law under the Clean Water Act and was subsequently picked up by other countries, including Canada (CEPA, 1994) and the European Union (European Commission, 2005).

1.2.2 *Carcinogenicity associated with PAHs*

Much of the interest in PAHs is based on their ability to induce tumours (malignant and benign) in humans and experimental animals. In fact, PAHs have played a seminal role in cancer epidemiology and in the establishment of toxicity testing methods for cancer endpoints. For example, the first recorded observation of an environmental exposure associated with cancer in humans was described in 1775 by Sir Percival Pott, an English surgeon who described the formation of tumours on the scrotums of chimney sweeps due to the accumulation of soot, a complex mixture containing PAHs, in the scrotal rugae (Pott, 1775), although he did not know that it was PAH-driven. Years later, in 1915, the first experimental evidence of chemical exposure associated with tumour formation in experimental animals was described by Yamagiwa and Ichikawa, two Japanese pathologists who observed tumours, pre-neoplastic lesions, and inflammatory changes on rabbit inner ears following dermal application of coal tar (Fujiki,

2014). Later, researchers at the Institute for Cancer Research in the United Kingdom isolated PAHs from coal tar and determined that they are carcinogenic (Cook et al., 1933). These pioneering studies stimulated a great deal of interest in the carcinogenicity of PAHs and PAH-containing mixtures, and several government and non-government agencies have classified PAHs and PAH-containing mixtures according to their potential to induce carcinogenesis. The most recognized example is the International Agency for Research on Cancer (IARC), an intergovernmental agency started in 1965; it has evaluated the carcinogenic hazard of almost 1,000 chemicals and chemical mixtures based on studies of cancer in humans, studies of cancer in experimental animals, and mechanistic data. As of June 26, 2015, 117 agents were classified by IARC as Group 1 (carcinogenic to humans), including BaP (IARC, 2012b), several PAH-containing mixtures (e.g. first-hand and second-hand tobacco smoke, coal-tar pitch, indoor emissions from household combustion of coal, diesel engine exhaust, and outdoor air pollution) (IARC, 2012a; 2012b; 2013a; 2013b), and multiple occupational settings for which PAH exposures have been documented (e.g. coal gasification, coke production, coal-tar distillation, aluminum production, iron and steel founding) (IARC, 2012a; 2012b). Several other individual PAHs are classified as Group 2A (probably carcinogenic to humans) (e.g., cyclopenta[*cd*]pyrene, DBahA, dibenzo[*al*]pyrene) or Group 2B (possibly carcinogenic to humans) (e.g., benz[*j*]aceanthrylene, BaA, BbF, benzo[*j*]fluorethene, BkF, benzo[*c*]phenanthrene, CHR, dibenzo[*ah*]pyrene, dibenzo[*ai*]pyrene, IP, 5-methylchrysene). Thus, PAHs and PAH-containing mixtures are internationally recognized for their carcinogenic potential.

A diverse array of tumour sites has been reported in experimental animals following exposure to different PAHs. Figure 1-4 summarizes the tumour sites reported for the priority PAHs and PAH-containing complex mixtures according to the route of exposure. PAH-induced

tumours have been reported in the gastrointestinal tract (following exposure to BaA, BaP, BbF, DBahA, and coal tar pitch), in the lung (BaA, BaP, BbF, BghiP, CHR, DBahA, IP, coal tar pitch, and coal flue gas), in the liver (BaA, BaP, BbF, BkF, CHR, FLA, and coal tar pitch), in the skin (ANT, BaA, BaP, BkF, CHR, DBahA, IP, PHE, asphalt, coal tar pitch, and coal flue gas), in the mammary glands (BaP and DBahA), and in the lymph nodes (BaA, BaP, and DBahA). Oral cancer bioassays, the most relevant to human exposure scenarios for PAHs, have only been conducted using ANT, BaA, BaP, DBahA, FLU, NAT, and PHE as the test chemicals. Nevertheless, the overall weight of evidence concerning the toxicological effects for PAHs deems that carcinogenicity should be the basis of human health risk assessments.

1.2.2.1 Mechanisms of PAH-induced carcinogenesis

PAH-induced carcinogenesis can be attributed to two major pathophysiologic mechanisms: (1)

DNA damage induced genetic damage and (2) aryl hydrocarbon receptor (AHR) activation.

(1) DNA damage induced genetic damage. Most PAHs are effectively metabolised and excreted from the body; however, some intermediate metabolites and by-products are highly reactive and can interact with DNA, leading to genotoxicity. A brief description of PAH metabolism will precede a description of the PAH genotoxicity pathways that have been described.

Metabolism. PAHs are considered xenobiotics, and as such, are metabolized for excretion via a complex series of biochemical reactions. Because of their lipophilicity, they must be converted to a more water soluble form and subsequently conjugated to an endogenous compound (e.g., glucuronic acid, glutathione) by multiple enzyme-catalyzed reactions loosely classified as phase I and phase II reactions. In phase I, enzymes convert the parent compound into more polar metabolites by catalyzing the addition or modification of functional groups required for metabolism by phase II enzymes. PAH metabolism begins with modification of the

parent compound by monooxygenase enzymes (e.g., CYP1A1, CYP1B1, CYP1A2) that catalyse the formation of an epoxide (Figure 1-5). The epoxides can spontaneously rearrange into phenols or they can be hydrolysed via epoxide hydrolase into dihydrodiols. The phenol metabolites can undergo glucuronide/sulfate conjugation for excretion or can be converted by prostaglandin H synthase (PHS) to quinones, which can be conjugated to glutathione (GSH) for excretion. The dihydrodiols when converted to quinones by aldo-keto reductases (AKRs) generate reactive oxygen species (ROS) via redox cycling (Penning et al., 1999). The dihydrodiols can be further conjugated by CYPs and PHS into dihydrodiol epoxides, which contribute significantly to the mutagenicity of PAHs (discussed below), or into phenol dihydrodiols that share the same fate as the phenol metabolites. Dihydrodiols themselves undergo glucuronidation or sulfation for excretion and the diol epoxides undergo GSH conjugation for excretion. The metabolite-induced genotoxicity is described below. Thus, PAHs undergo multiple biochemical reactions via different pathways in preparation for excretion.

Genotoxicity pathways. Three main genotoxicity pathways have been described for PAHs: (a) the diol epoxide pathway, (b) the radical cation pathway, and (c) the ROS pathway whereby ROS is generated as metabolic by-products of ortho-quinone generation.

(a) Diol epoxide pathway. The diol epoxide pathway is the most characterized and reportedly potent of the genotoxicity pathways. It is also referred to as the bay region dihydrodiol epoxide pathway. The bay region refers to the sterically-hindered region formed by angular benzo rings that consist of three carbon-carbon bonds (Figure 1-6). It is the region that inspired the “Bay Theory” (Jerina et al., 1976), which proposes that epoxides adjacent to the bay region yield highly stabilized carbocations and thus will be reactive and mutagenic. The “Bay Theory” extends to PAHs with fjord regions that consist of four carbon-carbon bonds. These are usually

more reactive than bay region diol epoxides, and this is thought to account for the increased mutagenic and carcinogenic properties associated with fjord region diol epoxides. Diol epoxides that form at bay or fjord regions are relatively resistant to phase II conjugation due to steric inaccessibility. The diol epoxide carbocations act as electrophiles that react with nucleophiles, such as DNA (usually with purines), to form stable covalent adducts. In most cases the adducts are repaired by nucleotide excision repair; however, in the presence of ineffective DNA repair or faulty DNA synthesis, they are converted into mutations.

The bay region diol-epoxide pathway is suggested to be responsible for the mutagenic and carcinogenic potential of all PAHs that are genotoxic carcinogens (IARC, 2010); however, experimental evidence is available only for BaP and BaA (i.e., BaP-7,8-diol-9,10-epoxide and BaA-3,4-diol-1,2-epoxide) (Buening et al., 1998; Thakker et al., 1982; Wislocki et al., 1978). BbF's bay region diol epoxide (BbF-9,10-diol-11,12-epoxide) cannot account for BbF's genotoxicity (Pfau et al., 1992); rather it is suggested that the triol epoxide metabolite of BbF (i.e., 5-OH-BbF-9,10-11,12-epoxide) contributes to its carcinogenicity (Weyand et al., 1993). Similarly, DBaA's bis-diol metabolite (DBaA-3,4:10,11-bis-diol) is suggested to contribute to DBaA's genotoxicity based on the abundance of bis-diol metabolites and adducts compared to bay region diol epoxide metabolites (Carmichael et al., 1993; Platt and Reischmann, 1987). More specifically, incubation of DBaA's cancer-causing bay region diol with liver microsomes prepared from rats treated with Aroclor 1254 did not identify any bay region diol epoxide metabolites (Platt and Reischmann, 1987); rather, the DNA adduct profile formed after application of DBaA-3,4:10,11-bis-diol produced a major adduct that matched those produced following treatment with DBaA and DBaA's bay region diol (Carmichael et al., 1993). Thus, PAH carcinogenesis is attributable to both bay region and non-bay region diol epoxides.

(b) Radical cation pathway. The monooxydation by P450 enzymes can also catalyze the removal of one π electron (an electron in the π bond of a double bond) to produce reactive electrophiles called radical cations. PAHs have a relatively low ionization potential, which allows the removal of an electron, and formation of a relatively stable radical cation. The PAH radical cations have positions of unequal charge localization that readily promote reactions with nucleophiles. Furthermore, their geometric configuration (e.g. angular rings) allows efficient DNA intercalation. Purines are most susceptible to nucleophilic attack by radical cations. PAH radical cations form unstable adducts with DNA that generate apurinic sites, in comparison with diol epoxides that form stable bulky adducts. These radical cations are thought to contribute to BaP's carcinogenesis as they make up 66% of adducts formed in BaP-exposed mouse skin (Chen et al., 1996); however, the precise mechanisms underlying their contribution to carcinogenesis are unclear.

(c) ROS and ortho-Quinones pathway. PAH exposure can also induce genotoxicity through the formation of PAH-*ortho*-quinones, which can form adducts with DNA, and cause DNA damage through the generation of ROS during activation to quinone metabolites. Quinones are generated via dehydrogenation of PAH dihydrodiols by AKRs, producing catechol and semi-quinone intermediates. *Ortho*-quinones are highly reactive electrophiles that can interact with nucleophiles, including DNA, to form adducts, and GSH to form conjugates. PAH *ortho*-quinones have been described for BaA and BaP (BaA-3,4-dione and BaP-7,8-dione, respectively); however, BaA-3,4-dione itself does not appear to be mutagenic in Ames test (Flowers-Geary et al., 1996), and despite the formation of stable adducts with deoxyguanosine and deoxyadenosine, BaP-7,8-dione is a poor direct-acting mutagen (Shou et al 1993). Thus,

adducts resulting from reactions with PAH-*ortho*-quinone metabolites do not appear to be responsible for PAH-induced mutagenicity.

ROS are generated through catechol-ortho-quinone redox cycling. In Ames assay tests under conditions that allow for redox cycling (e.g., presence of superoxide dismutase), both BaA-3,4-dione and BaP-7,8-dione have been found to be mutagenic (Flowers-Geary et al., 1996; Shou et al., 1993). It is thought that generated ROS can attack DNA bases and produce oxidized purines, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dGuo) (Henderson et al., 2003). In addition, free radicals can also be formed as by-products of phase I P450 metabolism, mitochondrial oxidation, reduction of oxygen, enzymes of peroxisomes that produce H₂O₂ as a metabolic product, and inflammation. Therefore, PAHs can cause DNA damage via direct interactions with DNA, as well as due to interactions between DNA and metabolic by-products generated by PAH metabolism (e.g., ROS).

(2) AHR activation. Receptor-mediated events, in particular AHR agonism, are the second major pathophysiologic mechanism underlying PAH-induced carcinogenesis. The AHR is a member of the basic-helix-loop-helix PAS (Per-Arnt-Sim)-domain-containing family of ligand-activated transcription factors controlling the expression of several genes encoding metabolism enzymes, as well as those encoding immune response and DNA damage response proteins. It has a particularly strong affinity for planar, hydrophobic compounds, including PAHs and halogenated aromatic hydrocarbons (HAHs). The HAHs, particularly TCDD, are considered high affinity ligands of the AHR and have a higher binding affinity compared to the PAHs; nM to μ M range for HAHs and pM to nM range for PAHs. PAH binding to the AHR induces its activity leading to transcription of several genes required for PAH metabolism. Metabolism (as described above) is integral to the generation of DNA reactive metabolites, and thus,

carcinogenesis. For example, mice deficient in AHR do not induce expression of cytochrome P450 enzymes and do not develop tumours following intraperitoneal injection or subcutaneous exposure to BaP (Shimzu et al., 2000). Thus, the role of AHR in PAH-induced carcinogenesis is significant.

1.2.3 Risk assessment of PAHs

The human health risk assessment process for PAHs involves the same four phases described above: hazard assessment, dose-response assessment, exposure assessment, and risk characterization (USEPA, 2005).

- Hazard assessment. PAHs and PAH-containing complex mixtures elicit a wide range of adverse health effects, including immunologic effects, reproductive and developmental effects, neurotoxic effects, cardiovascular effects, and cancer. For PAHs, cancer is the primary adverse outcome of concern due to strong epidemiological evidence and evidence in experimental animals (discussed in section 1.2.2). PAHs induce cancer via a genotoxic mode of action, starting with the binding of the parent PAH to the AHR, metabolism by P450 isozymes to reactive metabolites, covalent binding of the reactive metabolites with DNA, and, in the absence of proper repair mechanisms, formation of mutations that initiate the development of neoplasia (i.e., tumours). Since the toxicological database regarding the carcinogenic activity of most priority PAHs is incomplete, BaP is used as a representative PAH and point of reference. The use of BaP as a point of reference for PAH carcinogenicity is based on the assumption that carcinogenic PAHs act via the same mode of action. Overall, the primary hazard associated with PAHs is carcinogenicity via a genotoxic mode of action.
- Dose-response assessment. For BaP, the forestomach has the most frequently reported experimentally-induced tumours (i.e., of 13 BaP oral cancer bioassays, 12 were positive for forestomach tumours), and is the most sensitive tumourigenic organ (i.e., tumours occur at

lower doses in comparison with other organs). Therefore, data on BaP-induced forestomach tumour incidence are used to derive a POD. Genotoxic carcinogens are considered to not have a threshold due to the theoretical possibility that a single molecule of a genotoxic carcinogen may cause a mutation that could initiate carcinogenesis (European Commission, 2009).

Therefore, an oral cancer slope factor for BaP is calculated by drawing a straight line from the POD to zero (i.e., low dose linear extrapolation) (see Figure 1-1). The cancer slope factor will be used to determine the risk.

- Exposure assessment. For PAHs, oral ingestion of contaminated food and water makes up approximately 82–97% of human exposure (Hattemer-Frey and Travis, 1991; Phillips, 1999). The average daily intake of PAHs in the general population ranges from 1.2 to 17 µg/person/day for non-smokers (de Vos et al., 1990; Hattemer-Frey and Travis, 1991; Lodovici et al., 1995; Menzie et al., 1992; Phillips, 1999; Thomson et al., 1996). For BaP in particular, IARC reports US daily dietary intakes between 0.04 and 2.8 µg/person/day (IARC, 2010). Exposure estimates are used to inform the risk.
- Risk characterization. In the final step of the risk assessment for PAHs, BaP's oral cancer slope factor is used to determine the cancer risk at the relevant exposure levels.

1.2.4 Risk assessment of PAH-containing mixtures

As mentioned above, the application of the risk assessment process to complex mixtures requires additional considerations. Due to difficulties in applying a whole mixture risk assessment approach (described in section 1.1.2) the cancer risk assessment of PAH mixtures uses a component-based approach whereby the risk attributable to the PAH mixture is assumed to be equal to the incremental sum of the contribution of known priority PAHs in the mixture (i.e., the assumption of additivity). More specifically, the CA model of additivity is applied to PAHs based on the assumption that the PAHs act via the same mode of action. Additionally, for the

cancer risk assessment of PAH mixtures, the risk calculation only employs the oral slope factor for BaP; risks attributable to all other PAHs are scaled relative to BaP using potency equivalence factors (PEFs; discussed below). Thus, the human health risk assessment of PAH mixtures is conducted using a component-based approach under the assumption of concentration additivity.

Deviations from additivity determined according to the concentration addition paradigm have been investigated for tumourigenic effects. Examples include increased tumour incidence following dermal co-application of BaP with weakly or non-genotoxic PAHs, including benzo[*e*]pyrene, BghiP, FLU, and PYR (DiGiovanni et al., 1982; Slaga et al., 1979; van Duuren et al., 1973; van Duuren and Goldschmidt, 1976), and decreased tumour incidence following subcutaneous co-administration of BaP with BkF and CHR. Interestingly, co-administration of BaP with BghiP and IP resulted in no interactive behaviour (Falk and Kotin, 1964). Deviations from additivity have also been observed at the level of DNA adduct formation, P450 gene expression changes, induction of metabolic enzyme activity, and mutagenicity (Table 1-3), suggesting that these activities may be driving the observed deviations from additivity for tumour formation (Jarvis et al., 2014). Despite the observation of deviations from additivity, the precise mechanisms underlying the observations have yet to be defined.

1.2.4.1 The PEF approach for risk assessment of PAH mixtures

Assuming that the assumption of additivity according to CA is met, the major approach favoured by regulatory agencies such as Health Canada (CCME, 2010), the USEPA (USEPA, 1999; 2010), California EPA (Office of Environmental Health Hazard Assessment (OEHHA), 2005), the Netherlands (RIVM, 2000), and the United Kingdom (UK Environment Agency, 2002) for assessing the human health risks of PAH-containing mixtures relies on the use of PEFs (CCME, 2010), also known as the relative potency factor (RPF) (USEPA, 2010). This approach is

employed for classes of chemicals for which extensive information is available for one member of the chemical class (i.e., the reference chemical), and less is known about the other members. In this case, the overall carcinogenic risk of a mixture is determined using scaling factors (i.e., the PEFs or RFPs) and the toxic or carcinogenic potential of the poorly characterized chemicals is expressed as an equivalent dose of the reference chemical. For PAHs, the reference chemical is BaP and the excess lifetime cancer risk associated with exposure to the complex mixture is calculated as the sum of the risks for each PAH, after expressing each PAH as an equivalent dose of BaP, thereby permitting the use of the BaP cancer slope factor for each PAH. More specifically, organizations such as the Canadian Council of Ministers of the Environment have established eight PEFs relative to BaP (i.e., for BaA, BaP, BbF, BkF, BghiP, CHR, DBahA, and IP) that permit risk assessment of numerous PAHs; these values range from a high of 1 for DBahA (i.e., equally potent as BaP) to 0.01 for BghiP and CHR (i.e., less potent than BaP) (CCME, 2010). Thus, the risk assessment of PAH-containing mixtures is conducted using a component-based PEF approach, with the assumption that the PAHs share the same carcinogenic mode of action, and that their actions are additive.

1.3 Rationale for this thesis

Toxicological and risk assessment communities are faced with the challenging task of evaluating the human health and environmental risks of new and existing chemicals, as well as chemical mixtures of substances in complex environmental matrices (e.g., air, water, soil, etc.). The evaluation of thousands of chemicals in itself is a daunting challenge, and the magnitude of the challenge is augmented by the paucity of toxicological and/or exposure information on which to base chemical risk assessments (Maltby et al., 2006). The utility of traditional toxicity testing methods for routine toxicological evaluation of chemicals with little or no data is limited by the time-, cost-, and resource-intensive nature of the traditional toxicity testing paradigm (NRC,

2007a). Additionally, evaluations of chemical mixtures are especially problematic due to the complexity of their composition and lack of knowledge regarding the interactions of known components. Consequently, the toxicology community has embraced the implementation of new tools that can minimize animal use, time, and cost, thereby increasing the efficiency of the toxicological assessments required for human health risk assessments.

As an alternative to the traditional toxicity testing paradigm, the US NRC suggests an approach whereby short-term toxicity testing methods are complemented by mechanistic information generated using novel molecular biology tools such as toxicogenomics. These tools can, in principle, increase the efficiency of toxicological hazard assessment, reduce the uncertainty related to data interpretation, and improve direct extrapolation of animal data to humans (NRC, 2007b; Krewski et al., 2010). PAHs, which are common components of combustion emissions, are among the compounds that are cause for concern with respect to adverse human health effects. Considering that human exposures to PAHs are ubiquitous and associated with the initiation of carcinogenesis, this thesis evaluated the utility and applicability of toxicogenomics to expanding the current understanding of the toxic properties of individual PAHs as well as PAH-containing mixtures. Moreover, the thesis evaluated whether toxicogenomic data can be used to strengthen the scientific rationale underlying current approaches used for human health risk assessment of PAHs and PAH-containing mixtures.

Thus, the **general hypothesis** of this thesis is that experimental exposure to PAHs and/or PAH-containing mixtures induce statistically significant changes in transcription, and associated subcellular pathways, that are significantly correlated with an array of disease-related outcomes; and moreover, that this correlation yields mode of action information that can be incorporated into human health risk assessment. More specifically, the quantitative measurement of mRNA

levels at different doses of PAHs or PAH mixtures permits the accomplishment of the following **thesis objectives**: (a) to improve the understanding of mechanisms underlying the adverse, toxicological responses induced by individual PAHs in target tissues, and (b) to evaluate the contention that transcriptional profiles and pathway perturbation information can be used to critically examine interactions between individual PAHs in PAH-containing mixtures, and thus the assumption of additivity.

To realize these objectives, mice were exposed to three doses of eight individual priority PAHs, two defined synthetic PAH mixtures, and one complex PAH-containing mixture (coal tar extract), or vehicle daily for 28 consecutive days. Forestomach, liver, and lung tissues were collected three days following the final exposure for the following analyses; DNA microarray analysis for transcriptome profiling, ³²P-post-labeling analysis for determination of DNA adduct frequency, and P-gal positive selection assay analysis for the determination of *lacZ* mutant frequency. The resulting data was used to address the aforementioned thesis objectives; the specific rationale and objectives of each chapter are outlined below.

Chapters 2 and 3

BaP, which is frequently used as the point of reference for PAH carcinogenicity, is classified by IARC as a group 1 human carcinogen. Rodents exposed to BaP develop tumors in site-of-contact tissues (e.g., forestomach), as well as tissues more distant from the site-of-contact (e.g., liver and lung). Interestingly, the occurrence of tumors in rodent tissues is highly variable across published studies. For example, forestomach tumors are observed in 12 out of 13 BaP oral cancer bioassays, whereas observations of liver and lung tumors are inconsistent. It is also interesting to note that human cancer risk assessments for BaP, as well as other PAHs and PAH-containing mixtures, is based on the carcinogenic potency of BaP-induced forestomach tumors, despite the

fact that humans lack an analogous organ. Therefore, it is important to gain an improved understanding regarding the mechanisms underlying PAH-induced responses in forestomach; and more specifically, responses in forestomach that are mechanistically related to carcinogenesis in humans. As outlined below, chapters 2 and 3 employ gene expression profiling to investigate differences in tumor potency across tissues and the human relevance of forestomach tumors.

In **Chapters 2 and 3** the transcriptome from the forestomach, liver, and lung of mice exposed to BaP were compared. Additionally, the tissue-specific genotoxic effect of BaP was assessed via the frequency of DNA adducts and transgene mutations. The analyses were used to determine whether transcriptomic analyses can provide information that improves the understanding of the aforementioned differences in tissue-specific carcinogenic potency. In **Chapter 3** BaP-induced forestomach transcriptional profiles were compared with transcriptional profiles associated with a variety of human tumors. The analyses provide improved understanding regarding the relevance of rodent forestomach responses to understanding the risk of BaP-and/or PAH-induced human effects.

Chapters 4 and 5

Human health risk assessments of PAH-containing mixtures largely focus on the risks attributed to a targeted set of commonly monitored, unsubstituted PAHs known as the “priority PAHs”. As discussed earlier, cancer risk assessment of priority PAHs involves the use of the PEF approach, with BaP used as the point of reference. This component-based approach to risk assessment requires two important assumptions: (1) that the structurally similar PAHs in the mixture initiate carcinogenesis via the same mode of action as the reference chemical, and (2) that in a mixture, the total risk is the incremental sum of the contributions of the mixtures components (i.e., the

carcinogenic activity is additive). However, existing information regarding the carcinogenicity of PAHs and PAH-containing mixtures, and the underlying mechanisms, is woefully incomplete. Thus, there is a distinct lack of experimental support for the use of the PEF-based, reference chemical approach for risk assessment of PAH mixtures. Furthermore, although PAH additivity has been investigated using simple binary mixtures, there is relatively little published information regarding the effects of priority PAHs in defined or complex mixtures.

In an effort to understand whether transcriptomic profiling can be used to determine whether BaP is an appropriate point of reference for the other priority PAHs, **Chapter 4** examines and compares the transcriptomes of forestomach, liver, and lung from mice exposed to eight individual priority PAHs. More specifically, bioinformatics and pathway analysis was used to compare cancer-related gene expression changes induced by the individual PAHs with those induced by BaP. Additionally, DNA adduct and mutant frequency in lung tissue were assessed to confirm genotoxic phenotype, and the ethoxyresorufin-O-deethylase (EROD) assay was used to measure activation of relevant metabolic enzymes in the lung (i.e., CYP1A1 and 1A2). The analyses provide insight into the assumption that the PAHs share the same mode of action and the use of BaP as a point of reference for other priority PAHs. In an effort to understand if toxicogenomic profiling can be used to evaluate the assumption of PAH additivity **Chapter 5** compared the lung transcriptomes of mice exposed to each of the eight individual PAHs with the lung transcriptomes of mice exposed to two defined PAH mixtures and one complex PAH-containing mixture. More specifically, several component-based mathematical models of additivity were employed to evaluate the assumption of component additivity.

The resulting data presented in Chapters 2 through 5 will be used to evaluate the thesis hypothesis and objectives.

1.4 Tables

Table 1-1 Summary table of toxicogenomic studies investigating mixtures

Reference	Model	Tissue	Mixture components	Synthetic mixture type	Complex mixture	# dose groups	Mixture prediction
<i>In vivo</i>							
Kopec et al 2011	Mouse	Liver	TCDD, PCB153	Binary		8	Yes
Zucchi et al 2014	Zebrafish	Ovary and Brain	Drospirenone and Progesterone	Binary		3	Yes
Dondero et al 2011	Mussels	Gill tissue	Chlorpyrifos and Ni	Binary		5	No
Garcia-Reyero et al 2012	Daphnia magna	All	RDX, TNT, 2,4-DNT, 2,6-DNT, TNB, DNB	7 synthetic mixtures with at least five chemicals	Munition constituents	5: Components 1: Mixtures	No
Vandenbrouck et al 2009	Daphnia magna	All	Ni, Pb, Cd	Binary		4: Components 1: Mixture	No
Poynton et al 2014	Mussels	Gill tissue	Cd, Pb	Binary		1	No
Tilton et al 2011	Zebrafish	Olfactory tissue	Cu and Chlorpyrifos	Binary		3	No
Hendriksen et al 2007	Rat	Liver and Kidney	Methyl mercury, benzene, TCE	Binary and Ternary		3	No
Jordan et al 2012	Golfish	Liver and testes	DEHP, BPA, NP	Ternary			No
Hook et al 2008	Rainbow trout	Liver	ethynylestradiol, PBDE47, and CrVI,	Ternary		1	No
Silkworth et al 2008	Rat	Liver	TCDD		Aroclar 1254	3	No
Huang et al 2015	Zebrafish	Embryo	BaP and TBT	Binary		3	No

Krasnov et al 2007	Brown trout	Liver	Cd, CCl ₄ , PYR	Binary and Ternary	3	No
Siddens et al 2012	Mouse	Skin	TPA + BaP, DBaP	TPA + DE, DE + CT, DE + CT + CSE	1	No
Tilton et al 2015	Mouse	Skin	BaP and DBaP	DE, DE + CT, DE + CT + CSE	1	No
<i>In vitro</i>						
Staal et al 2007	Human	Liver cells	BaP, DBC, DBaH, BbF, FLA, 1-MPA	Binary with BaP or DBaH	3	Yes
Wei et al 2009	Minnow	Liver cells	PFOA, PFNA, PFDA, PFDoA, PFOS, 8:2FTOH	3 6-mixtures, 1 binary	1	No
Finne et al 2007	Rainbow trout	Liver cells	EE2, TCDD, PQ, NQO	Quaternary	1	No
Softeland et al 2014	Salmon	Liver cells	Endosulfan, PH, BaP, chlorpyrifos	Quaternary	5: Components 2: Mixtures	No
Staal et al 2008	Rat	Liver slices	BaP, DBaH, BbF, FLA, DBaP	Binary with BaP	1	No

Note. 1-methylphenanthrene (1-MPA), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 8:2fluorotelomer alcohol (8:2FTOH), Bisphenol A – 4,4'-isopropylidenediphenol (BPA), Di-(2-ethylhexyl)-phthalate (DEHP), 1,3-dinitrobenzene (DNB), 17-alphaethinyloestradiol (EE2), 2,2,4,4-tetrabromodiphenyl ether (PBDE47), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153), perfluorodecanoic acid (PFDA), perfluorododecanoic acid (PFDoA), perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), phenanthrene (PH), paraquat (PQ), 1,3,5-trinitroperhydro-1,3,5-triazine (RDX), tributyltin (TBT), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,7,8-tetra-chloro-dibenzofuran (TCDF), trichloroethylene (TCE), 1,3,5-trinitrobenzene (TNB), 2,4,6-trinitrotoluene (TNT), tetradecanoyl phorbol acetate (TPA).

Table 1-2 Physico-chemical properties of select PAHs

PAHs	Abbreviation	Chemical Formula	Molecular Weight	Aqueous Solubility (mg/L) ¹	Partition Coefficient (Log Kow)	Vapor Pressure (mm Hg) ¹	Henry's Law Constant (atm.m ³ /mol)	Total Rings
Napthalene	NAT	C ₁₀ H ₈	128	3.17E+01	3.4	8.50E-02	4.83E-04	2
Acenaphthylene	ACE	C ₁₂ H ₈	152	3.93E+00	4.1	9.12E-04	1.13E-05	3
Acenaphthene	ACN	C ₁₂ H ₁₀	154	4.00E+00	3.9	3.00E-01	1.55E-04	3
Fluorene	FLU	C ₁₃ H ₁₀	166	1.99E+00	4.2	3.20E-04	1.00E-04	3
Phenanthrene	PHN	C ₁₄ H ₁₀	178	1.15E+00	4.5	1.12E-04	2.33E-05	3
Anthracene	ANT	C ₁₄ H ₁₀	178	4.30E-02	4.5	2.67E-06	1.93E-05	3
Pyrene	PYR	C ₁₆ H ₁₀	202	1.35E+00	4.6	2.45E-06	1.10E-05	4
Fluoranthene	FLA	C ₁₆ H ₁₀	202	2.60E-01	5.2	9.22E-06	9.45E-06	4
Benz(a)anthracene	BaA	C ₁₈ H ₁₂	228	9.40E-03	5.7	3.05E-08	3.35E-06	4
Chrysene	CHR	C ₁₈ H ₁₂	228	2.00E-03	5.7	6.23E-09	9.46E-05	4
Benzo(a)pyrene	BaP	C ₂₀ H ₁₂	252	1.60E-03	6.0	5.49E-09	1.13E-06	5
Benzo(b)fluoranthene	BbF	C ₂₀ H ₁₂	252	1.50E-03	6.2	5.00E-07	1.11E-04	5
Benzo(k)fluoranthene	BkF	C ₂₀ H ₁₂	252	8.00E-04	6.2	2.00E-09	8.29E-07	5
Benzo(ghi)perylene	BghiP	C ₂₂ H ₁₂	276	2.60E-05	6.7	1.00E-10	1.41E-07	6
Indeno(123,cd)pyrene	IP	C ₂₂ H ₁₂	276	2.20E-05	6.6	1.00E-10	1.60E-06	6
Dibenz(ah)anthracene	DBahA	C ₂₂ H ₁₄	278	2.49E-03	6.5	1.00E-10	1.47E-08	5

Note. PAHs ordered from low to high molecular weight. Data taken from CHEMFATE database created on behalf of the USEPA and PubChem. Fluorene partition coefficient taken from Miller et al. (1985).

¹ At room temperature

Table 1-3 Effect of binary and ternary mixtures of PAHs on DNA adduct formation, enzyme activity, and enzyme gene expression in vivo and in vitro

Reference	Model	Tissue	Endpoint Measured	Mixture Components	Additive/ Antagonistic/ Synergistic
In vivo					
DiGiovanni et al., 1982	Mouse	Skin	DNA adducts	BaP:BeP	Additive
Hughes and Phillips, 1990	Mouse	Skin	DNA adducts	DBaP:BaP/DBaP	Additive
Slaga et al., 1979	Mouse	Skin	DNA adducts	DMBA: BeP	Antagonistic
Smolarek et al., 1987	Mouse	Skin	DNA adducts	DMBA: BeP	Antagonistic
Hughes and Phillips, 1990	Mouse	Skin	DNA adducts	DBaP:BaP:DBaP	Antagonistic
Rice et al., 1984	Mouse	Skin	DNA adducts	BaP:FLU/PYR	Synergistic
Rice et al., 1988	Mouse	Skin	DNA adducts	BaP:FLU/PYR	Synergistic
DiGiovanni et al., 1982	Mouse	Skin	DNA adducts	DMBA: BeP	Synergistic
Smolarek et al., 1987	Mouse	Skin	DNA adducts	BaP:BeP	Synergistic
Slaga et al., 1979	Mouse	Skin	DNA adducts	BaP:BeP/PYR/FLU	Synergistic
Hughes and Phillips, 1990	Mouse	Skin	DNA adducts	BaP:DBaP	Synergistic
In vitro					
Falahatpisheh et al., 2004	Rat	Kidney	EROD	BaP:CHR	Antagonistic
Falahatpisheh et al., 2004	Rat	Kidney	EROD	BaP:ANT	Antagonistic
Harris et al., 2013	Human	Colon (HT-29)	Metabolite levels	BaP:FLU	Synergistic
Cherng et al., 2006	Human	Liver (HEPG2)	CYP450 gene expression	BaP:1-NP	Antagonism
Falahatpisheh et al.,	Rat	Kidney	CYP450 gene	BaP:CHR	Antagonism

2004			expression		
Gabelova et al., 2013	Rat	Liver (WB-F344)	CYP450 gene expression	BaP:DBcgC	Antagonism
Staal et al., 2008	Rat	Liver slices	CYP450 gene expression	BaP:BbF/DBahA/DBalP	Antagonism
Staal et al., 2008	Rat	Liver slices	CYP450 gene expression	DBahA:DBalP/FLU	Antagonism
Cherng et al., 2001	Human	Liver (HEPG2)	CYP450 gene expression	BaP:BghiP	Synergism
Staal et al., 2007	Human	Liver (HEPG2)	CYP450 gene expression	BaP:BbF/DBahA/DBalP/1-MP	Synergism
Gabelova et al., 2013	Human	Liver (HEPG2)	CYP450 gene expression	BaP:DBcgC	Synergism
Falahatpisheh et al., 2004	Rat	Kidney	CYP450 gene expression	BaP:ANT	Synergism
Staal et al., 2008	Rat	Liver slices	CYP450 gene expression	BaP:FLU	Synergism
Tarantini et al., 2011	Human	Liver (HEPG2)	DNA adducts	BaP:BaA/BghiP/CHR/FLU/PYR	Additive
Cherng et al., 2006	Human	Liver (HEPG2)	DNA adducts	BaP:1-NP	Antagonism
Sevastyanova et al., 2007	Human	Liver (HEPG2)	DNA adducts	BaP:BkF	Antagonism
Tarantini et al., 2011	Human	Liver (HEPG2)	DNA adducts	BaP:BkF	Antagonism
Binkova and Sram, 2004	Human	Liver (HEPG2)	DNA adducts	BaP:BaA/BbF/BkF/BghiP/CHR/DBahA/IP	Antagonism
Binkova and Sram, 2004	Human	Liver (HEPG2)	DNA adducts	DBalP:BaA/BbF/BkF/BghiP/CHR/DBahA/IP	Antagonism
Sevastyanova et al., 2007	Human	Liver (HEPG2)	DNA adducts	BaP:BaA/BbF/BkF/BghiP/CHR/DBahA/IP	Antagonism
Gabelova et al., 2013	Rat	Liver (WB-F344)	DNA adducts	BaP:DBcgC	Antagonism
Staal et al., 2008	Rat	Liver slices	DNA adducts	BaP:BbF/DBahA/DBalP	Antagonism
Staal et al., 2008	Rat	Live slices	DNA adducts	DBahA:DBalP/FLU	Antagonism

Cherng et al., 2001	Human	Liver (HEPG2)	DNA adducts	BaP:BghiP	Synergism
Staal et al., 2007	Human	Liver (HEPG2)	DNA adducts	BaP:BbF/DBahA/DBaIP/1-MP	Synergism
Staal et al., 2007	Human	Liver (HEPG2)	DNA adducts	BaP:FLU	Synergism
Sevastyanova et al., 2007	Human	Liver (HEPG2)	DNA adducts	BaP:BaA/BbF/BghiP/CHR/DBahA/IP	Synergism
Tarantini et al., 2011	Human	Liver (HEPG2)	DNA adducts	BaP:BbF/DBahA/IP	Synergism
Staal et al., 2008	Rat	Liver slices	DNA adducts	BaP:FLU	Synergism
Smolarek and Baird, 1984	Hamster	Embryo (SHE)	DNA adducts	BaP:BeP	Synergism
Gabelova et al., 2013	Human	Liver (HEPG2)	DNA adducts	BaP:DBcgC	Antagonism/ Synergism*

* depending on ratio of mixture components

Note. Benzo[*e*]pyrene (BeP), dibenzo[*ae*]pyrene (DBaP), dibenzo[*cg*]chrysene (DBcgC), dimethylbenzanthracene (DMBA), 1-methylphenanthrene (1-MP), 1-(-1-naphthyl)piperazine (1-NP).

1.5 Figures

Low-dose extrapolation approaches

Purpose: extrapolate the POD to a dose level that is unlikely to begin to cause an adverse effect in humans

Basis: chemical's toxic mode of action

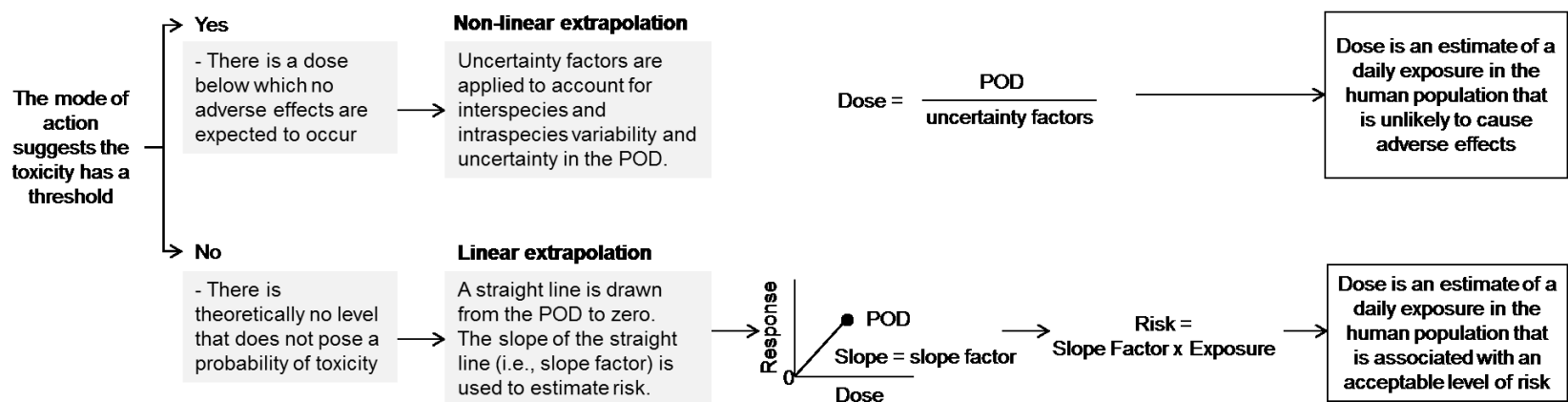


Figure 1-1 Low-dose extrapolation approaches

Schematic describing options for low-dose extrapolation approaches in human health risk assessment that depend on the chemical's mode of action. A non-linear low-dose extrapolation approach is employed if the mode of action suggests that the toxicity has a threshold (e.g., saturation of metabolism). Here, the experimental POD is divided by a composite uncertainty factor to determine an estimated dose of a daily exposure in a human population that is unlikely to cause adverse health effects to humans. A linear extrapolation approach is employed if the mode of action suggests that the toxicity has no threshold (e.g., genotoxic carcinogens). Here, a straight line is drawn from the experimental POD to zero and the slope of the straight line (i.e., the slope factor) is measured. An estimate of risk of developing the adverse health effect resulting from exposure to the chemical is calculated using the slope factor. An acceptable dose can be determined from this approach by establishing an acceptable level of risk to the population.

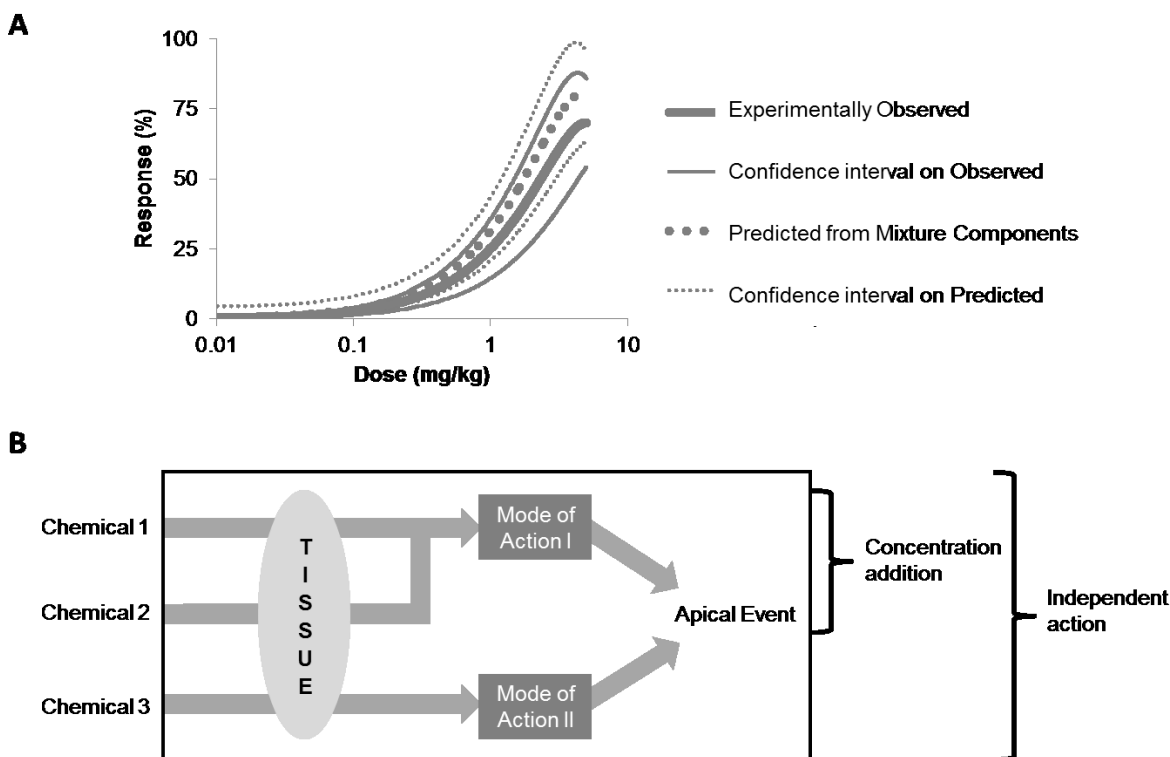


Figure 1-2 Schematic describing the predictive reference models of additivity

(a) Representative plot using hypothetical data showing a comparison of the observed response elicited by a mixture and the response predicted using mathematical models of additivity. When the confidence interval around the predicted dose-response curves generated using mathematical models of additivity overlap with the confidence intervals around the observed dose-response curves, then the assumption of additivity according to that specific model is considered validated. When the confidence intervals do not overlap, the assumption of additivity according to that model is considered invalid, which suggests chemical interactions occurring. (b)

Conceptual schematic showing the responses induced by three chemicals where the same apical event in the same target tissue is reached via different modes of action. The combined response of these chemicals can be described by either concentration addition or independent action. Modified from Altenburger et al. (2012).

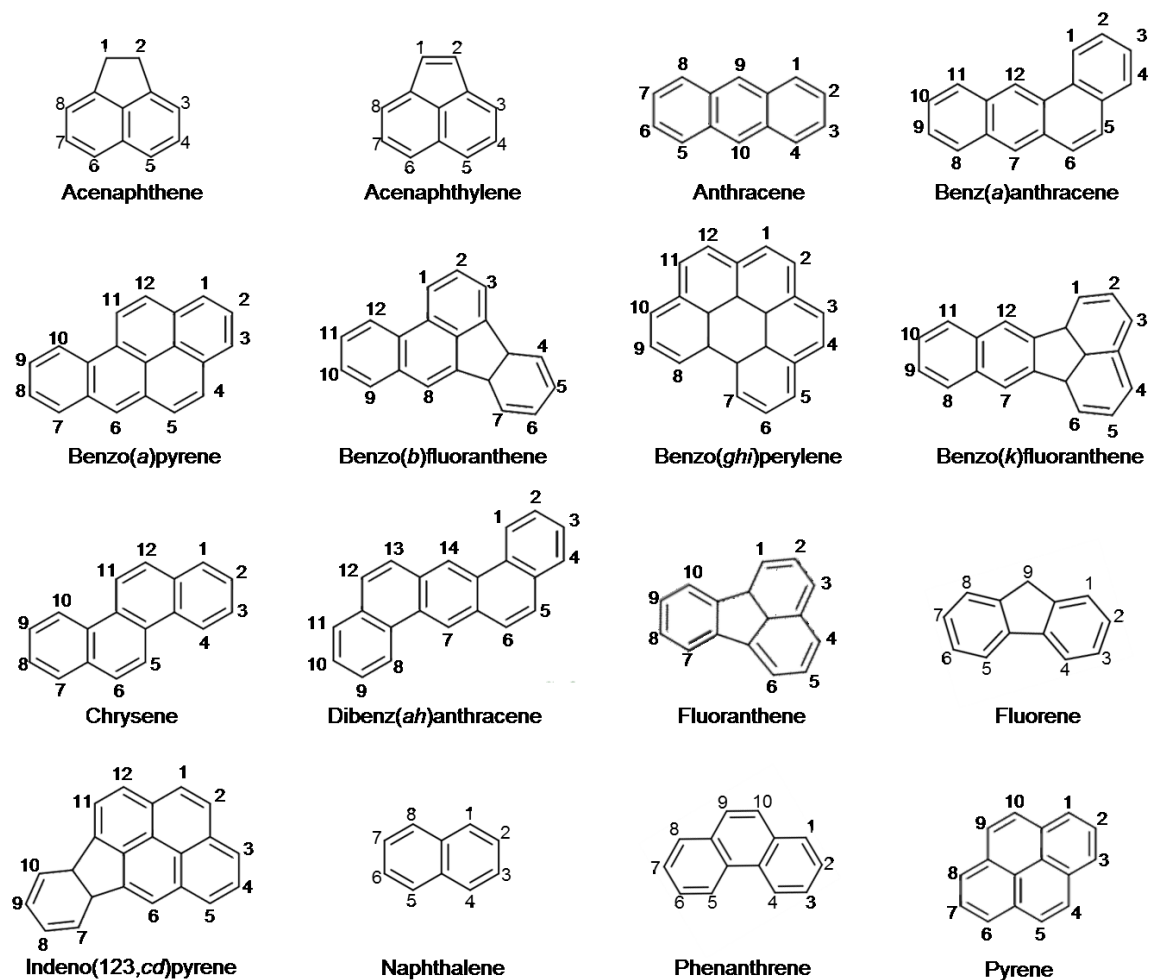


Figure 1-3 Structure of PAHs

Figures were produced using ChemDoodle 2D Sketcher

(<https://web.chemdoodle.com/demos/sketcher/>).



Figure 1-4 Rodent carcinogenicity of PAHs and PAH-containing mixtures, by route of exposure

Heatmap showing the different cancers known to be induced by the 16 priority PAHs and complex mixtures containing PAHs. LV – liver, LG – lung, GI – gastrointestinal tract including stomach and intestines, SK – skin, MG – mammary gland, LN – lymph nodes. Note that for coal tar pitch and coal flue gas, inhalation studies were conducted rather than intra-pulmonary as indicated in the figure.

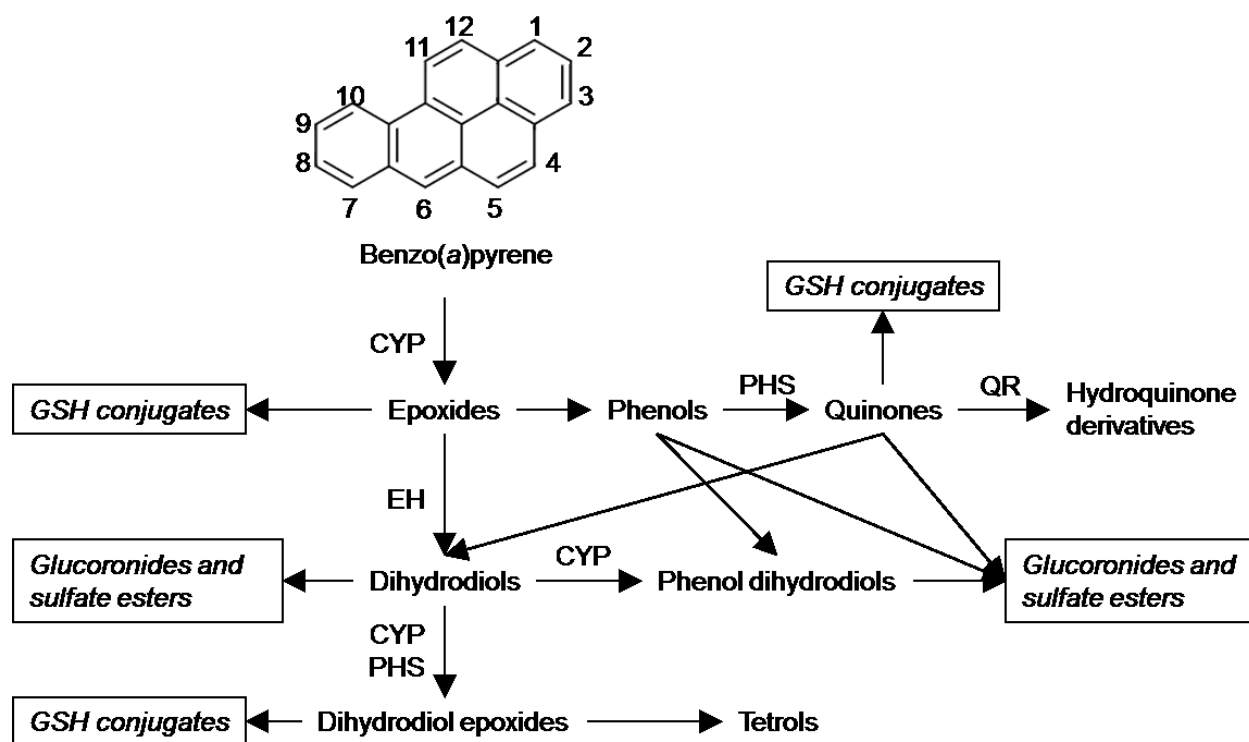


Figure 1-5 Schematic showing mammalian metabolism of BaP

Figure shows the complex series of metabolic reactions leading to BaP bioactivation and excretion. CYP – cytochrome P450 enzymes, EH – epoxide hydrolase, PHS – prostaglandin H synthase, QR – quinone reductase, GSH – glutathione. Metabolites conjugated for excretion are enclosed in black boxes. Adapted from IARC monograph, volume 92 (IARC, 2010).

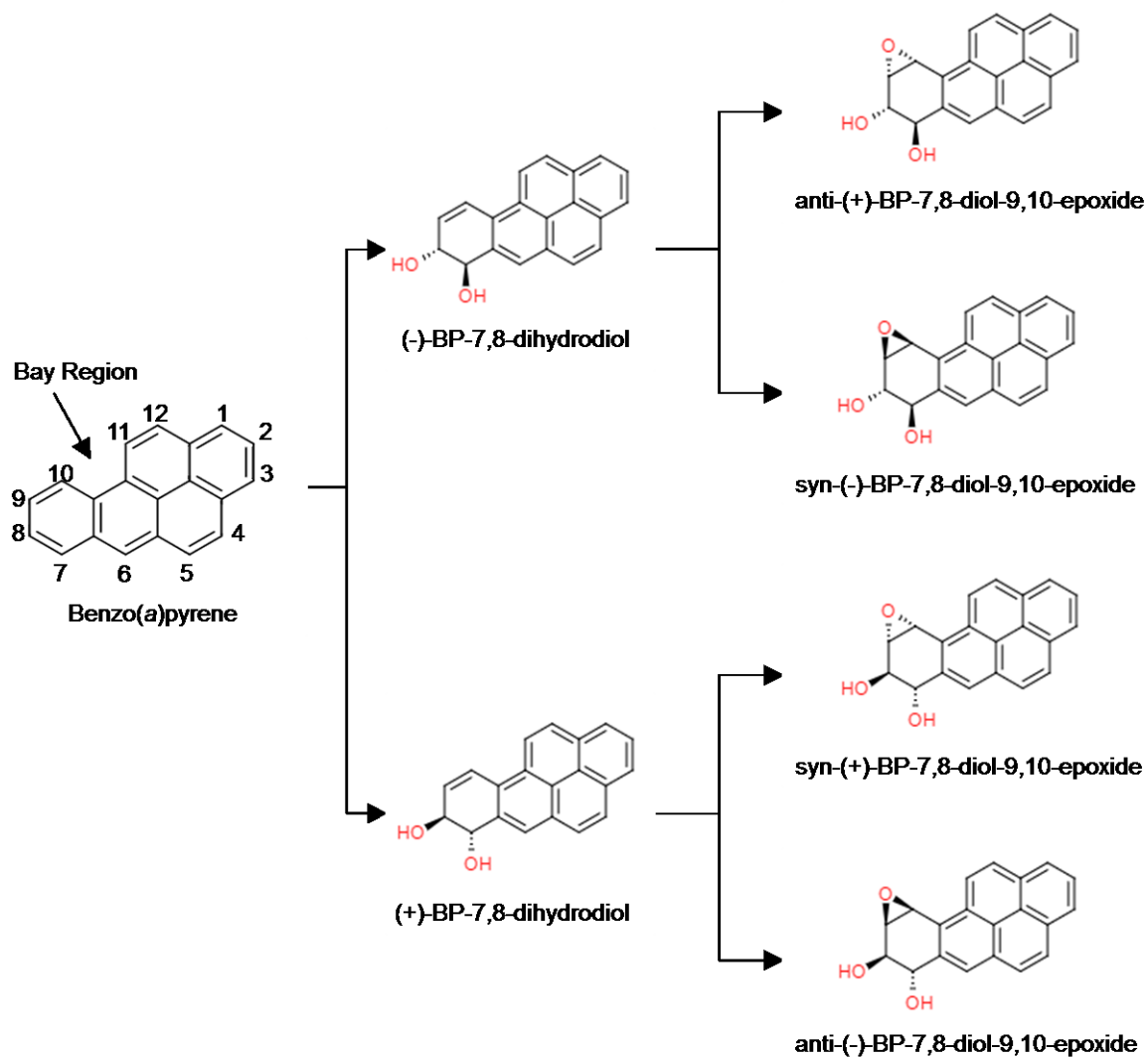


Figure 1-6 Formation of Bay region and DNA-reactive BaP metabolites.

Figure shows location of Bay region in BaP and stereoselective reactive metabolites. Figure was produced using ChemDoodle 2D Sketcher (<https://web.chemdoodle.com/demos/sketcher/>).

2 Chapter: Subchronic Oral Exposure to Benzo(a)pyrene Leads to Distinct Transcriptomic Changes in the Lungs that are Related to Carcinogenesis

Modified from: Labib, S., Yauk, C.L., Williams, A., Arlt, V.M., Phillips, D.H., White, P.A., Halappanavar, S. (2012) Toxicological Sciences 129(1):213-224. Reprinted with permission from Oxford University Press.

2.1 Abstract

We have previously shown that acute oral exposure to the environmental carcinogen benzo(a)pyrene (BaP) elicits comparable levels of DNA adducts, but distinct transcriptomic changes, in mouse lungs and livers, the two main BaP bioactivating organs. Oral BaP exposure is predominantly associated with lung cancer and not hepatic cancer in some animal models, suggesting that gene expression differences may provide insight into the drivers of tissue-specific carcinogenesis. In the present study, we examine pulmonary DNA adduct formation, *lacZ* mutant frequency, and mRNA profiles in adult male MutaTMMouse following sub-chronic (28 day) oral exposure to BaP (0, 25, 50, and 75 mg/kg/day) and sacrificed 3 days post-exposure. The results are compared to those obtained from livers of the same mice (previously published). Although there was a 1.8- to 3.3-fold increase in the levels of DNA adducts in lung compared to liver, the *lacZ* transgene mutant frequency was similar in both tissues. At the transcriptomic level, a transition from activation of the DNA damage response p53 pathway at the low dose to the induction of genes involved in angiogenesis, evasion of apoptosis and growth signals at the high doses was evident only in the lungs. These results suggest that tissue DNA adducts and mutant frequency are sensitive markers of target tissue exposure and mode of action, whereas, early changes in gene expression may provide a better indication of the likelihood of

carcinogenic transformation in selected tissues. Moreover, the study provides new information on the underlying mechanisms that contribute to tissue specific responses to BaP.

2.2 Introduction

Benzo(a)pyrene (BaP) is a well-studied polycyclic aromatic hydrocarbon (PAH) that is found in cigarette smoke, indoor and outdoor air, as well as in charred meats. Metabolic activation of BaP by cytochrome P450 (CYP) enzymes, namely CYP1A1 and CYP1B1, results in the generation of several BaP metabolites, including BaP-7,8-diol-9,10-epoxide (BPDE), a highly reactive form of BaP. BPDE is the primary metabolite of BaP that covalently binds DNA and forms DNA adducts (Baird et al., 2005). These DNA adducts, if left unrepaired, can cause mutations in genes involved in xenobiotic metabolism, tumour suppression, or oncogenes leading to tumour development. The International Agency for Research on Cancer (IARC) has recently upgraded their classification of BaP to a Group 1 (i.e., known human) carcinogen (Baan et al., 2009). Exposure to BaP leads to tumour formation in animal models, and epidemiologically, a link between BaP exposure and cancer incidence in humans has been established (OEHHA, 2010). In addition to being carcinogenic and mutagenic, BaP is a known immunosuppressant (Dean et al., 1983). BaP can also alter cell cycle progression (Solhaug et al., 2005), induce inflammation (Qamar et al., 2012), and impair DNA repair and apoptotic processes (Solhaug et al., 2005) leading to aberrant cellular functioning.

The primary site of BaP metabolism is the liver; however, hepatocarcinogenesis is rare in humans and inconsistent in animals (OEHHA, 2010). Lung carcinogenesis, on the other hand, is prevalent in mice exposed orally to BaP (Stoner et al., 1984; Wattenberg and Leong, 1970), including MutaTMMouse (Hakura et al., 1998). This is explained by greater BaP retention in the lung (Galván et al., 2005; Harrigan et al., 2004), higher induction of CYP1A1 and CYP1B1 in

the lung tissue (Harrigan et al., 2006), and higher levels of BPDE-DNA adducts in the lungs compared to the liver (Harrigan et al., 2004). Indeed, the levels of BaP-induced DNA adducts in any tissue at a given time is a function of metabolic conversion of BaP to its DNA-reactive metabolites via phase I xenobiotic metabolism, phase II detoxification, the rate of adduct repair, and the cell turnover rate, collectively suggesting that tissue dynamics and BaP response between lung and liver tissues likely differs.

Our previous work examined global gene expression changes in the lungs of adult male mice following acute exposure to 150 and 300 mg/kg BaP by oral gavage for three consecutive days and sacrificed four hours post-exposure (Halappanavar et al., 2011). The pulmonary transcriptomic response included changes in biological pathways involved in B-cell receptor (BCR) signalling, inflammation, and DNA damage response. We also noted that the response in the lung tissue was different, and in some respects, much more pronounced than livers from the same mice. Oxidative stress, xenobiotic metabolism, aryl hydrocarbon receptor (AHR) signalling, and glutathione metabolism were among the common pathways that were affected in both tissues, whereas negative regulation of the BCR signalling, indicative of potential primary immunodeficiency, was unique to the lung tissue. However, both tissues exhibited similar levels of DNA adducts, suggesting that evidence of higher levels of tissue DNA adducts are not sufficient to predict the observed transcriptomic differences in lungs and livers. Although the study was conducted at very high doses of BaP, the findings support the hypothesis that tissue-specific mechanisms are at work, and that differential regulation of the known biological pathways associated with cancer formation could explain the selective targeting of lungs for carcinogenesis.

In the present study, we explore further the potential of early BaP-induced gene expression changes to identify the tissue-specific events that may eventually lead to carcinogenic transformation following BaP exposure. We globally profile the pulmonary transcriptome and measure in parallel the induced frequency of mutations and DNA adducts (known predictors of genotoxicity-mediated cancer) in the lung tissues of adult male mice repeatedly exposed to 25, 50 and 75 mg/kg BaP for 28 consecutive days by oral gavage and sacrificed three days post-exposure. The results are compared and interpreted in light of the differential biological responses reported for the lungs and livers of mice following both acute (Halappanavar et al., 2011; Yauk et al., 2011) and sub-chronic (Malik et al., 2012) exposures to BaP.

2.3 Materials and Methods

2.3.1 Animal Treatment

Adult (25 weeks-old) male MutaTMMouse (transgenic mouse strain 40.6) were exposed to BaP (Sigma Aldrich, Canada) as described previously (Malik et al., 2012). In brief, animals were dosed daily via oral gavage for 28 days with varying doses of BaP (0, 25, 50, 75 mg/kg body weight/day) dissolved in olive oil. Each dose group contained five animals. Mice were sacrificed by cardiac puncture under isoflurane anaesthesia 72 hours following the final exposure. The right lobe of the lung was excised, flash frozen in liquid nitrogen, and stored at -80°C until use. For the duration of the experiment, food (2014 Teklad Global standard rodent diet) and water were provided ad libitum and mice were caged individually in plastic film isolators (Harlan Isotec, UK) on a 12 hour light/12 hour dark cycle. Mice were bred, maintained and treated in accordance with the Canadian Council for Animal Care Guidelines and approved by Health Canada's Animal Care Committee.

2.3.2 Tissue Selection

The major exposures to BaP occur via the oral route (drinking and feed) (Hetttemer-Frey and Travis, 1991). The organs directly affected by BaP as a consequence of oral exposure include stomach, esophagus, tongue, and larynx (Culp et al., 1998). However, studies conducted by Stoner et al. (1984) and Wattenberg and Leong (1970) show lung and liver to be equally impacted by BaP (oral gavage) and moreover, revealed sensitivity of lung for tumour development in comparison with liver. In alignment with these reports, our previous work (Halappanavar et al., 2011; Yauk et al., 2011) revealed lung-specific regulation of the biological processes known to be associated with cancer formation. Thus, in the present study we have investigated transcriptional responses in lung and liver. The results of the study may also help address the importance of considering the multi organ toxicity in calculating the risk associated with chemicals, such as BaP.

2.3.3 Tissue DNA Extraction

The frozen lung and liver tissue was sliced randomly. Genomic DNA was isolated from a random tissue section for measuring the levels of DNA adducts and transgene mutant frequency. In brief, lung tissue was minced and de-gassed to remove all traces of air in the alveoli. Livers were homogenized in ice cold TMST buffer (50 mM Tris, pH 7.6, 3 mM magnesium acetate, 250 mM sucrose, 0.2% Triton X-100) as described in Douglas et al. (1994). The minced tissue was washed twice in cold phosphate buffered saline and lysed in 10 mM Tris pH7.6, 10 μ M EDTA, 100 μ M NaCl, 1% SDS overnight at 37°C on a rotating platform. The lysate was digested with proteinase K (1 mg/ml lysis buffer). DNA was isolated using a serial phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) extraction (Renault et al., 1997). DNA

was precipitated in ethanol and dissolved in Tris-EDTA buffer (10 mM Tris pH7.6, 500 mM EDTA). DNA was stored at 4°C until use.

2.3.4 DNA Adduct Analysis

DNA adduct formation in each sample was determined using the nuclease P1 digestion enrichment version of the ³²P-postlabelling assay as described previously (Phillips and Arlt, 2007) with minor modifications. Briefly, 4 µg of DNA was digested overnight with micrococcal nuclease (288 mUnits, Sigma, cat. no. N3755) and calf spleen phosphodiesterase (1.2 mUnits, MP Biomedicals, cat. no. 100977), enriched and labelled as described elsewhere (Phillips and Arlt, 2007). Radiolabelled adducted nucleotide biphosphates were separated by thin-layer chromatography on polyethyleneimine-cellulose plates (Macherey-Nagel, Düren, Germany) with the following chromatographic conditions (Arlt et al., 2008): D1, 1.0 M sodium phosphate, pH 6; D3, 4.0 M lithium-formate, 7.0 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris, 8.5 M urea, pH 8. Chromatographs were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (relative adduct labelling) were calculated from the adduct counts per minute (cpm), the specific activity of [γ -³²P]ATP, and the amount of DNA (pmol of DNA-P) used. An external BPDE-DNA standard was used for identification of BaP-DNA adducts. Results are expressed as DNA adducts/108 nucleotides.

2.3.5 LacZ Mutant Frequency (Positive Selection)

The transgene lacZ mutant frequency in lungs was determined using the P-gal (phenyl- β -D-galactopyranoside) positive selection assay as described in Vijg and Douglas (1996) and Lambert et al. (2005). The λ gt10lacZ DNA was rescued from the genomic DNA using the TranspackTM lambda packaging system (Stratagene, USA). The packaged phage particles were mixed with host bacterium (*Escherichia coli* lacZ⁻, galE⁻, recA⁻, pAA119 with galT and galK), plated on

minimal medium containing 0.3% (w/v) P-Gal and incubated overnight at 37°C. Total plaque-forming units (pfu) were measured on concurrent titres that did not contain P-Gal. Mutant frequency is expressed as the ratio of the number of mutant pfu to total pfu. Mutant frequency data analysis was performed as described previously (Malik et al., 2012).

2.3.6 RNA Extraction and Purification

Total RNA was isolated for gene expression analysis and qRT-PCR validation as described previously (Halappanavar et al., 2011). Briefly, total RNA was extracted from the lungs using TRIzol reagent (Invitrogen, USA) and purified using RNeasy Mini Kit (Qiagen, Canada). The RNA quantity and purity was checked using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Canada). The RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada). The samples showing A260/A280 ratios between 2.1 and 2.2 and having RNA Integrity Number above 7.5 were used for further analysis.

2.3.7 Microarray Hybridization and Analysis

Double-stranded cDNA and cyanine-labelled cRNA was synthesized (Agilent Linear Amplification Kits, Agilent Technologies, Canada) from 250 ng of total RNA from each sample and universal reference total RNA (Stratagene, Canada). Cyanine-labelled cRNA targets were in vitro transcribed using T7 RNA polymerase and purified by RNeasy Mini Kit (Qiagen, Canada). From each (sample and reference) labelled sample 825 ng of cRNA was hybridized to Agilent 4X44K oligonucleotide microarrays (Agilent Technologies, Canada) at 60°C overnight (16 hours) in the Agilent SureHyb hybridization chamber. Arrays were washed and scanned on an Agilent G2505B Scanner according to manufacturer's recommendations. Feature extraction software version 10.7.3.1 (Agilent Technologies, Canada) was used to extract the data.

A reference design (Kerr and Churchill, 2001) was used to analyse mRNA expression as described previously (Malik et al., 2012). All analyses were conducted in the R (R-Development-Core-Team, 2010) environment using the MAANOVA library (Wu, 2010). The background fluorescence was measured using the negative control (-)3xSLv1 probes; probes with median signal intensities less than the trimmed mean (trim = 5%) plus three trimmed standard deviations of (-)3xSLv1 probe were flagged as absent (within the background signal). Probes were considered present if at least four of the five samples within a condition had signal intensities greater than three trimmed standard deviations above the trimmed mean of the (-)3xSLv1 probes (background signal). Data were normalized using the `transform.madata()` function using the `glowess` option with a span of 0.1. Ratio intensity plots and heat maps for the raw and normalized data were constructed to identify outliers. One sample (50 mg/kg group) was removed from the analysis based on clustering. The statistical model for this analysis included fixed effects of array and treatment condition and was applied to the log₂ of the absolute intensities. Differentially expressed transcripts (upregulated or downregulated relative to olive oil treated control mouse lung samples) were determined using the `Fs` statistic option in the `matest()` function. The p values for all statistical tests were estimated by the permutation method with residual shuffling and false discovery rate (FDR) adjusted p values were estimated using the `adjPval()` function. The fold change calculations were estimated as described previously (Malik et al., 2012). Significant genes were selected based on a FDR adjusted p value < 0.05 for any BaP exposed versus control contrast.

2.3.8 qRT-PCR Array Validation

Mouse pathway-specific PCR array (cancer-PAMM-033, SABiosciences™, USA) and custom PCR arrays consisting of 172 genes in total were employed to validate the microarray results.

Genes for the custom array were selected based on their implication in biological processes relevant to lung carcinogenesis. These genes included statistically significant differentially expressed genes (FDR adjusted $p < 0.05$), differentially expressed genes that exhibited high fold changes (fold rank only) but were not statistically significant, and genes that were differentially regulated (FDR adjusted $p < 0.05$) in the livers from the same mice (Malik et al., 2012). In brief, 0.8 μ g of total lung tissue RNA ($n=5$ /group) from each sample was reverse transcribed using RT2 First Strand Kit (SABiosciencesTM, USA). Real-time PCR was performed using RT2 SYBR Green PCR Master Mix on a CFX96 real-time detection system (Bio-Rad, Canada). Threshold cycle values for each well were averaged. Relative gene expression was determined according to the comparative Ct method and normalized to reference RNAs Hprt1, Gapdh, and Actb housekeeping genes for the Cancer Pathway array and to reference RNAs Gusb and Hprt1 housekeeping genes for the custom arrays. Transcripts were further normalized by subtracting the median delta Ct value for each sample. Differential expression was determined with a two-sample bootstrap test using R software (R-Development-Core-Team, 2010). The fold change was estimated using the ratio of the arithmetic mean of the treated sample to the mean of the control samples. Standard errors for the fold change values were estimated using the bootstrap test (Efron and Tibshirani, 1993).

2.3.9 Bioinformatics

All mRNA data are deposited in the NCBI Gene Expression Omnibus database under accession numbers GSE35718 (lung) and GSE24910 (liver). Following normalization, biological functions perturbed in response to BaP were identified using functional annotation clustering in the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000).

The biological and molecular functions of genes that were significantly differentially expressed (FDR adjusted $p \leq 0.05$, or FDR adjusted $p \leq 0.1$ and fold change ≥ 1.5) following exposure to BaP treatment were analyzed and explored in IPA (Ingenuity® Systems, Redwood City, CA). Molecular relational networks of genes modulated by BaP in lung tissue enriched for cancer function were generated using IPA. Each molecule was overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks were generated based on their connectivity. All relationships are supported by at least one reference from the literature.

2.4 Results

Daily exposure to 25, 50, and 75 mg/kg BaP for 28 consecutive days caused no overt signs of toxicity and there was no significant body weight loss in any of the exposed mice compared to vehicle treated controls.

2.4.1 *BaP-induced DNA adduct formation in lung and liver tissue*

The presence of DNA adducts indicates the development of pre-mutagenic lesions following a chemical exposure. The ³²P-postlabelling technique was employed to measure BaP-induced DNA adducts in the lung tissues of mice exposed to 0, 25, 50, and 75 mg/kg BaP for 28 consecutive days (Figure 1a). BaP-7,8-diol-9,10-epoxide-N2-deoxyguanosine (dG-N2-BPDE) was identified as the main adduct as reported previously (Lemieux et al., 2011). No adducts were detected in mice dosed with vehicle control. DNA adduct formation by BaP was dose-dependent. Although liver DNA adducts were analyzed in these samples previously (Lemieux et al., 2011; Malik et al., 2012), these data were not used. Instead, ³²P post-labelling was repeated in these samples in order to ensure that the protocols in both liver and lung were identical and performed in the same lab. Similar to results observed in the lungs, dG-N2-BPDE was the main adduct

identified in liver, and adduct levels increased in a dose-dependent manner (Figure 1a).

However, the frequency of DNA adducts in liver was significantly lower than in lung tissue for each dose (Bonferonni-corrected p value ≤ 0.05), suggesting that lung tissue is more susceptible to DNA damage caused by BaP.

2.4.2 *Mutagenic activity of BaP in the lung tissue*

The MutaTMMouse contains around 29 +/- 4 (Shwed et al., 2010) copies of λ gt10lacZ shuttle vector stably integrated in the mouse genome, thus permitting in vivo lacZ mutant frequency analysis. In agreement with the DNA adduct results, a dose-dependent increase in mutant frequency was observed (Figure 1b) in lung tissues. Compared to the vehicle controls, a 5.2-, 13- and 18.6-fold increase was seen in the 25, 50 and 75 mg/kg dose groups, respectively. Mice gavaged with pure olive oil (vehicle) yielded a low spontaneous lacZ mutant frequency. No significant differences between the lung and liver tissues were observed for lacZ mutant frequency.

2.4.3 *General overview of pulmonary gene expression profiles*

Repeated oral gavage of mice with BaP induced significant changes in the expression of many genes in the lung tissue. MAANOVA analysis revealed 20, 145, and 373 unique probes that were differentially expressed (up or downregulated) with a fold change ≥ 1.5 in either direction, and an FDR adjusted $p \leq 0.05$ in the 25, 50 and 75 mg/kg exposure groups compared to controls, respectively (Supplementary Table 2-1). The complete lung microarray dataset is available through the Gene Expression Omnibus at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>), accession number GSE35718. Hierarchical cluster analysis on all differentially expressed genes (FDR $p \leq 0.05$, fold change ± 1.5) revealed that all treatment groups clustered separately from the control

(Supplementary Figure 2-1), thus, a clear treatment effect was observed as a result of exposure to BaP.

Among the differentially expressed genes, 297 were upregulated and 90 were downregulated. Cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), growth differentiation factor 15 (*Gdf15*), carboxylesterase 5 (*Ces5*), pleckstrin homology-like domain, family A, member 3 (*Phlda3*), multimerin 2 (*Mmrn2*), cyclin G1 (*Ccng1*), serum amyloid A 3 (*Saa3*) and sestrin 2 (*Sesn2*) were the most upregulated genes in all the dose groups. The most downregulated genes included Sarcolipin (*Sln*), myomesin 2 (*Myom2*), tropomyosin 1, alpha (*Tpm1a*) in the 75 mg/kg group, chitinase 3-like 3 (*Chi3l2*) and gastrin releasing peptide (*Grp*) in the 50 mg/kg group, and sineoculis-related homeobox 1 homolog (*Six1*) (*Drosophila*) and ras homolog gene family, member U (*Rhou*) in the 25 mg/kg dose group. Twenty-five probes representing 23 genes were commonly altered between the dose groups with an FDR $p \leq 0.05$ in at least one dose group (Table 2-1). The observed transcriptomic response was dose-dependent.

Gene ontology analysis was employed to assign biological processes and functional categories to significant genes (FDR $p \leq 0.05$, fold change ≥ 1.5) in DAVID. Biological functions perturbed in response to BaP were identified using functional annotation clustering in DAVID

(Supplementary Table 2-2a). Significant functional enrichment (score > 2.0) was found for biological processes including extracellular signalling, cell adhesion, angiogenesis, apoptosis, and regulation of cell growth in all dose groups. Further combined analysis employing DAVID and KEGG pathways revealed that the altered genes are associated with specific biological pathways, including p53 signalling, molecular mechanisms of cancer, small cell lung cancer, extracellular membrane receptor interaction, and focal adhesion (Supplementary Table 2-3a).

In addition to the analysis described above, we relaxed the FDR adjusted p value to include genes that exhibit FDR $p \leq 0.1$ and fold change ≥ 1.5 to see if we had missed any important genes in the pathways described above as a consequence of the stringent MAANOVA analysis. Using this criterion, 47, 181, and 429 genes in the 25, 50, and 75 mg/kg dose groups, respectively, were differentially altered. Fifty-one additional genes were identified by relaxing the p -value cut-off (Supplementary Table 2-5). DAVID analysis (Supplementary Table 2-2b and 2.3b) as described above revealed three new functional categories: cell-to-cell and cell-to-matrix interactions, protein maturation, and inflammatory response. An in-depth functional analysis using IPA on these genes (FDR $p \leq 0.1$ and fold change ≥ 1.5) revealed that the altered genes were associated with multiple functions, such as cancer, cell death, cell cycle, cellular growth and proliferation, cellular movement, DNA replication and repair, inflammatory response, and tumour morphology (Figure 2-2a). Since the present work is focused on finding perturbations in pathways associated with carcinogenic transformation in the lung tissue, a sub-analysis on specific genes that were grouped under the functional group ‘cancer’ was performed. The sub-analysis revealed that a majority of the genes from this group were mainly associated with the p53 signalling pathway and were implicated in several biological processes regulated by p53, such as cell cycle arrest, apoptosis, inhibition of cellular growth and proliferation, and DNA repair. Many of these genes were common across the dose groups (Tables 2.1, 2.2, and 2.3) and showed a clear dose-response. The other affected pathways included the molecular mechanisms of cancer, AhR signalling, BCR signalling, and angiogenesis (Table 2-3). A similar functional analysis using IPA on liver (Malik et al., 2012) revealed that the altered genes were associated with lipid metabolism, cancer, molecular transport, inflammatory response, and cell death (Figure 2-2b). Sub-analysis of the cancer-associated genes in the liver reveals that in addition to

the p53 signalling pathway, this category includes genes involved in metabolism and inflammation, which are indirectly linked to carcinogenesis.

Visualization of the relationship between these genes in a biological network created in IPA (Figure 2-3) revealed that at the low dose the immediate downstream effectors of p53, such as *Cdkn1a*, *Ccng1*, *Bax*, and *Tp53inp1* mainly involved in DNA damage recognition, cell cycle arrest, and apoptosis were upregulated. However, with increasing dose, genes known to be the suppressors of p53 signalling pathway, such as *Mdm2* were upregulated along with other genes implicated in proliferation and growth, evasion of apoptosis, promotion of angiogenesis, and cell invasion and metastasis pathways, all of which are the hallmarks of cancer.

2.4.4 RT-PCR validation of microarray results

Exposure to BaP resulted in altered expression of a number of genes involved in the molecular mechanisms of cancer (Table 2-3). Perturbation of this pathway was confirmed using a cancer pathway-specific PCR array (Mouse Cancer Pathway Finder PCR array, SABiosciences™) containing 84 genes. In addition, 61 more genes implicated in BCR signalling, calcium signalling, TGF β signalling, cell cycle, and DNA damage repair pathways were also analyzed (Table 2-3). From each dose group five individual samples were analyzed.

In total, 57 of the 145 genes were differentially expressed with $p \leq 0.1$ in at least one treatment group compared to controls (Table 2-3, Supplementary Table 2-4); 23 of these correlated with the microarray results. Thirty-one genes were significantly differentially expressed as measured by the PCR arrays but not by microarrays. This is presumably due to the enhanced dynamic range and sensitivity of RT-PCR. These genes included baculoviral IAP repeat-containing 5 (*Birc5*), tumour necrosis factor receptor superfamily member 1A (*Tnfrsf1a*), jun proto-oncogene (*Jun*), c-fos-induced growth factor (*Figf*), insulin-like growth factor 1 (*Igf1*), interferon beta 1

fibroblast (Ifnb1), vascular endothelial growth factors a and c (Vegfa and Vegfc), plasminogen activator urokinase (Plau), integrin alpha 3 (Itga3), platelet-derived growth factors a and b (Pdgfa and Pdgfb), and transforming growth factor beta 1 (Tgfb1) (Table 3).

2.5 Discussion

BaP is the only PAH classified by IARC as a known human carcinogen (Baan et al., 2009). The ability to induce DNA damage and mutations in key genes involved in tumour suppression or tumour promotion is one of the postulated mechanisms of BaP-induced carcinogenesis (Denissenko et al., 1996; Ross and Nesnow, 1999). The metabolic activation of BaP by phase I CYP enzymes is a prerequisite for its potentially carcinogenic interaction with DNA (Arlt et al., 2008; Rubin, 2001). Several clinical and epidemiological studies have shown a link between the levels of DNA adducts and exposure-induced carcinogenesis (Gunter et al., 2007; Tang et al., 2001). Thus, the presence of DNA adducts may be a risk factor for developing cancer. Although many tissues are capable of metabolizing BaP to its carcinogenic intermediate, which is associated with the development of mutations, carcinogenesis is specific to certain tissues. For example, BaP is predominantly metabolized in the liver; however, hepatocarcinogenesis is relatively less frequent in some experimental animal models and in humans compared to pulmonary carcinogenesis (Hakura et al., 1998; Stoner et al., 1984; Wattenberg and Leong, 1970). Several factors are suggested to play a vital role in the tissue-specific transformation process, including a balance between the metabolic activation and detoxification processes and the relative rates of DNA damage, DNA repair and individual susceptibility.

The presence of tissue DNA adducts and genetic mutations in genes is often used as a measure of the carcinogenic potential of a chemical (Mei et al., 2006; Wang et al., 2011). Although these markers of exposure and effect, respectively, classify a chemical as genotoxic

and non-genotoxic, they may not accurately predict with certainty their carcinogenic potential. We have recently shown that acute exposure to BaP by oral gavage leads to similar levels of DNA adducts in the lungs and livers, two BaP bioactivating organs (Halappanavar et al., 2011). However, despite similar levels of DNA damage (reflective of bioavailable BaP) in both the tissues, the transcriptomic responses in lungs were different and included lung-specific perturbations of several biological pathways associated with molecular mechanisms of cancer (Halappanavar et al., 2011). The present study was undertaken to further explore the molecular mechanisms underlying such distinct tissue-specific responses and their implication in cancer formation employing a sub-chronic exposure model.

In agreement with the published literature (Baird et al., 2005), we found that daily exposure to BaP for 28 days resulted in significant genotoxicity as measured by a dose-dependent increase in DNA adduct formation and lacZ transgene mutant frequency in the lungs of exposed animals (Figure 2-1). Compared to the livers from the same mice there was a statistically significant 1.8- to 3.3-fold increase (Bonferonni-corrected p value ≤ 0.05) in DNA adduct formation in the lung tissues. In alignment with these results, mRNA expression of DNA polymerase Kappa (PolK), necessary for the error-free bypass of the (+)-trans-BPDE-N2-dG DNA adduct (Ogi et al., 2002), was significantly upregulated in the lungs (Table 2-3) but not the liver. In contrast, levels of DNA adducts in liver and lung were comparable in C57BL/6 mice acutely exposed to very high doses of BaP (Halappanavar et al., 2011) (i.e., 150 mg/kg and 300 mg/kg BaP per day for three days, sampled 4 and 24 hours after the last exposure). The discrepancy in DNA adduct levels between the acute and sub-chronic repeated dose study could be simply be due to the amount of total BaP gavaged over time. Compared to the hepatic tissue, rate of clearance of BaP from the lungs occurs slowly resulting in longer retention of BaP in lungs (Harrigan et al., 2004),

suggesting that the repeated exposure to BaP over 28 consecutive days may have led to accumulation of BaP in lungs resulting in higher DNA adduct levels. Studies have shown accumulation of DNA adducts in some organs that are resistant to repair or following chronic repeated exposures. It has also been suggested that at higher doses metabolic activation/detoxification of BaP could reach a threshold, which in turn may limit the speed with which reactive metabolites are detoxified in the organ compared to the speed of the translocation of these metabolites from the primary site (e.g., liver) to other distant organs (e.g., lungs) .

Although we report significant differences in DNA adduct formation between the lung and liver, the transgene mutant frequency in both tissues was not significantly different, which may suggest more efficient DNA repair in the lungs compared to livers (Figure 2-2, high dose). The elevated mutant frequency observed in livers could be attributed to the higher rates of cellular metabolism (as reflected by the number of differentially altered genes in metabolic pathways) potentially resulting in an increase in by-products of reactive oxygen species. These results suggest that in addition to mutations arising as a result of unrepaired bulky DNA adducts, other factors such as alterations in critical molecular processes implicated in cancer formation may play a role in the selective targeting of the lung tissue for carcinogenesis following BaP exposure.

Our previous work (Halappanavar et al., 2011) on mice acutely exposed to BaP revealed suppression of B-cell receptor (BCR) signalling pathway and an elaborated DNA damage response in the lungs, whereas the livers from the same mice showed subtle changes in only a few genes associated with DNA damage response (Halappanavar et al., 2011). Repressed BCR signalling, leading to primary immunodeficiency, is potentially implicated in cancer initiation (Jumaa et al., 2005). In the present study we observed similar suppression of a few of the key

genes in the BCR signalling pathway in the lungs. However, the response was only observed in the high dose group (Table 2-2), suggesting that the collective downregulation of the BCR signalling pathway may be a potential mechanism that operates in the lungs following exposure to high doses of BaP over a very short period of time.

Exposure to BaP for 28 consecutive days induced a large transcriptomic response in the lungs sampled 3 days post-exposure compared to the livers. MAANOVA analysis of liver tissue revealed differential expression of 6, 7, and 121 unique probes compared to 20, 145, and 373 probes in the lungs, in the 25, 50 and 75 mg/kg exposure groups respectively (Malik et al., 2012). The p53 signalling pathway was commonly affected in both tissues (Table 2 and 5) (Malik et al., 2012). P53 is a tumour suppressor protein and mutations in the TP53 gene have been found in many types of human cancers linked to environmental exposures (Kucab et al., 2010) including lung cancers. The p53 protein is a transcription factor that mediates expression of several genes, including Cdkn1a, an inhibitor of cyclin-dependent kinases, Bax, a pro-apoptotic gene, Gadd45, a DNA damage repair gene, which is also involved in cell cycle arrest, and many inflammatory modulators and growth factors. Transgenic mice devoid of TP53 gene (p53 knockout) or mice in which p53 activity is chemically inhibited, exhibit increased susceptibility to develop spontaneous and chemically induced tumours (Donehower et al., 1992). Cellular DNA damage response to BaP is regulated by p53 (Park et al., 2006). Furthermore, increased rates of lung cancer following exposure to BaP arise in transgenic mice expressing lung-specific dominant-negative mutant p53 (Tchou-Wong et al., 2002), suggesting an important role for the p53 signalling pathway in the development of BaP-induced lung cancer.

Since the focus of the present study was to understand the molecular perturbations in lungs that are associated with tissue-specific tumour development (i.e, relative to the liver), we

compared the expression changes of several genes in KEGG pathways associated with cancer in both tissues. Table 2-4 shows a list of 41 genes whose expression was significantly differentially expressed ($\text{FDR } p \leq 0.05$, fold change ≥ 1.5) in lungs but not in the livers. These genes are known for their function in biological pathways such as apoptosis, cell cycle, cytokine-cytokine receptor interaction, extracellular membrane receptor interactions, focal adhesion, mitogen-activated protein kinase (MAPK) signalling, p53 signalling, peroxisome proliferating agent receptor (PPAR) signalling, transforming growth factor beta ($\text{TGF}\beta$) signalling, and Wnt signalling. In contrast, the liver showed expression changes in fewer genes, and these were mainly the downstream effectors of p53 signalling; Apaf1, Trp63, MyoD, Egr1, and Btg2 involved in induction of apoptosis and negative regulation of cellular proliferation. Distinctly affected in the lungs were Sestrin 2 (Sesn2), Pmaip1, and Trp53inp1, associated with the p53-mediated DNA damage response, Mdm2, involved in the negative regulation of p53 signalling, and Bcl2, implicated in cell survival.

Carcinogenic transformation in the lung following BaP exposure appears to be a dose-dependent process. Figure 2-3 is a molecular relational network consisting of differentially altered genes in lung tissues enriched for cancer function (using IPA functional categorization) in response to BaP exposure in our model. Interestingly, nearly all genes in the network were found directly or indirectly connected to the transcription factor p53, which is the main node in the network. At the lowest dose, genes associated with an early response to DNA damage, such as Cdkn1a and Ccng1 involved in cell cycle arrest and Bax and Tp53inp1 involved in the apoptotic process, were upregulated, indicating cellular efforts to halt replication until the damage is repaired. With increasing dose, the p53 DNA damage response pathway continued to be activated. However, activation of pathways and genes implicated in silencing p53 function also

became evident. This silencing may be related to AhR-dependent induction of Mdm2 expression (Pääjärvi et al., 2005), which is a negative regulator of p53 signalling; Mdm2 was upregulated in the two higher doses. While an apparent dose-dependent transition in the p53 DNA damage response pathway was observed, at the highest dose many genes involved in angiogenesis (Angpt2, Bdnf, Cyr61), evasion of apoptosis (Bcl2l1, Cryab, Lcn2, Rela), growth signals (Pdgfb, Pdgfd, Itga1, Notch4) and invasion and metastasis (Il1b, Mmp3, Pdgfra, Shh) were upregulated. These high dose effects are indicative of a shift in the fine cellular dynamics from a protective repair mode to a cellular replication and proliferation mode, which may suggest cellular transformation, potentially a key event in the progression to lung carcinogenesis.

One limitation of the present study is that the responses were investigated in whole tissue. The observed changes reflect the responses of over 40 different cell types. It is clear from the lung DNA adduct and mutant frequency data that the observed changes in molecular pathways are the direct results of BaP metabolism in the lung tissue. However, it should be noted that the influence of circulating factors from other target sites may also contribute to the pulmonary responses.

In conclusion, we show that the BaP-induced DNA adduct levels in lung tissue are comparatively higher than in livers after 28 consecutive days of exposure to increasing doses of BaP by oral gavage. However, the occurrence of fixed mutations was comparable in both tissues suggesting that for certain carcinogens traditional markers of genotoxicity, i.e., tissue DNA adduct levels and mutant frequency may only predict the exposure to the carcinogen and not the carcinogenic outcome. In contrast, dose-dependent transition in p53 DNA-damage response pathway that included tissue protective (adaptive) responses at the lowest dose (for e.g., genes involved in cell cycle and apoptosis) to growth and proliferative responses at the highest dose

(e.g., growth signals and pro-proliferation) was distinct to lungs only, suggesting such key biological pathway perturbations occurring early after the exposure may be causal to carcinogenesis in lungs compared to livers.

2.6 Tables and Figures

Table 2-1. Agilent probes with FDR adjusted *P* value ≤ 0.05 common to all three dose groups as measured by microarrays on lung tissue.

Agilent Probe	Accession #	Gene Symbol	Function	25 mg/kg/day	50 mg/kg/day	75 mg/kg/day
A_52_P612803	NM_009831	<i>Ccng1</i>	Cell Cycle	↑ 1.8	↑ 3.3	↑ 4.6
A_51_P363947	NM_007669	<i>Cdkn1a</i>	Cell Cycle	↑ 2.5	↑ 5.7	↑ 9.3
A_51_P472751	NM_007915	<i>Ei24</i>	Apoptosis	↑ 1.4	↑ 1.8	↑ 2.1
A_51_P508289	NM_010145	<i>Ephx1</i>	Xenobiotic Metabolism	↑ 1.7	↑ 2.3	↑ 2.9
A_52_P532982	NM_011819	<i>Gdf15</i>	Cellular Differentiation and Proliferation	↑ 2.6	↑ 6.9	↑ 9.1
A_51_P468173	NM_008251	<i>Hmgn1</i>	DNA Damage Repair	↑ 1.3	↑ 1.5	↑ 1.5
A_51_P167489	XM_140451	<i>Lama3</i>	Cell Adhesion	↑ 1.6	↑ 1.5	↑ 1.5
A_51_P484111	NM_016762	<i>Matn2</i>	Unknown Function	↓ 1.5	↓ 1.5	↓ 1.9
A_51_P463562	NM_008620	<i>Mpa2</i>	Possibly Cellular Differentiation	↑ 1.4	↑ 1.5	↑ 1.8
A_52_P13389	NM_026793	<i>Myct1</i>	Possibly Apoptosis	↑ 1.6	↑ 1.8	↑ 1.9
A_51_P195875	NM_010929	<i>Notch4</i>	Cellular Differentiation	↑ 1.6	↑ 1.7	↑ 1.8
A_51_P363801	NM_023217	<i>Pgpep1</i>	Proteolysis	↑ 1.3	↑ 1.6	↑ 1.5
A_51_P329928	NM_013750	<i>Phlda3</i>	Apoptosis	↑ 2.1	↑ 3.8	↑ 5.1
A_51_P224564	NM_176833	<i>Ppm1f</i>	Apoptosis	↑ 1.4	↑ 1.5	↑ 2.0
A_51_P227392	NM_133955	<i>Rhou</i>	Cell Cycle	↓ 1.6	↓ 1.6	↓ 1.5
A_51_P246903	NM_026467	<i>Rps27l</i>	Apoptosis/DNA Damage Repair	↑ 1.4	↑ 1.8	↑ 2.5
A_51_P319572	NM_001037709	<i>Rusc2</i>	Unknown Function	↑ 1.3	↑ 1.5	↑ 1.7
A_51_P415755	AK052232	<i>Sema6d</i>	Cellular Differentiation	↑ 1.4	↑ 1.4	↑ 1.5
A_52_P154710	AK170547	<i>Sesn2</i>	DNA Damage Repair	↑ 1.7	↑ 2.7	↑ 3.8
A_52_P220810	NM_144551	<i>Trib2</i>	Cellular Proliferation	↑ 1.4	↑ 1.9	↑ 2.1
A_51_P175580	NM_021897	<i>Trp53inp1</i>	Apoptosis	↑ 1.6	↑ 2.5	↑ 2.9
A_52_P503387	NM_021897	<i>Trp53inp1</i>	Apoptosis	↑ 1.6	↑ 2.3	↑ 2.9
A_51_P509609	NM_053082	<i>Tspan4</i>	Cell Signaling	↑ 1.7	↑ 1.7	↑ 1.8
A_51_P155052	NM_053247	<i>Xlkd1</i>	Metastasis	↑ 2.2	↑ 2.3	↑ 3.2
A_52_P686785	NM_053247	<i>Xlkd1</i>	Metastasis	↑ 2.1	↑ 2.1	↑ 3.6

Note. ↑ indicates upregulated. ↓ indicates downregulated. Fold changes relative to control are shown. The most commonly associated gene function is provided.

Table 2-2 p53 regulated genes that were significantly differentially expressed with microarrays and real-time PCR in lungs.

Gene Symbol	25 mg/kg/day		50 mg/kg/day		75 mg/kg/day	
	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR
Cdkn1a	↑ 2.5	↑ 3.2	↑ 5.7	↑ 8.1	↑ 9.3	↑ 12.6
Ccng1	↑ 1.7	↑ 2.2	↑ 3.1	↑ 3.0	↑ 4.6	↑ 4.3
<i>Trp53inp1</i>	↑ 1.6	↑ 1.8	↑ 2.4	↑ 2.1	↑ 2.9	↑ 3.2
<i>Sesn2</i>	-	↑ 2.9	↑ 2.2	-	↑ 2.7	↑ 3.4
Bax	↑ 1.5	↑ 1.9	↑ 1.9	↑ 2.5	↑ 2.5	↑ 3.4
<i>Pmaip1</i>	-	-	↑ 1.7	↑ 1.7	↑ 1.7	↑ 1.8
<i>Mdm2</i>	-	-	-	↑ 1.8	↑ 1.6	↑ 2.7
<i>Bcl2</i>	-	-	-	-	-	↑ 1.5
<i>Ccnb2</i>	-	-	-	↑ 1.7	-	↑ 1.9
<i>Tnfrsf10b</i>	-	↑ 2.0	-	↑ 2.7	-	↑ 4.4
Zmat3	-	↑ 1.8	↑ 1.8	↑ 1.7	↑ 2.7	↑ 2.5

Note. ↑ indicates upregulated. – indicates no change. Only genes with FDR adjusted $P \leq 0.1$ and fold change ≥ 1.5 are shown. Gene symbols in bold were also differentially expressed in liver (Malik *et al.* 2012).

Table 2-3 Genes validated using real-time PCR arrays divided based on functional association in lungs.

Function	Gene Symbol	25 mg/kg/day		50 mg/kg/day		75 mg/kg/day	
		Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR
P53 Signalling	<i>Cdkn1a</i>	↑ 2.5	↑ 3.2	↑ 5.7	↑ 8.1	↑ 9.3	↑ 12.6
	<i>Tnfrsf10b*</i>	-	↑ 2.0	-	↑ 2.7	-	↑ 4.4
	<i>Ccng1</i>	↑ 1.7	↑ 2.2	↑ 3.1	↑ 3.0	↑ 4.6	↑ 4.3
	<i>Bax</i>	↑ 1.5	↑ 1.9	↑ 1.9	↑ 2.5	↑ 2.5	↑ 3.4
	<i>Sesn2</i>	-	↑ 2.9	↑ 2.2	-	↑ 2.7	↑ 3.4
	<i>Trp53inp1</i>	↑ 1.6	↑ 1.8	↑ 2.4	↑ 2.1	↑ 2.9	↑ 3.2
	<i>Mdm2</i>	-	-	-	↑ 1.8	↑ 1.6	↑ 2.7
	<i>Zmat3</i>	-	↑ 1.8	↑ 1.8	↑ 1.7	↑ 2.7	↑ 2.5
	<i>Ccnb2*</i>	-	-	-	↑ 1.7	-	↑ 1.9
	<i>Pmaip1</i>	-	-	↑ 1.7	↑ 1.7	↑ 1.7	↑ 1.8
	<i>Bcl2*</i>	-	-	-	-	-	↑ 1.5
B cell Receptor Signalling	<i>Cd3g*</i>	-	-	-	-	-	↓ 1.7
	<i>Cxcr5*</i>	-	-	-	-	-	↓ 1.5
	<i>Dock2*</i>	-	-	-	-	-	↓ 1.5
	<i>Tnfsf10</i>	-	↑ 1.5	↑ 1.8	-	↑ 1.8	↑ 1.5
Calcium Signalling	<i>Penk*</i>	-	↑ 2.5	-	-	↓ 2.4	↑ 2.4
	<i>Cyr61</i>	-	↑ 1.5	-	↑ 1.8	↑ 1.9	↑ 1.8
	<i>Adrb1</i>	-	↑ 1.7	-	-	↑ 1.9	↑ 1.7
	<i>Slc30a1</i>	-	-	↑ 1.5	-	↑ 1.5	↑ 1.7
	<i>S100a8*</i>	-	↓ 1.6	-	-	-	-
	<i>Pln*</i>	-	↓ 2.4	-	-	-	-
	<i>Per1*</i>	-	↑ 2.4	-	-	-	-
Transforming Growth Factor Signalling	<i>Ccnb2</i>	-	-	-	↑ 1.7	-	↑ 1.9
	<i>Ctgf</i>	-	↑ 1.7	-	↑ 1.6	↑ 1.8	↑ 1.9
	<i>Cyr61</i>	-	↑ 1.5	-	↑ 1.8	↑ 1.9	↑ 1.8
	<i>Eng*</i>	-	↑ 1.8	-	↑ 1.5	-	↑ 1.6
	<i>Jun*</i>	-	↑ 1.5	-	-	-	↑ 1.6
	<i>Igf1*</i>	-	-	-	-	-	↑ 1.5
	<i>Tgfb1*</i>	-	-	-	-	-	↑ 1.5
Cell Cycle and DNA Damage Repair	<i>Cdkn1a</i>	↑ 2.5	↑ 3.2	↑ 5.7	↑ 8.1	↑ 9.3	↑ 12.6
	<i>Sesn2</i>	-	↑ 2.9	↑ 2.2	-	↑ 2.7	↑ 3.4
	<i>Mdm2</i>	-	-	-	↑ 1.8	↑ 1.6	↑ 2.7
	<i>Mgmt*</i>	-	-	-	↑ 2.0	-	↑ 2.7

	<i>Ccnd1</i>	-	-	↑ 1.7	↑ 1.8	↑ 2.3	↑ 2.5
	<i>Polk</i>	-	-	↑ 1.6	↑ 1.6	↑ 1.9	↑ 1.8
	<i>Tgfb1</i>	-	-	-	-	-	↑ 1.5
	<i>Prc1*</i>	-	↑ 1.5	-	-	-	-
Apoptosis	<i>Tnfrsf10b*</i>	-	↑ 2.0	-	↑ 2.7	-	↑ 4.4
	<i>Bax</i>	↑ 1.5	↑ 1.9	↑ 1.9	↑ 2.5	↑ 2.5	↑ 3.4
	<i>Trp53inp1</i>	↑ 1.6	↑ 1.8	↑ 2.4	↑ 2.1	↑ 2.9	↑ 3.2
	<i>Bcl2l1</i>	-	-	-	↑ 1.7	↑ 1.6	↑ 2.3
	<i>Tnfrsf1a*</i>	-	-	-	-	-	↑ 1.7
	<i>Bcl2*</i>	-	-	-	-	-	↑ 1.5
	<i>Tgfb1*</i>	-	-	-	-	-	↑ 1.5
	<i>Tnfsf10</i>	↑ 1.5	↑ 1.5	↑ 1.8	-	↑ 1.8	↑ 1.5
	<i>Cidea*</i>	-	↑ 2.7	-	-	-	-
	<i>Card6*</i>	-	↑ 1.5	-	-	-	-
Angiogenesis	<i>Col18a1</i>	-	-	-	↑ 1.5	↑ 1.8	↑ 2.1
	<i>Cyr61</i>	-	↑ 1.5	-	↑ 1.8	↑ 1.9	↑ 1.8
	<i>Kdr*</i>	-	-	-	-	-	↑ 1.8
	<i>Pdgfa*</i>	-	↑ 1.7	-	↑ 1.5	-	↑ 1.8
	<i>Vegfc*</i>	-	↑ 1.6	-	-	-	↑ 1.7
	<i>Pecam1*</i>	-	-	-	-	-	↑ 1.7
	<i>Igf1</i>	-	-	-	-	-	↑ 1.5
	<i>Pdgfb</i>	-	↑ 1.5	-	-	↑ 1.6	↑ 1.5
	<i>Tgfb1*</i>	-	-	-	-	-	↑ 1.5
	<i>Vegfa*</i>	-	↑ 1.5	-	-	-	-
	<i>F2*</i>	-	↑ 2.9	-	-	-	-
	<i>Plg*</i>	-	↑ 1.9	-	-	-	↑ 1.8
Invasion and Metastasis	<i>Serpine1</i>	-	↑ 1.8	↑ 2.0	↑ 3.3	↑ 2.8	↑ 5.0
	<i>Plau*</i>	-	↑ 2.8	-	↑ 2.9	-	↑ 3.1
	<i>Mmp2</i>	-	-	-	-	↑ 1.5	↑ 1.9
	<i>Twist1*</i>	-	↓ 2.9	-	-	-	-
Adhesion	<i>Pecam1</i>	-	-	-	-	-	↑ 1.7
	<i>Itga3*</i>	-	-	-	-	-	↑ 1.5
Signal Transduction	<i>Jun</i>	-	↑ 1.5	-	-	-	↑ 1.6
	<i>Myc</i>	-	-	-	↓ 1.5	-	-

Note. ↑ indicates upregulated, ↓ indicates downregulated, - indicates no change. Only genes with FDR adjusted $p \leq 0.1$ and fold change ≥ 1.5 are shown. Genes with an asterisk (*) indicate genes significantly differentially expressed by the PCR array but not significant by microarray. Please note that some genes appear more than once because they are implicated in several functions.

Table 2-4 Cancer pathway (KEGG) associated genes significantly differentially expressed in microarrays in the lung and not in the liver.

Gene Symbol	25 mg/kg/day	50 mg/kg/day	75 mg/kg/day	Gene Symbol	25 mg/kg/day	50 mg/kg/day	75 mg/kg/day
<i>Bax</i>	-	↑ 1.9	↑ 2.5	<i>Map4k4</i>	-	-	↑ 1.8
<i>Bcl2l1</i>	-	-	↑ 1.6	<i>Mdm2</i>	-	↑ 1.7	↑ 2.2
<i>Bdnf</i>	-	-	↑ 1.5	<i>Myc</i>	-	-	↓ 1.6
<i>Bmp6</i>	-	-	↑ 1.5	<i>Nkd1</i>	-	↑ 1.7	↑ 1.9
<i>Ccl5</i>	-	-	↓ 2.1	<i>Ntrk2</i>	-	-	↓ 1.6
<i>Cd47</i>	-	-	↓ 1.5	<i>Pdgfd</i>	-	-	↑ 1.5
<i>Col4a1</i>	-	-	↑ 1.7	<i>Pdgfd</i>	-	-	↑ 1.5
<i>Col4a2</i>	-	-	↑ 1.7	<i>Pdgfra</i>	-	-	↓ 1.5
<i>Cpt1b</i>	-	-	↓ 1.6	<i>Pmaip1</i>	-	↑ 1.7	↑ 1.8
<i>Cpt1c</i>	-	-	↑ 1.9	<i>Ptpm</i>	-	-	↑ 1.6
<i>Dcn</i>	-	-	↓ 2.4	<i>Rasgrp3</i>	-	-	↑ 1.8
<i>Ei24</i>	-	↑ 1.8	↑ 2.1	<i>relA</i>	-	-	↑ 1.6
<i>Flnc</i>	-	-	↓ 1.7	<i>Serpine1</i>	-	↑ 2.0	↑ 2.8
<i>Gp9</i>	-	↑ 1.8	↑ 1.5	<i>Sesn2</i>	↑ 1.7	↑ 2.7	↑ 3.8
<i>Itga1</i>	-	-	↑ 1.5	<i>Smurf2</i>	-	↑ 1.6	-
<i>Kitl</i>	-	↑ 1.6	↑ 1.6	<i>Tnfrsf12a</i>	-	-	↑ 1.7
<i>Lama3</i>	↑ 1.6	↑ 1.5	↑ 1.5	<i>Tnfsf10</i>	-	↑ 1.8	↑ 1.8
<i>Lamb3</i>	↑ 1.5	↑ 1.5	↑ 1.6	<i>Ubc</i>	-	-	↑ 1.5
<i>Lamc2</i>	-	-	↑ 1.6	<i>Wnt10a</i>	-	↓ 1.6	↓ 1.6
<i>Ltb</i>	-	-	↓ 1.5	<i>Wnt7a</i>	-	-	↑ 1.8
<i>Map4k1</i>	-	-	↓ 1.5				

Note. ↑ indicates upregulated, ↓ indicated downregulated, - indicates no change. Only genes with FDR adjusted $p \leq 0.05$ and fold change ≥ 1.5 are shown.

Table 2-5 p53 regulated genes identified in liver by Malik et al. (2012) were cross-validated in the lung using real-time PCR arrays.

Gene Symbol	25 mg/kg/day		50 mg/kg/day		75 mg/kg/day	
	Lung	Liver	Lung	Liver	Lung	Liver
<i>Cdkn1a</i>	↑ 3.2	-	↑ 8.1	↑ 6.8	↑ 12.6	↑ 16.9
<i>Tnfrsf10b</i>	↑ 2.0	-	↑ 2.7	↑ 3.7	↑ 4.4	↑ 7.8
<i>Egr</i>	-	-	-	↑ 4.1	-	↑ 6.4
<i>Btg2</i>	-	-	-	↑ 2.2	-	↑ 3.4
<i>Ccnb2</i>	-	-	↑ 1.7	-	↑ 1.9	↑ 2.4
<i>Zmat3</i>	↑ 1.8	-	↑ 1.7	↑ 1.6	↑ 2.5	↑ 2.4
<i>Apaf1</i>	-	-	-	↑ 1.8	-	↑ 2.2
<i>Prc1</i>	-	-	-	-	-	↑ 1.9
<i>Ccng1</i>	↑ 2.2	-	↑ 3.0	-	↑ 4.3	↑ 1.8
<i>Birc5</i>	-	-	-	-	-	↑ 1.8
<i>Myod</i>	-	↓ 2.1	-	↓ 1.6	-	↓ 1.6
<i>Bax</i>	↑ 1.9	-	↑ 2.5	-	↑ 3.4	↑ 1.7
<i>Trp63</i>	-	↓ 2.1	-	↓ 2.5	-	-

Note. ↑ indicates upregulated, ↓ indicates downregulated, - indicates no change. Only genes with FDR adjusted $p \leq 0.1$ and fold change ≥ 1.5 are shown.

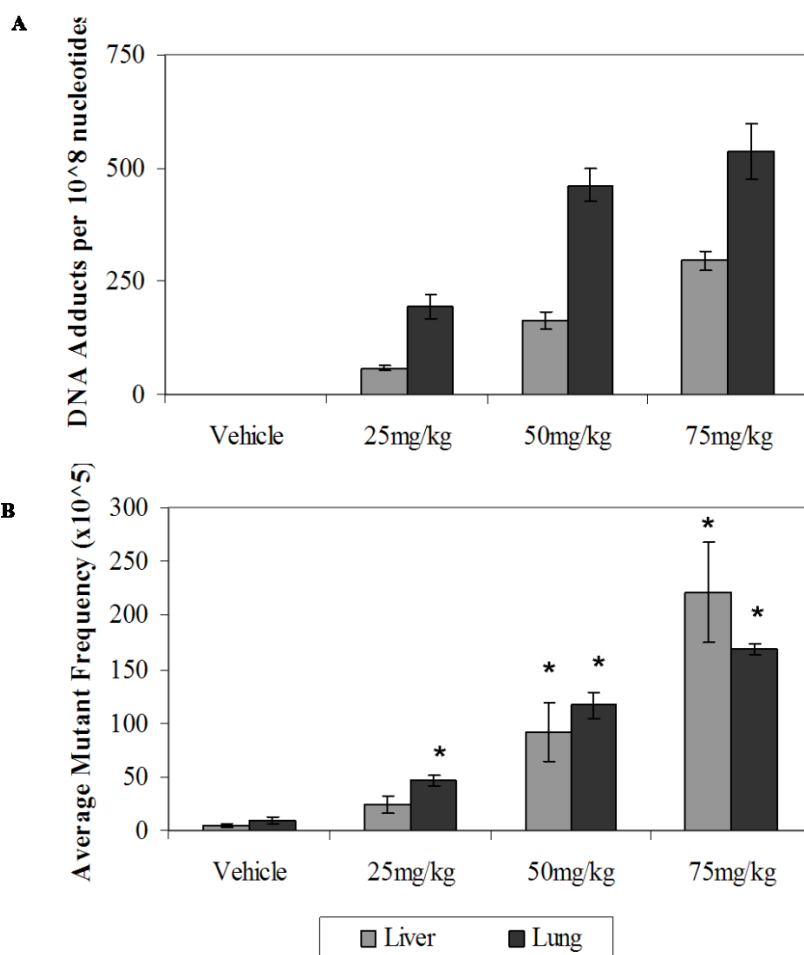


Figure 2-1 (a) DNA adduct formation and (b) *lacZ* mutant frequency in the lungs and livers from Muta™ Mouse subchronically exposed to BaP.

Levels of dG- N^2 -BPDE adducts were determined using the nuclease P1 enrichment version of the ^{32}P -postlabeling method. Data are represented as average $\pm$ SEM ($n = 5$ mice/group). ND, not detected. Average *lacZ* mutant frequency was determined using the P-Gal positive selection assay. Values shown are average frequencies $\times 10^5 \pm$ SEM. Asterisk (*) indicates significance by Student's t-test ($P \leq 0.01$) compared with controls. Transgene mutant frequency data for the liver are from the study by Malik et al. (2012).

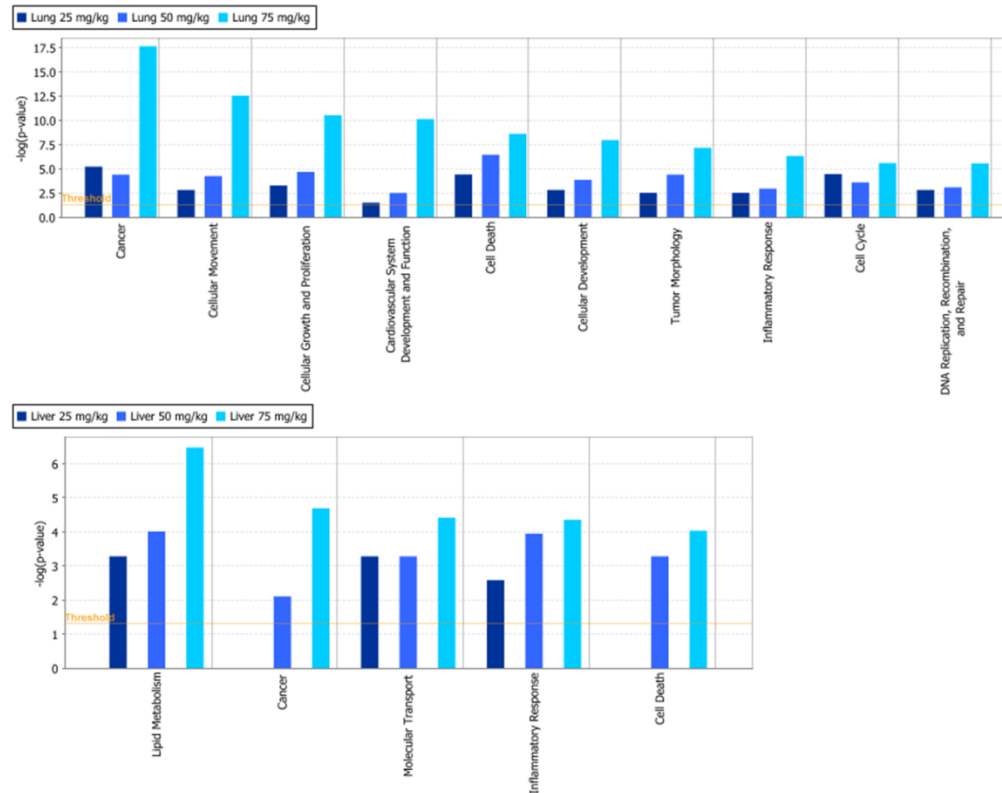


Figure 2-2 Functional analysis of differentially expressed genes using IPA for (a) lung and (b) liver.

The Ingenuity Pathway Analysis Knowledge Base was used to identify the biological functions and/or diseases that were significantly over-represented among the differentially expressed genes (FDR adjusted $P \leq 0.1$ and fold change ≥ 1.5 in either direction). Right-tailed Fisher's exact test was used to calculate a P value determining the probability that each biological function and/or disease assigned to that dataset was due to chance alone (threshold set at P value ≤ 0.05).

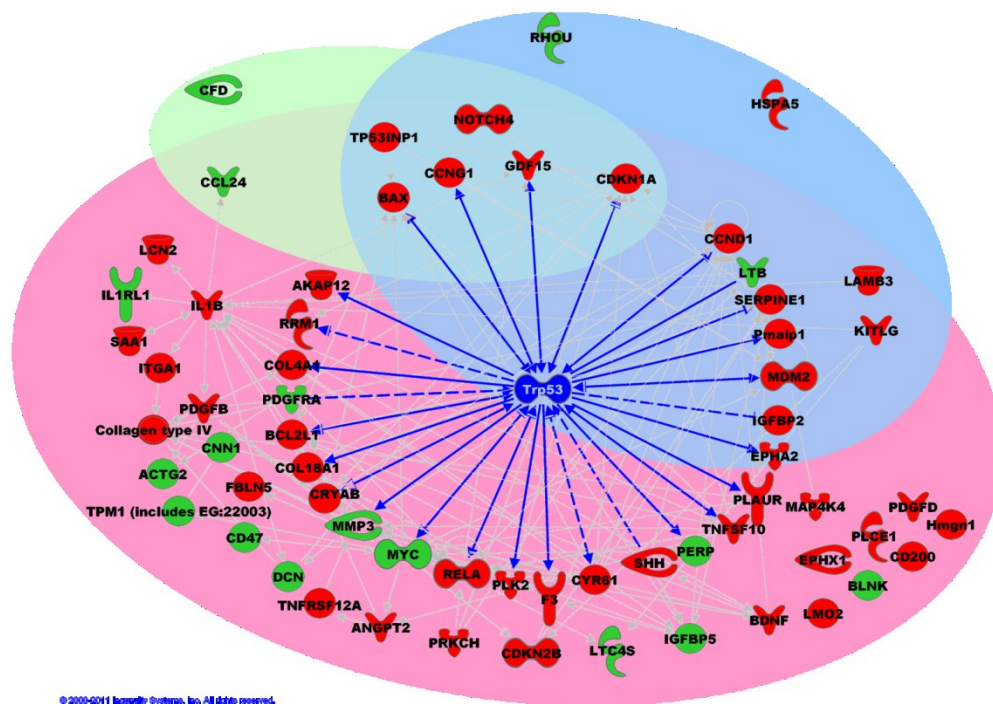


Figure 2-3 Molecular relational network of genes modulated by BaP in lung tissues enriched for cancer function using IPA for all three doses with p53 at the central node.

A network is a graphical representation of the molecular relationships between molecules. Molecules are represented as nodes and the biological relationship between nodes is represented as a line. The gene list was derived from cancer-associated genes determined in the Ingenuity Pathway Analysis functional analysis of significantly differentially expressed genes (Fig. 2). The networks were generated from cancer-associated genes differentially regulated in the 25 mg/kg-bw/day (green circle), 50 mg/kg-bw/day (blue circle), and 75 mg/kg-bw/day (pink circle) dose groups. Red color denotes upregulation and green color denotes downregulation.

3 Chapter: Toxicogenomic Outcomes Predictive of Forestomach

Carcinogenesis Following Exposure to Benzo(a)pyrene: Relevance to Human Cancer Risk

Modified from: S Labib, CH Guo, A Williams, CL Yauk, PA White, S Halappanavar. (2013). Toxicology and Applied Pharmacology. 273(1), 269-280. Reprinted with permission from Elsevier.

3.1 Abstract

Forestomach tumours are observed in mice exposed to environmental carcinogens. However, the relevance of this data to humans is controversial because humans lack a forestomach. We hypothesize that an understanding of early molecular changes after exposure to a carcinogen in the forestomach will provide mode-of-action information to evaluate the applicability of forestomach cancers to human cancer risk assessment. In the present study we exposed mice to benzo(a)pyrene (BaP), an environmental carcinogen commonly associated with tumours of the rodent forestomach. Toxicogenomics tools were used to profile gene expression response in the forestomach. Adult MutaTM Mouse males were orally exposed to 25, 50, and 75 mg BaP/kg-body-weight/day for 28 consecutive days. Forestomach was collected three days post-exposure. DNA microarrays, real-time RT-qPCR arrays, and protein analyses were employed to characterize responses in the forestomach. Microarray results showed altered expression of 414 genes across all treatment groups (± 1.5 fold; False Discovery Rate adjusted $P \leq 0.05$). Significant downregulation of genes associated with phase II xenobiotic metabolism and increased expression of genes implicated in antigen processing and presentation, immune

response, chemotaxis, and keratinocyte differentiation were observed in treated groups in a dose-dependent manner. A systematic comparison of the differentially expressed genes in the forestomach from the present study to differentially expressed genes identified in human diseases including human gastrointestinal tract cancers using the NextBio Human Disease Atlas showed significant commonalities between the two models. Our results provide molecular evidence supporting the use of the mouse forestomach model to evaluate chemically-induced gastrointestinal carcinogenesis in humans.

3.2 Introduction

A review of the Carcinogenic Potency Database (<http://toxnet.nlm.nih.gov/cpdb/>) and the US National Cancer Institute/National Toxicology Program (<http://ntp-server.niehs.nih.gov>) databases reveals that exposure to approximately 120 different substances results in the development of cancerous lesions in the rodent forestomach (Proctor et al., 2007). Indeed, the forestomach is a target for carcinogenesis in rodents following oral exposure to various environmental chemicals. However, the human health relevance of this type of cancer is unclear because humans lack a forestomach (Proctor et al., 2007).

Anatomically, the rodent forestomach exhibits similarities to both the human esophagus and stomach. Detailed analyses of cancers of the forestomach reveal that these tumours are initiated by hyperplasia of the forestomach squamous epithelial cells that form preneoplastic lesions (Fukushima et al., 1997). These lesions progress into benign papillomas and metastatic carcinomas over time, which histologically resemble squamous cell carcinomas of the human esophagus (Nyrén and Adami, 2002), stomach (Callery et al., 1985), colon (Landau et al., 2007), and anal canal (Szmulowicz and Wu, 2012). However, forestomach tumours are strictly of squamous cell origin, whereas esophageal cancers can also originate from glandular columnar

epithelial cells in the form of adenocarcinomas, suggesting multiple mechanisms in the development of esophageal tumours in humans (Proctor et al., 2007). In addition, more than 95% of human stomach cancers are adenocarcinomas that originate from glandular columnar epithelial cells or poorly differentiated cells (Tsukamoto et al., 2007), and are thus distinct from rodent forestomach tumours (Proctor et al., 2007). Although there appear to be some fundamental differences between the aforementioned tumour types, it seems reasonable to contend that the molecular initiating events leading to cancers in the rodent forestomach and the human stomach share commonalities. A detailed and systematic characterization of the carcinogenic modes of action in the rodent forestomach will provide an improved context for evaluating the biological relevance of rodent forestomach tumour data for human health risk assessment.

Benzo(a)pyrene (BaP), a known human carcinogen (IARC, 2012), is a polycyclic aromatic hydrocarbon (PAH) that is produced during the incomplete combustion of organic materials from various sources including wood and tobacco smoke, vehicle exhaust, residential heating, electric power, and cooking. Human exposure to BaP occurs primarily through oral consumption of BaP-containing foods (Hettmer-Frey and Travis, 1991). BaP requires metabolic activation by members of the cytochrome P450 family of enzymes, which can generate several DNA-reactive metabolites including BaP-7,8-dihydrodiol-9,10-epoxide (BPDE). These metabolites are capable of forming covalent adducts with proteins and DNA. If left unrepaired, DNA damage can cause mutations leading to impaired gene function. We and others have demonstrated the formation of DNA adducts following exposure to BaP in a variety of tissues, including the gastrointestinal tract (Lemieux et al., 2011), which is a primary site of contact following oral gavage, and also in distant organs, such as the lung and liver (Halappanavar et al., 2011; Labib et al., 2012; Malik et

al., 2012). In experimental animals, exposure to BaP via feed or gavage predominantly and consistently leads to the development of squamous cell papillomas in the forestomach that primarily originate from the epithelial cell lining (Culp et al., 1998; Wester et al., 2012). Although genotoxicity is the primary mode of action of BaP in virtually all tissues, we previously used toxicogenomics to demonstrate that the nature and extent of molecular responses at the gene and protein level are somewhat different between tissues (Halappanavar et al., 2011; Labib et al., 2012; Malik et al., 2012; Yauk et al., 2011). For example, a comparison of gene expression profiles in mouse lung and liver following acute BaP exposure (i.e. 150 or 300 mg/kg-body-weight/day by oral gavage for 3 days) revealed broad commonalities, including the activation of pathways involved in oxidative stress, xenobiotic metabolism, AhR signalling, and the DNA damage response (Halappanavar et al., 2011). However, significant inhibition of B-cell receptor signalling was a predominant perturbation uniquely found in the lungs of BaP-exposed mice (Halappanavar et al., 2011). In addition, the magnitude of the overall transcriptional response (fold changes and the total number of genes) is much greater in the lung than the liver. We also recently demonstrated that DNA adduct levels are higher in the lungs of BaP treated mice as compared with liver, but that mutation frequencies in these two tissues are quite similar (Labib et al., 2012). In alignment with our findings, Uno et al. (2004) showed tissue-specific roles of Cyp1a1 using Cyp1a1 knockout mice. These authors showed that Cyp1a1 expression in the intestine plays an important role in detoxification, whereas Cyp1a1 activation in the livers is associated with DNA damage-induced liver carcinogenesis (Uno et al., 2004). These studies collectively demonstrate that although genotoxicity is a primary component of the mode of action of BaP-mediated carcinogenesis, differences in the underlying molecular responses to BaP likely contribute to the observed tissue-specificity during tumourigenesis.

The present study employed genomics and bioinformatics tools to identify early molecular initiating events that contribute to tumourigenesis in the forestomach, with the overarching objective of exploring the biological relevance of forestomach tumours for human health risk assessment. More specifically, we employed global gene expression profiling of forestomach tissue from mice exposed to BaP at doses that are known to induce tumours in the mouse forestomach (Culp et al., 1998). Adult male MutaTMMouse were exposed to 25, 50, and 75 mg/kg-bw/day of BaP for 28 consecutive days via oral gavage, and the top dose has previously been shown to cause squamous cell carcinomas, papillomas, and hyperplasia in the forestomach (Hakura et al., 1998). Mice were sacrificed 3 days after the last exposure. Global transcription response was analyzed in detail in order to identify the expression changes in biological pathways associated with cancer formation in the forestomach. BaP-induced gene expression profiles were compared with transcriptomic profiles of human diseases (e.g., human gastrointestinal tract cancer) using the NextBio Human Disease Atlas.

3.3 Methods

3.3.1 *Animal treatment*

Mouse exposures and sample collection procedures are described in detail previously (Labib et al., 2012; Lemieux et al., 2011; Malik et al., 2012). The MutaTMMouse contains around 29 +/- 4 copies of λ gt10lacZ shuttle vector, a non-transcribed insert, stably integrated in the mouse genome (Shwed et al., 2010), thus permitting in vivo lacZ mutant frequency analysis. The transgenic insert, which contains lacZ, is employed as an in vivo mutation target. Briefly, 25-week old male MutaTMMouse were individually housed in plastic film isolators, provided with water and food (2012 Teklad Global standard rodent diet) ad libitum, and were subjected to a 12 hr light/12 hr dark cycle. Mice were divided into four experimental groups consisting of 5

animals each: 0 (control), 25 (low dose), 50 (medium dose) and 75 (high dose) mg/kg-bw/day of BaP (Sigma-Aldrich, Oakville, ON, Canada) dissolved in olive oil. Mice were exposed daily by oral gavage for 28 consecutive days. The control group received only olive oil, the vehicle control. We acknowledge that the doses used in the present study are high compared to the expected daily intake of BaP via food in the United States (Hattemer-Frey and Travis, 1991). However, these high doses permit a toxicogenomic investigation of toxicological mechanisms underlying BaP-induced effects at much early post-exposure time points. The animals were sacrificed by cardiac puncture under isoflurane anaesthesia on day 3 following the last exposure. The forestomach was excised, flash-frozen in liquid nitrogen, and stored at -80°C. Care and maintenance of the mice in this experiment were approved by the Health Canada Animal Care Committee.

3.3.2 Tissue RNA extraction and purification

Total RNA was isolated from a random section of the forestomach tissue as described in Labib et al. (2012). In brief, a small random section of the mouse forestomach was homogenized immediately in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using the Retsch Mixer MM 400. The RNA was isolated using chloroform and precipitated using isopropyl alcohol. The RNA was subsequently purified using RNeasy Mini Plus kits (Qiagen, Mississauga, ON, Canada). All RNA samples showed an A260/280 ratio between 2.0 and 2.2, and an A260/230 ratio between 1.7 and 2.3. The integrity of the RNA samples was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). All samples had an RNA integrity number above 6.6 and were all used for microarray analysis.

3.3.3 *Microarray hybridization and analysis*

Total RNA (200 ng) from each individual mouse forestomach sample in each treatment group and Universal Mouse Reference RNA (UMRR, Stratagene, Mississauga, ON, Canada) was used to synthesize cDNA and cyanine-labelled cRNA using the Agilent Linear Amplification Kit (Agilent Technologies Inc., Mississauga, ON, Canada). Cyanine-labeled cRNA was in vitro transcribed using T7 RNA polymerase and purified using RNeasy Mini Kits (Qiagen, Mississauga, ON, Canada); experimental samples were labelled with Cyanine-5 and the UMRR was labelled with Cyanine-3. 300 ng of labelled cRNA from each experimental sample was hybridized with the same amount of labelled reference RNA to Agilent Sureprint G3 Mouse GE 8x60K microarrays (Agilent Technologies Inc., Mississauga, ON, Canada) at 65°C overnight (17 hours) in the Agilent SureHyb hybridization chamber. The arrays were washed and scanned on an Agilent G2505B Scanner according to the manufacturer's recommendations. Data were extracted using Feature Extraction 10.7.3.1 (Agilent Technologies, Inc., Mississauga, ON, Canada).

Data normalization and analyses were conducted in the R environment (R-Development-Core-Team, 2010). Briefly, the background fluorescence was measured using the negative control 3xSLv1 probes; probes with median signal intensities less than the trimmed mean (trim = 5%) plus three trimmed standard deviations of 3xSLv1 probe were flagged as absent (i.e. within the background signal). Probes were considered present if at least four of the five samples within a condition had signal intensities greater than three trimmed standard deviations above the trimmed mean of the 3xSLv1 probes (background signal). Data were normalized using LOWESS (Yang et al., 2002), and ratio intensity plots and heat maps for the raw and normalized data were constructed to identify outliers. Three samples were removed from the analysis based on clustering (one sample in each of the 25, 50, and 75 mg/kg-bw/day dose groups). The final

sample size consisted of n=5 in control, n=4 in 25 mg/kg-bw/day, n=4 in 50 mg/kg-bw/day, n=4 in 75 mg/kg-bw/day groups. Differentially expressed transcripts (upregulated or downregulated relative to olive oil treated control mouse forestomach) were determined using the MAANOVA library in R. The statistical model included the fixed effects slide and treatment condition, and was applied to the log2 of the relative intensities. The Fs statistic (Cui et al., 2005) was used to test for treatment effects. The P values for all statistical tests were estimated by the permutation method using residual shuffling, followed by adjustment for multiple comparisons using the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995). The fold change calculations were estimated as described previously (Malik et al., 2012). Significant genes were selected based on FDR-adjusted P value ($\text{FDR } P \leq 0.05$ for any BaP exposed versus control contrast. All microarray data have been deposited in the NCBI Gene Expression Omnibus database and can be accessed through the accession number GSE43438.

3.3.4 *Bioinformatics and pathway analysis*

Following microarray normalization, all genes that passed the $\text{FDR } P \leq 0.05$ and fold change ≥ 1.5 cut-off were considered for further analysis using various bioinformatics and pathway analysis tools to identify the biological functions or processes in the forestomach perturbed in response to BaP. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang da et al., 2009a; Huang da et al., 2009b) Functional Annotation Tools (clustering and charts) were used to identify gene ontology (GO) terms associated with the differentially expressed gene list. Genes significantly differentially expressed in each of the significant clusters were plotted on polar plots using SigmaPlot for Windows version 12.0 build 12.0.0.182. In addition to the functional classification, DAVID was used to visualize the differentially expressed genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG)

pathways. The biological and molecular functions of the significantly differentially expressed genes following BaP treatment in the forestomach were further analyzed and explored in Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) and MetaCore (Thomson Reuters, <http://www.genego.com/metacore.php>). The significance of the association between the data set and the canonical pathways and functions in IPA was measured using the Fisher's exact test. IPA Upstream Regulator Analysis was used to identify the cascade of upstream transcriptional regulators that explain the observed gene expression changes in the forestomach. Only regulators with a Z-score above 2.0 were considered in this analysis. MetaCore process networks identified the networks associated with GO processes that were most significant in this dataset.

3.3.5 Mining of public databases for human disease correlation

All statistically significant and differentially expressed genes from all dose groups were mined against the genomics data repositories in NextBio (<http://nextbio.com>). Disease prediction was performed using the 75 mg/kg-bw/day dose group. Data were compared to curated datasets available in NextBio to identify published studies of diseases with similar gene profiles, gene ranking, and consistency in fold change directionality. Pairwise gene signature correlations and rank-based enrichment statistics were employed to calculate the NextBio scores for each disease. The disease that ranked the highest in comparison with BaP exposure was given a score of 100 and the rest were normalized accordingly. The meta-analysis function was used to compare the significantly differentially expressed genes associated with the most enriched gene ontologies from BaP exposed forestomach to 59 gastrointestinal tract cancer biosets from curated databases available in NextBio (Supplementary Table 3-1), including esophageal cancers, gastric cancers, intestinal cancers, and colon cancers.

3.3.6 Quantitative Real Time (qRT)-PCR array validation

Eighty-four genes were selected for further validation by quantitative real-time RT-qPCR using custom RT² Profiler PCR Arrays and a BioRad CFX96 Real-Time PCR detection system. Genes were selected based on their statistical significance, and their relevance to biological phenomena of interest and relevance to the project. A custom RT² Profiler PCR Array plate, the RT² First Strand Kit and RT² SYBR[®] Green qPCR Mastermix (QIAGEN Sciences, Maryland, USA) were used. Reference genes for normalization were selected based on their stable expression levels in the treated and control samples. Ct values for each gene were normalized to actin β (Actb) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh), the most stable reference genes. A threshold value was set to 10². Only samples that were included in the microarray analysis were used for validation. Furthermore, an additional 2 samples (one in each of the 50 and 75 mg/kg-bw/day dose groups) were removed based on improper clustering. Given this criteria, the final validation group consisted of sample size of n=5 in control, n=4 in 25 mg/kg-bw/day, n=3 in 50 mg/kg-bw/day, n=3 in 75 mg/kg-bw/day groups.

3.3.7 Western blot analysis

Total protein from the frozen mouse forestomach experimental and control tissues was extracted using Bio-Plex Cell Lysis Kits (BioRad Laboratories, Mississauga, ON, Canada) containing a protease inhibitor cocktail (1:200) (Sigma Aldrich, Oakville, ON, Canada) and a phosphatase inhibitor cocktail (PhosSTOP, ROCHE, Laval, QC, Canada). Total protein was quantified using a Bradford protein assay kit (BioRad Laboratories, Mississauga, ON, Canada). 30-50 μ g of total protein was run on 4-15% SDS/PAGE gels (BioRad Laboratories, Mississauga, ON, Canada) and was transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) by wet transfer for 1 hour at 100V. Anti-proteasome 20S LMP2 (1 μ g/ml; Abcam Inc., Toronto,

ON, Canada) and anti-MECL-1 (E20) (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used to test for immunoproteasome activation. Anti-20S proteasome β 1 (FL-241) (1:200; Santa Cruz Biotechnology) and anti-proteasome 20S β 2 subunit (MCP165) (1:1000; Enzo Life Sciences, Brockville, ON, Canada) were used to monitor changes in proteasome composition. All expression values were normalized relative to β -actin (1:2,500; Cell Signaling Technologies, Danvers, MA, USA) in Image Lab software version 4.1 build 16 (BioRad Laboratories, Mississauga, ON, Canada).

3.4 Results

Daily exposure to 25, 50, and 75 mg/kg-bw/day BaP for 28 consecutive days elicited no overt signs of toxicity and there was no significant loss in body weight for any of the exposed mice compared to vehicle-treated controls (Lemieux et al., 2011).

3.4.1 *General overview of forestomach gene expression profiles*

Repeated oral gavage with BaP induced significant changes in the expression of many genes in the forestomach. MAANOVA analysis revealed a total of 497 probes associated with 414 specific genes differentially expressed (up- or down-regulated) with a fold change ≥ 1.5 in either direction, and an FDR $P \leq 0.05$ in at least one treatment group (Supplementary Table 3-2).

Compared to the vehicle controls, 12, 158, and 487 unique probes associated with 9, 135, and 408 unique genes were significantly differentially expressed in the 25, 50, and 75 mg/kg-bw/day dose groups, respectively (Supplementary Figure 3-1). Hierarchical cluster analysis of all significant, differentially expressed genes (FDR $P \leq 0.05$ and fold change ≥ 1.5) revealed that the treatment groups clustered separately from controls, thus a clear treatment effect was observed as a result of exposure to BaP (Supplementary Figure 3-2).

3.4.2 *Biological function and pathway analysis of significantly differentially expressed genes*

GO and pathway analyses were employed to assign biological processes and functional categories to genes whose expression was significantly affected by BaP in DAVID, KEGG, IPA, and MetaCore.

Using the functional annotation tool in DAVID, significantly differentially expressed genes were categorized into specific biological processes, cellular compartments, and molecular functions. The resulting clusters consisting of GO terms were sorted based on DAVID enrichment score and were considered significant only if they exhibited Benjamini-corrected $P \leq 0.05$ (Figure 3-1). With this cut-off, four main GO clusters were identified: antigen processing and presentation [GO:0019882], immune response [GO:0006955], chemotaxis [GO:0006935], and epithelial cell differentiation [GO:0030855]. Each of these biological processes was further expanded to identify the extent of enrichment and coverage.

The immune response classification consisted of 27 and 45 genes in the 50 and 75 mg/kg-bw/day dose groups, respectively, and was represented by defense response [GO:0006952], immune response [GO:0006955], and response to virus [GO:0009615]. The antigen processing and presentation classification was enriched with a total of 3, 14, and 40 genes in the 25, 50, and 75 mg/kg-bw/day dose groups, respectively. Within this classification, processes such as antigen processing and presentation *via* major histocompatibility complex (MHC) class I [GO:0002474], response to virus [GO:0009615], cell surface [GO:0009986], antigen processing and presentation [GO:0019882], MHC protein complex [GO:0042611], MHC class I protein complex [GO:0042612], antigen processing and presentation of peptide antigen [GO:0048002] were over represented. The epithelial cell differentiation consisted of 10 and 19 differentially expressed genes in the 50 and 75 mg/kg-bw/day dose groups, respectively, and was enriched with GO

terms such as cornified envelope [GO:0001533], ectoderm development [GO:0007398], epidermis development [GO:0008544], epidermal cell differentiation [GO:0009913], keratinocyte differentiation [GO:0030216], epithelial cell differentiation [GO:0030855], keratinization [GO:0031424], intermediate filament-based process [GO:0045103], intermediate filament cytoskeleton organization [GO:0045104], and epithelial development [GO:0060429]. Lastly, the chemotaxis classification was associated with 2, 5, and 24 genes in the 25, 50, and 75 mg/kg-bw/day dose groups, respectively, and was represented by chemotaxis [GO:0006935], inflammatory response [GO:0006954], chemokine activity [GO:0008009], response to wounding [GO:0009611], taxis [GO:0042330], and chemokine receptor binding [GO:0042379]. Detailed analysis of the entire list of differentially expressed genes implicated in these processes revealed a toxic response gradient involving activation of immune response and inflammation, thus reflecting a host defense response to BaP exposure. Induction of the keratinization process was observed primarily in the high dose group and is suggestive of activation of cellular transformation processes normally observed during carcinogenic transformation. Figure 3-1 demonstrates this gradual transition. GO clusters in Figure 1 display genes associated with all child GO terms. Genes common to more than one GO cluster are displayed with the most biologically relevant cluster.

Pathway analysis of all the differentially expressed genes in all dose groups was conducted using KEGG pathways in DAVID, IPA Canonical Pathways, and MetaCore Network Processes (Supplementary Figure 3-3). A total of seven KEGG pathways showing a significant (Benjamini corrected $P \leq 0.05$) enrichment according to DAVID, ten IPA canonical pathways ($P \leq 0.05$), and nine MetaCore network processes ($P \leq 0.05$), were significantly enriched in at least two treatment groups (Supplementary Figure 3-3). Unique pathways in each dose group included

inflammation-related signaling at the 25 mg/kg-bw/day dose group, natural killer T (NKT) cell mediated cytotoxicity at the 50 mg/kg-bw/day dose group, and p53 signaling and glutathione metabolism at the 75 mg/kg-bw/day dose group. Supplementary Figure 3 shows all of the pathways identified using each analysis tool.

Detailed analysis in IPA revealed that all of the differentially expressed genes and the associated biological processes are regulated by a finite number of upstream regulators, including cytokines, transcriptional factors, kinases, and receptors (Supplementary Table 3-3). The primary upstream regulator was interferon γ (IFN γ), which is a cytokine with immunoregulatory properties. IFN γ was associated with 69 differentially expressed genes in our dataset, and the expression profiles of these genes reflect IFN γ activation (Figure 3-2). The analysis also revealed the inhibition of the transcriptional regulator tripartite motif containing 24 (Trim24), which is associated with 28 differentially expressed genes, and p53, which is associated with 21 genes.

3.4.3 Disease prediction

In order to identify the expression patterns in BaP-exposed forestomach that may be relevant to human diseases, differentially expressed genes from the 75 mg/kg-bw/day BaP dose group were analyzed using the NextBio disease atlas. This analysis revealed bacterial infectious diseases, respiratory disorders, parasitic diseases, and viral infectious diseases as the top four human diseases correlated with mouse BaP exposed forestomach expression profiles (Supplementary Table 3-4). Cancer was the 18th most correlated disease, supported by 641 studies. We then queried all of the significant genes (FDR $P \leq 0.05$ and fold change ≥ 1.5) in BaP-treated forestomach associated with antigen processing and presentation, immune response, chemotaxis, and keratinocyte differentiation against curated studies in NextBio related to human esophageal

(score 60), gastric (score 15), intestinal (score 64), and colon (score 38) cancers. Several genes associated with the aforementioned top GO processes were common between the BaP exposed forestomach responses and the aforementioned types of human cancers (Table 3-1), suggesting strong similarity between gene expression in the BaP-exposed rodent forestomach and various human cancers.

3.4.4 qRT-PCR Validation of Representative Genes

A total of 84 genes were validated using RT-qPCR arrays (Supplementary Table 3-5). These genes included: (a) 54 involved in antigen processing and presentation, immune and inflammatory response, and keratinocyte differentiation; (b) seven involved in xenobiotic metabolism; (c) eight involved in DNA damage response; (d) three growth factors; and (e) 12 genes showing the highest fold changes.

Fifty six of the 84 genes were differentially expressed with $P \leq 0.05$ in at least one treatment group compared to matched controls (Table 3-2) including: 16 related to antigen processing and presentation, 14 in the immune and inflammatory processes, 14 involved in keratinocyte differentiation, six in DNA damage response pathways, and six involved in xenobiotic metabolism. Three genes showed statistically significant differential expression by RT-qPCR, but not by microarrays. These included murine double minute 2 (*Mdm2*), C-type lectin domain family 2 member g (*Clec2g*), and amphiregulin (*Areg*).

3.4.5 Protein Validation

In addition to the changes described above, our gene expression analysis also revealed distinct upregulation of immunoproteasome subunits. Specifically, proteasome subunit beta type 9 (Psmb9: 2.8 and 3.5-fold change in 50 and 75 mg/kg-bw/day dose groups, respectively) and 10 (Pmb10: 1.6 and 1.5-fold change in 50 and 75 mg/kg-bw/day dose groups, respectively) were

perturbed by oral BaP exposure. These genes encode the immunoproteasome catalytic subunits $\beta 1i$ and $\beta 2i$, respectively, which are activated in place of the standard proteasome subunits $\beta 1$ and $\beta 2$ (Ferrington and Gregerson, 2012) under conditions of oxidative stress or IFN γ induction (Heink et al., 2005). Since immunoproteasome activation is specific to the presentation of MHC class I antigens, and since they are the upstream regulators of the immune response pathways altered in the present study, we investigated the biological relevance of the observed changes in Psmb9 and Psmb10 gene expression at the protein synthesis level. Western blot analysis showed a dose-dependent increase in immunoproteasome subunits $\beta 1i$ (from 1.7–3.6 fold) and $\beta 2i$ (from 1.9–4.6-fold) protein expression relative to matched controls, respectively (Figure 3-3). However, we saw no change in subunits $\beta 1$ and $\beta 2$, the normal proteasome subunits, suggesting specific BaP-induced activation of immunoproteasome in the forestomach.

3.5 Discussion

We analyzed global gene expression profiles in the forestomach of mice exposed to the genotoxic carcinogen BaP in order to explore the molecular mechanisms associated with forestomach cancer. Mice were exposed via oral gavage for 28 consecutive days to 25, 50 and 75 mg/kg-bw/day BaP. The top dose is known to induce forestomach tumours in the mouse strain examined (Hakura et al., 1998). Systematic analysis of all differentially expressed genes revealed three major features: 1) activation of DNA damage response pathways; 2) activation of immune response including antigen presentation pathways and immunoproteasome; and 3) potential for oncogenic transformation reflected by induction of genes involved in keratinization in the high dose. We present results that delineate the molecular dynamics of dose-dependent transitions in the response to BaP, explore the relevance of the BaP-induced gene expression profiles to human

disease, and finally, discuss the applicability of rodent forestomach tumour data for human health risk assessment.

3.5.1 BaP-induced DNA damage response in the forestomach

The most established mechanism underlying BaP-induced carcinogenesis involves the metabolic activation of BaP, and subsequent interaction of BaP metabolites with DNA, leading to DNA adducts and mutations in tumour suppressor genes or oncogenes (IARC, 2012). However, rates of BaP metabolism, levels of DNA adducts, and mutation frequencies do not correlate with the extent of tumour formation in the tissues studied (Godschalk et al., 2003), suggesting that additional tissue-specific factors may contribute to carcinogenic events. Although we did not directly measure DNA adducts in the forestomach, a previous study by our group did note a dose-dependent increase in DNA adducts and lacZ mutant frequency in the glandular stomach of mice exposed to BaP (Lemieux et al., 2011), and previous studies have established that levels of DNA adducts in the glandular stomach and forestomach from mice orally exposed to BaP for 24 hours and five days are comparable (Arlt et al., 2008). Although the finding of Arlt et al. (2008) refer to acute exposure effects, it should be acknowledged that the differences between the rate of cellular proliferation and the metabolic capacity to form DNA-reactive BaP metabolites in the forestomach and glandular stomach, could all influence the extent of DNA damage and the outcome following 28 days of repeated exposure. For instance, basal cellular proliferation rates, as determined by BrdU incorporation and/or Ki67 staining (marker of proliferation) in the forestomach are approximately 50-67% that of the glandular stomach (Shibata et al., 1990; Tanaka et al., 2011). While not the same endpoint, other studies have used hyperplasia as marker of increased cellular proliferation, and have shown increased hyperplasia in the forestomach following BaP oral exposure. Other studies comparing cellular proliferation rates after oral

exposure to okadaic acid-like compounds (non-PAHs) have shown higher proliferation levels in the forestomach compared to the glandular stomach (Yuasa et al., 1994). These results imply that upon stimulation by BaP for 28 consecutive days, cellular proliferation could be expected to significantly increase in the forestomach compared to 24 hours or five days of exposure. Another determinant of the potential differences between the two tissues that plays an important role in the DNA damage induced by BaP could be differences in phase I metabolism by cytochrome P450 enzymes, which is necessary for the generation of DNA reactive BaP metabolites, such as BPDE. Indeed, it has been shown that, following five days of oral BaP exposure, the mRNA and protein levels of Cyp1a1 and Cyp1b1 are higher in the forestomach compared to the glandular stomach (Uno et al., 2008). In toto, we can reasonably assert that following 28 days of repeated exposure, due, at least in part, to plausible increases in the cellular proliferation and higher metabolic capacity, the forestomach would be expected to show comparatively higher DNA adduct levels and increases in the lacZ mutant frequency. However, further studies are needed to conclusively define the mechanisms underlying the similarities and differences in the responses of forestomach and glandular stomach to oral PAH exposures.

In alignment with the observed levels of DNA adducts and induction of mutations in the glandular stomach, analysis of all differentially expressed genes and perturbed biological pathways in the forestomach revealed activation of the DNA damage response. The cellular DNA damage response to BaP exposure is regulated by p53 (Park et al., 2006), a transcription factor that suppresses tumour formation by modulating the expression of genes involved in cell cycle control, DNA repair, inflammatory modulators, and growth factors. Our previous work has shown that the p53-regulated DNA damage response is a primary pathway induced in the lungs and the livers of the same mice as those studied here (Labib et al., 2012; Malik et al., 2012).

Genes within the p53-pathway exhibited a dose-dependent response in liver and lung, indicating the important role of p53 mediated responses in coping with increasing amounts of DNA damage in these tissues. Analysis of the forestomach transcriptome revealed alteration of 21 p53-responsive genes (Figure 3-2) in all of the doses combined. Seven of these genes are directly involved in the DNA damage response, including the cell cycle regulatory genes cyclin dependent kinase inhibitor 1a (Cdkn1a) and cyclin-G1 (Ccng1), the DNA repair enzyme O-6-methylguanine-DNA methyltransferase (Mgmt), and pro-apoptotic genes Pmaip1, Trp53inp1, Trp3, and Trp73. The p53 transcription factor was ranked 47th of 139 upstream regulators in the analyses conducted here (see Supplementary Table 3-3). These results imply that the DNA damage and activation of DNA repair mechanisms may be involved in the onset and progression of cancer in the forestomach; however, additional perturbations of other biological processes such as activation of immune response, and antigen processing and presentation pathways are likely contributing to the development and progression of forestomach tumours.

3.5.2 BaP activates antigen processing and presentation pathway

The other predominantly affected pathways in BaP-exposed forestomach involved the activation of genes involved in immunoproteasome activation, immune response, inflammatory response, and antigen presentation. Specifically, significant enrichment was observed for MHC class genes involved in antigen processing and presentation (Table 3-2). MHC molecules (class I and II) are cell surface glycoproteins that are necessary for the presentation of peptide antigens to T lymphocytes, including T helper cells, cytotoxic T lymphocytes, and NKT cells (Garcia-Lora et al., 2003). Antigenic peptides are generated from degraded endogenous proteins during lysis or degradation of an invading pathogen's cellular components by specialized multi-catalytic protease complexes called the immunoproteasomes (Ferrington and Gregerson, 2012). Antigen

presentation is the mechanism by which MHC class molecules bind antigens in the form of peptides on the surface of bacterial or virus-infected cells and present them for recognition and removal by the immune cells. Activated MHC class I molecules in nucleated cells and MHC class II molecules on antigen presenting cells and lymphocytes initiate activation of cytotoxic T lymphocytes and NKT cells (class I), and T helper cells (class II). Thus, initiated T cell-mediated signaling leads to a cascade of events ranging from cytokine production, cellular proliferation, and target cell lysis (Weltzien et al., 1996). Excessive T cell activation and cytokine production will ultimately lead to apoptosis of cells probably consisting of cellular debris or chemical metabolites. Phagocytic engulfment of such damaged cells will ultimately lead to the efficient removal of the threat.

Various chemicals trigger an immune response via T cell activation (Weltzien et al., 1996). Several studies have demonstrated the activation of T cells following exposure to BaP. For example, peritoneal macrophages from mice exposed to BaP are shown to have an increased ability to present MHC class II antigens (Myers et al., 1987). Anderson et al. (1995) showed that AhR binding and phase I metabolism of dimethylbenz(a)anthracene (another immunogenic PAH) is essential for the induction of immune response involving CD8⁺T cells that is mediated by MHC class I molecules in C3G/HeN mice. Transcriptomic profiling of peripheral blood mononuclear cells from healthy 25-30 year-old non-smoking humans exposed for 20 hours to three concentrations (10-fold dilution series) of eight genotoxic carcinogens (4-hydroxy-2-nonenal, malondialdehyde, acrylamide, 2-amino-3-methyl-3H-imidazo[4,5-F]quinolone, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, aflatoxin B1, dimethylnitrosamine, and BaP) and three non-genotoxic carcinogens (ethanol, polychlorinated biphenyl 153, and 2,3,7,8-tetrachlorodibenzo-p-dioxin), showed that genes related to immune response activation were

uniquely found in the transcriptomic fingerprints associated with eight genotoxic carcinogens, including BaP (Hochstenbach et al., 2012). These results suggest that this type of MHC class-induced response is common to a diverse array of xenobiotics, and they raise important questions about the role of immune responses in the recognition and removal of toxicants.

3.5.3 MHC class I-mediated detoxification of BaP

Cellular debris arising from the degradation of invading pathogens generates antigenic peptides that are bound by MHC molecules and presented to the immune cells for recognition and removal by the phagocytes; however, it isn't clear why or how xenobiotics, or BaP in particular, stimulate this response. What is clear is that the metabolism of PAHs is a prerequisite for activation of the immune response (Anderson et al., 1995). Therefore, one possibility is that the accumulation of DNA- or protein- reactive metabolites stimulates the generation of antigenic peptides. The initial metabolism of BaP involves its oxidation to epoxides by members of the cytochrome P450 family of enzymes (IARC, 2012) that are further metabolized by epoxide hydrolases into reactive BaP diol epoxides that can accumulate if not removed immediately. Downregulation of the phase II enzymes sulfotransferase family 1E member 1 and UDP-glucuronosyltransferase 2 family polypeptide B34, which conjugate a sulfonyl group and glucuronic acid to electrophilic phase I BaP metabolites, respectively, that was observed in the forestomach of mice treated with high doses of BaP, could contribute to the accumulation of hydrophobic reactive metabolites. If the metabolites are not cleared efficiently, they can form covalent adducts with DNA and protein, the latter of which can induce BaP-specific antibody production and immune response (Schellenberger et al., 2012). BPDE can also bind the tripeptide glutathione (GSH), the most abundant cellular antioxidant, leading to its depletion. Glutathione-s-transferases (GST) mediate the formation of GSH-BPDE adducts by catalyzing the

binding of the sulfhydryl group of GSH to electrophilic BaP metabolites (Dostal et al., 1988; Singh et al., 2004). Although the process of GSH conjugation effectively decreases the cellular pool of reactive BaP metabolites, excessive binding of GSH to BPDE depletes the cellular pool of free glutathione by 20 to 30% (Romero et al., 1997), and this can signal increased activation of MHC class I antigen presentation (Makhadiyeva et al., 2012). MHC molecules can bind these GSH-BPDE adducts or BPDE directly and present them to phagocytes for their ultimate removal. In toto, these arguments suggest that activation of MHC class I molecules in the forestomach following oral BaP exposure serves primarily as a detoxification mechanism.

Another interesting detoxification pathway in the forestomach of BaP-treated mice appears to involve the immunoproteasome. The primary role of the normal proteasomal degradation machinery is the removal of abnormally folded proteins, peptides, cellular debris, and adducted proteins (Hill et al., 2007; Sommerburg et al., 2009). The immunoproteasome is a specialized proteasome complex activated during an immune response that plays a specific role in the generation of peptides that can fit into MHC class I molecules, leading to the proteasome-mediated degradation of cellular debris (Ferrington and Gregerson, 2012; Krüger and Klotzel, 2012). Significant and specific increases in the expression of genes encoding the $\beta 1i$ and $\beta 2i$ subunits (Psm9 and Psm10, respectively) and the corresponding protein products (in the higher dose groups), supports the idea that immune response-mediated removal of BaP plays a major role in the forestomach. In support of this, Schellenberger et al. (2012) showed that BaP-peptide adducts induce the production of BaP-specific antibodies, whereas BaP bound to bulky proteins results in an immune response directed primarily against the protein rather than against BaP (Grova et al., 2009). Antibodies generated against the peptide adduct complexes are able to sequester BaP and its metabolites, resulting in reduced excretion in the urine and feces

(Schellenberger et al., 2012). These results collectively suggest that the immune response is a predominant route by which BaP is detoxified in the forestomach, and that this process involves activation of the immunoproteasome and immune response. Figure 4 shows the systematic arrangement of differentially expressed genes in the BaP treated forestomach depicting the sequential activation of the aforementioned processes that we propose lead to the removal of BaP.

3.5.4 Human relevance

In order to investigate the relevance of gene expression changes in the rodent forestomach to effects that relate to human health, we conducted meta-analyses of the altered gene sets (Figure 1, antigen processing and presentation, immune response, chemotaxis, and keratinocyte differentiation) using the NextBio Human Disease Atlas tool. The analysis revealed a strong similarity between the dataset of the present study and genes altered during human bacterial infectious diseases, parasitic disease, respiratory disease, and viral infectious disease (Supplementary Table 3-4), supported by 85, 82, 27, and 117 studies, respectively. Melo and Ruvkun (2012) used the nematode *C elegans* model to demonstrate that exposure to chemicals/xenobiotics or pathogens leads to disruption of the same essential cellular activities such as mitochondrial function, ribosome activities, protein translation, mRNA processing, etc. These disruptions collectively serve as the physiological cues that lead to activation of defence mechanisms, including detoxification pathways and immune responses. The results presented here suggest that the recognition and removal of BaP and/or its reactive metabolites from the forestomach involves the same pathogen recognition mechanisms that defend the host against microbial attack.

The other highlight of the present data set is the significant upregulation of a large number of genes involved in keratinocyte differentiation (Table 3-2), especially at the 50 and 75 mg/kg-bw/day doses (Figure 3-1). We observed dose-dependent increases in the expression of numerous keratins including *Krt6a*, *Krt6b*, *Krt12*, *Krt17*, and *Krt18*, as well as factors that are involved in the promotion of keratinocyte differentiation, such as small proline-rich proteins *Sprr1b*, *Sprr2a2*, *Sprr2d*, *Sprr2e*, *Sprr2g*, *Sprr2h*, *Sprr2i*, and *Sprr2k*. Analysis of this gene set in the NextBio Human Disease Atlas Tool showed strong similarities between the BaP-exposed forestomachs and malignant tumours of the intestine and gastric cancers in humans (Table 3-1).

In the forestomach, the transformation of cuboidal epithelial cells into stratified squamous cells, which produce large amounts of keratins, is the first indication of initiation of carcinogenic events (Kossoy et al., 2006). The process is referred to as keratinocyte differentiation and is routinely observed during the transformation of stomach epithelial cells in adenocarcinomas caused by *Helicobacter pylori* (*H. pylori*) infection in humans (where chronic infection eventually leads to intestinal metaplasia) in humans. Keratins in squamous epithelia serve to protect the cells from injury and are regulated by many cancer-promoting stimuli to promote cell growth, survival, and motility. Excessive levels of keratins are strongly associated with the development of various human cancers (Cao et al., 2011; Mosca et al., 2010; Somji et al., 2008) and are correlated with poor clinical outcome (Ide et al., 2012). A thick layer of keratin is noted on pre-malignant papillomas and on the epithelial surface of the forestomach in BaP fed mice (Rigdon and Neal, 1966; Rigdon and Neal, 1969). Enhanced keratinocyte differentiation and dysplasia is also observed in mice exposed to dimethylbenz(a)anthracene (Kossoy et al., 2006) via feed. Dimethylbenz(a)anthracene, an alkyl-substituted PAH, has also been shown to induce similar carcinogenic effects in the forestomach of exposed mice. In another study, Shi et al.

(2010) found squamous cell carcinoma in the preputial gland duct of Cyp1a1 and Cyp1b1 double knockout mice following 12 weeks of oral BaP exposure that showed increased levels of keratin deposition. In a follow-up study using microarrays, these authors further demonstrated a significant upregulation of genes associated with keratinization in the preputial gland duct after 8 weeks of exposure to BaP (Gálvez-Peralta et al., 2013). Although the role of keratinocyte differentiation in carcinogenesis is not well understood, Krt17 is known to positively regulate cell size and growth of keratinocytes by binding to the adaptor protein 14-3-3 sigma and by stimulating mTOR signaling (Kim et al., 2006), which is involved in sustaining proliferative signaling (a hallmark of cancer) (Hanahan and Weinberg, 2011). These results collectively suggest that the key events leading to cancer are different in the forestomach and somewhat distinct from those observed in other tissues, and include a strong immune response, persistent inflammation, and initiation of keratinocyte differentiation. The strong similarities observed between our data set and those that are associated with human stomach diseases, including cancers, provides evidence for use of forestomach tumour data for assessments of human cancer risks associated with PAHs such as BaP.

An acknowledged shortcoming of the present study is that we did not compare the transcriptomic responses between the forestomach and glandular stomach, target and non-target organs for BaP-induced cancers in rodents. Although the rodent glandular stomach is analogous to the human gastric stomach, it does not develop tumours after exposure to high doses of BaP (oral, intraperitoneal, or intramammary route) that induce significant DNA damage. Out of the 570 two-year rodent cancer bio-assays conducted by the National Toxicology Program (NTP), very few resulted in glandular stomach cancers (Chandra et al., 2010). Therefore, it is quite possible that we will see different transcriptomic profiles in the two tissues; however, we do

expect to see similar changes in genes that respond to DNA damage. Our previous work comparing transcriptomic responses in lung and liver tissues exposed to BaP have rightly pointed out the distinct modes of action within each tissue that relate to their inherent function. For example, despite comparable levels of DNA damage between the lung and liver tissues, our findings showed a rather subtle transcriptomic response in the liver (target organ for carcinogenesis by BaP) that was not directly suggestive of cancer even at the higher doses. On the other hand, transcriptomics did reveal changes in the expression of important DNA damage responsive genes consistent with the observed DNA adducts in the liver. In the lung tissue, a dramatic response was observed at all doses tested, with incremental dose-related changes related to carcinogenesis. These results suggest that the inherent functions of tissues, and the concomitant balance between biological pathways leading to enhanced cellular proliferation and pathways leading to cell death, play important roles in chemically-induced carcinogenesis. Based on these arguments we should expect to see 1) changes in the expression of DNA damage response genes in the glandular stomach similar to those observed in the forestomach, lung (Labib et al., 2012), and liver (Malik et al., 2012) of mice exposed to BaP, and 2) parallel activation of cellular proliferation and cell death genes. In addition, in accordance with the reported mechanisms underlying the formation of glandular stomach tumours (i.e. the inhibition of gastric acid secretion leading to hypergastrinemia and hyperplasia (Chandra et al., 2010)), we may expect to observe induction of pathways counteracting these processes. Since due to lack of available tissue, the present study could not provide evidence to substantiate the aforementioned assertions, additional experiments are warranted.

3.5.5 Conclusion

We present a model for the rodent forestomach responses to BaP and propose key pathways that we believe underlie BaP-induced forestomach carcinogenesis. We find that the gene expression signatures in BaP-exposed forestomach are broadly similar to human cancers of the gastrointestinal tract, and that general detoxification pathways including sulfation and glucuronidation are negatively regulated in the BaP-exposed forestomachs, potentially resulting in the accumulation of BaP and BaP metabolites. Excessive accumulation of DNA- or protein-reactive metabolites then serve as cues leading to the activation of other detoxification/defence mechanisms such as the immunoproteasome, antigen processing and presentation pathways, T cell activation, cytokine-mediated inflammation, and interferon γ signalling. Exposure to very high levels of BaP (75 mg/kg-bw/day) leads to unremitting activation of immune responses and inflammation, and ultimately activation of keratinocyte differentiation, an important biological alteration that is considered a hallmark of forestomach carcinogenesis. Despite functional and histological differences between the rodent forestomach and the human stomach, there appear to be common tissue-specific alterations in core cellular responses to invading pathogens/toxicants and the identity and functions of individual genes that are disrupted in the forestomach of mice exposed to BaP for 28 days are consistent with the development of human cancers of the esophagus and gut. Thus, our results provide support for the relevance and use of rodent forestomach tumour data in human cancer risk evaluation. More studies involving testing of other known forestomach carcinogens with a wide range of doses and time points will be necessary to further confirm these findings.

3.6 Tables and Figures

Table 3-1 Meta-analysis in NextBio of significant genes in BaP-treated mouse forestomach and human profiles in which esophageal, gastric, intestinal, or colon cancer was a phenotype.

Disease	Antigen processing and presentation ^a	# Studies ^b	Immune Response	# Studies	Chemotaxis	# Studies	Keratinocyte differentiation	# Studies
Esophageal cancers	B2M	3	Ifi35	8	Ccl11	2	Col11a1 (III)	7
	H2-K1	4	Irf1	3	Ccl7	1	Krt17	6
	H2-q10	6	Irf9	4	Ccl8	1	Trp63	5
	H2-t23	2	Mx1	3	Ccr2	1		
	Nlrc5	2	Socs1	3	Cxcl10	3		
	Tap1	5	Stat2	4	Cxcl16	2		
	Psmb9	5	Tap1	5	Cxcl9	4		
	Psmb10	6	Clec3b	1	Stat2	4		
			Clec2f	1				
Gastric cancers	H2-q10 (IIIB)	1	Ifi35	1	Ccl8	4	Aldh1a3	6
	H2-t23	2	Irf1	1	Cxcl10	5	Col11a1	5
	Nlrc5	2	Irf9	1	Cxcl16	5	Krt17	7
	H2-Aa (IIIB)	3	Socs1	2	Cxcl9 (IIIA, IIIB)	6	Krt6b	1
	Tap1 (IB, IVA)	7	Stat2 (IIIB, IVA)	3	Il20ra	1	Sprr1b (IIIB)	2
	Psmb9	3	Tap1 (IB, IVA)	7	Rasgrp2	1	Sprr2g (IIIB)	1
			Clec3b (IIIB)	1	Stat2 (IIIB, IVA)	3	Trp63 (IIIB)	1
					Tnsfrsf16 (IIIA, IIIB)	2		
Intestinal cancers	H2-Aa (IA, IIIA)	2	Irf9	1	Cxcl10 (IB, II, IIIA)	4	Agpat6	1
	Tap1 (IA, IB, II, IIIA, IVA)	4	Mx1 (IB)	1	Cxcl16 (IB, II, IIIA, IIIB)	4	Col11a1 (IB, II, IIIB, IVA, IVB)	5
			Stat2 (II, IIIA, IIIB)	4	Cxcl9 (IA, IB, II, IIIA, IIIB, IVA)	6	Krt17 (IB, II)	2
			Tap1 (IA, IB, II, IIIA, IVA)	4	Stat2 (II, IIIA, IIIB)	4	Tbx3 (IB, IIIA)	3

			Clec2f (IIIA, IVB) Clec10a (IA)	2 1			Trp63 (IVB)	1
Colon cancer	H2-K1	1	Irf1	4	Ccl11	2	Aldh1a3	8
	H2-q10	4	Irf9	4	Ccl7	2	Col11a1	17
	H2-t23	2	Mx1	6	Ccl8	1	Ddr1	3
	Nlrc5	3	Socs1	9	Cxcl10	14	Krt17	11
	H2-Aa	1	Stat2	2	Cxcl16	19	Krt6b	20
	Tap1	7	Tap1	7	Cxcl9	12	Krt6a	10
	Psmc9	8			Il20ra	12	Pou3f1	1
					Rasgrp2	5	Sprr1b	2
					Stat2	2	Sprr2a2	1
					Tnfrsf11b	1	Sprr2d	2
					Tnfrsf16	1	Tbx3	1
							Trp63	1

^a The genes listed were significantly differentially expressed (FDR $P \leq 0.05$ and fold change ≥ 1.5) in our dataset and similarly differentially expressed in the curated studies in NextBio.

^b The number of studies refers to the total number of studies used in the meta-analysis that are positively correlated with our dataset. The individual studies used in the meta-analysis can be found in Supplementary Table 1.

Table 3-2 Genes validated by real-time PCR in the forestomach of BaP treated mice divided by function.

	25 mg/kg/day vs Control		50 mg/kg/day vs Control		75 mg/kg/day vs Control	
	PCR array	Microarray	PCR array	Microarray	PCR array	Microarray
Antigen Processing and Presentation						
<i>B2m</i>	-	-	↑8.6	↑2.1	↑10.3	↑3.1
<i>Clec10a</i>	-	-	↑5.4	↑1.7	↑5.8	↑1.8
<i>Clec2e</i>	-	-	↑4.6	-	-	↑1.9
<i>Clec2g</i>	↓2.5	-	-	-	↓7.8	-
<i>Clec4a1</i>	-	-	↑8.2	-	-	↑1.6
<i>H2-Aa</i>	-	-	↑2.5	-	↑2.8	↑2.6
<i>H2-DMb1</i>	↑1.9	-	↑4.7	-	↑8.7	↑2.0
<i>H2-K1</i>	↑2.1	-	↑3.2	↑2.4	↑3.7	↑3.7
<i>H2-M2</i>	-	-	↑7.5	↑2.3	↑2.7	↑5.0
<i>H2-Q7</i>	↑1.9	-	↑2.6	↑2.2	↑2.4	↑3.2
<i>H2-Q8</i>	-	-	↑7.2	↑3.0	↑12.3	↑5.1
<i>H2-T10</i>	↑2.6	-	↑7.4	↑2.0	↑11.6	↑2.7
<i>H2-T23</i>	↑1.7	-	↑5.3	↑2.1	↑6.6	↑2.8
<i>H2-T9</i>	↑1.9	-	↑3.6	↑1.6	↑3.9	↑2.2
<i>Klra15</i>	-	-	↑8.1	↑1.6	-	↑1.7
<i>Tap1</i>	↑3.9	-	↑11.1	↑1.8	↑7.6	↑2.6
DNA Damage Response						
<i>Bst2</i>	↑2.1	-	↑3.0	↑2.7	↑5.3	↑3.2
<i>Mdm2</i>	-	-	↑1.9	-	↑3.3	-
<i>Mgmt</i>	-	-	↑2.3	↑1.9	↑3.1	↑1.7
<i>Pmaip1</i>	↑2.9	-	↑15.4	↑1.8	↑18.9	↑2.1
<i>Trp53inp1</i>	↑1.7	-	↑1.8	↑1.7	↑2.3	↑1.8
<i>Trp63</i>	↑1.6	-	-	-	↑1.4	↑1.5

Immune/Inflammatory Response						
<i>Ccl11</i>	-	-	↑2.4	-	↑4.8	↑1.8
<i>Ccl12</i>	↑2.2	↑2.0	↑11.4	↑2.1	↑19.9	↑2.8
<i>Ccl7</i>	↑1.8	-	↑9.9	-	↑18.7	↑1.8
<i>Ccl8</i>	↑7.1	↑6.6	↑53.4	↑13.8	↑87.6	↑16.7
<i>Cxcl10</i>	-	-	↑8.9	↑3.0	↑17.2	↑4.3
<i>Cxcl16</i>	-	-	-	↑1.4	↑1.9	↑1.6
<i>Cxcl9</i>	-	-	↑6.4	-	↑10.8	↑2.9
<i>Ifi35</i>	-	-	↑2.3	↑1.4	↑2.9	↑1.6
<i>Irf1</i>	-	-	↑2.7	↑1.9	↑2.4	↑1.8
<i>Irf9</i>	↑1.8	-	↑5.1	↑2.1	↑7.8	↑2.8
<i>Isg15</i>	↑4.3	-	↑26.6	↑4.6	↑82.1	↑8.8
<i>Saa1</i>	-	-	↑3.7	↓2.4	-	↓3.2
<i>Saa2</i>	-	-	-	↓3.0	↓3.4	↓4.4
<i>Socs1</i>	-	-	-	-	↑2.6	↑1.6
Keratinocyte Differentiation						
<i>Areg</i>	-	-	↑3.0	-	↑4.8	-
<i>Gbp2</i>	↑1.9	-	↑12.3	↑2.3	↑4.7	↑2.4
<i>Klk14</i>	-	-	↑3.8	-	↑8.6	↑2.5
<i>Krt16</i>	-	-	↑2.3	↑7.3	↑2.4	↑13.3
<i>Krt6a</i>	-	-	-	↑3.2	↑5.4	↑4.5
<i>Krt6b</i>	-	-	↑12.0	↑2.9	↑24.1	↑4.5
<i>Oas1a</i>	-	-	↑16.3	↑5.1	↑28.4	↑8.9
<i>Spr1b</i>	-	-	↑2.9	↑3.7	↑4.4	↑8.5
<i>Spr2e</i>	-	-	↑5.4	↑2.0	↑6.5	↑2.4
<i>Spr2f</i>	-	-	↑2.5	↑3.0	↑4.2	↑7.2
<i>Spr2g</i>	-	-	-	↑2.7	↑23.0	↑4.6
<i>Stfa1</i>	-	-	-	↑9.9	↑308.1	↑78.5

<i>Stfa2</i>	↑4.1	-	↑32.1	↑4.4	↑136.6	↑15.5
<i>Stfa2l1</i>	-	-	↑2.4	↑2.8	-	↑11.4
Xenobiotic Metabolism						
<i>Aldh1a3</i>	↑1.8	-	↑5.1	-	↑4.7	↑1.8
<i>Gsta1</i>	-	↑1.9	↑6.6	↑2.1	↑15.8	↑2.5
<i>Gsta2</i>	-	-	↑11.7	-	-	↑2.0
<i>Gstm4</i>	-	-	↑4.3	-	↑6.7	↑1.5
<i>Gsto1</i>	-	-	↑2.9	-	↑5.0	↑1.6
<i>Ugt2b34</i>	-	-	↓1.8	-	↓2.0	↓3.1

Note. All genes shown were significantly differentially expressed with a FDR $P \leq 0.05$ and fold change ≥ 1.5 in either direction. ↑ indicates upregulated; ↓ indicates downregulated, - indicates no change.

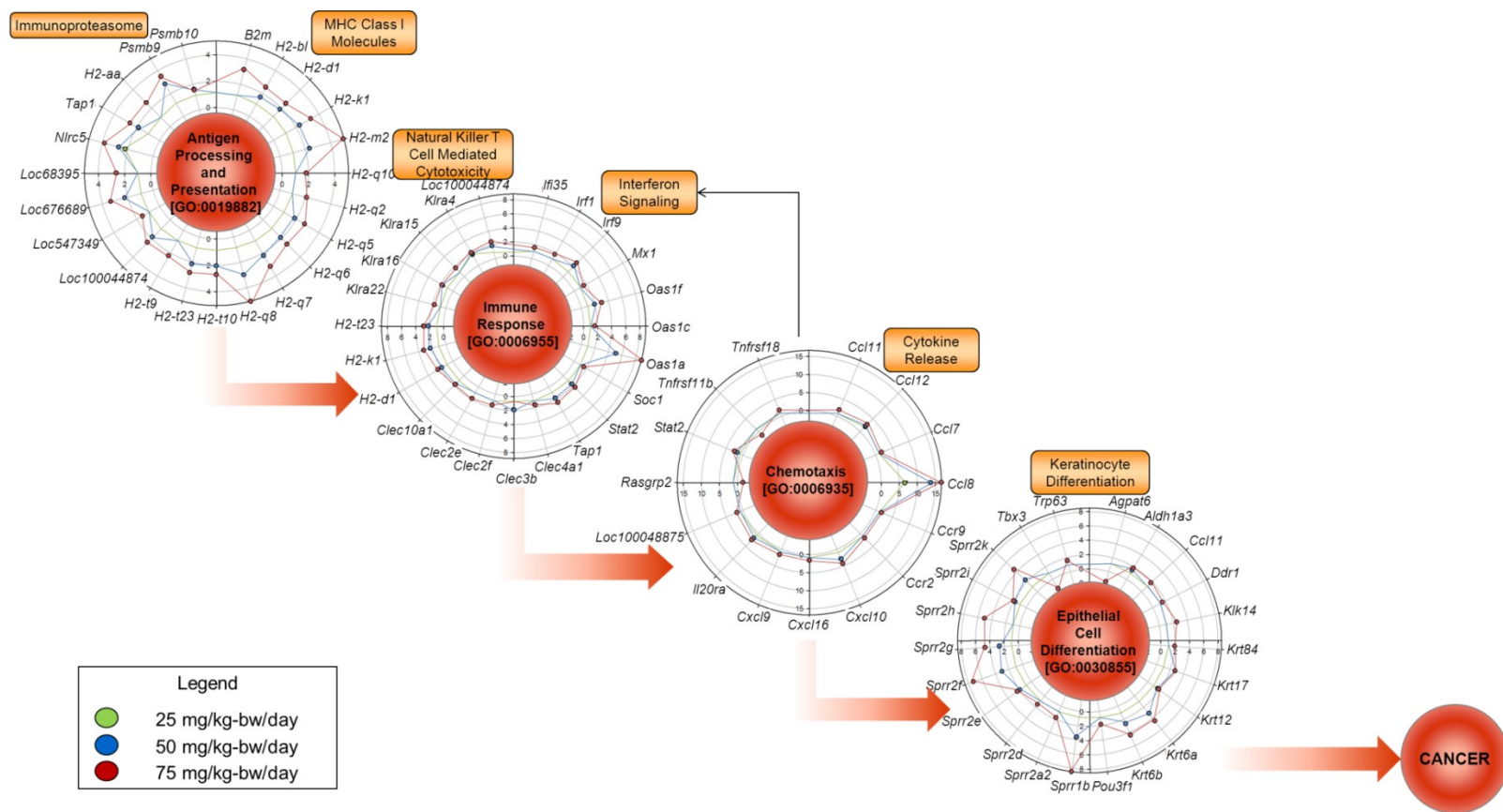


Figure 3-1. Step-wise organization of the most significant gene ontology processes perturbed in BaP-exposed forestomach showing dose-dependent transition in the response from antigen processing and presentation to epithelial cell differentiation.

Polar plots show the fold changes associated with the significantly differentially expressed genes in each ontology category (red circle) subdivided into individual pathways/networks associated with the genes (orange rectangles). Arrows indicate causal relationships between categories. Green lines and circles indicate response in 25 mg/kg-bw/day, blue in 50 mg/kg-bw/day, and red in 75 mg/kg-bw/day dose groups.

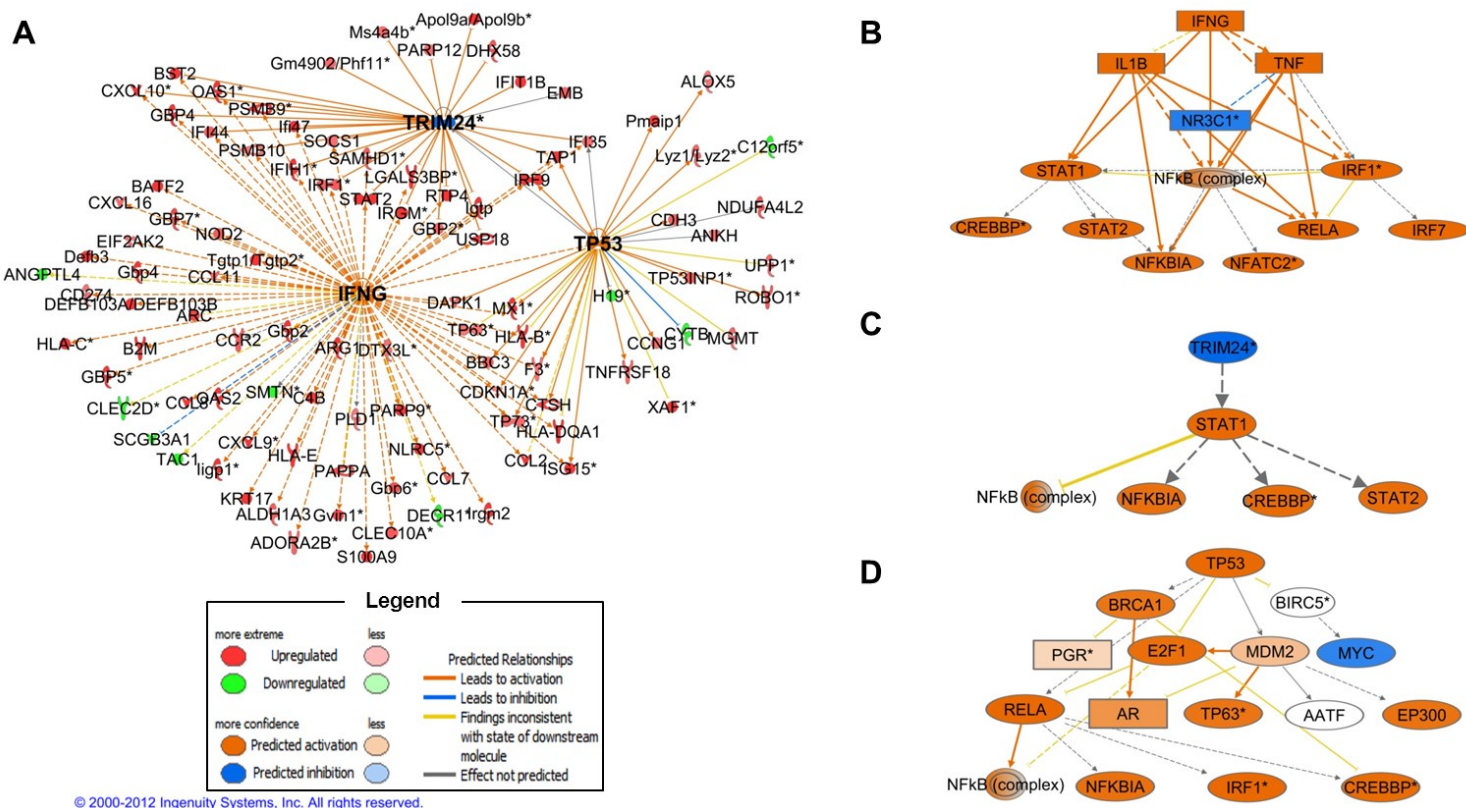


Figure 3-2. Upstream regulator analysis.

(a) Graphical representation of upstream regulators and the significantly differentially expressed genes associated with them. The graph was generated by IPA's Upstream Regulator analysis. The IFN γ , P53, and NF κ B regulators were selected for their relative significance in the context of the dataset as determined by the Z-score. (b-d) Mechanistic Network (IPA) analysis of activated and inhibited regulators significant in the BaP exposed forestomach showing interconnections between regulators in the gene set. Dashed lines indicate indirect interaction.

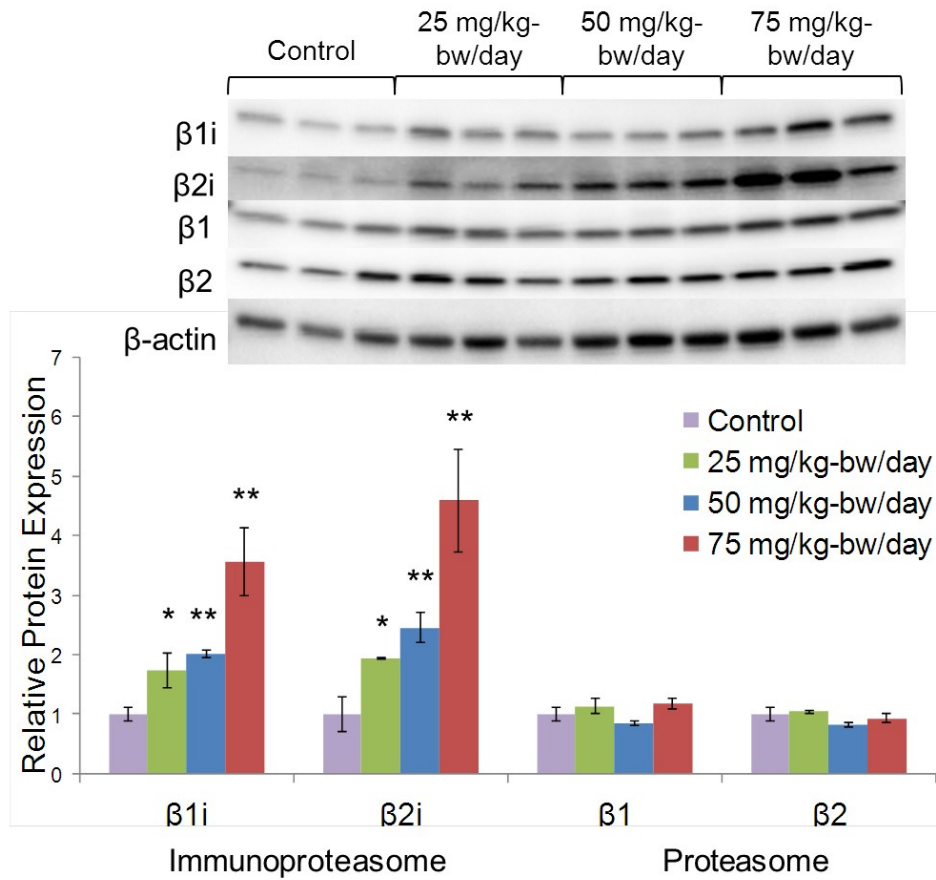


Figure 3-3. Activation of the immunoproteasome in BaP exposed forestomach.

Western blot analysis confirming the expression of immunoproteasome subunits β1i and β2i and proteasome subunits β1 and β2 in MutaTMMouse forestomach exposed to 25 (green), 50 (blue), and 75 (red) mg/kg-bw/day BaP. The band intensities were normalized to β-actin. * $P \leq 0.1$, ** $P \leq 0.05$.

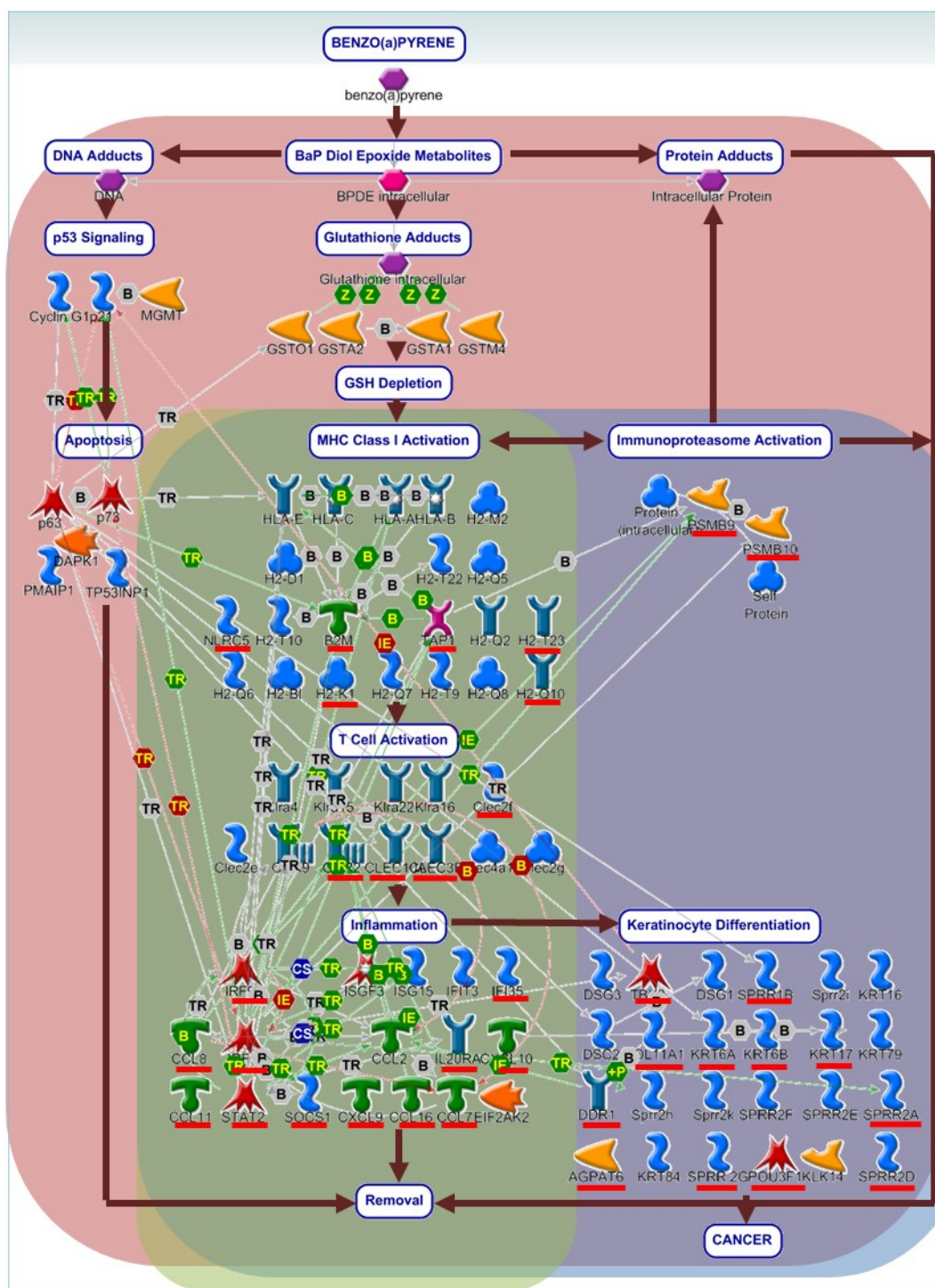


Figure 3-4. Molecular relational network of genes modulated by BaP in the forestomach using MetaCore for all three doses.

A network is a graphical representation of the molecular relationships between molecules.

Biological relationships between two molecules are represented by a line; green lines denote

positive effect, red lines denote negative effect, and grey lines represent unspecified effect.

Biological processes are presented in causal order of BaP-induced damage removal and eventually carcinogenic transformation. Hexagons represent physical and functional interactions: B – binding, +P – phosphorylation, Z – catalysis, TR – transcription regulation, IE – influence on expression, CS – complex subunit. The processes include genes that were differentially regulated in the 25 mg/kg-bw/day (green rectangle), 50 mg/kg-bw/day (blue rectangle), and 75 mg/kg-bw/day (red rectangle) dose groups.

4 Chapter: Comparative Transcriptomic Analyses to Scrutinise the Assumption That Genotoxic PAHs Exert Effects via a Common Mode of Action

Modified from: Labib, S., Williams, A., Guo, C.H., Leingartner, K., Arlt, V.M., Schmeiser, H.H., Yauk, C.L., White, P.A., Halappanavar, S. (*In Press*) Archives of Toxicology. Reprinted with permission from Springer Berlin Heidelberg.

4.1 Abstract

In this study, the accuracy of the assumption that genotoxic, carcinogenic polycyclic aromatic hydrocarbons (PAHs) act via similar mechanisms of action as benzo(a)pyrene (BaP), the reference PAH used in the human health risk assessment of PAH-containing complex mixtures, was investigated. Adult male MutaTMMouse were gavaged for 28 days with seven individual, genotoxic PAHs. Global gene expression profiles in forestomach, liver, and lung (target tissues of exposure) were determined at three days post-exposure. The results are compared with our previously published results from mice exposed to BaP *via* the same exposure regimen. Although all PAHs showed enhanced ethoxyresorufin-O-deethylase activity, DNA adduct formation, and *lacZ* mutant frequency in the lungs, the unsupervised cluster analysis of differentially expressed genes revealed that the transcriptional changes are both PAH- and tissue-specific, with lung showing the most response. Further bioinformatics/pathway-based analysis revealed that all PAHs induce expression of genes associated with carcinogenic processes, including DNA damage response, immune/inflammatory response, or cell signaling processes; however, the type of pathways and the magnitude of change varied for each PAH and were not the same as those observed for BaP. Benchmark dose modeling showed transcriptomic data closely reflected the

known tumour incidence for the individual PAHs in each tissue. Collectively, the results suggest that the underlying mechanisms of PAH-induced toxicity leading to tumourigenesis are tissue-specific and not the same for all PAHs; based on the tissue type considered, use of BaP as a reference chemical may over- or underestimate the carcinogenic potential of PAHs.

4.2 Introduction

Many regulatory agencies recommend the use of a reference chemical approach to assess the hazards posed by chemical mixtures in complex environmental matrices (EFSA, 2008; Health Canada, 2010; Health Protection Agency, 2010; USEPA, 2010). For example, the current human health risk assessment approach for evaluating the carcinogenic risks of soils contaminated with mixtures of polycyclic aromatic hydrocarbons (PAHs) involves examination of the cumulative risk posed by a targeted set of PAHs. Benzo(a)pyrene (BaP) is the most extensively studied of the carcinogenic PAHs, and is used as the point of reference for the carcinogenic potency of the other PAHs.

Human health risk assessment of PAH-contaminated complex environmental mixtures considers the incremental carcinogenic risk of a targeted set of PAHs as toxicological equivalents of BaP (relative potency factor approach). However, to be able to apply this approach, all structurally similar PAHs in a mixture must elicit similar toxicological responses via similar modes/mechanisms-of-action. The most well-established mode of action of genotoxic, carcinogenic PAHs involves binding and activating the aryl hydrocarbon receptor (AhR), the expression of genes associated with xenobiotic metabolism, and the concurrent metabolism of PAHs. This in turn leads to the production of reactive intermediates (e.g. diol epoxides) that covalently bind DNA and form bulky DNA adducts. When left unrepaired, these DNA adducts can result in mutations in tumour suppressor genes or oncogenes, which can

eventually lead to carcinogenesis, implying a genotoxic mode of action in cancer. Consequently, measurement of DNA adducts and DNA mutations have long been used as measurable markers of the genotoxic mode of action of PAHs. However, the existing toxicological database (showing evidence for carcinogenicity and the details pertinent to the underlying mechanisms of carcinogenicity) for the individual PAHs is incomplete and thus lacks experimental support for the use of the reference chemical method (Bostrom et al., 2002). In order to strengthen the application of the reference chemical approach, the USEPA (USEPA, 2010) has outlined certain considerations that are important for PAH-mixture cancer risk assessment, which includes a detailed characterization of toxicological responses elicited by individual PAHs in the mixture.

Recent work from our laboratory examined the levels of DNA damage, mutation and global gene expression changes in the forestomach (Labib et al., 2013), livers (Malik et al., 2012) and lungs (Labib et al., 2012) of MutaTMMouse males exposed to BaP by oral gavage for 28 consecutive days. The results of these studies revealed that 1) the levels and relationship between bulky DNA adducts and transgene mutant frequencies differ between tissues, and 2) BaP-induced alterations in gene/protein expression associated with a diverse array of cellular signaling pathways/biological functions, including carcinogenesis and the magnitude of change, varied between the tissues. Moreover, compared to the amount of DNA damage and mutations, the observed extent of the transcriptomic response was more closely related to previously reported tumour incidence in each of these tissues (Hakura et al., 1998). These results suggest that the carcinogenic potency and the underlying mode of action (including metabolism, DNA damage, mutation, and pathway perturbations) for BaP differs based on the type of the tissue investigated. These results also implied that the current approach of human health risk

assessment, which assumes that the toxicological activities of PAHs reflect a single mode of action may be overgeneralized.

In the present study, we sought to explore the underlying carcinogenic modes of action of several individual PAHs to specifically investigate whether the assumption of similar mode of action is accurate. We exposed MutaTMMouse males to three doses of seven PAH congeners or vehicle for 28 consecutive days via oral gavage. These seven PAHs are known to be genotoxic and constitute the panel of PAHs recommended for routine measurement in contaminated soils for the protection of human and environmental health in the Canadian soil quality guidelines (CCME, 2010) and in contaminated food samples (EFSA, 2008). Forestomach, livers, and lungs were collected 3 days after the final exposure and DNA microarrays were employed to generate the gene expression profiles of these tissues. The results were compared to gene expression profiles in forestomach, livers, and lungs of mice previously exposed to BaP (Labib et al., 2013; Labib et al., 2012; Malik et al., 2012). The data were used to (a) identify the cancer-associated biological functions and pathways affected following exposure to different PAHs, (b) evaluate the assumption that all structurally similar PAHs with similar toxicological outcome (i.e., cancer) act via the same mode of action, and (c) determine whether BaP as a reference chemical appropriately represents the toxicity of the other 7 PAHs. In addition to the gene expression profiling, DNA adducts and lacZ transgene mutant frequencies were measured in lungs to confirm the genotoxic phenotype, and activation of metabolic enzymes in the lungs of mice was measured using the ethoxyresorufin-O-deethylase (EROD) assay.

4.3 Methods

4.3.1 *Materials and dose selection*

Seven PAHs were individually analyzed in this study: benz(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(g,h,i)perylene (BghiP), benzo(k)fluoranthene (BkF), dibenz(a,h)anthracene (DBahA), indeno(123-c,d)pyrene (IP) (Cambridge Isotopes Laboratories, USA), and chrysene (CHR) (Sigma Aldrich Canada Ltd, Canada). All of these PAHs are classified as possible or probable human carcinogens by IARC and the USEPA (IARC, 2010; USEPA, 2011), except for BghiP which is classified as a genotoxic PAH. Maximum doses were initially selected based on the maximum tolerated dose of each PAH in a dose-range-finding study. This dose was diluted two times, twofold, to obtain two lower doses. The specific doses were as follows: BaA (20, 40, 80 mg/kg-bw/day), BbF (25, 50, 100 mg/kg-bw/day), BghiP (6.25, 12.5, 25 mg/kg-bw/day), BkF (25, 50, 100 mg/kg-bw/day), CHR (17.5, 50, 150 mg/kg-bw/day), DBahA (6.25, 12.5, 25 mg/kg-bw/day), and IP (12.5, 25, 50 mg/kg-bw/day). The results are compared to data derived from experiments using an identical experimental design for BaP exposures (25, 50, 75 mg/kg-bw/day); results from the BaP study were published in Malik et al. (2012), Labib et al. (2012), and Labib et al. (2013). Data from DBahA-exposed livers were also published previously in Malik et al. (2013).

4.3.2 *MutaTMMouse exposures*

Animal care, exposures, and tissue collection procedures were described previously (Labib et al., 2012; Lemieux et al., 2011). In brief, adult male MutaTMMouse (transgenic mouse strain 40.6) were exposed by oral gavage for 28 consecutive days to olive oil (vehicle control) or three doses of the seven individual PAHs dissolved in olive oil, according to the established Organisation for Economic Co-operation and Development (OECD) guideline for transgenic rodent mutation

assays (OECD, 2011). Each treatment group contained five animals. Mice were killed by cardiac puncture under isofluorene anaesthesia 3 days after the final exposure. No mice showed overt signs of toxicity during the exposure period. Five mice from the BkF group and one from the IP group died as a result of accidental pulmonary puncture during oral gavage. Thus, the final sample size for BkF was 4, 3, 4, and 4 in the control, low-, medium-, and high-dose groups, respectively, at necropsy. The IP control group consisted of only 4 mice at necropsy. The forestomach, left lobe of the liver, and the right lobe of the lung were collected, flash-frozen in liquid nitrogen, and stored at -80°C until use. Mice were bred, maintained, and treated in accordance with the Canadian Council for Animal Care Guidelines, and all protocols were approved by Health Canada's Animal Care Committee.

4.3.3 *Tissue RNA extraction and purification*

Total RNA was isolated for gene expression analysis using TRIzol reagent (Invitrogen, Canada) and RNeasy Mini Kits following the protocols previously published (Halappanavar et al., 2011; Labib et al., 2013; Labib et al., 2012). For detailed description of methods, please see Supplementary File 4.1A.

4.3.4 *Microarray hybridization and analysis*

RNA samples were hybridized to Agilent Sureprint G3 Mouse GE 8 x 60 K microarrays (Agilent Technologies, Canada) following previously published protocols (Labib et al., 2013; Malik et al., 2012) and scanned on the Agilent G2505B scanner. Sample sizes of 5 were used for each treatment and control group, except for the BkF and IP exposures. LOWESS was used to normalize the signal intensities, MAANOVA was used to identify differentially expressed genes, and multiple comparisons were adjusted for using the false discovery rate (FDR) approach, as described in Labib et al. (2013) (Supplementary File 4.1B). All microarray results were

deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE51321. Differentially expressed genes (DEGs) were defined as genes with FDR-adjusted P values less than 0.05 ($\text{FDR } P \leq 0.05$) from the pairwise analysis to controls with absolute fold change estimates greater than or equal to 1.5 in either direction (fold change ± 1.5). Hierarchical cluster analysis was applied to the filtered data and visualized using a heatmap using (R Development Core Team, 2010) (Supplementary File 4.1C).

4.3.5 *Bioinformatics and pathway analysis*

The lists of DEGs ($\text{FDR } P \leq 0.05$, fold change ± 1.5) from each PAH were independently analyzed to identify biological functions or processes perturbed in response to the treatment. DAVID (Huang da et al., 2009) Functional Annotation Charts were used to identify gene ontology (GO) terms (biological processes and cellular compartments) associated with the significant genes and to classify the DEGs into biological pathways using KEGG pathways (Kanehisa and Goto, 2000). MetaCore (Thomson Reuters, <http://www.genego.com/metacore.php>) pathway analysis and network process analysis were used for functional classification. The canonical pathway analysis, biological function analysis, and network analysis within Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) were used to identify biological pathways and functions associated with the DEGs. Since there was a large redundancy in the annotation of the perturbed biological processes, pathways, and functions, the results were further grouped under parent pathways/processes (Supplementary Table 4-1). IPA's Upstream Regulator analysis was performed to determine the regulatory mechanisms (transcription factors and receptors) operating in response to individual PAHs, and these were compared to BaP. Supplementary File 4.1D describes the criteria used to

determine the statistical significance of altered pathways, functions, biological processes, or upstream regulators.

The NextBio meta-analysis function (<http://nextbio.com>) was used to compare the transcriptomic responses following exposure to individual PAHs and following exposure to BaP. A less stringent non-FDR P value cut-off of less than 0.05 and a fold change of ± 1.2 was applied. A pairwise comparison was made for each gene between the two datasets (for example, BaP vs BbF). A rank-based enrichment statistic was applied to determine the final correlation score. If the direction of change in the expression of a gene following exposure to a congener PAH was the same as the direction of change in expression of that gene following exposure to BaP, then the correlation was positive.

Since the focus of the study was to compare the carcinogenic potential of PAHs, significantly perturbed pathways and processes were reorganized according to their association with the 6 hallmarks of cancer (activating invasion and metastasis, enabling replicative immortality, evading growth suppressors, inducing angiogenesis, resisting cell death, sustained proliferative signaling), 2 emerging hallmarks (deregulating cellular energetics, avoiding immune destruction), and 2 enabling characteristics (genome instability and mutation, tumour-promoting inflammation), as described in Hanahan and Weinberg (2011). Re-classification was determined by conducting a literature review using search terms related to each significant pathway and each hallmark of cancer. Pathways or processes that could not be classified as cancer related using these criteria were not used in the subsequent analysis and interpretation of the results.

4.3.6 DNA extraction from lung tissue

Frozen lung tissues were sliced randomly for isolation of genomic DNA by phenol/chloroform extraction as described previously (Labib et al., 2012; Renault et al., 1997) (Supplementary File 4.1E).

4.3.7 LacZ mutant frequency in lung tissue

The lacZ mutant frequency analysis in lung tissues using the phenyl- β -D-galactopyranoside (P-Gal) positive selection assay was conducted as described previously (Labib et al., 2012; Lemieux et al., 2011) (Supplementary File 4.1F). Mutant frequency was expressed as the ratio of mutant plaque forming units to total plaque forming units.

4.3.8 DNA adduct analysis in lung tissue

DNA adduct formation in each sample was determined using the nuclease P1 digestion enrichment version of the ^{32}P -post-labeling assay as described previously (Phillips and Arlt, 2014; Phillips and Arlt, 2007) with minor modifications described in Labib et al. (2012) (Supplementary File 4.1G). The results are expressed as DNA adducts per 108 nucleotides.

4.3.9 Enzymatic analysis of lung Cyp1a activity

Enzymatic analysis of pulmonary CYP1A activity was assessed via the ethoxyresorufin-*O*-deethylase (EROD) assay. Since the available amount of tissue from each treatment group was small, samples from the same treatment group were pooled (Supplementary File 4.1H).

4.3.10 Benchmark dose (BMD) calculations

BMD and BMDL (lower confidence limit of BMD) values were modeled for apical endpoint measurements (EROD activity, DNA adduct formation, lacZ mutant frequency) in lungs.

Further, BMDs were modeled for the total number of DEGs, the number of DEGs related to the

hallmarks of cancer, and for the number of hallmarks of cancer perturbed by each PAH in the forestomach, liver, and lung tissues using the USEPA's Benchmark Dose software BMDS version 2.5.0 (<http://www.epa.gov/ncea/bmds/>) (Davis et al., 2011). Continuous data were run against five models (Exponential, Hill, Power, Polynomial, and Linear), and proportional (dichotomous) data were run against eight models (Gamma, Logistic, LogLogistic, Probit, LogProbit, Weibull, Multistage, QuantalLinear). The benchmark response (BMR) was set to 10 % extra risk as recommended by the Benchmark Dose Technical Guidance document (USEPA, 2012). The best model for each dataset was selected based on the lowest Akaike's Information Criterion (AIC) value, excluding models with BMD greater than the highest dose, BMD/BMDL ratios >10, and goodness of fit $P < 0.1$.

4.4 Results

Adult male MutaTMMouse were exposed to three doses of individual PAHs (BaA, BbF, BghiP, BkF, CHR, DBahA, or IP) for 28 consecutive days *via* oral gavage. The exposure regimen did not cause any overt signs of toxicity, and there was no significant body weight loss in any of the exposed mice compared to vehicle-treated controls. Forestomach, liver, and lung tissues were collected 3 days after the final exposure.

4.4.1 General overview of the microarray results

The transcriptomic profiles derived from the present study were compared with the profiles of mice exposed to BaP (following the same exposure regimen described for other seven PAHs in this study) to investigate whether BaP, as a reference PAH, accurately represents the toxicity posed by other individual PAHs. Global transcriptomic changes in the forestomach, liver, and lung tissue were characterized.

Table 4-1 summarizes the total number of DEGs (FDR $P \leq 0.05$, fold change ± 1.5) for each tissue and the different doses tested. Supplementary Table 4-2 provides the full lists of DEGs including the gene names, gene accession numbers, P values, and fold changes for the forestomach, liver, and lung. The results are presented by tissue type below.

4.4.1.1 Forestomach transcriptional response

Responses to each individual PAH were analyzed separately, and the results were then compared across all PAHs. Of all the PAHs tested, BaP induced the largest response in forestomach.

Hierarchical cluster analysis was conducted using 1008 DEGs that showed differential expression in at least one dose group for at least one PAH (Figure 4-1A). The analysis revealed that the samples generally clustered first by PAH (with dose groups together) rather than by dose. BaP clustered along with BbF and BaA (1) on one branch. CHR, DBahA, and IP clustered on a separate branch (2). However, there was no clear clustering observed for BghiP and BkF, which were scattered between the two branches. A VENN analysis of the DEGs from all PAHs revealed that there were no genes in common to all PAHs studied, supporting the results of the cluster analysis. The number of DEGs common between BaP and the other PAHs tested was as follows: 5 for BaA, 8 for BbF, 4 for BghiP, 7 for BkF, 9 for CHR, 0 for DBahA, and 1 for IP (Figure 4-1B). Figure 1B also shows the genes in common for each pairwise comparison across all of the PAHs, revealing remarkably little in common.

In addition to comparison of DEGs by VENN analysis, a meta-analysis of all datasets was conducted using NextBio. Since most of the PAHs induced very subtle responses at the low or medium doses, the meta-analysis was conducted on the high-dose groups of each PAH (Figure 4-1C). This analysis employed a less stringent statistical model (ANOVA $P \leq 0.05$) compared to the FDR-adjusted P value approach and used a ranking of genes based on their fold changes to

explore the correlation of the trends in gene ranking. The results revealed a strong correlation between BaP, BbF, and BaA, whereas BghiP and IP were least correlated with BaP. CHR was negatively correlated with BaP.

Similarities in the forestomach response relative to BaP. Figure 4-1D summarizes the molecular pathways, biological processes, and molecular functions that are altered in forestomach following exposure to BaP as compared to the other seven PAHs. This analysis includes all significantly altered gene ontologies (EASE $P \leq 0.05$), KEGG pathways (EASE $P \leq 0.05$), IPA canonical pathways ($P \leq 0.05$), and MetaCore process networks ($P \leq 0.05$). Since there was a large redundancy in the annotation of the perturbed biological processes, pathways, and functions, the results were further grouped under parent pathways/processes (Supplementary Table 1-1).

Five main functional categories of pathways and processes were perturbed in forestomach following exposure to BaP: DNA damage response, altered cell signaling, immune and inflammatory response, altered metabolism, and cytoskeletal organization (Figure 4-1D). Specifically, in the DNA damage response, DEGs were mainly associated with p53 signaling; AhR signaling, endocytosis, epithelial cell differentiation, and ubiquitination were the predominant pathways under altered cell signaling; xenobiotic metabolism, glutathione metabolism, melatonin degradation, and estrogen biosynthesis were related to altered metabolism, and cell division control protein 42 homolog (CDC42) signaling was the most significantly affected under the cytoskeletal organization category. The immune/inflammatory response category was the most enriched, and the DEGs were mainly associated with antigen processing and presentation, pattern recognition receptors, innate to adaptive immune cell communication (immune cell communication), interferon signaling, OX40 signaling, cytotoxic T

cell signaling, TREM1 signaling, cytokine-cytokine receptor interaction, and nod-like receptor (NLR) signaling.

To focus on the cancer mode of action, pathway perturbations that functioned in processes related to the published hallmarks of cancer were examined in detail (Hanahan and Weinberg 2011). Specifically, the number and types of pathways associated with the hallmarks of cancer that were perturbed by each PAH were compared to BaP. In the forestomach, BaP exposure perturbed all ten hallmarks of cancer across all doses (Figure 4-1E). The number of pathways associated with each hallmark ranged from one to five; the five pathways were associated with the emerging hallmark “avoiding immune destruction” and the enabling characteristic of “tumour-promoting inflammation”.

Of the 20 distinct biological pathways altered in response to BaP, only endocytosis was perturbed by more than one PAH (BghiP and BkF). Other BaP-perturbed pathways such as ubiquitination, antigen processing and presentation (APP), pattern recognition receptors, xenobiotic metabolism, and epithelial cell differentiation were altered following exposure to BaA, BbF, BbF or BkF respectively. CHR, DBahA, and IP did not have any pathways in common with BaP. We note that although endocytosis was altered by both BaP and BkF treatment, the direction of change was different; endocytosis appeared to be upregulated following BaP exposure and downregulated following BkF exposure, suggesting that the final toxic outcome of this change may be different for these PAHs (Figure 4-2A). Other examples of discrepancies in pathway regulation include epithelial cell differentiation (BaP↑, BkF↓) and protein ubiquitination (BaP↑, BaA ↓). It was difficult to determine the directionality of change of endocytosis in BghiP, since 50% of DEGs in this group suggested upregulation of the pathway and 50% suggested downregulation. Compared to the BaP-induced response, BaA perturbed

only two hallmarks of cancer, with one altered pathway each; BbF perturbed seven hallmarks of cancer with one to two pathways each; CHR perturbed five hallmarks of cancer with one to five pathways each, the strongest response associated with “activating invasion and metastasis”; DBahA and IP did not perturb any hallmarks.

We performed an Upstream Regulator Analysis in IPA to identify upstream biological molecules (transcription factors, membrane receptors, cytokines, etc.) responsible for regulating the expression of DEGs (Supplementary Figure 4-1) in the forestomach for each of the PAHs. Very few commonalities were observed between BaP and the other PAHs. The results suggest that the transcriptomic changes induced in forestomach by each of the PAHs are mediated by different regulatory molecules.

Differences in the forestomach response relative to BaP. Figure 2B shows a range of cellular processes and functions including thrombin signaling, estrogen receptor signaling, transcription, and extracellular membrane interactions that were not altered by BaP, but were altered by exposure to at least one of the other PAHs. Indeed, several pathways (e.g., retinoid X receptor (RXR) activation, lipid metabolism, and integrin signaling) were altered in response to more than one PAH. However, the relevance of these pathways to cancer formation in forestomach is unclear.

4.4.1.2 Hepatic transcriptional response

Of the PAHs tested, BbF, BaA, and DBahA induced the most changes in hepatic gene expression. Hierarchical cluster analysis was conducted using 977 DEGs that were differentially expressed in at least one dose group for at least one PAH. Cluster analysis revealed that each PAH clustered separately, suggesting PAH-specific transcriptomic responses in the liver (Figure 3A). Except for the high dose of BbF, each PAH clustered along with the other dose groups for

that PAH. Three main clusters were observed: cluster 1 consisting of BaP, cluster 2 consisting of BkF, IP, BbF, and DBahA, and cluster 3 consisting of BaA, CHR, and BghiP. Similar to the forestomach results, a VENN analysis of all hepatic DEGs revealed that there were no genes in common to all the PAHs studied. The number of DEGs that were in common between BaP and the other PAHs included: 10 for BaA, 30 for BbF, 2 for BghiP, 9 for BkF, 1 for CHR, 11 for DBahA, and 2 for IP (Figure 4-3B). Figure 3B also shows the genes in common for each pairwise comparison across all of the PAHs, revealing little in common.

A meta-analysis of all DEGs from the high-dose group of all the PAHs was conducted in NextBio (Figure 4-3C). Transcriptomic profiles of BbF and BaP showed a strong positive correlation, whereas BkF, DBahA, and IP did not correlate with BaP. BaA and CHR were negatively correlated with BaP.

Similarities in the hepatic response relative to BaP. BaP-induced hepatic transcriptomic responses were functionally separated into four main categories of pathways and processes: DNA damage response, altered cell signaling, immune and inflammatory response, and altered metabolic signaling. Category 1 (DNA damage response) DEGs were mainly associated with p53 signaling. Category 2 (cell signaling pathways) included DEGs associated with RXR, AHR, nuclear factor-like 2 (NRF2), peroxisome proliferator-activated receptors (PPAR), phosphate and tensin homolog (PTEN), nuclear factor kappa light-activated enhancer of activated B cells (NFkB), lipid, signal transducer and activator of transcription 3 (STAT3), and 5' adenosine monophosphate-activated protein kinase (AMPK) signaling pathways. Subcategories of cell signaling pathways related to immune/inflammatory response (Category 3) such as acute phase response, T helper cell signaling, and interleukin signaling were also altered. Category 4 (metabolic signaling pathways) included xenobiotic metabolism, glutathione metabolism, and

retinol metabolism. In the liver, BaP exposure activated all ten hallmarks of cancer (Figure 4-3E). The number of pathways associated with each hallmark ranged from one to five; the five pathways were associated with “sustained proliferative signaling” and the enabling characteristic of “tumour-promoting inflammation”.

Of the 16 biological pathways altered in response to BaP, RXR signaling and xenobiotic metabolism were commonly perturbed by 3 other PAHs (BbF, BkF, and DBahA). Pathways common to 2 other PAHs included p53 signaling, AHR signaling, and retinol metabolism common to BbF and BkF, and lipid signaling common to BbF and DBahA. Although the p53 signaling pathway was altered following treatment with BaP, BbF, and BkF, it was upregulated in response to BaP, whereas it was downregulated in response to BbF and BkF (Figure 4A). For many other pathways, it was difficult to determine the directionality of change: RXR activation (BaP↑, BbF↓), lipid signaling (BaP↑, DBahA↓), xenobiotic metabolism signaling (BaP↑, BbF↓), and retinol metabolism (BaP↑, BbF↓). Six upstream regulators were identified that were common to three of the PAHs including BaP (Online Resource 4). BbF, BkF, and DBahA each perturbed nine, ten, and six hallmarks of cancer, respectively. The majority of the hallmarks were associated with one pathway; however, “resisting cell death” for BbF was associated with five pathways, “avoiding immune destruction” for BkF associated with six pathways, and “sustained proliferative signaling” for DBahA associated with five pathways. BghiP activated “deregulating cellular energetics” with one pathway, and CHR and IP did not activate any hallmarks of cancer.

Differences in the hepatic response relative to BaP. Figure 4B shows a range of cellular processes and functions such as apoptosis, retinoic acid receptor (RAR) signaling, and circadian rhythm that were not perturbed following exposure to BaP, but were for at least one of the other PAHs. A few of these pathways were altered in response to more than one PAH, including cell

cycle, wingless integration 1 (WNT) signaling, antigen processing and presentation, amino acid metabolism, steroid biosynthesis, and melatonin degradation. PAH-specific cellular responses were also noted, and they included cell cycle and apoptosis signaling for BbF, immune and inflammatory response (B cell receptor signaling (BCR), antigen processing and presentation, immune cell communication, cytotoxic T cell signaling, immune-mediated apoptosis) for BkF, and circadian rhythm for DBahA.

4.4.1.3 Pulmonary transcriptional response

A robust transcriptomic response was observed in lungs compared to forestomach and liver for most of the PAHs. Hierarchical cluster analysis was conducted using 2324 DEGs that were differentially expressed in at least one dose group of at least one PAH. Consistent with the observations in the forestomach and liver, transcriptomic patterns were unique in the lungs, exhibiting separate clustering of each PAH along with their dose groups (Figure 4-5A). Three main branches were observed: BkF, CHR, and BghiP were clustered on branches 1, 2, and 3, respectively, BaP, DBahA, and BbF on branch 4A, and IP and BaA on 4B. Analysis of all DEGs for commonalities among the PAHs by VENN analysis revealed no genes in common to all PAHs studied. The number of DEGs in common between BaP and the other PAHs was 28 for BaA, 145 for BbF, 13 for BghiP, 28 for BkF, 6 for CHR, 126 for DBahA, and 32 for IP (Figure 4-5B). NextBio meta-analysis of all of the DEGs from the high-dose groups of each PAH revealed that DBahA and BbF were the most correlated with the transcriptomic changes induced by BaP, followed by BkF and BaA. Consistent with the observations in forestomach and liver, CHR was negatively correlated with BaP (green bar) (Figure 4-5C).

Similarities in the pulmonary responses relative to BaP. Although BaP induced a robust pulmonary transcriptomic response, the main categories of pathways and processes perturbed in

the lungs were the same as those in forestomach and livers, with the exception of alterations in homeostasis (Figure 4-5D). However, subcategories of pathways and processes varied. For example, for DNA damage response, BaP-induced DEGs were associated with apoptosis, cell cycle, and p53 signaling. Altered cell signaling was enriched and included several subcategories of pathways and processes: angiogenesis, neuronal signaling, lipid signaling, as well as WNT, PPAR, RXR, PTEN, phosphoinositide-3-kinase protein kinase B (PI3K/AKT), insulin-like growth factor 1 (IGF-1), AHR, and platelet-derived growth factor (PDGF) signaling pathways. The most significantly altered immune and inflammatory pathways were B cell receptor signaling, complement activation, innate-to-adaptive immune cell communication, and interleukin signaling. Circadian rhythm was the only pathway perturbed under the category ‘homeostasis’. In the lungs, BaP exposure activated pathways associated with all ten hallmarks of cancer (Figure 4-5E). The number of pathways associated with each hallmark ranged from one to ten pathways; the ten pathways were associated with “sustained proliferative signaling”.

Although 16 pathways or biological processes were perturbed by BaP and at least one other PAH, the collective pathway response induced by the other PAHs varied significantly, which is clearly shown in Figure 4-6A. For example, apoptosis, cell cycle, and p53 signaling pathways reflecting DNA damage response were also induced by BbF, DBaA, and IP. Of the 11 distinct pathways in the “altered cell signaling” category perturbed in response to BaP, only 7 were significant following BbF exposure, and less than 4 were altered in response to the other PAHs. Similarly, only BbF, BaA, and IP showed significant changes in at least 3 of the four pathways associated with immune and inflammatory response. In addition, among the commonly regulated pathways, the directionality of change in pathways reflecting their functionality was different for each PAH. A few examples of this include p53 signaling (BaP↑, BaA↓), apoptosis

(BaP↑, BkF↓, CHR↓), and complement system activation (BaP↓, BbF↑). For several others, it was difficult to determine directionality: apoptosis (BaP↑, BaA↓, IP↑), p53 signaling (BaP↑, BbF↑, DBahA↑, IP↑), angiogenesis (BaP↑, CHR↓), Wnt signaling (BaP↑, BbF↓, BghiP↑, DbahA↓), neuronal signaling (BaP↑, BbF↓, BkF↓, DBahA↓), lipid signaling (BaP↑, BbF↓), and complement activation (BaP↑, CHR↓). The other PAHs each affected pathways associated with at least eight hallmarks of cancer, with the exception of BghiP that induced two with one pathway each. BbF perturbed the greatest magnitude (number of pathways) of response with two hallmarks associated with 12 significant pathways, “resisting cell death” and “sustained proliferative signaling” and one hallmark associated with eleven significant pathways, “activating invasion and metastasis”.

Fourteen upstream regulators were associated with the genes altered in response to 3 of the PAHs and BaP in lungs (Supplementary Figure 4-1), including endogenous mammalian chemicals, protein complexes and groups, enzymes, growth factors, ligand-dependent nuclear receptors, and transcription regulators. All of these regulators (except β -estradiol) were connected by a single node - p53 - a transcription factor commonly implicated in PAH-induced carcinogenesis.

Differences relative to BaP. Antibody production, circadian rhythm, calcium signaling, xenobiotic metabolism, G protein signaling, and cytoskeletal organization were among the pathways altered following exposure to PAHs that were not perturbed by BaP exposure (Figure 4-6B). Although these differences were not found for BaP, pathways such as cytoskeletal organization, immune-mediated apoptosis, circadian rhythm, and calcium signaling were perturbed in response to more than three PAHs, potentially suggesting a non-BaP-like response shared by other PAHs. Some of these pathways were unique to a single PAH, including:

exocytosis signaling, RNA processing, and antibody production for BaA; altered cell signaling (ABC transporters, protein kinase A signaling, thrombin signaling, endothelin signaling, 14-3-3 signaling, inhibitor of P70 S6 kinase (P70S6KI) signaling, hypoxia signaling, ephrin receptor signaling), acute phase response, extracellular membrane interactions, and inflammatory response for BbF; altered metabolism (retinol metabolism, melatonin degradation, steroid biosynthesis) for BkF; altered cell signaling (cell-to-cell signaling, nitric oxide signaling) and cytoskeletal reorganization (focal adhesion kinase (FAK) signaling, paxillin signaling) for CHR; transforming growth factor β (TGF β) signaling for DBahA; and altered cell signaling (aldosterone signaling, ubiquitination) and NLR signaling for IP.

4.4.2 Apical responses to PAHs in the lungs

Since the lung transcriptome was the most affected of the three tissues studied, in the present study, we evaluated DNA adduct formation, *lacZ* mutant frequency and EROD activity for lungs. The purpose is to exclusively compare the PAH-specific responses for these apical endpoints within the most affected tissue.

4.4.2.1 Pulmonary DNA adduct formation

The presence of bulky DNA adducts indicates the potential mutagenicity of exposure to a chemical. Using the ^{32}P -post-labeling technique, DNA adducts were measured in mouse lungs following exposure to each of the individual PAHs (Figure 4-7A). No adducts were detected in controls treated with vehicle only. The DNA adduct profiles obtained are shown in Supplementary Figure 4-2. Although every PAH tested induced adducts in all dose groups, the adduct levels for all of these PAHs were lower than BaP. BaA, BbF, BghiP, and DBahA showed a dose-dependent increase in adduct levels similar to BaP; however, the levels of DNA adducts formed following exposures to BkF, CHR, and IP did not show dose-specific trends. It is

important to note that the DNA adduct spots for the BghiP and IP samples were close to the origin on the thin-layer chromatography plate, which may have impacted quantitation. Thus, the observed increases in the adduct levels for these two PAHs are classified as semiquantitative (Supplementary Figure 4-2).

4.4.2.2 Pulmonary *lacZ* mutant frequency

A dose-dependent increase in pulmonary *lacZ* mutant frequency compared to vehicle controls was observed for BaP (Labib et al. 2012) and DBahA (Figure 4-7B). Significant increases in *lacZ* mutant frequencies were observed in at least one dose group of BbF and BkF (ANOVA: $P \leq 0.0001$), and BghiP and IP (ANOVA: $P \leq 0.01$). *LacZ* mutant frequency was marginally increased for BaA and CHR despite the presence of DNA adducts (ANOVA: $P \leq 0.1$).

4.4.2.3 EROD enzymatic activity analysis

We measured enzymatic activity of CYP1A1 *via* the EROD assay in the lungs of mice exposed to all of the PAHs (Figure 4-7C). A dose-dependent increase in the activity of CYP1A1 was observed for BaP, BaA, BbF, BghiP, CHR, DBahA, and IP. BkF also showed an increase in EROD activity, but there was no apparent dose-response. BbF showed the maximum activity at the high dose with a 43.8-fold increase compared to vehicle controls. BghiP was the lowest inducer at all doses and showed maximum activity at the high dose with 2.8-fold increase compared to vehicle controls.

4.4.3 BMD analysis

The relative potency of the PAHs was calculated using BMD analysis. BMD and BMDL values for each apical endpoint in the lung tissue (DNA adduct formation, *lacZ* mutant frequency, and EROD activity) and for the number of hallmarks of cancer activated by each PAH in the forestomach, liver, and lung were obtained. BMD values derived from gene expression data were

compared to BMDs of the apical endpoints (Figure 4-7D-I). In general, the BMD models were able to fit the dose-response relationships exhibited by the apical endpoints and the hallmarks of cancer for the eight PAHs studied. Visual inspection of the curves confirmed this, and BMD/BMDL ratios <4 were obtained for all datasets.

The BMD values derived from the pulmonary DNA adduct data ranged from 1.5 mg/kg-bw/day for DBahA to 15.4 mg/kg-bw/day for BaP. The BkF and CHR data could not be modeled. In order of lowest to highest BMD values: DBahA < BghiP < BbF < BaA < IP < BaP; however, the differences between BMD and BMDL (lower confidence intervals) for each PAH suggest that the potencies are not statistically significant, with the exception of DBahA, which is substantially lower. The BMD values derived from the *lacZ* mutant frequency data ranged from 0.5 mg/kg-bw/day for DBahA to 23.5 mg/kg-bw/day for BghiP, compared to 2.6 mg/kg-bw/day for BaP. The BaA, BbF, BkF, and CHR data could not be modeled. In order of lowest to highest BMD values: DBahA < BaP < IP < BghiP; however, the BghiP and IP lower confidence intervals overlap. The BMD values derived from the pulmonary EROD data ranged from 0.06 mg/kg-bw/day for DBahA to 48.2 mg/kg-bw/day for BaP. The BbF and BkF data could not be modeled. In order of lowest to highest BMD values: DBahA < IP < CHR < BghiP < BaA < BaP. Note that the lower confidence intervals for DBahA and IP overlap.

The BMDL values derived from the hallmarks of cancer in the forestomach tissue ranged from 25.3 mg/kg-bw/day for BghiP to 150.0 mg/kg-bw/day for CHR, compared to 56.5 mg/kg-bw/day for BaP. The forestomach data for BaA, DBahA, and IP could not be modeled because of lack of response at the transcriptional level. In order of lowest to highest, the BMDL values were BghiP < BaP < BkF < BbF < CHR; however the lower confidence intervals overlap for all PAHs. The BMD values derived from the hallmarks of cancer in the liver ranged from 17.0

mg/kg-bw/day for DBahA to 71.9 mg/kg-bw/day for BaP. The data for BaA and BghiP could not be modeled due to lack of model fit, and the data for CHR, and IP could not be modeled due to lack of response. In order of lowest to highest, the BMD values were DBahA < BkF = BbF < BaP; however, the lower confidence intervals for all PAHs except for DBahA overlap. The BMD values derived from the hallmarks of cancer in the lung ranged from 7.53 mg/kg-bw/day for DBahA to 139.2 mg/kg-bw/day for CHR, compared to 34.5 mg/kg-bw/day for BaP. In order of lowest to highest, the BMD values were DBahA < BkF < IP < BbF < BaP < BaA < CHR. Note that the lower confidence intervals for all PAHs overlap except for DBahA. The data for BghiP could not be modeled due to lack of fit.

4.5 Discussion

BaP is commonly accepted as a reference PAH in the estimation of excess lifetime cancer risk posed by genotoxic PAHs or PAH-containing mixtures (CCME, 2010; EFSA, 2008; Health Canada, 2010). This is primarily based on the hypothesis that the genotoxic PAHs, such as those studied herein, act via similar modes/mechanisms of action. However, the results from several recent studies have challenged this paradigm (Jung et al., 2013; Song et al., 2012; Tilton et al., 2015). In the present study, we tested this hypothesis using a toxicogenomic approach that enables investigation of global changes in the expression of genes and associated pathways in a tissue in a single experiment. We specifically characterized the gene expression changes occurring in mouse forestomach, liver, and lung following exposure to seven individual PAHs (CCME, 2010) and compared the expression changes in these tissues to those induced by exposure to BaP in previous studies (Labib et al., 2013; Labib et al., 2012; Malik et al., 2013). Systematic bioinformatics analysis of all DEGs and pathways perturbed in response to each individual PAH revealed three major features: (1) all PAHs induce expression changes in genes

associated with a wide variety of carcinogenic pathways, processes, and functions, (2) all PAHs induce changes in the expression of genes implicated in DNA damage response conventionally described for genotoxic PAHs; however, the response varies significantly from PAH to PAH in the number of DEGs and the magnitude of the response (level of expression of each gene), and (3) PAHs induce transcriptional responses that are PAH- and tissue-specific. The significant differences noted in the global pathway perturbations across PAHs imply that, in contrast to the assumptions made in mixture risk assessment, the underlying toxicity pathways, some of which have been implicated in carcinogenesis, are not the same for all of the PAHs investigated and vary across tissues.

The accepted ways by which carcinogenic PAHs induce DNA damage and mutations involve: (1) induction of the dihydrodiol epoxide pathway catalyzed by CYP enzymes leading to stable pro-mutagenic DNA adducts, (2) the oxidation of PAH dihydrodiols by dihydrogenases to PAH-derived ortho-quinones leading to stable DNA adducts and formation of reactive oxygen species that may further contribute to the DNA damage, and (3) binding and activation of AhR resulting in intensified transcriptional activation of not only CYP1A1 but also sulfotransferases and other conjugation enzymes, which also influence DNA adduct formation. The formation of DNA adducts, an immediate consequence of PAH metabolism, is considered an early key event of PAH-mediated carcinogenesis (Moffat et al., 2015). However, several studies have shown that DNA adducts may not accurately predict the carcinogenic risk of genotoxic PAHs (Pottenger et al., 2009). For example, it has been shown that DNA adducts are present in most of the tissues of rats (Kroese et al., 2001) and mice (Zuo et al., 2014) exposed to BaP, including the tissues that eventually develop cancer as well as those that do not. Similar observations of lack of tumours in tissues that showed high levels of DNA adducts following exposure to BaP were also noted in

mice (Culp et al., 1998) and in rats (Weyand et al., 1995). Furthermore, tissue-specific rates of CYP induction, levels of DNA adducts, and mutation frequencies do not correlate with the extent of tumour formation in the tissues studied (Godschalk et al., 2003). These findings suggest that additional factors may be involved in PAH-induced tissue-specific oncogenesis. While Kroese et al. (2001) suggested that local cellular proliferation may be one such additional factor required for cancer development, here we show that perturbations of several additional biological pathways that are directly favorable to carcinogenesis may be operating in parallel with DNA damage/mutation to influence the probability of developing tissue-specific tumours in mice (Figure 4-8).

In the present study, exposure to each of the PAHs caused significant increases in both DNA adducts and transgene mutant frequency in lung compared to matched controls. However, considerable differences in lung responses were observed at the pathway level for each PAH examined. PAH-specific pathway changes were also observed in the liver and forestomach. To determine whether consideration of apical endpoints and perturbations in a number of key pathways that are favorable to cancer formation would help improve cancer predictivity, we compared the BMDs for the lung apical endpoints and genomics endpoints for each of the PAHs examined. The known hallmarks of cancer were used as a directional tool to effectively classify a wide variety of pathways perturbed by the PAHs into functional groups and to highlight the altered tissue microenvironment following exposure. BMD calculation for individual apical endpoints and cancer-specific pathways varied depending on the type of data considered. The DNA adduct-derived BMDs predicted BghiP to be the second most potent, which is contrary to the available literature and its recent classification as a PAH lacking evidence for carcinogenicity by the USEPA and IARC (IARC, 2010; USEPA, 2011). These results further support the

complexities related to the predictive value of DNA adducts for carcinogenic potency of PAHs discussed above. BMD values derived from the results of mutant frequency ranked DBahA and BaP as the most potent with respect to their ability to induce mutation in a non-transcribed transgene. BaA and CHR data could not be modeled due to their poor ability to induce mutation as observed by the lack of response. It was also not possible to model BkF data due to lack of dose response. However, at the low and medium dose, BkF did show increased mutant frequency. Similarly, cancer pathway-based BMDs ranked DBahA as the most potent PAH able to elicit hallmark events and CHR as the least potent, which is in line with IARC's classification of CHR as group 3 non-carcinogen (IARC, 2010; USEPA, 2011). BaP was consistently ranked less potent than DBahA for all endpoints considered, in line with the results of an earlier study that showed DBahA as a more potent carcinogen in rats compared to BaP (Wenzel-Hartung et al., 1990). The importance of the temporal coordination of the hallmarks of cancer should be noted. The activation of angiogenesis or invasion and metastasis are later events in tumour progression; however, identification of perturbations in related signaling pathways at early stages may enhance the tumour predictivity when considered in conjunction with conventional toxicological endpoints. Although, it is not general practice to derive BMDs for DNA adducts, mutations (e.g., lacZ mutant frequency), or gene pathways for regulatory purposes, in the absence of rodent cancer bioassay data, BMDs for these toxicological endpoints may serve to set regulatory limits that would protect against effects that are mechanistically or empirically linked to carcinogenesis and prioritize compounds for further testing by long-term cancer bioassays. Recently, in a comprehensive case study, Moffat et al. (2015) compared BMDs derived from traditional cancer bioassays and those derived from pathway-based analyses indicative of carcinogenesis in BaP-exposed tissues. The authors showed a high concordance between the

points of departures derived from both apical and gene expression endpoints, as well as cancer bioassays for BaP. However, it is not possible to make direct comparisons between the BMDs derived from cancer bioassays and the results from the present study since not all the PAHs examined have cancer bioassay results available (IARC, 2010). Although further studies are needed to conclusively demonstrate the use of such non-cancer bioassay data for regulatory purposes, in the immediate term, the gene expression results obtained from the present and the study by Moffat et al. can be used to support the decision to use non-cancer data in a regulatory setting and to help reduce the uncertainties associated with that approach. Collectively, these results suggest that depending on the endpoint investigated, the potency ranking of each of these PAHs, including BaP, will differ for each tissue; a detailed knowledge of pathway perturbations will reduce the uncertainties involved in making such predictions of future carcinogenesis and will increase the accuracy of these rankings.

With the exception of BghiP, we observed that the key pathways involved in DNA adduct formation and DNA damage in lungs were all activated in response to the individual PAH exposures. These results suggested that six of the PAHs tested induce DNA damage responses similar to those observed in response to BaP. However, activation of several other cellular signaling pathways indicative of hallmarks of cancer, including neuronal signaling (required for activation of invasion and metastasis), activation of angiogenesis (required for blood vessel formation in tumours), activation of PI3K/AKT signaling pathway (involved in resisting cell death), cell cycle progression (required for cellular proliferation in the presence of damaged DNA), circadian rhythm signaling (associated with the evasion of growth suppressors), inhibition of PPAR γ signaling (required for inhibition of neoplastic growth of cancer cells), avoiding immune destruction, and tumour-promoting inflammation, varied between the PAHs.

The other pathways uniquely altered only in response to congener PAHs in the present study, such as calcium signaling (BaA, BbF, and CHR), endocytosis (BbF, CHR, and DBahA), chemokine signaling (BbF, BghiP, and BkF), and cytoskeletal reorganization (BbF, BkF, CHR, and IP), have previously been linked to BaP exposure (Ba et al., 2015; Laupeze et al., 2002; Mayati et al., 2012; van Grevenynghe et al., 2003). For example, G-protein coupled receptor signaling mediates alterations in calcium signaling in endothelial HMEC-1 cells and human kidney HEK293 cells following exposure to BaP (Mayati et al., 2012), as demonstrated by the inhibition of BaP-induced signaling in the presence of suramin, a potent inhibitor of G protein. Chemokine signaling induced by BbF, BghiP, and BkF was observed following BaP-induced inflammation in primary human macrophages (Lecureur et al., 2005; Sparfel et al., 2010). Similarly, endocytic function is inhibited in macrophages and dendritic cells derived from human monocytes exposed to BaP (Laupeze et al., 2002; van Grevenynghe et al., 2003). However, the implications of these pathways in cancer formation in lungs are not clear. Thus, it is not apparent whether a combination of DNA adduct formation, mutation induction, and the perturbation of pathways mentioned above is sufficient to trigger cancer formation following exposure to PAHs. One possibility is that the PAHs that induced low levels of DNA damage or mutations and subtle responses at the pathway levels could be acting as tumour promoters rather than initiators of cancer. For instance, it is known that altered calcium signaling helps sustain cellular proliferation and foster metastatic environment (Prevarskaya et al., 2011; Resende et al., 2013), and defective endocytosis has been linked to increased metastatic function in cancer cells (Mosesson et al., 2008). Cytoskeletal organization has been linked to metastasis and invasion of early tumour cells (Donald et al., 2001), and it is known that BaP exposure promotes cell migration and invasion in both in vivo and in vitro models of human hepatocellular carcinoma (Ba et al., 2015). This may

be required for expansion of genetically damaged cells and subsequent cancer formation.

Inflammation involving chemokine signaling is also proposed to promote conditions conducive to cancer in a genotoxic as well as in non-genotoxic scenarios (Lu et al., 2006). Thus, in addition to the DNA damage response pathways induced by BaP and the other PAHs examined, activation of distinct signaling pathways induced by the other PAHs suggests greater contribution of alternative carcinogenic mode/mechanisms.

In addition to the induction of cancer-related pathways, the results of the present study demonstrate that the tissue-specific transcriptional responses to PAHs are distinct, suggesting that the carcinogenic potency of each individual PAH will vary based on the type of tissue investigated. Of the forestomach, liver, and lung tissues, the greatest response at the transcriptional level was induced in the lung, which we previously showed for BaP in both acute exposure (B6C3F1 mice orally exposed to BaP for 3 days with sample collection 4 h post-exposure) (Halappanavar et al., 2011) and sub-chronic studies (Labib et al., 2013; Labib et al., 2012). Indeed, the BMD values for transcriptional pathways associated with the hallmarks of cancer for seven of the eight PAHs indicated that the pulmonary response was more sensitive than the liver and forestomach (BaP, BaA, BbF, BkF, CHR, DBahA, and IP). For BghiP, only the forestomach data could be modeled, possibly indicative of lower potential for adverse outcomes in the lung and liver compared to the forestomach for this PAH. Similar observations of enhanced pulmonary response were noted in mice exposed to oral BaP in studies conducted by Halappanavar et al. (2011); Harrigan et al. (2006). Other recent studies have also shown tissue-specific gene expression changes in a multi-tissue analysis following BaP exposure (Zuo et al., 2014). One of the explanations for the observed tissue-specific discrepancies could be the different rates of metabolism, clearance and translocation or tissue distribution of these PAHs

from the targeted tissues. It is a possibility that some PAHs, such as DBahA and BbF, are quickly distributed to distant organs (e.g., lungs) from the forestomach (site of contact), which may result in reduced impact on the forestomach. This in turn may induce differential responses at the molecular level that are related to the likelihood of eventual cancer formation.

4.5.1 Conclusions

The present study explored the validity of the assumption that BaP is an acceptable reference PAH for genotoxic, carcinogenic PAHs by examining the global gene expression changes in three separate tissues. Our results showed that all of the PAHs investigated were able to induce genotoxicity in a dose-dependent manner. However, these results were not comparable with the known relative potencies calculated based on BaP as a reference chemical (CCME, 2010). The results also revealed a response at the transcriptional level following exposure to the PAHs that varied according to agent, dose, and tissue. Moreover, the gene expression results clearly demonstrated that each PAH induces molecular alterations (signaling pathways) related to carcinogenesis that are distinct from those observed for BaP, thus contradicting the assumption that they all act through similar mechanisms. We show differences in the ability of PAHs to induce major key events, as well as other molecular changes related to carcinogenesis, which suggests that the relative potency approach, which is estimated based on the current default assumptions, may not be accurate. These distinct tissue-specific transcriptional responses are indicative of the need to exercise caution when deriving potency equivalence factor (PEF) values; depending on what endpoint/tissue is evaluated in coming up with a PEF value, these differences could lead to overestimation or underestimation of the PEF, which would result in overestimation or underestimation of the total risk estimate.

Thus, the results of the present study show that BaP alone may not accurately reflect the toxicity imparted by other PAH congeners and suggest that consideration of toxicity induced by

more than one PAH be used to represent the class of PAHs. Finally, moving forward, an integrated approach that involves multiple endpoints, including genomics, and multiple tissues may help increase the accuracy of the assay-derived relative potencies for each PAH and the predictive potential of the relative potency approach.

4.6 Tables and Figures

Table 4-1 Total numbers of differentially expressed genes in forestomach, liver, and lung tissues.

FORESTOMACH		*BaP	BaA	BbF	BghiP	BkF	CHR	DBahA	IP
LOW	Up	9	10	0	0	1	3	0	0
	Down	0	0	2	3	0	2	0	2
	Total	9	10	2	3	1	5	0	2
MED	Up	119	3	2	4	7	37	1	0
	Down	16	2	2	8	128	17	2	1
	Total	135	5	4	12	135	54	3	1
HIGH	Up	311	5	15	95	12	166	3	5
	Down	97	11	6	14	143	52	2	3
	Total	408	16	21	109	155	218	5	8
TOTAL		414	27	23	119	213	244	6	9
LIVER									
LOW	Up	2	7	16	2	5	2	29	0
	Down	3	70	5	3	1	2	27	0
	Total	5	77	21	5	6	4	56	0
MED	Up	9	22	122	2	96	3	20	1
	Down	1	172	37	2	28	3	37	5
	Total	10	194	159	4	124	6	57	6
HIGH	Up	109	51	201	20	111	3	188	6
	Down	21	163	83	40	58	1	121	13
	Total	130	214	284	60	169	4	309	19
TOTAL		139	340	352	69	224	13	334	24
LUNG									
LOW	Up	14	22	219	1	7	3	165	6
	Down	3	7	37	1	3	3	27	25
	Total	17	29	256	2	10	6	192	31
MED	Up	104	152	222	27	61	0	254	41
	Down	22	40	131	9	55	1	46	120
	Total	126	192	353	36	116	1	300	161
HIGH	Up	240	409	586	51	91	92	415	140
	Down	77	214	340	12	108	67	151	132
	Total	317	623	926	63	199	159	566	272
TOTAL		329	675	1136	84	248	162	624	319

Note. Gene expression profiles for BaP in the forestomach, liver, and lung were previously published* (Labib et al., 2012; Labib et al., 2013).

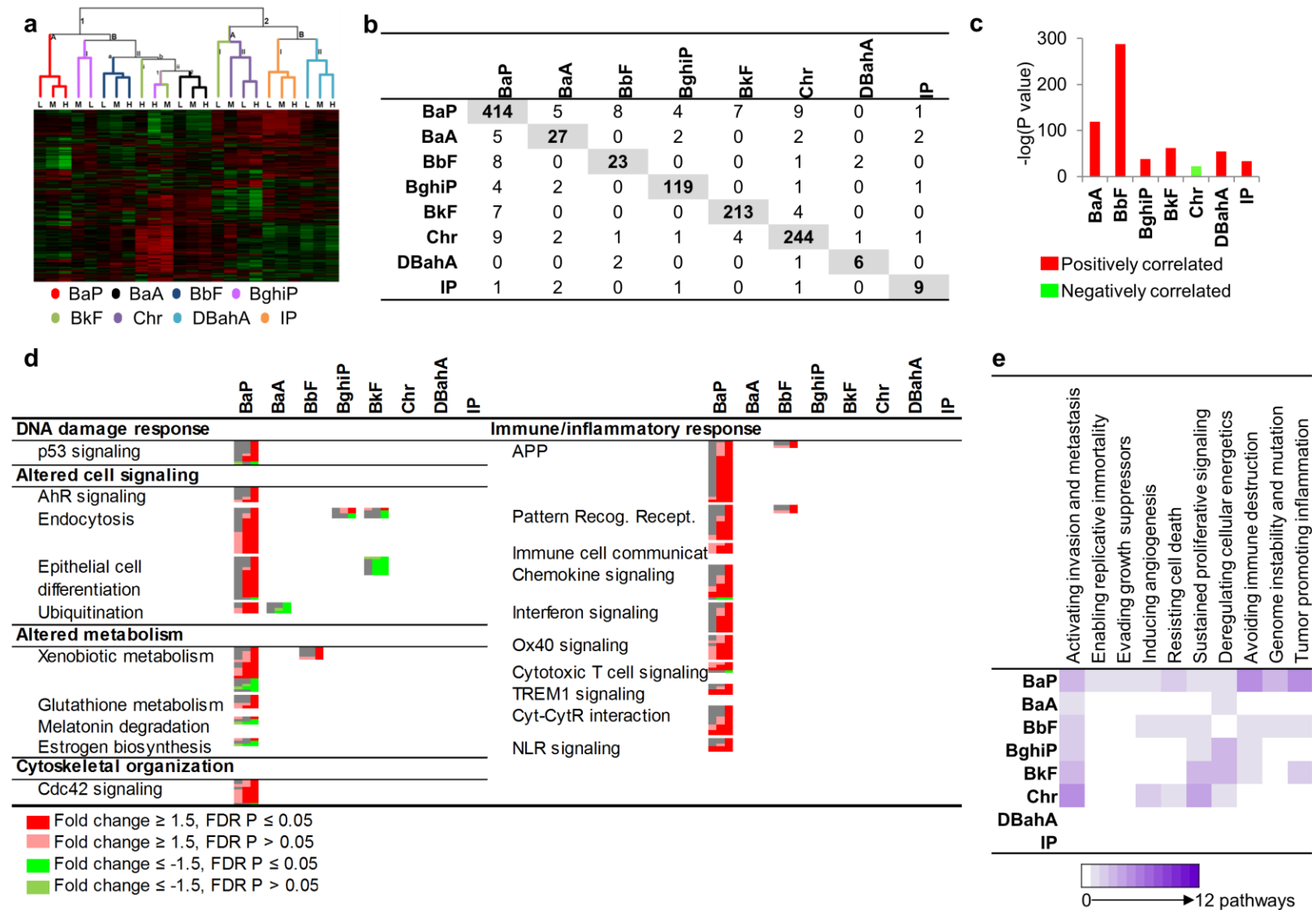


Figure 4-1 Forestomach data analyses part I.

(a) Hierarchical cluster analysis comparing forestomach gene expression data from adult male MutaTM Mouse exposed to three doses (low – L, medium – M, high – H) of eight PAHs. Branches are color coded for PAH treatment. Genes were included if they reached significance ($\text{FDR } P \leq 0.05$, fold change ± 1.5 in either direction) in at least one treatment group. Groups represent an average of all mice in our group. (b) Table of forestomach DEGs that are common in at least one dose group. Cells highlighted in grey represent the total number of DEGs for that PAH. (c) Degree of correlation between BaP DEGs and DEGs for each PAH in the forestomach using the NextBio Meta-analysis tool. The height of each vertical bar represents the degree of correlation between the PAH and BaP ($-\log(P \text{ value})$), which is associated with the directionality of the gene expression changes. Red color denotes positive correlation with BaP and green color denotes negative correlation. (d) All pathways significantly enriched for by BaP in the forestomach and the commonalities with other PAHs. Each column represents a dose group for the denoted PAH and each row represents a gene. All colored cells represent genes with fold change ≥ 1.5 in either direction. (e) Perturbation of the hallmarks of cancer, emerging hallmarks, and enabling characteristics by each PAH in the forestomach based on transcriptomic data. The purple cells represent the relative contribution of pathways associated with each hallmark of cancer.

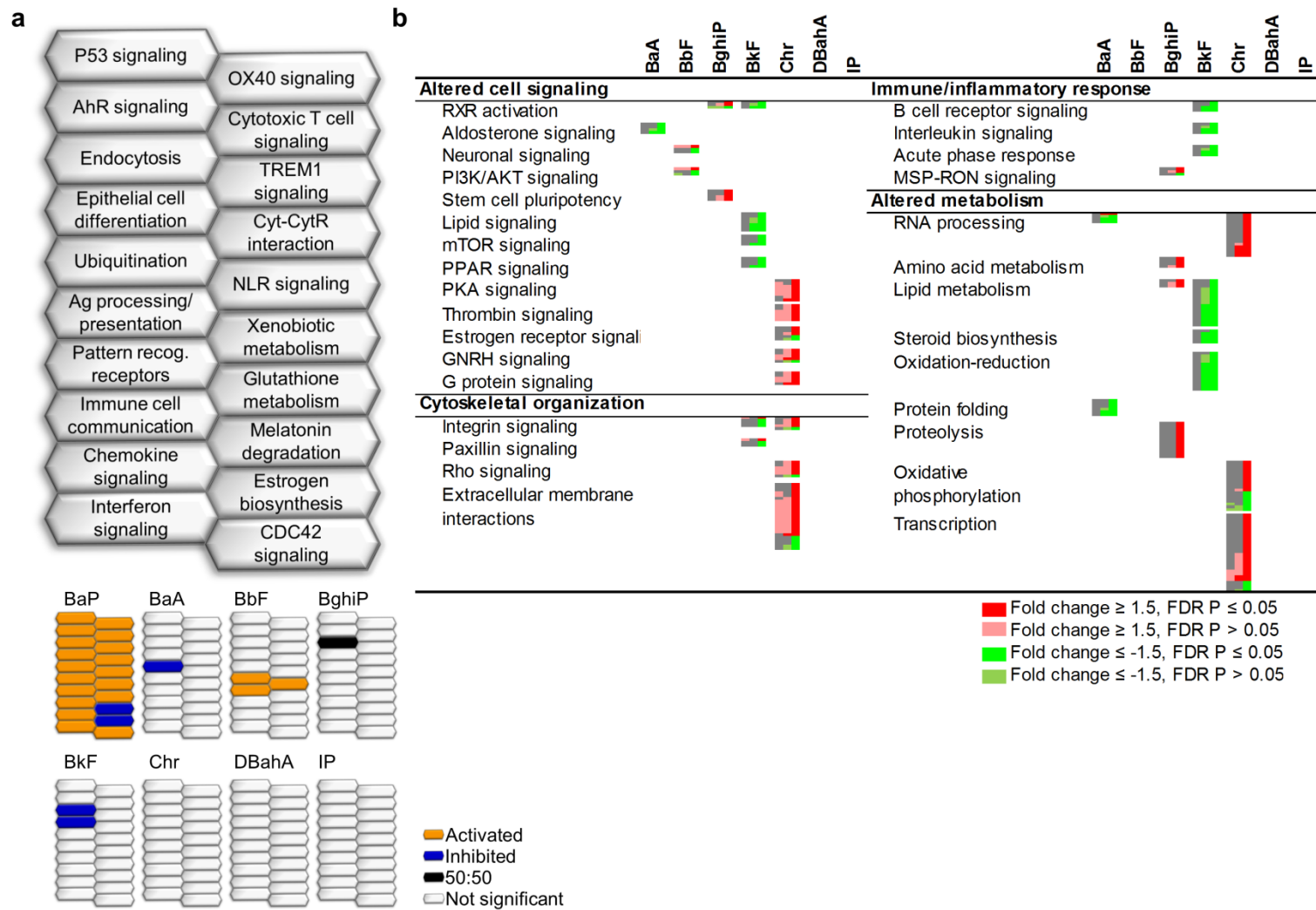


Figure 4-2 Forestomach data analyses part II.

(a) Honeycomb diagram showing direction of regulation of pathways commonly enriched for by BaP and other PAHs. Orange honeycombs represent pathways that are predicted to be activated based on the direction of regulation of the genes and blue cells represent those predicted to be inhibited. Black cells represent those in which 50% of the genes indicate activation and 50% of the genes indicate inhibition. White cells represent pathways not significant. (b) Pathways enriched for by at least one PAH and not by BaP. Each column represents a dose group for the denoted PAH and each row represents a gene. All colored cells represent genes with fold change ≥ 1.5 in either direction.

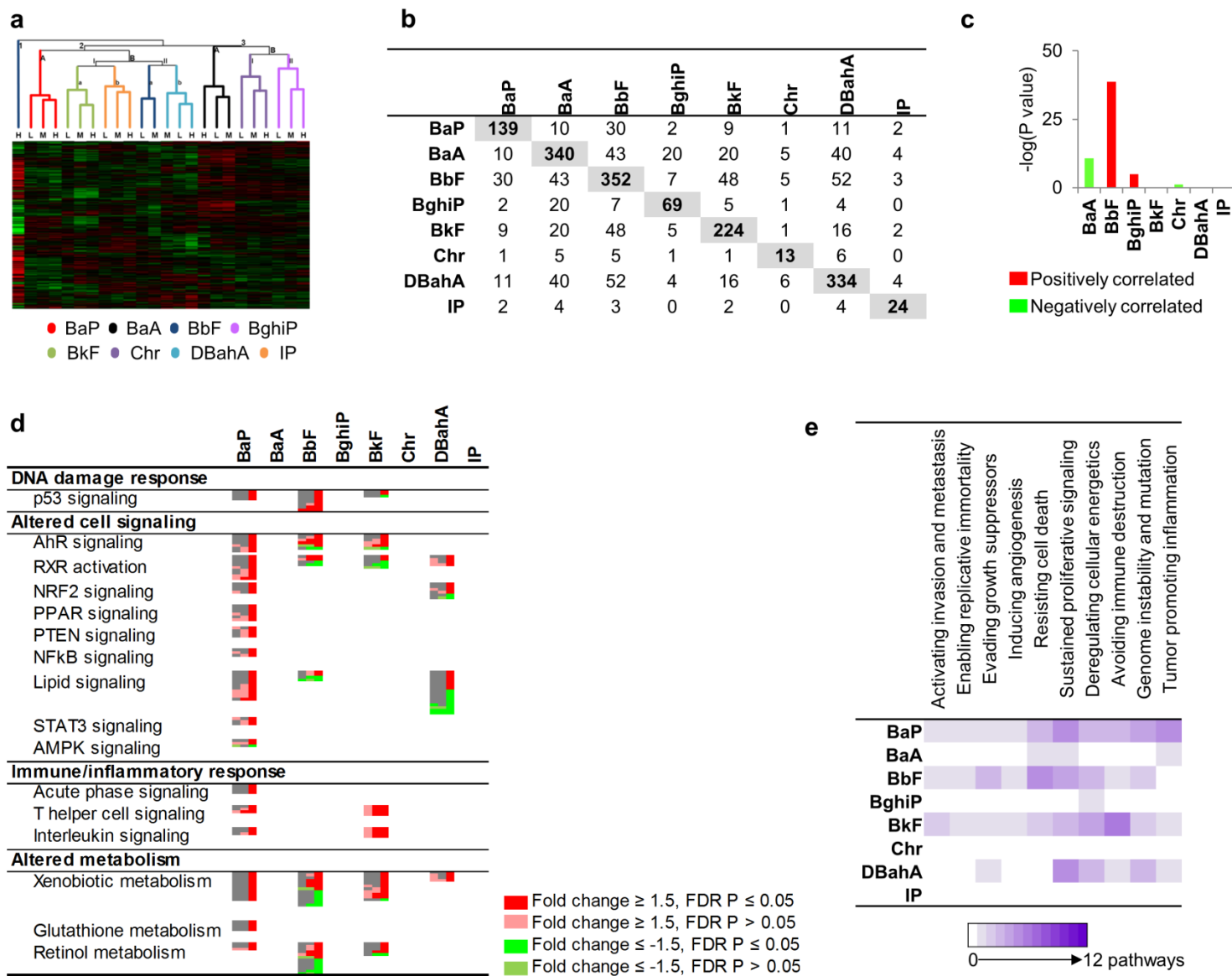


Figure 4-3 Liver data analyses part I.

(a) Hierarchical cluster analysis comparing hepatic gene expression data from adult male MutaTM Mouse exposed to three doses (low – L, medium – M, high – H) of eight PAHs. Branches are color coded for PAH treatment. Genes were included if they reached significance ($FDR\ P \leq 0.05$, fold change ± 1.5 in either direction) in at least one treatment group. Groups represent an average of all mice in our group. (b) Table of hepatic DEGs that are common in at least one dose group. Cells highlighted in grey represent the total number of DEGs for that PAH. (c) Degree of correlation between BaP DEGs in the liver and DEGs for each PAH in the liver using the NextBio Meta-analysis tool. The height of each vertical bar represents the degree of correlation between the PAH and BaP ($-\log(P\text{ value})$), which is associated with the directionality of the gene expression changes. Red color denotes positive correlation with BaP and green color denotes negative correlation. (d) All pathways significantly enriched for by BaP in the liver and the commonalities with other PAHs. Each column represents a dose group for the denoted PAH and each row represents a gene. All colored cells represent genes with fold change ≥ 1.5 in either direction. (e) Perturbation of the hallmarks of cancer, emerging hallmarks, and enabling characteristics by each PAH in the liver based on transcriptomic data. The purple cells represent the relative contribution of pathways associated with each hallmark of cancer

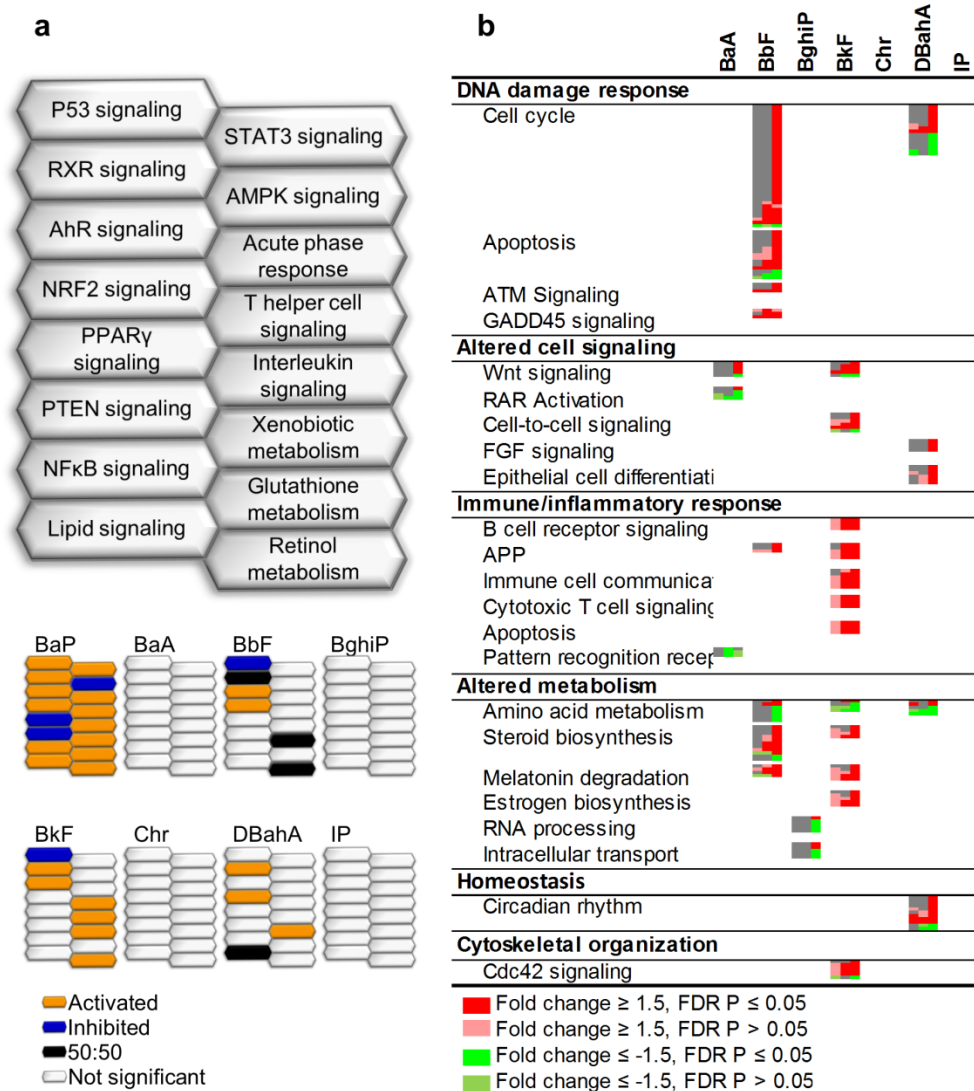


Figure 4-4 Liver data analysis part II.

(a) Honeycomb diagram showing direction of regulation of pathways commonly enriched for by BaP and other PAHs. Orange honeycombs represent pathways that are predicted to be activated based on the direction of regulation of the genes and blue cells represent those predicted to be inhibited. Black cells represent those in which 50% of the genes indicate activation and 50% of the genes indicate inhibition. White cells represent pathways not significant. (b) Pathways enriched for by at least one PAH and not by BaP. Each column represents a dose group for the denoted PAH and each row represents a gene. All colored cells represent genes with fold change ≥ 1.5 in either direction.

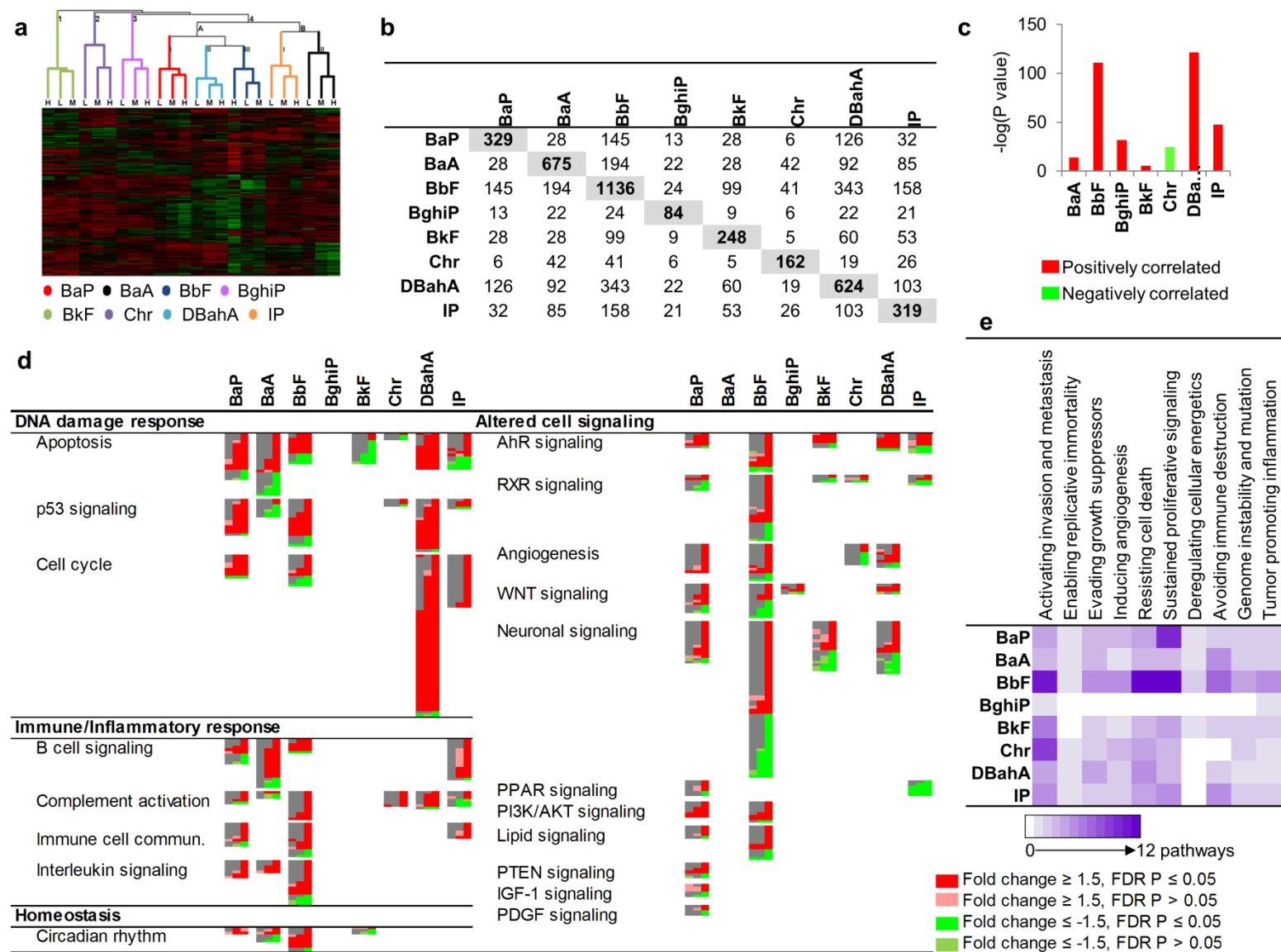


Figure 4-5 Lung data analysis part I.

(a) Hierarchical cluster analysis comparing pulmonary gene expression data from adult male MutaTMMouse exposed to three doses (low – L, medium – M, high – H) of eight PAHs. Branches are color coded for PAH treatment. Genes were included if they reached significance ($FDR\ P \leq 0.05$, fold change ± 1.5 in either direction) in at least one treatment group. Groups represent an average of all mice in our group. (b) Table of pulmonary DEGs common in at least one dose group. Cells highlighted in grey represent the total number of DEGs for that PAH. (c) Degree of correlation between BaP DEGs in the lung and DEGs for each PAH in the lung using the NextBio Meta-analysis tool. The height of each vertical bar represents the degree of correlation between the PAH and BaP ($-\log(P\text{ value})$), which is associated with the directionality of the gene expression changes. Red color denotes positive correlation with BaP and green color denotes negative correlation. (d) All pathways significantly enriched for by BaP in the lung and the commonalities with other PAHs. Each column represents a dose group for the denoted PAH and each row represents a gene. All colored cells represent genes with fold change ≥ 1.5 in either direction. (e) Perturbation of the hallmarks of cancer, emerging hallmarks, and enabling characteristics by each PAH in the lung based on transcriptomic data. The purple cells represent the relative contribution of pathways associated with each hallmark of cancer

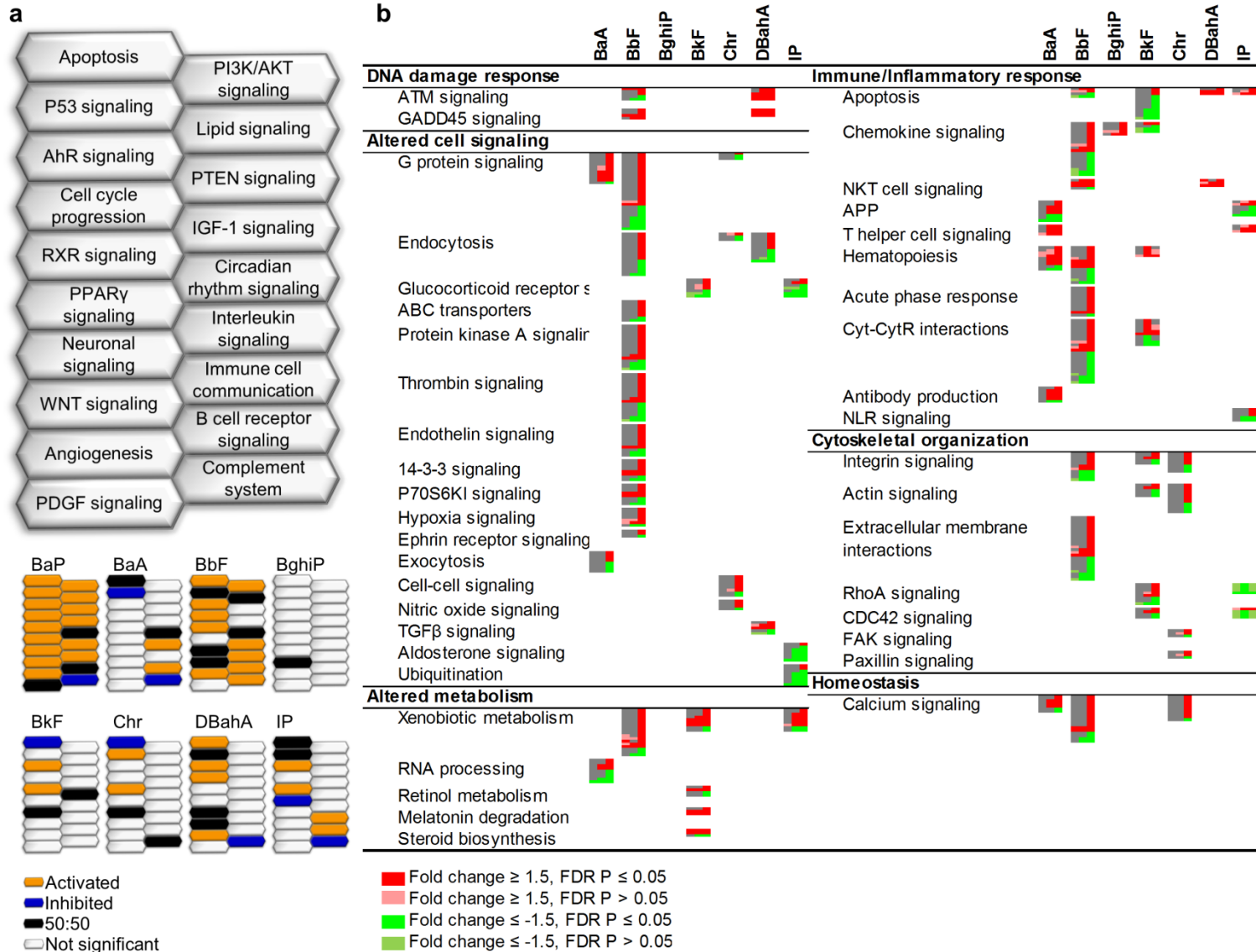


Figure 4-6 Lung data analysis part II.

(a) Honeycomb diagram showing direction of regulation of pathways commonly enriched for by BaP and other PAHs. Orange honeycombs represent pathways that are predicted to be activated based on the direction of regulation of the genes and blue cells represent those predicted to be inhibited. Black cells represent those in which 50% of the genes indicate activation and 50% of the genes indicate inhibition. White cells represent pathways not significant. (b) Pathways enriched for by at least one PAH and not by BaP. Each column represents a dose group for the denoted PAH and each row represents a gene. All colored cells represent genes with fold change ≥ 1.5 in either direction

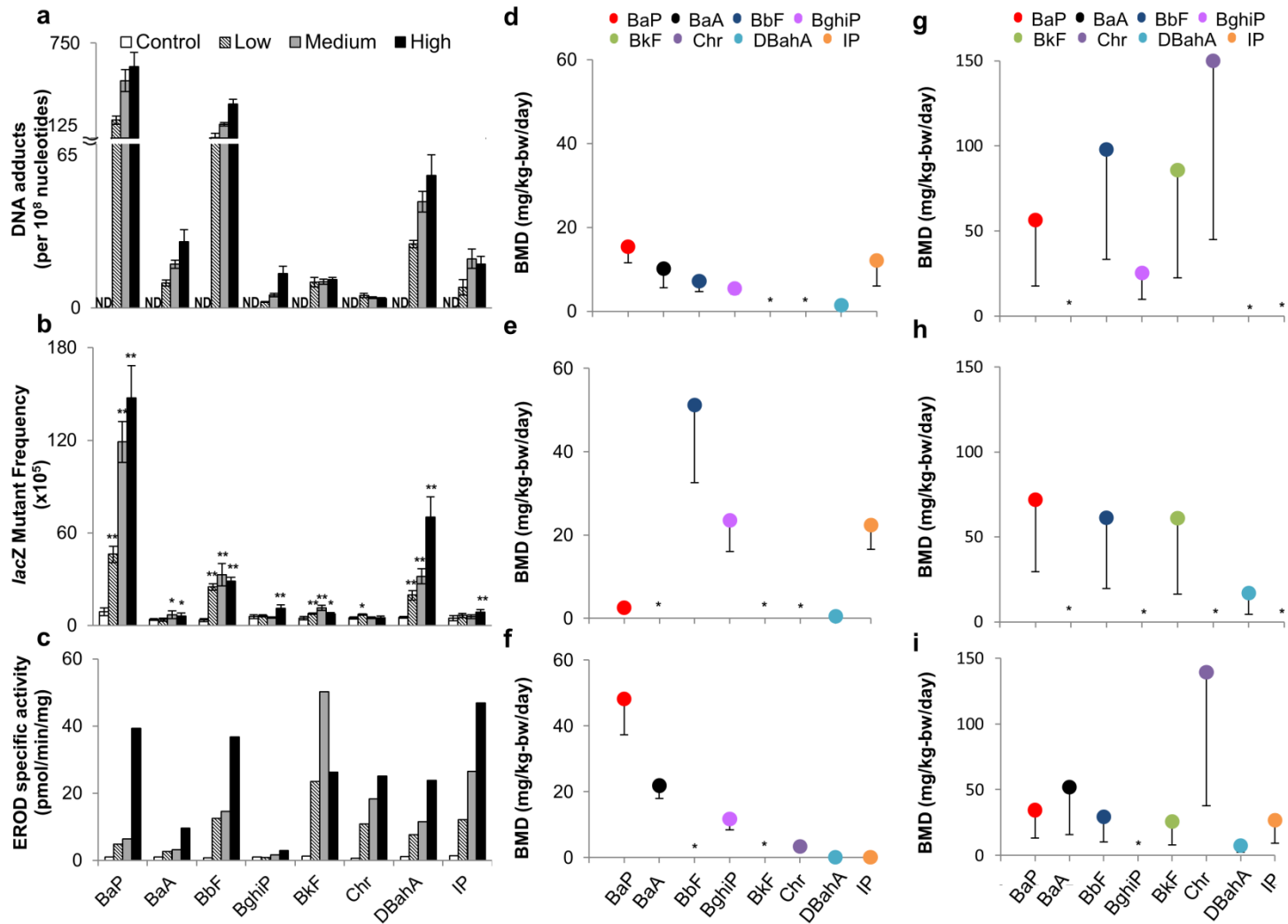


Figure 4-7 Apical endpoints and BMD analyses.

(a) DNA adduct formation, (b) *lacZ* mutant frequency, and (c) EROD activity in the lungs from MutaTM Mouse subchronically exposed to BaP, BaA, BbF, BghiP, BkF, CHR, DBahA, and IP. Levels of PAH-DNA adducts were determined using the nuclease P1 enrichment version of the ³²P-postlabeling method. Data are represented as average $\pm$ SEM. Average *lacZ* mutant frequency was determined using the P-Gal positive selection assay. Values shown are average frequencies $\times 10^5 \pm$ SEM. Asterisk * denotes $P < 0.1$, ** denotes $P < 0.01$ compared to vehicle controls. EROD activity was determined by pooling the tissue for all mice in each dose group. No significance can be calculated. DNA adduct and *lacZ* mutant frequency for BaP were previously reported (Labib et al 2012). ND, not detected. (d-i) Charts of BMD (-BMDL) values in mg/kg-bw/day for each PAH based on data for (d) DNA adducts, (e) *lacZ* mutant frequency, and (f) EROD activity, and the number of hallmarks of cancer associated with the pathways in the (g) forestomach, (h) liver, and (i) lung. * indicates the data could not be modeled

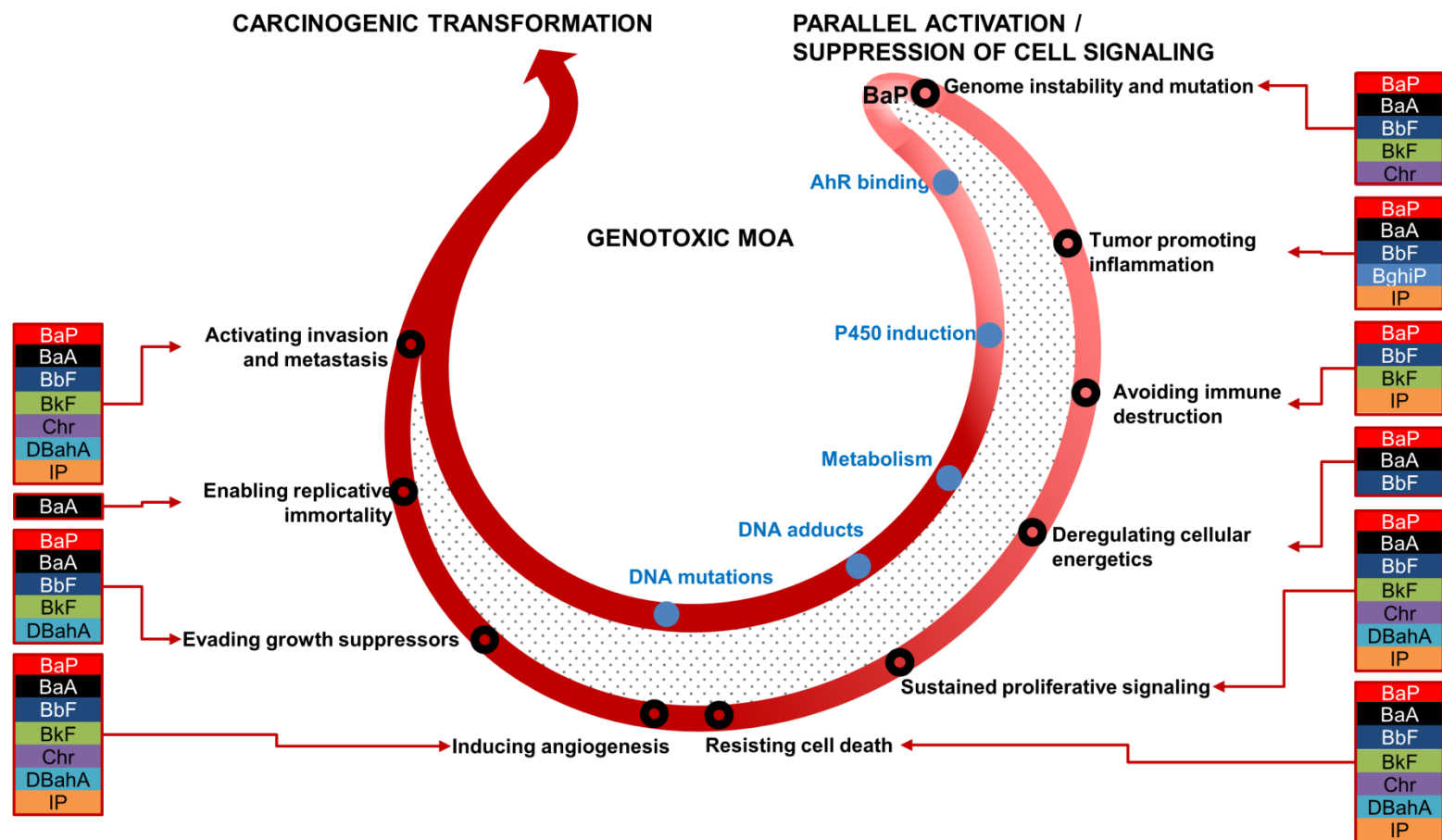


Figure 4-8 Activation or suppression of cell signaling pathways in support of pulmonary carcinogenic transformation.

This schematic describes the contribution of each PAH to the carcinogenic mode of action in the form of the hallmarks of cancer. These signaling events are vital to the progression of cancer and are occurring in parallel to the genotoxic MOA key events. The order of the hallmarks in the figure does not reflect any temporal coordination or cooperation with the existing key events. BaP's carcinogenic MOA key events are shown in blue font.

5 Chapter : A framework for the use of single chemical transcriptomics data in predicting the hazards associated with complex mixtures of polycyclic aromatic hydrocarbons

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5.1 Abstract

The assumption of additivity applied in the risk assessment of environmental mixtures containing carcinogenic polycyclic aromatic hydrocarbons (PAHs) was investigated using transcriptomics.

MutaTMMouse were gavaged for 28 days with three doses of eight individual PAHs, two defined mixtures of PAHs, or coal tar, an environmentally ubiquitous complex mixture of PAHs.

Microarrays were used to identify differentially expressed genes (DEGs) in lung tissue collected three days post-exposure. Cancer-related pathways perturbed by the individual or mixtures of PAHs were identified and dose-response modeling of the DEGs was conducted to calculate gene/pathway benchmark doses (BMDs). Individual PAH-induced pathway perturbations (the median gene expression changes for all genes in a pathway relative to controls) and pathway BMDs were applied to models of additivity (i.e., concentration addition (CA), generalized concentration addition (GCA), and independent action (IA)) to generate predicted pathway-specific dose-response curves for each PAH mixture. The predicted and observed pathway dose-response curves were compared to assess the sensitivity of different additivity models.

Transcriptomics-based additivity calculation showed that IA accurately predicted the pathway perturbations induced by all mixtures of PAHs. CA did not support the additivity assumption for the defined mixtures; however, GCA improved the CA predictions. Moreover, pathway BMDs derived for coal tar were comparable to BMDs derived from previously published coal tar-induced mouse lung tumor incidence data. These results suggest that in the absence of tumour

incidence data, individual chemical-induced transcriptomics changes associated with cancer can be used to investigate the assumption of additivity and to predict the carcinogenic potential of a mixture.

5.2 Introduction

Human exposures to environmental chemicals occur via contact with complex mixtures rather than single chemicals in isolation. Currently, quantitative hazard and risk assessment of chemical mixtures is conducted using two approaches: (a) a whole mixture approach or (b) a component-based approach that only examines prioritized mixture components. The whole mixture approach is used when data are available for the mixture in question or for a surrogate mixture that is sufficiently similar to the mixture under investigation. The whole mixture approach is preferred by several regulatory agencies such as Health Canada and the United States Environment Protection Agency (CCME, 2010; USEPA, 2010); however, due to difficulties in processing mixtures (e.g., atmospheric particulate matter) and the lack of standards for positive controls, the whole mixture approach cannot be used for routine mixture toxicity testing and risk assessment. Similarly, comparing each newly identified mixture to a sufficiently similar mixture is complicated because of the relative differences in mixture composition that may influence the overall toxicity of the mixture. Thus, assuming that the total risk and hazard associated with a mixture is the sum of the contributions from the known priority components, the component-based approach has been deemed the most appropriate and practical for routine assessments. This approach is also referred to as the additivity approach, and it is currently employed by multiple regulatory agencies worldwide, including Health Canada (2010), the USEPA (2010), and the European Commission (2001).

The component-based approach relies on a series of established reference models centered on the assumption that individual components of the mixture do not interact, do not influence

each other's toxicity, and that their toxicological activity is additive. Accordingly, the toxicity of mixtures composed of similarly acting chemicals (e.g., those with similar biochemical mechanisms of toxicity or carcinogenic modes of action) is calculated as the sum of the concentrations/doses of selected chemicals, each adjusted using a relative potency scalar to convert amounts to equivalents of a potent reference substance (i.e., the concentration addition or CA model). For mixtures of dissimilarly acting chemicals, the total toxic response of the mixture is calculated by summing the toxic responses of each chemical component (i.e., the independent action or IA model, also known as response addition and effect addition models). Although pragmatic, the CA approach is limited in scope since it can only estimate the combinatorial toxicity of the mixture up to the maximal toxic response of the weakest mixture component. Consequently, recent advances in mixtures toxicology have led to the development of alternative models, such as generalized concentration addition model (GCA) (Howard and Webster, 2009), which considers the maximal toxic response of each mixture component. The GCA model has been used effectively in predicting toxicological responses of mixtures of aryl hydrocarbon receptor (AHR) agonists (Howard et al., 2010). Together, these models have proven useful for identifying interactive behaviours in defined mixtures (i.e., deviations from additivity), as well as for understanding the influence of unknown substances on the additive behaviour of known mixture components (European Commission, 2012; Kortenkamp et al., 2011).

As the toxicology community embarks upon a paradigm shift towards more mechanistic testing strategies (NRC, 2007), applications of 'omics tools, such as transcriptomic studies investigating global changes in gene expression, are providing insight into the toxic action of chemicals and comprehensive information on the biological pathways and processes disturbed by environmental chemicals. However, despite noteworthy advantages, few studies have applied

transcriptomics to investigate the joint effects of chemicals in mixtures. The advantages of transcriptomic tools for mixture assessments relate to the high-content data that are generated, and the utility of such data for identifying and discriminating pathways and processes affected by individual mixture components and the mixtures themselves. Such analyses can determine if structurally similar chemicals indeed act via a common mechanism and can provide predictive insight regarding the likelihood of chemical interactions.

Polycyclic aromatic hydrocarbons (PAHs) are a class of structurally-similar chemicals, many of which have been characterized as genotoxic carcinogens. They are formed during the incomplete combustion of organic materials, and are ubiquitously found in many environmentally-relevant complex mixtures to which humans are regularly exposed. Human health risk assessments of PAH mixtures commonly employ the CA approach that incorporates quantitative contributions from the 16 PAHs that were previously selected by the USEPA as priorities for concern and control (i.e., priority PAHs). In a recent study, we selected 8 of the 16 priority PAHs that are genotoxic carcinogens and applied global transcriptomic profiling, in combination with other apical endpoint analyses, to determine if these 8 PAHs share a similar mode of action (Labib et al., 2015). The results revealed that the eight structurally-similar PAHs all induced DNA damage, mutation, and enzyme activity; however, at the transcriptional level, the response was markedly different. Indeed, the individual PAHs induced alterations in the expression of genes associated with diverse arrays of biological functions and processes that were not common to all eight compounds. Furthermore, the transcriptional profiles were tissue-specific (Labib et al., 2012; Labib et al., 2013; Labib et al., 2015). The results suggested that the underlying genotoxic and carcinogenic mechanisms of action for these eight PAHs are not

identical. This led us to hypothesize that the assumption regarding a similar mode of action may be inaccurate.

The primary objective of the present study is to examine the utility of transcriptomic data for investigating the combined effects of chemicals in mixtures. Since we have tissue-specific transcriptomic data for the eight individual PAHs, this study examined PAH-containing mixtures. More specifically, whole genome transcriptional profiling was used to examine pathways/processes perturbed by exposure to two defined mixtures of four and eight PAHs, and a coal tar extract (a highly complex mixture of PAHs). Since the lung transcriptome was the most perturbed of the three tissues studied in our previous work in terms of transcriptomic responses and DNA damage induction, in this study the pulmonary transcriptomes following exposure to the mixtures were compared to the pulmonary transcriptomes from our previous studies of eight PAHs individually (Labib et al., 2012; Labib et al., 2013; Labib et al., 2015). Furthermore, the comparisons were used to evaluate the CA, GCA, and IA models, to investigate interactions between priority PAHs, and lastly, to determine the influence of unknown substances on the activity of the complex mixtures via comparison of the simple and complex mixtures. The analyses specifically examined pathways associated with cancer since the PAHs examined are known or suspected carcinogens. The data were further used to assess whether the predicted and actual perturbations of cancer-associated pathways elicited by coal tar exposures are aligned with the lung tumour incidence observed in the Culp et al. murine carcinogenicity study (Culp et al., 1998).

5.3 Methods

5.3.1 Chemicals

BaP and chrysene (CHR) were purchased from Sigma-Aldrich Canada Ltd. (Canada).

Benz(*a*)anthracene (BaA), benzo(*b*)fluoranthene (BbF), benzo(*ghi*)perylene (BghiP),

benzo(*k*)fluoranthene (BkF), dibenz(*ah*)anthracene (DBahA), and indeno(123,*cd*)pyrene (IP) were purchased from Cambridge Isotopes Laboratories (USA). All compounds had purity $\geq 98.8\%$.

5.3.2 Preparation of PAH mixtures and animal exposures

Coal tar extract (CT-Mix) was prepared as described previously (Wise et al., 1988; Long et al., 2016b). The analytical assessment of the CT-Mix for volatile organic compounds and PAH content was conducted by Paracel Laboratories Ltd. (Ottawa, ON, Canada), a commercial laboratory accredited according to ISO/IEC 17025:2005 by the Canadian Association for Laboratory Accreditation, by gas chromatography mass spectrometry (EPA reference methods 624 and 625) (further details can be found in Supplementary Table 5-1). The relative content of the eight priority PAHs in the CT-Mix was 23983 mg PAH/kg CT-Mix. The relative concentration of each of these eight PAHs relative to total PAH content in the CT-Mix used in this study was within 0 - 1% of that found in the coal tar sample used in a previously published 2-year feeding study with coal tar (Culp et al., 1998) (Supplementary Table 5-1); the content of the eight PAHs in the Culp et al. (1998) study was 12499 mg PAH/kg coal tar.

The proportion of each PAH in the CT-Mix was then used to prepare two defined mixtures containing only four PAHs (4PAH-Mix) or only eight PAHs (8PAH-Mix). The PAHs selected for the 4PAH-Mix and 8PAH-Mix constitute the panel of four and eight PAHs recommended for routine examination of food samples by the European Food Safety Authority because they are the genotoxic PAHs that were measured in coal tar mixtures used in oral carcinogenicity studies (EFSA, 2008). Exposure doses for the defined mixtures and the CT-Mix were selected based on the maximum tolerated dose of each mixture determined in a dose range-finding study. This stock was diluted twice, by two-fold each, to obtain two lower doses. The specific doses in the 4PAH-Mix are as follows: BaP (9.9, 19.9, 39.7 mg/kg-day), BaA (12, 23.9, 47.8 mg/kg-day),

BbF (12.6, 25.3, 50.6 mg/kg-day), and CHR (11.4, 22.7, 45.5 mg/kg-day). This provided doses of 12.5, 25, and 50 mg BaP equivalents/kg-day based on the CCME PAH-specific potency equivalence factors (CCME, 2010). The specific doses of the 8PAH-Mix are as follows: BaP (10.5, 20.9, 41.9 mg/kg-day), BaA (12.6, 25.2, 50.4 mg/kg-day), BbF (13.3, 26.7, 53.4 mg/kg-day), CHR (12.0, 24.0, 48.0 mg/kg-day), BghiP (5.7, 11.4, 22.7 mg/kg-day), BkF (6.9, 13.7, 27.5 mg/kg-day), DBahA (1.2, 2.4, 4.8 mg/kg-day), and IP (5.2, 10.4, 20.9 mg/kg-day). This provided doses of 15, 30, and 60 mg BaP equivalents/kg-day. The CT-Mix was diluted to provide doses of 1.3, 2.5, and 5.1 mg BaP equivalents/kg-day.

The animal care, exposures, and tissue collection procedures have been previously described (Labib et al., 2012; Lemieux et al., 2011). Mice were bred, maintained, and treated in accordance with the Canadian Council for Animal Care Guidelines, and all protocols were approved by Health Canada's Animal Care Committee. In brief, adult, male MutaTMMouse (transgenic mouse strain 40.6 on BALB/C-DBA/2 background) were exposed by oral gavage for 28 consecutive days to olive oil (vehicle control) or to three doses of each of the PAH-containing mixtures. The dosing regime follows the established Organisation for Economic Co-operation and Development (OECD) guideline for transgenic rodent mutation assays (i.e., TG #488) (OECD, 2011). Each treatment group contained five animals. Three days after the final dose animals were sacrificed by cardiac puncture under isofluorene anaesthesia. Two mice from the 8PAH-Mix died as a result of accidental pulmonary puncture during oral gavage. Thus, the final sample size for the 8PAH-Mix experiment was 5, 5, 4, and 4 in the control, low, medium, and high-dose groups, respectively. The right lobe of the lung was collected, flash frozen in liquid nitrogen, and stored at -800C until use..

5.3.3 Tissue RNA extraction and purification

Total RNA for gene expression analysis was isolated as described previously (Halappanavar et al., 2011; Labib et al., 2012; Labib et al., 2013). Briefly, total RNA was extracted from a random slice of lung using TRIzol reagent (Invitrogen, Canada) and purified using RNeasy Mini Kit (Qiagen, Canada) following manufacturer's recommendations. All samples met quality control standards for RNA with A260/A280 ratios between 2.0 and 2.2 determined using a NanoDrop Spectrophotometer (ThermoFisher Scientific, Canada) and RNA integrity numbers above 8.0 determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Canada).

5.3.4 Microarray hybridization and analysis

A detailed description of the microarray hybridization and statistical analysis protocols have been published previously (Labib et al., 2013). With the exception of the 8PAH-Mix (see above), 5 replicates were examined for each PAH mixture treatment group. In brief, 200 ng of total RNA from each individual sample and 200 ng of Universal Mouse Reference RNA (Stratagene, Canada) were used to synthesize cDNA and cyanine-labeled cRNA using the Agilent Linear Amplification Kit (Agilent Technologies Inc., Canada). The labeled cRNAs (i.e., Cyanine-5 for experimental samples and Cyanine-3 for reference RNA) were purified using RNeasy Mini Kits (Qiagen, Canada). 300 ng of labeled cRNA from each experimental sample was hybridized with the same amount of labeled reference RNA to Agilent Sureprint G3 Mouse GE 8x60K microarrays (Agilent Technologies Inc., Canada) at 65°C for 17 hours in the Agilent SureHyb hybridization chamber. The arrays were washed and scanned on an Agilent G2505B Scanner according to the manufacturer's recommendations. Data were extracted using Feature Extraction 10.7.3.1 (Agilent Technologies Inc., Canada).

Details of the statistical normalization methods for the transcriptomic data were published previously (Labib et al., 2013; Labib et al., 2015). Briefly, a reference design (Kerr and

Churchill, 2001; Kerr and Churchill, 2007) was used to analyze microarray data. Non-background median signal intensities were normalized using LOWESS (Yang et al., 2002) using the R (R Development Core Team, 2010) platform. A transcript was considered to be a differentially expressed gene (DEG: upregulated or downregulated relative to the vehicle treated controls) using the MAANOVA library in R (Wu et al., 2003). The Fs statistic was used to test for treatment effects (Cui et al., 2005). The P values for all statistical tests for each probe ID were estimated by the permutation method using residual shuffling followed by adjustment for multiple comparisons using the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995). The fold change calculations were based on the least-square means (Goodnight and Harvey, 1978; Searle et al., 1980). All microarray results are available in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE87691.

Biological replicates of each experimental condition were collapsed to an average expression value for each gene and normalized to the median of the control samples. This dataset was then filtered using the DEGs from the three independent MAANOVA analyses. Hierarchical clustering was then applied to the filtered data using the one minus correlation dissimilarity metric using the Spearman correlation with average linkage. Data were visualized using a heatmap.

5.3.5 Bioinformatics and pathway analysis

The list of DEGs (FDR $P \leq 0.05$, fold change ± 1.5 in at least one dose group) from each PAH congener (Labib et al., 2012; Labib et al., 2015), the two defined PAH mixtures, and the coal tar extract were independently analyzed to identify biological functions or processes perturbed in response to the treatments. DAVID (Huang et al., 2009) Functional Annotation Charts were used to identify gene ontology (GO) terms (biological processes and cellular compartments) associated with the significant genes, and to classify the DEGs into biological pathways using

KEGG pathways (Kanehisa and Goto, 2000). Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) Canonical Pathway analysis, Biological Function analysis, and Network analysis were used to identify biological pathways and functions associated with the DEGs. Based on the tool used, different criteria were used to determine the significance of pathways, functions or biological processes perturbed. Any pathway or process that was associated with more than 3 DEGs was included in the interpretation of the results. In addition, an EASE score (right-tailed Fisher's test) cut-off of $P \leq 0.05$ was applied to DAVID ontologies and KEGG pathways, and $P \leq 0.05$ to IPA canonical pathways. Redundancy in pathways and processes were reduced by collapsing multiple pathways or processes implying perturbation of the same biological function as described in Labib et al. (2015). Since our previous studies (Labib et al. 2012; Labib et al., 2013; Labib et al., 2015) focussed on carcinogenic effects of PAHs, pathways pertinent to cancer formation were the focus of the analysis in the present study.

5.3.6 *Transcriptional benchmark dose (BMD) analysis*

BMDExpress version 1.4.1 (Yang et al., 2007) was used to perform BMD analysis on the transcriptomic data. Only genes that had “present” calls in at least one dose group (i.e., 4 out of 5 biological samples within at least one experimental group with signal intensities above the background non-murine control probes by at least three standard deviations), fold change $\geq \pm 1.5$, and ANOVA $P < 0.05$ were modeled. Hill, Power, Linear, and Polynomial (20 and 30) models were used to fit the gene expression dose-response data. For each gene, the best fitting model was selected based on (1) a nested Chi-square test (cut-off of 0.05) to choose between linear and polynomial models, (2) the lowest Akaike Information Criterion (AIC) values for the nested, Hill, and power models, and (3) curve goodness-of-fit $P > 0.1$. Genes with BMD values higher than the highest dose were excluded. The resulting gene BMD datasets were mapped to

all pathways/processes described in Labib et al. (2015). Median BMD and BMDL (lower confidence limit) were reported for pathways with at least three DEGs.

5.3.7 Application of the component-based reference models to evaluate additivity

The transcriptomic data for individual PAHs (including the degrees of pathway perturbations induced by each PAH and transcriptional BMDs for each gene and pathway induced by each PAH) were used to generate predicted dose-response curves for the perturbed pathways for each PAH-containing mixture using three models of additivity (CA, GCA, and IA). Thus, when we refer to predicted pathway perturbation or predicted dose-response curves, we are referring to those generated using the CA, GCA, and IA models. The complete strategy is described in detail in Figure 5-1.

Step 1 – Analysis of pulmonary transcriptional data for mice exposed to individual PAHs. In the first step, lung-specific transcriptional changes for eight individual PAHs were analyzed at each dose to identify significantly altered pathways associated with pulmonary carcinogenesis (analyses are described previously by Labib et al. (2012, 2013, 2015)); of these pathways, eight were identified as specifically important for PAH-induced carcinogenesis, and these were considered for mathematical modelling as described below.

Step 2 - Pathway selection for quantitative prediction of mixture responses. For the selection of pathways to be used in the additivity modeling, we first assessed if these eight pathways were also significantly perturbed following exposure to the three PAH-containing mixtures (concordance analysis). Since alteration in expression of individual genes can be associated with several redundant biological functions and processes, the ability of a PAH or a PAH mixture to perturb biological pathways (reflective of collective expression changes in several genes) was selected as the metric for prediction calculations (Figure 5-1). More specifically, the median percent change in gene expression for genes annotated to a pathway (that passed the

BMDExpress filter), relative to controls, was used as the effect level to represent pathway perturbations for comparisons of predicted and observed responses. For example, a 50% change in gene expression corresponds to an absolute 1.5 fold-change. The maximal effect level was defined as the largest median percent gene expression change for a particular pathway at any of the doses tested (this value was necessary for the GCA model described below). BMD modeling was used to conduct pathway dose-response assessment as described above, and pathway median BMD values were used in lieu of ED50 values that are traditionally used in predictive models of mixture toxicity.

Step 3 - Mixture prediction analyses using mathematical models of additivity. For each selected pathway, CA (also known as dose addition), GCA, and IA models were then employed to predict pathway perturbations associated with mixture exposures. The formulas and methodologies employed for CA, GCA, and IA modeling are described below.

1. The CA model is used to predict the concentrations of the mixture that would elicit a predetermined effect. In principle, concentrations of individual mixture components that are capable of eliciting the same effect on their own are added, after being multiplied by a scaling factor that accounts for potency differences. CA predictions were modeled according to equation 1.

$$D_{pred.mix} = \left(\sum \frac{p_{PAH}}{d_{PAH}^e * PEF_{PAH}} \right)^{-1} \quad (\text{equation 1})$$

Where $D_{pred.mix}$ is the predicted dose of the mixture across a range of effect levels, which were determined starting with the maximal effect level of the weakest inducer for that pathway and declining down to zero (the effect levels correspond to the benchmark response (BMR) used to generate BMDs). d_{PAH}^e is the dose level (BMD) at which each PAH on its own exerts the effect level (BMR); PEF_{PAH} is the potency equivalence factor (PEF) for each PAH scaled for

potency relative to BaP (CCME, 2010) (BaP=1; BaA=0.1; BbF=0.1; Chr=0.01; BghiP=0.01; BkF=0.1; DBahA=1; and IP=0.1); and p_{PAH} is the fraction of each PAH in the mixture.

$D_{pred.mix}$ values were calculated for a range of effect levels (up to the maximal effect level of the weakest inducer of that pathway) and a prediction dose-response curve was established. In the case of PAHs for which selected pathway BMDs could not be calculated (due to a lack of genes that passed the BMDExpress filters described above) mathematical modeling proceeded without data input for that PAH. Statistical uncertainties for the predicted effects were presented as the most conservative estimate of the upper and lower bounds for each mixture component using two-sided confidence intervals for the BMD.

2. The GCA model is an extension of the CA model that accounts for the maximal effect level of the mixture components by including a parameter (e_{PAH}^{max}) for the maximum effects induced by each mixture component. GCA predictions were modeled according to equation 2.

$$E_{pred.mix} = \frac{\sum \left(e_{PAH}^{max} * [PAH] / d_{PAH} \right)}{1 + \sum ([PAH] / d_{PAH})} \quad (\text{equation 2})$$

Where $E_{pred.mix}$ is the predicted pathway perturbation (as above, the percent change in gene expression for responding genes in a pathway) induced by the mixture at a specific dose; e_{PAH}^{max} is the maximal effect level, which is the maximum pathway perturbation (median percent change in gene expression) induced by each PAH mixture component; $[PAH]$ is the concentration of the individual PAH in the mixture at a specific mixture concentration; d_{PAH} is the dose level (BMD) at which each PAH exerts a 50% change in gene expression, which corresponds to an absolute 1.5-fold change. $E_{pred.mix}$ values were calculated for the mixture concentrations and a prediction

dose-response curve was established. In the case of PAHs for which selected pathway BMDs could not be calculated (due to a lack of genes that passed the BMDExpress filters described above) mathematical modeling proceeded without data input for that PAH. Statistical uncertainties for the predicted effects were expressed as the most conservative estimate of the upper and lower bounds for each mixture component using two-sided confidence intervals for the BMDs.

3. The IA model is commonly used for prediction of the mixture toxicity when the mixture under consideration contains compounds with diverse modes of action. IA predictions were modeled according to equation 3.

$$E_{pred.mix} = 1 - \prod(1 - e_{PAH} * p_{PAH}) \quad (\text{equation 3})$$

Where $E_{pred.mix}$ is the predicted pathway perturbation (as above, the percent change in gene expression for responding genes in a pathway) induced by the mixture at a specific mixture concentration; e_{PAH} is the median percent change in gene expression for responding genes in a pathway for each PAH at that concentration; and p_{PAH} is the fraction of each PAH in the mixture. For a range of mixture concentrations, $E_{pred.mix}$ values were calculated and a prediction curve was established. Statistical uncertainties for the predicted effects were expressed as 95% confidence bands.

Predictivity analysis. For each of the pre-selected pathways, the predicted dose-response curves were compared to the actual, observed mixture dose-response curves (Figure 5-1). If the confidence bands around the predicted dose-response curve generated using each model overlapped with the 95% confidence band around the observed dose-response curve, then the assumption of additivity under that specific model was considered validated. If the confidence

bands did not overlap, the model was considered invalid with respect to the assumption of additivity, which suggests chemical interactions.

BMD comparisons. As a quantitative measure of similarity or dissimilarity between dose-response curves, BMD and BMDL values calculated for the predicted dose-response curves generated by each of the models were compared with the observed BMD values. BMD values were calculated using the USEPA's Benchmark Dose Software BMDS version 2.5.1 (<http://www.epa.gov/ncea/bmds/>) (Davis et al., 2011). Data-points (doses and effect levels) across the predicted dose-response curves generated using the CA, GCA, and IA models were selected from the mathematical modeling output for BMD modeling. These data were modeled as continuous data and were run against Exponential, Hill, Power, Polynomial, and Linear models of dose-response. The BMR was set to 50% corresponding to a 1.5-fold change in gene expression, which is thought to represent a biologically meaningful critical effect level for gene expression changes. The best model for each dataset was selected based on the lowest AIC value, excluding models with a goodness-of-fit $P < 0.1$.

5.3.8 BMD analysis of lung tumour incidence data

The transcriptomic data were further used to assess whether the predicted and actual perturbations of cancer-associated pathways following exposures to CT-Mix are reflective of the actual lung tumour incidence observed in mice exposed to coal tar. The lung tumour incidence data from a published 2-year feeding study in mice (Culp et al., 1998) was used. In this study, coal tar extracts were added to the feed of 5-week old female B6C3F1 (C57BL/ 6N/ X/ C3H/ HeNMTV) mice at 0, 0.01, 0.03, 0.1, 0.3, 0.6, and 1.0% per day, or 0, 0.22, 0.66, 2.2, 6.6, 13.4, 22.0 mg BaP equivalents/kg-bw/day (Benford et al., 2010). Tumours formed in the tongue, oesophagus, forestomach, liver, and lung tissues. The lung tumour incidence numbers as reported by Culp et al. (1998) were used to model the cancer dose-response using BMDS. Since the

tumour incidence plateaus in the highest dose groups (13.4 and 22.0 mg BaP equivalents/kg-bw/day), those dose groups were removed from the BMD analysis to improve model fit. The data were modeled as dichotomous tumour incidence and were run against all degrees of the multistage model. The BMR was set to 10% extra risk as recommended by the Benchmark Dose Technical Guidance document (USEPA, 2012). The best model for each dataset was selected based on the lowest AIC value, excluding models with a BMD higher than the highest dose, a BMD/BMDL ratio greater than 5.0, and a goodness-of-fit $P < 0.1$.

5.4 Results

In three separate studies adult male MutaTMMouse specimens were administered three doses of three PAH-containing mixtures (i.e., 4PAH-Mix, 8PAH-Mix, and CT-Mix) for 28 consecutive days by gavage. For the defined mixtures, the PAHs were mixed according to their proportional amounts in the complex coal tar extract. The exposure regime did not cause any overt signs of toxicity and there was no significant body weight loss in any of the exposed mice compared to vehicle treated controls. Lung tissue was collected three days after the final exposure and global changes in the lung transcriptome were assessed. PAH mixture-specific transcriptomic profiles were established and the results were quantitatively compared with transcriptomic profiles from pulmonary tissue of mice similarly exposed to eight individual PAH congeners that are constituents of the mixtures examined herein (Labib et al. 2012; Labib et al., 2015). The comparisons of the transcriptomes were used to investigate whether individual mixture components (i.e., individual PAHs) induce transcriptomic responses that are similar to those induced by PAH mixtures, and whether pathway perturbations induced by individual PAHs and an assumption of additivity can be used to predict the extent of pathway perturbations elicited by the examined mixtures.

5.4.1 *General overview of microarray results*

The 4PAH-Mix, 8PAH-Mix, and CT-Mix induced significant changes in the expression of many genes in lung tissue. MAANOVA analysis revealed 720, 921, and 460 unique DEGs in at least one dose group following exposures to the 4PAH-Mix, 8PAH-Mix, and CT-Mix, respectively (Figure 5-2A). Supplementary Table 5-2 provides details of all DEGs, including gene names, gene accession numbers, P values, and fold changes. Hierarchical cluster analysis on all DEGs revealed that each PAH mixture group clustered separately from the other (Supplementary Figure 5-1A), thus a distinct treatment effect was observed as a result of exposure to each of the mixtures. A VENN analysis of the DEGs from all treatment groups revealed 235 DEGs common between the 4PAH-Mix, 8PAH-Mix, and CT-Mix exposures (Figure 5-2B).

Figure 5-2C summarizes all of the molecular pathways and biological processes that are altered in lung tissue following exposure to each PAH mixture. This analysis includes all significantly altered gene ontologies (EASE $P \leq 0.05$), KEGG pathways (EASE $P \leq 0.05$), and IPA canonical pathways ($P \leq 0.05$), as described in the Methods. Six main functional categories of pathways and processes were perturbed: DNA damage response, altered cell signaling, altered metabolism, immune/inflammatory response, cytoskeletal reorganization, and cellular homeostasis.

Clustering analysis of DEGs induced by the mixtures and individual PAH exposures (Labib et al. 2012; Labib et al., 2015) revealed that each PAH mixture clustered separately from the individual PAHs (Supplementary figure 5-1B), indicating that the mixture effect is distinct from that elicited by the individual PAHs and that the mixtures are more similar to each other than any other individual PAHs. Furthermore, the three mixtures clustered on the same branch as DBahA

and BbF, indicating that there is greater similarity between these two PAHs and the mixtures compared to the other PAHs tested.

5.4.2 Prediction of mixture pathway perturbations

Step 1 - Transcriptomic profiles of individual PAHs. The pulmonary transcriptome induced following exposures to eight individual PAHs contained in the 4PAH-Mix and 8PAH-Mix, including details of perturbed pathways and processes, are published previously by Labib et al. (2012, 2013, 2015). Of the many pathways enriched by the individual PAHs, eight biological pathways and processes, which were significantly enriched in at least one individual PAH-treated group, were identified as being relevant to PAH-induced carcinogenesis (i.e., AHR Signaling, Angiogenesis Signaling, Apoptosis Signaling, B Cell Receptor Signaling, Cell Cycle Signaling, Circadian Rhythm Signaling, P53 Signaling, and Xenobiotic Metabolism Signaling pathways). These pathways can be grouped under five main functional categories: DNA damage response, altered cell signaling, altered metabolism, immune/inflammatory response, and cellular homeostasis, all of which play an important role in initiation and/or promotion of cancer. These pathways are employed for the quantitative analyses in steps 2 and 3 below.

Step 2 - Pathway selection for quantitative prediction of mixture responses. Pathways associated with cancer formation that were identified in our earlier PAH studies (Labib et al., 2012; Labib et al., 2015) were scrutinised to determine if they were also perturbed following exposures to the 4PAH-Mix, the 8PAH-Mix, and the CT-Mix. Each of the eight cancer-related pathways were perturbed by at least one PAH-containing mixture, with both similarities and differences noted in terms of the identity of the pathways perturbed, and the number of genes perturbed for each pathway (Figure 5-3A). Of the eight pathways, Angiogenesis Signaling was significantly affected only by the 4PAH-Mix and the 8PAH-Mix, B Cell Receptor Signaling was affected only by the 4PAH-Mix, and P53 Signaling, Apoptosis Signaling, Cell Cycle Signaling, AHR

Signaling, Circadian Rhythm Signaling, and Xenobiotic Metabolism Signaling were significantly affected by all three PAH-mixtures. Thus, the six latter pathways, which were perturbed by all three types of mixtures, were selected for further analysis.

Following selection of the relevant pathways, the median percent change in gene expression relative to controls for genes associated with a pathway and the maximal effect levels (i.e., largest median percent change in gene expression relative to controls for genes associated with a pathway across all tested doses) for each pathway were derived for each treatment.

Supplementary Table 5-3 presents the median and mean gene expression changes for each PAH for all doses and pathways, as well as the fold change of the most affected gene for each pathway. Differences in maximal effects at the doses tested ranged from 48% for CHR and BghiP to 108% for BbF for AHR Signaling, from 56% for BkF to 71% for IP for Apoptosis Signaling, from 59% for BghiP, BkF, and CHR to 75% for BaP for Cell Cycle Signaling, from 58% for DBahA to 167% for IP for Circadian Rhythm Signaling, from 62% for BaA to 174% for CHR for the P53 Signaling, and from 48% for BaA to 106% for IP for Xenobiotic Metabolism Signaling.

Curve fitting and BMD analysis for both probes and pathways altered following exposure to individual PAHs was conducted using BMDEpress. BMD analysis was conducted for a range of BMRs based on the maximum effect levels for the individual PAHs, and median pathway BMD values were calculated for each BMR. Median BMD values at BMR 10% are shown in Figure 3B. The median BMD values for AHR Signaling ranged from 2.77 mg/kg-bw/day for DBahA to 106.6 mg/kg-bw/day for CHR; Apoptosis Signaling from 3.83 mg/kg-bw/day for DBahA to 94.71 mg/kg-bw/day for CHR; Cell Cycle Signaling from 3.11 mg/kg-bw/day for DBahA to 79.74 mg/kg-bw/day for CHR; Circadian Rhythm Signaling from 3.61 mg/kg-bw/day

for DBahA to 42.15 mg/kg-bw/day for BkF; P53 Signaling from 2.92 mg/kg-bw/day for DBahA to 39.51 mg/kg-bw/day for CHR; and Xenobiotic Metabolism Signaling from 5.02 mg/kg-bw/day for DBahA to 68.73 mg/kg-bw/day for CHR.

Step 3 - Mixture prediction using mathematical models of additivity. In order to determine if pathway perturbations elicited by exposures to individual PAHs, and an assumption of additivity, can be used to realistically predict pathway perturbations elicited by PAH mixtures, we compared predicted mixture pathway perturbations obtained using CA, GCA, and IA component-based models to pathway perturbations induced by the three tested mixtures. Next, the best predictive model was identified by comparing the BMD values for the predicted and observed dose-response functions. The model (CA, GCA, or IA) that yielded a predicted BMD that is closest to the observed BMD was denoted the best model. The results obtained using each of the component-based models are outlined below.

Predictivity analysis. As expected, the CA model (equation 1) predicted dose-responses for pathway perturbations up to the maximal effect induced by the least responding individual PAH in the mixture (i.e., weak inducer of gene expression changes). For the 4PAH and 8PAH simple mixtures (Figures 5-4A and B), the CA model-predicted dose-response curves are positioned to the left (at lower doses) of the observed dose-response curve for all six pathways tested, and the confidence bands for the predicted and observed pathway perturbations do not overlap. The CA predicted dose-response curves for the CT-Mix were also shifted to the left of the observed pathway perturbations induced by the CT-Mix (Figure 5-5); however, the CA model predictions correspond with the observed pathway perturbations of the AHR Signaling, Circadian Rhythm Signaling, P53 Signaling, and Xenobiotic Metabolism Signaling pathways. Additionally, there is a tendency for additivity for the Apoptosis Signaling and Cell Cycle Signaling pathways as the

confidence bands between the predicted and observed responses are approaching each other in the low dose regions.

Next we used the GCA model (equation 2) to predict mixture-induced pathway perturbations (Figures 5-4 and 5-5). This model yielded curves across the full range of doses for each mixture. For the 4PAH-Mix, the GCA model prediction corresponds with the observed pathway perturbations for all six pathways tested (Figure 5-4A). For the 8PAH-Mix, the GCA model prediction corresponds with the observed pathway perturbations for AHR Signaling, Apoptosis Signaling, Cell Cycle Signaling, and Xenobiotic Metabolism Signaling pathways (Figure 5-4B). For the CT-Mix, GCA model predictions correspond with the observed perturbations for the Circadian Rhythm Signaling pathway and a tendency for additivity was observed for the P53 Signaling and Xenobiotic Metabolism Signaling pathways as the confidence bands between the predicted and observed responses are approaching each other (Figure 5-5).

Finally, we predicted the mixture-induced pathway perturbations using the IA model (equation 3) generally used for chemicals with dissimilar modes of action. The IA model provided reasonable predictions that corresponded to observed pathway perturbations for all six pathways (Figures 5-4 and 5-5) for the 4PAH-Mix, 8PAH-Mix, and CT-Mix.

Please note that in order to assess the impact of filtering the transcriptome data by P value and fold change Steps 2 and 3 were repeated without the application of any filters. More specifically, as in the analysis described above, pathway perturbations were measured as the change in gene expression for all genes annotated to a pathway that passed the BMDEpress filter relative to controls; however, only genes that had a “present” call in at least one dose group were modeled. The results of this analysis were comparable with the results detailed above (Supplementary Figure 5-2). Briefly, the CA model predicted dose-response curves were

positioned to the left of the observed dose-response curves for all pathways and mixtures tested, the GCA model predictions corresponded with the observed pathway perturbations for several pathways for the defined mixtures but did not correspond with the observed pathway perturbations for the CT-Mix, and the IA model yielded the most reasonable predictions that corresponded to the observed pathway perturbations for all pathways and mixtures tested.

BMD comparisons. For the CA model, the predicted BMD50 values for the 4PAH-Mix ranged from a factor of 2.7- (AHR Signaling) to 9.3-fold (P53 Signaling) less than the observed values. For the 8PAH-Mix the predicted BMD50 values ranged from a factor of 14.5- (Cell Cycle Signaling) to 30.2-fold (Circadian Rhythm Signaling) less than the observed values. For the CT-Mix the predicted BMD50 values ranged from a factor of 1.3- (AHR Signaling) to 3.5-fold (Circadian Rhythm Signaling) less than the observed values (Table 5-1).

For the GCA model, the predicted BMD50 values for the 4PAH-Mix and 8PAH-Mix were close to the observed BMD50 values. For the 4PAH-Mix, the BMD50 values ranged from a factor of 1.3- (P53 Signaling) to 1.4-fold (Circadian Rhythm Signaling) less than the observed values to a factor of 3.2- (Xenobiotic Metabolism Signaling) to 5.6-fold (Cell Cycle Signaling) greater than observed. For the 8PAH-Mix, the BMD50 values ranged from a factor of 1.1- (AHR Signaling) to 4.1-fold (Circadian Rhythm Signaling) less than observed to 1.1-fold (Apoptosis Signaling and Cell Cycle Signaling) greater than the observed values. For the CT-Mix, the predicted BMD50 values ranged from a factor of 2.1- (Circadian Rhythm Signaling) to 5.2-fold (Xenobiotic Metabolism Signaling) greater than observed.

For the IA model, the predicted BMD50 values for the 4PAH-Mix, 8PAH-Mix, and CT-Mix were close to the observed BMD50 values. Specifically, for the 4PAH-Mix BMD50 values ranged from a factor of 1.2- (Circadian Rhythm Signaling) to 3.5-fold (AHR Signaling) greater.

For the 8PAH-Mix, the predicted BMD50 values were the same as observed for Circadian Rhythm Signaling and 1.1- (Xenobiotic Metabolism Signaling) to 2.4-fold (Cell Cycle Signaling) greater than the observed values. Similarly, for the CT-Mix, the predicted BMD50 values ranged from a factor of 1.3-fold (AHR Signaling, Circadian Rhythm Signaling) less than observed to 1.2- (Cell Cycle Signaling) to 1.7-fold (P53 Signaling, Xenobiotic Metabolism Signaling) greater than observed.

5.4.3 BMD modeling of published cancer data

BMD modeling was conducted using the coal tar-induced lung tumour incidence data from the Culp et al. (1998) study. BMD and BMDL values of 2.47 and 1.50 mg/kg-bw/day, respectively, were determined using the best fit model (i.e., Multistage-Cancer 20).

5.5 Discussion

In this study, we analyzed the pulmonary transcriptome of mice administered defined- and complex-PAH mixtures by gavage daily for 28 days, and compared these results with pulmonary transcriptomes of mice similarly exposed to individual PAHs (Labib et al., 2015) to evaluate several component-based models of PAH additivity. We found that (1) PAH-containing mixtures induce expression changes in genes associated with a wide variety of pathways and processes that may be implicated in carcinogenesis, and that (2) with few exceptions, the perturbed pathways were similar to those observed following exposure to individual PAHs. We then used our earlier work on individual PAHs to evaluate the ability of three component-based models of additivity to predict mixture responses at the pathway level. More specifically, we compared pathway perturbations predicted using these models with observed pathway perturbations following exposures to the PAH-containing mixtures. The results were used to assess the validity of the assumptions related to the toxicological additivity of PAHs in simplified and complex PAH mixtures.

Previous studies have used transcriptomic data to evaluate the validity of additivity assumptions and to provide evidence to support toxicological interactions of mixture components (Kopec et al., 2011; Staal et al., 2007; Zucchi et al., 2014). For example, Kopec et al. (2011) applied individual gene expression data to evaluate additivity models using a select set of genes (*Nqo1*, *Dysf*, *Pla2g12a*, *Serpib6a*, and *Srxn1*) differentially expressed in livers of C57BL/6 mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153), or a binary mixture of the two. The authors applied statistical modeling of the gene expression data and noted non-additive gene expression responses for several of the individual genes. Similarly, Zucchi et al. (2014) employed individual gene expression values from the ovaries and brains of female zebrafish exposed to drospirenone, progesterone, and a binary mixture of the two to investigate additivity of the substances. The study reported additive and less than additive effects for the three transcripts selected for analysis. In addition to single-gene data, Staal et al. (2007) employed gene expression changes for all DEGs from human hepatoma cells (HEPG2) exposed to binary mixtures of BaP and either DBaA, BbF, fluoranthene, or 1-methylphenanthrene to evaluate an additivity model based on the sum of the gene expression ratios (treated:controls) for individual mixture components. Depending on the genes examined, the authors found additive, antagonistic, and synergistic effects. These studies provide excellent examples of early applications of toxicogenomics in mixtures toxicology, but they fail to address issues related to concentration and effect additivity. Indeed, the concentration-effect additivity dichotomy is germane to mixtures toxicology, and an improved understanding of the toxicological hazards of mixtures must examine both CA and IA approaches, thereby scrutinising toxicological behaviours of substances present in complex mixtures and the biological processes driving these behaviours.

Although single genes may play important roles in different biological events, they do not reflect the complex interactions and associations that occur among and between genes that trigger biological signaling cascades and cellular pathways. Analysis at the pathway level reduces the complexity of large transcriptomic datasets by relating gene expression changes to mechanistically relevant events. This approach reduces the dimensionality of the data to facilitate comparisons between different exposure scenarios by categorizing individual genes that are related to specific functions. Thus, in the present study we used pathway perturbations to scrutinize assumptions of additivity by evaluating several models of additivity using simple and complex mixtures of PAHs.

As stated earlier, ED50 values are traditionally used in these types of analyses; however, ED50 calculations require knowledge of the maximal effect level of a chemical. Thus, this can pose challenges when comparing chemicals with different capacities to induce a maximal effect. Given these limitations, in place of ED50 modeling we used BMD modelling of the gene and pathway data, which does not require a maximal response and allows the generation of values based on non-asymptotic models (Burgoon and Zacharewski 2008). Thus, BMD analysis using a constant BMR enables comparisons of both strong and weak inducers of a measured effect.

5.5.1 Pathway perturbations induced by PAHs or PAH-containing mixtures

We found that several key pathways involved in PAH-induced responses, including AHR binding, metabolic activation, DNA damage, and increased cellular proliferation, were all affected at the transcription level by individual PAHs, the 4PAH-Mix, the 8PAH-Mix, and the CT-Mix (Figure 5-3A). The results suggest that, in general, the core transcriptomic responses elicited by exposures to PAHs are similar to the responses observed following exposures to the PAH-containing mixtures and supported the original hypothesis that carcinogenic PAHs and PAH mixtures act via mutagenic mode of action. However, some non-mutagenic pathways were

also observed to be perturbed. For example, PPAR Signaling, PI3K/AKT Signaling, PTEN Signaling, IGF-1 Signaling, Glucocorticoid Receptor Signaling, Steroid Biosynthesis, Integrin Signaling, FAK Signaling, and Paxillin Signaling were only perturbed by individual PAHs; whereas, Collagen Metabolism (4PAH-Mix), Leukocyte Migration (4PAH-Mix, 8PAH-Mix), T Cell Signaling (CT-Mix), and mTOR Signaling (CT-Mix) were uniquely perturbed by one or more of the mixtures examined. These pathways have been previously linked to exposures to BaP and other PAH-containing complex mixtures. For example, increased collagen synthesis, which is suggested to play an early role in carcinogenesis, also occurs in lung organ cultures exposed to BaP (Bhatnagar et al., 1980). Leukocyte migration, also known as transepithelial leukocyte migration, was induced by CT-Mix and is altered in human umbilical vein endothelial cells (HUVEC) following exposure to cigarette smoke, a complex mixture containing PAHs (Shen et al., 1996). T cell signaling, which was induced by the CT-Mix, is known to be disrupted by BkF, a PAH that is known to reduce T cell counts (CD4+ and CD8+) in mouse spleen (Jeon et al., 2005). MTOR signaling induced by the CT-Mix has been shown to play an important role in BaP-induced cellular responses (Baumann et al., 2014), as demonstrated by proteomic analysis of BaP-exposed Jurkat T cells. In addition, application of the mTOR inhibitor rapamycin reduced BaP-induced lung tumour formation, growth, and progression in A/J mice (Yan et al., 2006); however, the role of this signaling in BaP-induced tumour formation remains unclear. Thus, in addition to the pathways related to known mutagenic modes of action, perturbation of distinct signaling pathways uniquely induced by individual PAHs, or alternatively, by PAH-containing mixtures, suggests contributions of additional sub-cellular phenomena in PAH-induced carcinogenesis. The perturbation of pathways and mechanisms

other than those consistent with the mutagenic mode-of action may explain non-additive behaviour of the PAHs observed or predicted by the concentration addition model.

5.5.2 *Predicting pathway perturbations for mixtures of PAHs*

In risk assessment, the carcinogenic risks associated with incidental ingestion of materials contaminated with PAHs are typically estimated using the CA approach, which requires an assumption that structurally similar PAHs with similar toxicological outcomes share a common mode of action; and moreover, that their contributions to the toxicological properties of the mixture are additive. The approach employs the concentration of each PAH, and its potency relative to the well characterised reference compound BaP, to determine the contribution of each PAH to the overall toxicity of the mixture. However, the mathematical modeling of pathway perturbations using the CA model described herein, which applied pathway-level gene expression data to the traditional CA model, did not support the assumption that the effective doses of the 4PAH-Mix and the 8PAH-Mix can be determined by the contributions of each PAH in the mixture. This is consistent with previous studies reporting that the CA model can over-estimate the effective concentration (e.g., BaP equivalents) of a PAH mixture relative to the actual toxicological response elicited by the mixture itself. For example, Lemieux et al. (2015) noted that the effective concentration determined by the CA approach yields a level of BaP equivalents that exceed that required to elicit effects observed in cultured cells. In other words, the actual toxicological properties of the mixture are far lower than those predicted by summing the contributions from priority PAHs. Such deviations from additivity emphasize the likely occurrence of antagonistic chemical interactions at the doses employed. Whereas Lemieux et al. (2015) argued that the sub-additive responses of cultured cells to complex PAH mixtures from contaminated soils are indicative of metabolic insufficiency related to competition for the enzymatic machinery required to generate reactive PAH metabolites, comparison of the

predictivity of the CA model for the synthetic mixtures and CT-Mix in this study suggests the potential presence of unknown mixture components that are skewing the estimates for the mixture. As such, PAHs make up only 15% of the entire CT-mix studied and in specific, the 8 PAHs attribute to a small fraction (2 %) of the CT-mix. In this case, the CA model tended towards additivity in the degree of pathway perturbations associated with CT-Mix exposures, whereas it over-estimated the degree of pathway perturbations in the synthetic mixtures. The eight PAHs included in this study cover a wide range of genotoxic and carcinogenic potencies, and the published PEFs for these PAHs span two orders of magnitude (USEPA, 1993; CCME, 2010). In addition, their ability to induce DNA damage and mutations is also variable (Labib et al., 2015; Long et al., 2016a). However, previous studies have also shown that other PAHs such as dibenzo[*def,p*]chrysene (Chepelev et al., 2016; Long et al., 2016a), which are not regularly measured in PAH-contaminated samples, are more mutagenic than the priority PAHs investigated in the present study. The presence of such PAHs in the CT-Mix may explain the poor predictive power of CA for the CT-Mix, which can result in inaccurate risk estimates for materials contaminated with PAHs.

The GCA model is an extension of the original CA model that takes into consideration the inability of CA model to account for differences in maximal effects between mixture components (Howard and Webster, 2009). This approach includes a function that accounts for maximal effect level of each component as well as the Hill slope values applicable to receptor-mediated interactions. Howard et al. (2010) showed that this model was able to accurately predict joint effects of full agonist, partial agonist, and near-competitive antagonist combinations of AHR ligands. In this study, the GCA model yielded improved predictivity compared to the CA model for the synthetic mixtures, but was a poor predictor of the response for the CT-Mix. In

the context of the current work, binding and activation of the AHR by PAHs results in transcriptional activation of cytochrome P450 genes 1A1 and 1B1, which encode enzymes that catalyze the formation of dihydrodiol epoxides that react with DNA to form mutagenic bulky adducts. In addition, PAH-related AHR binding induces perturbations in a variety of toxicity and biochemical pathways involved in carcinogenesis (Dietrich and Kaina, 2010). Unfortunately, the addition of a metric to represent the maximal effect level may be a limitation of the GCA model in predicting complex mixture responses. The poor predictivity of the GCA model for the CT-Mix may reflect the presence of unknown mixture components with potentially higher maximal effect levels. Thus, despite GCA's inability to predict the CT-Mix pathway perturbations, for synthetic mixtures it provided improved predictions, relative to the CA approach.

The IA model provides predictions of mixture outcomes by summing the toxic responses of each mixture component. In this study, the IA model provided the best predictions of pathway perturbations for both synthetic and complex PAH mixtures. More specifically, comparison of predicted and observed dose-response curves and BMD values across each of the selected pathways showed the closest correspondence in comparison to what was observed for the CA and GCA models. The IA model assumes that the individual mixture components act via dissimilar modes of action, and this contention is supported by our previous PAH study that provided evidence of distinct compound-specific biochemical mechanisms leading to carcinogenesis (Labib et al., 2015). The combined results from this study and our previous studies suggest that application of CA alone may not be sufficient to predict the effects of the PAHs in mixtures and question the suitability of CA in human health risk assessment of PAH-containing mixtures. Thus, the results of this study support the use of the IA model for PAH-containing mixtures.

It is important to note that although the dose of the coal tar mixture was far below the doses used for the synthetic PAH mixture (in BaP equivalents), at the molecular level they induced similar perturbations of pathways related to carcinogenesis. This suggests that the mechanisms underlying the carcinogenicity of PAH-containing mixtures are concordant. Previously, we showed that the pathways perturbed by the individual PAHs varied across PAHs and tissues (Labib et al., 2015), yet the commonality in the pathways perturbed between the PAH mixtures, as well as between the mixtures and the individual components of the mixtures, suggests that the transcriptomic responses are sensitive enough to identify differences while still being specific enough to tease out commonalities related to carcinogenic transformation.

The identification of which PAH (if any) is the main contributor to a PAH-containing mixture's carcinogenicity is of particular interest. BaP has traditionally been used as a point of reference in the estimation of excess lifetime cancer risk posed by PAH-containing mixtures (CCME, 2010; EFSA, 2008; Health Canada, 2010). This is primarily based on the fact that BaP's carcinogenicity and toxicological properties are well-characterized and because of the abundance of information related to its occurrence in environmental media. In the present study, hierarchical cluster analysis showed that DBahA and BbF co-clustered on the same branch as the three mixtures, whereas BaP clustered on an adjacent branch. These results suggest that DBahA and BbF may play a strong role in the response induced by the PAH-containing mixtures compared to the other PAHs tested, including BaP, and that perhaps BaP may not be an adequate representative for PAH-containing mixtures. These results are in alignment with the conclusions of our previously published study stating that BaP may not be an appropriate representative of carcinogenic PAHs (Labib et al., 2015). Thus, comparison of the pulmonary transcriptomes from

mice exposed to individual PAHs and PAH-containing mixtures showed that DBahA and BbF may have greater influence in PAH-mixture induced toxicological response.

5.5.3 Quantitative comparisons of coal tar-induced mouse pulmonary tumours with transcriptomic-based pathway perturbations using predictive models of additivity

This work scrutinized the utility of transcriptomic data for evaluating different models of additivity to assess the toxicological properties of PAHs and PAH mixtures. Since the analyses are based on pathway perturbations related to carcinogenesis, the results have implications for the quantitative risk assessment of PAH-containing mixtures. To enhance the utility of transcriptomic profiling for quantitative risk assessment we also compared the transcriptional BMD values with those for a relevant apical endpoint (e.g. coal tar-induced lung tumour incidence). The CT-Mix transcriptional BMDs for the cancer-related pathways (Table 5-1) observed here (ranging from 1.61 to 3.02 mg/kg-day) were similar to the Culp et al. (1998) lung tumour BMD value of 2.47 mg/kg-bw/day. Similarly, the BMDs for the cancer-related pathways generated by the IA model (ranging from 1.70 to 3.69 mg/kg-day) were also close to the lung tumour BMD value. It is important to note that the proportion of non-PAH content in the coal tar used in the Culp et al. (1998) study is not available and thus, it is difficult to accurately conclude if the observed carcinogenic responses in the feeding study are reflected in the transcriptomic responses. Moreover the background of the B6C3F1 mouse strain used in the Culp et al. (1998) study differs from the background of the MutaTMMouse (BALB/C-DBA/2) used in this study. Despite of the stated differences, there was a high degree of concordance between the transcriptional and lung tumor BMD values. Thus, although based on only one PAH-containing mixture, the concordance between the predicted cancer-pathway BMDs and lung tumour BMDs suggests that this type of cancer-related pathway data predicted using the IA model of additivity

involving the eight genotoxic-carcinogenic PAHs might be a useful screening tool for the initial estimation of a PAH-containing mixture's cancer risk..

5.5.4 Conclusions

This study used transcriptomics-derived pathway perturbations induced by individual PAHs to evaluate the applicability of such data in deriving predictions of pathway perturbations for synthetic and complex mixtures of PAHs. We demonstrate a strategy that used pathway-level transcriptional data and conventional mathematical models to evaluate several approaches to predict the effects of PAH-containing mixtures. In contrast to the assumption of dose additivity that is commonly employed for PAH-containing mixtures (i.e., CA-based approach), our results imply that perturbations of toxicity pathways, some of which have been implicated in carcinogenesis, are best predicted by a model that assumes dissimilar modes of action for mixture components (i.e., IA-based approach). The median change in expression value for DEGs associated with perturbed pathways, and dose-response analyses of the gene expression data for perturbed pathways, were successfully incorporated into existing predictive models of additivity. The transcriptional BMDs generated using the mathematical models of additivity resulted in values consistent with lung tumour development induced by coal tar (BMD: 2.5 mg/kg-bw/day; BMDL: 1.5 mg/kg-bw/day). Since the pathway perturbations noted in our study are biologically linked to PAH-induced carcinogenesis, it is biologically plausible that the doses at which these pathways are affected might approximate the dose at which adverse outcome would be expected. As the public repository of genomics datasets for individual chemicals is populated, and the underlying mechanisms of toxicity are revealed, these types of data can be used for prioritizing mixtures for further toxicity testing.

Our comparisons of observed and predicted responses under different additive models indicate that CA, which is traditionally used for risk assessment of PAH mixtures, over-estimates

the responses compared to what is actually observed. This suggests that the traditional additive paradigm, which employs CA and PEFs to determine the effective amount of a reference compound (e.g., BaP), may yield conservative risk estimates. Additional work examining other PAH mixtures would be required to validate this conclusion.

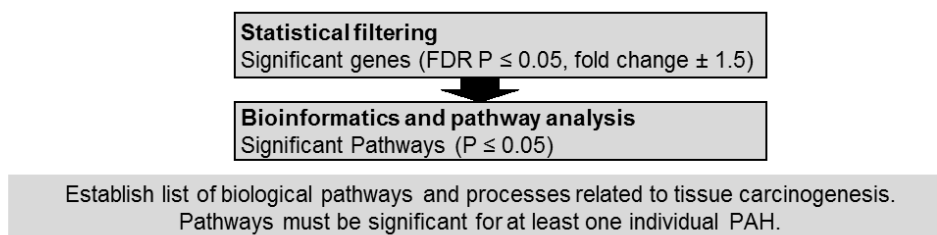
5.6 Tables and Figures

Table 5-1 Predicted and observed benchmark dose (BMD50) values for selected pathways perturbed by each mixture.

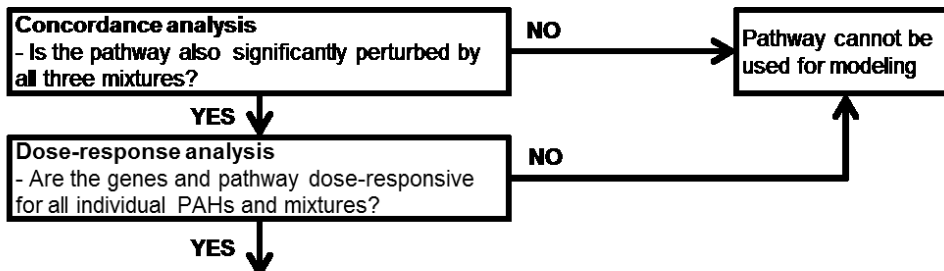
Pathway	Observed (mg/kg-day)		CA (mg/kg-day)		GCA (mg/kg-day)		IA (mg/kg-day)	
	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
4PAH-Mix								
AHR Signaling	8.30	5.60	3.04	2.30	27.54	13.24	29.04	19.57
Apoptosis	15.84	9.54	3.41	2.76	68.29	14.73	37.13	30.12
Cell Cycle	15.07	8.67	3.72	3.01	84.43	26.59	36.17	26.23
Circadian Rhythm	14.53	6.93	1.61	1.30	10.71	5.59	16.79	12.58
P53 Signaling	16.06	9.94	1.73	1.40	12.47	8.21	24.03	20.80
Xenobiotic Metabolism	20.97	12.16	3.63	2.77	66.67	46.38	41.25	28.22
8PAH-Mix								
AHR Signaling	25.71	16.04	1.63	0.85	24.27	15.39	31.05	22.07
Apoptosis	32.33	22.24	1.13	0.73	34.50	15.64	40.84	30.96
Cell Cycle	18.63	10.93	1.28	1.04	20.97	1.36	43.96	31.57
Circadian Rhythm	19.54	13.77	0.65	0.53	4.78	1.53	20.07	15.57
P53 Signaling	23.68	16.09	1.04	0.88	11.03	5.34	34.19	28.97
Xenobiotic Metabolism	32.86	15.90	1.13	0.86	26.86	15.25	36.41	24.86
CT-Mix								
AHR Signaling	2.24	1.32	1.71	0.79	7.19	5.03	1.73	1.28
Apoptosis	2.53	1.30	1.14	0.80	10.88	6.14	3.52	2.47
Cell Cycle	3.02	1.47	1.29	1.05	11.11	6.19	3.69	2.78
Circadian Rhythm	2.23	1.21	0.64	0.52	4.72	3.22	1.70	1.34
P53 Signaling	1.61	1.03	1.09	0.92	7.29	5.08	2.71	2.32
Xenobiotic Metabolism	1.85	1.09	1.18	0.90	9.67	5.87	3.24	2.24

Note: BMDL is the lower 95% confidence limit on the BMD

STEP 1: Analysis of Pulmonary Transcriptomes for Mice Exposed to Individual PAHs



STEP 2: Pathway Selection for Quantitative Prediction of Mixture Responses



STEP 3: Mixture Prediction Analyses Using Mathematical Models of Additivity

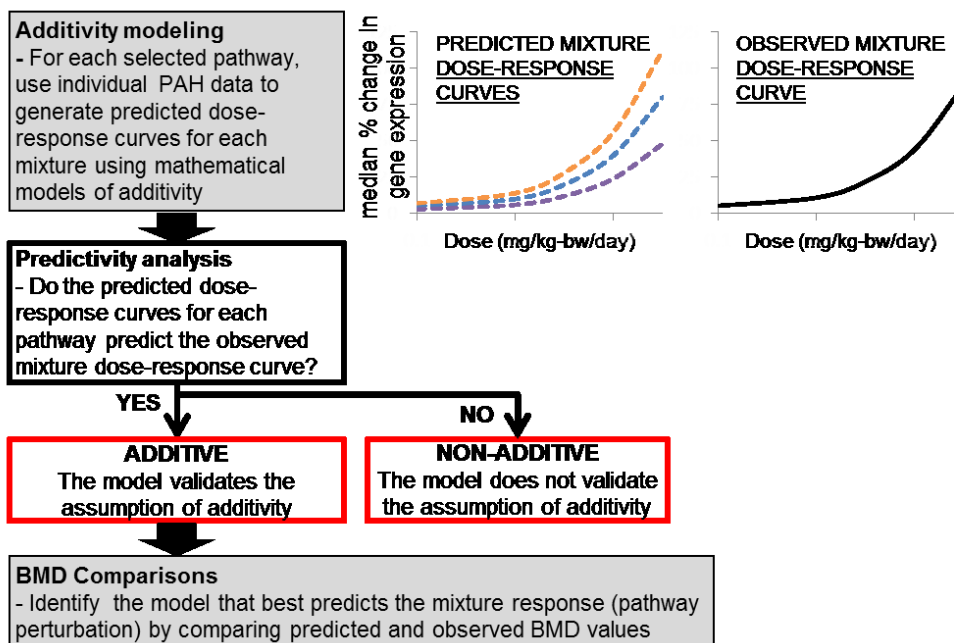


Figure 5-1 Workflow to select pathways for mathematical modeling and mixture prediction analyses

In step 1: analysis of pulmonary transcriptomes for mice exposed to individual PAHs, following statistical filtering and bioinformatics and pathway analysis, pathways were selected based on cancer-related biological pathways and processes perturbed by the individual PAHs examined in

Labib et al. (2015). The number of significant genes and significant pathways induced by each PAH are shown. In step 2: pathway selection for quantitative prediction of mixture responses, pathways were selected following concordance and dose-response analyses (white boxes). Pathways that did not pass concordance and dose-response analyses were not used for mathematical modeling. In step 3: mixture prediction analyses using mathematical models of additivity, the individual PAH data was used to generate predicted dose-response curves for each mixture using three established mathematical models of additivity – concentration addition (CA), generalized concentration addition (GCA), and independent action (IA). The predicted dose-response curves and the BMDs for the predicted dose-response curves were compared with the observed mixture dose-response curves and BMDs for the observed dose response curves in the predictivity analysis and BMD comparison.

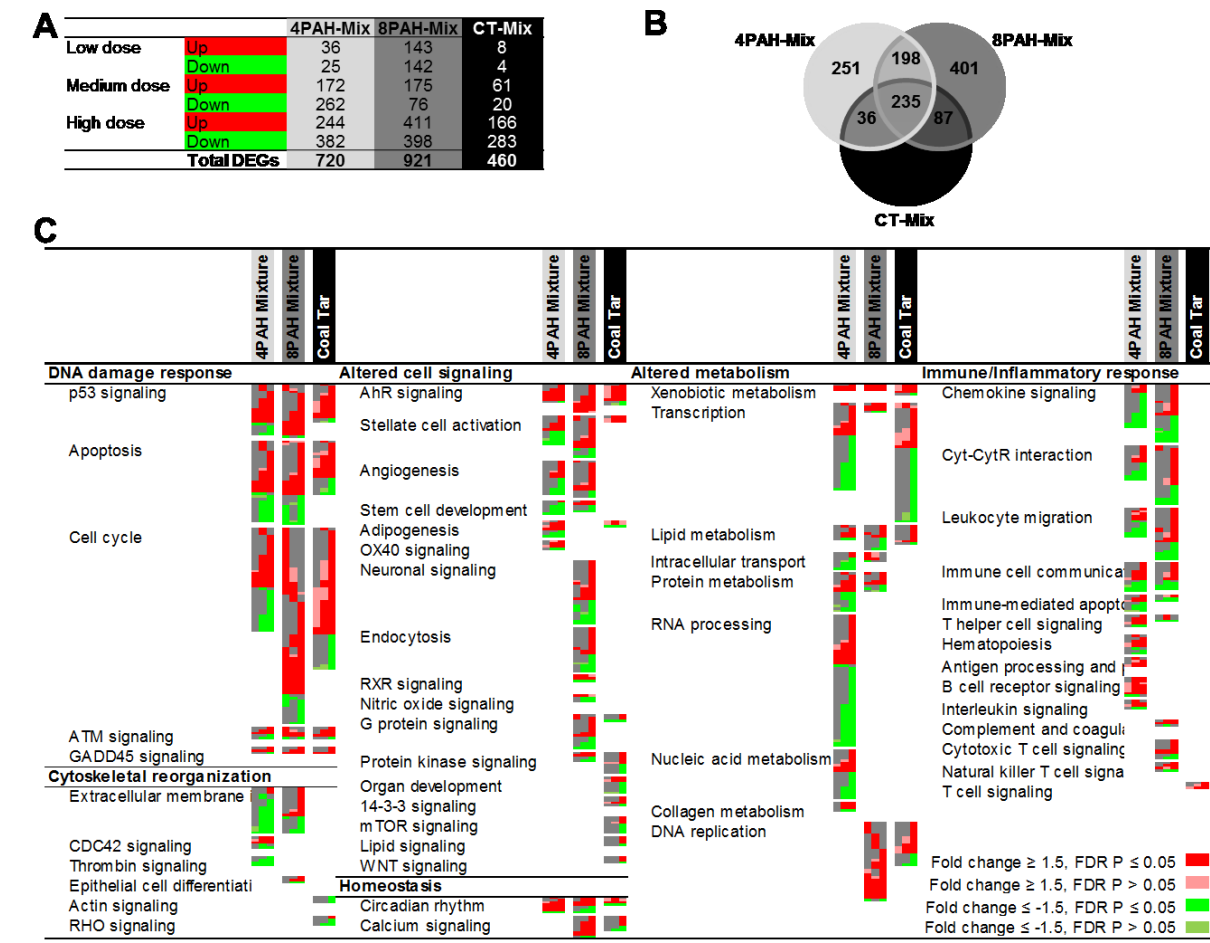


Figure 5-2 General overview of microarray results data analysis.

(A) Table of pulmonary DEGs for each dose group of each PAH-containing mixture. (B) VENN diagram showing overlap between DEGs in at least one dose group of the three PAH-containing mixtures. (C) All pathways significantly enriched by each of the PAH-containing mixtures, and their commonalities. Each column represents a dose group and each row represents a gene. All red and green colored cells represent genes with fold change ≥ 1.5 in either direction.

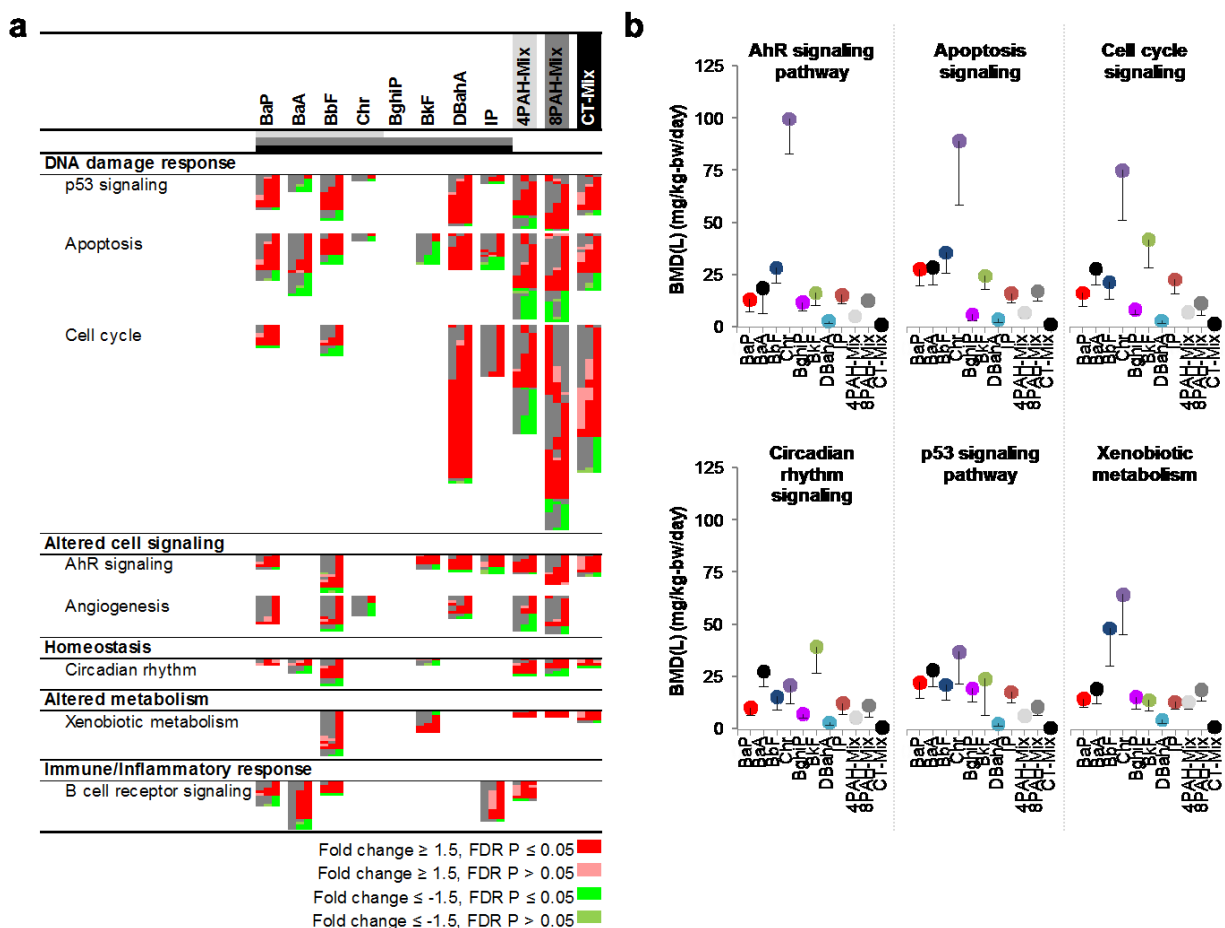


Figure 5-3 Comparison of cancer-related pathways for individual PAH and PAH-mixtures

(A) Selected cancer-related pathways significantly enriched by each of the individual PAHs and PAH-containing mixtures, and their commonalities. Each column represents a dose group and each row represents a gene. All red and green colored cells represent genes with fold changes ≥ 1.5 in either direction. (B) BMD10 values in mg/kg-bw/day for each PAH and PAH-containing mixture derived using BMDExpress. The circles represent the BMD and the lower bars represent the BMDL.

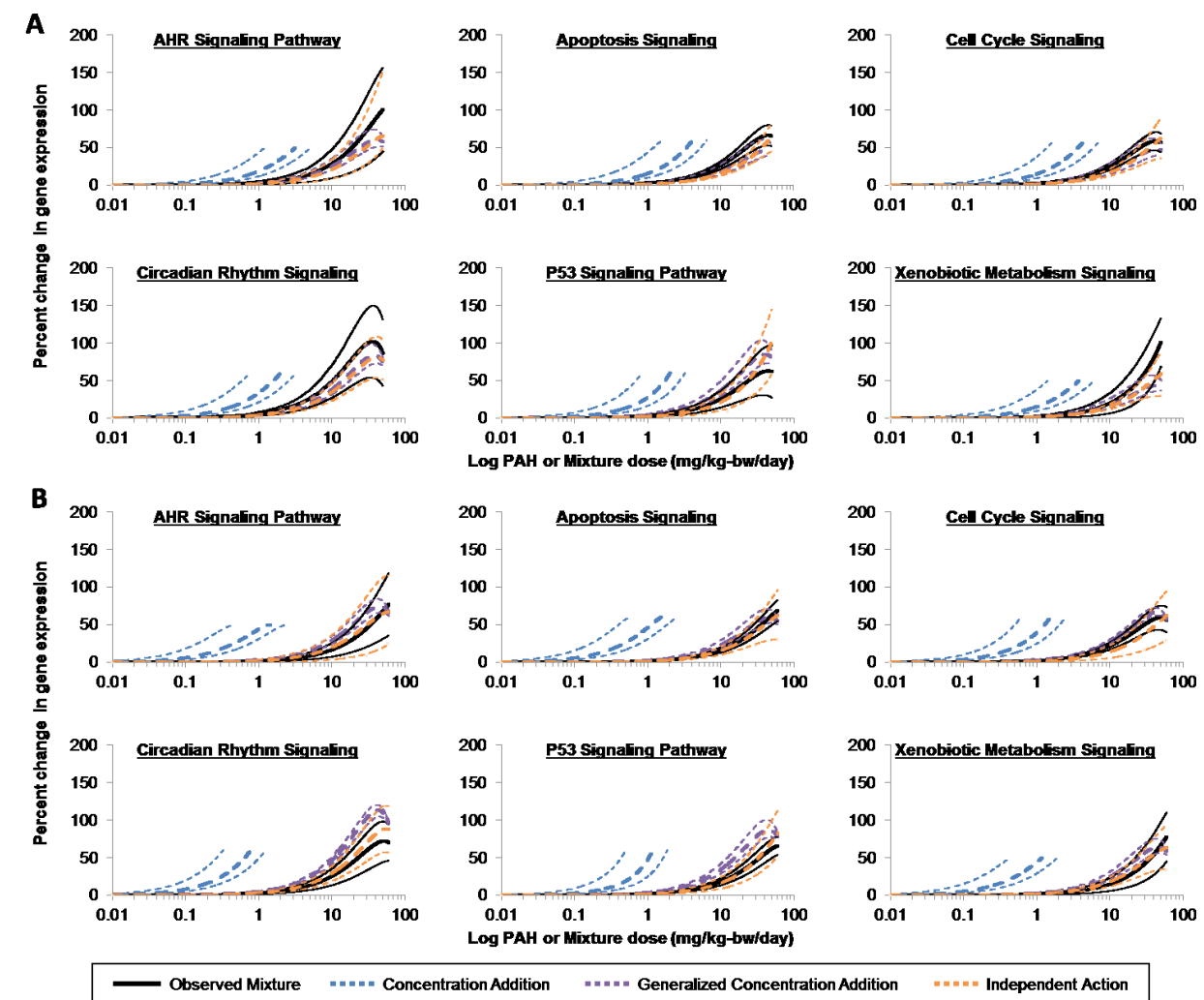


Figure 5-4 Comparisons of predicted and observed mixture effects for (A) a mixture of 4 PAHs and (B) a mixture of 8 PAHs.

Using individual, single-PAH dose-response relationships, additive effects were predicted using the models of concentration addition (CA; blue dotted lines), generalized concentration addition (GCA; purple dotted lines), and independent action (IA; orange dotted lines). Observed dose-response curves are represented by solid black lines. Thin lines represent 95% confidence bands.

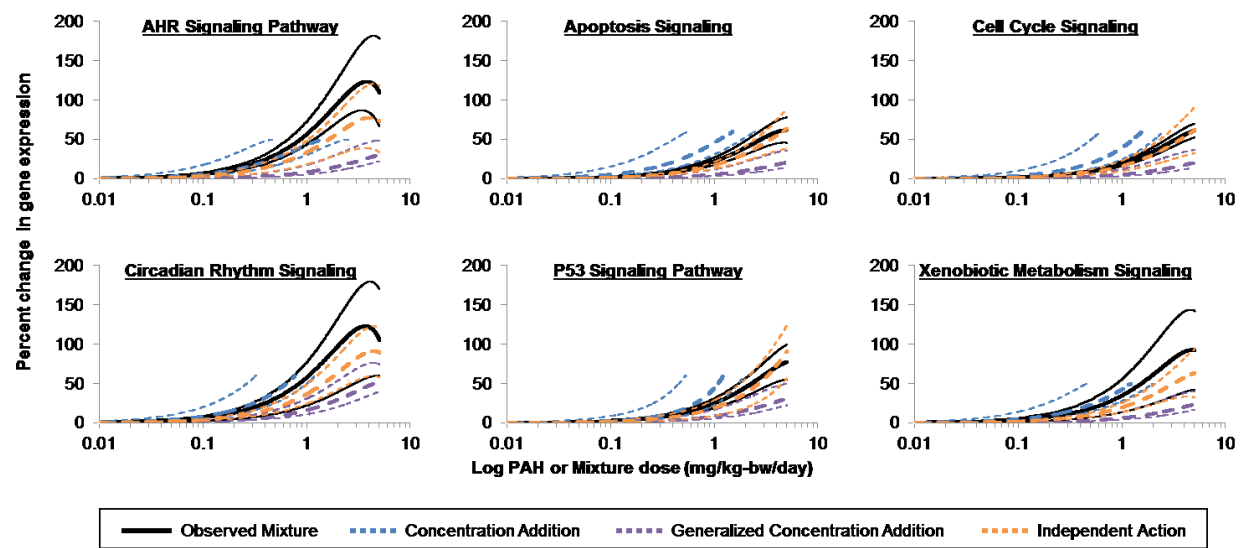


Figure 5-5 Comparison of predicted and observed effects of a PAH-containing coal tar extract

Based on the individual single-PAH dose-response relationships, additive effects were predicted using the models of concentration addition (CA; blue dotted lines), generalized concentration addition (GCA; purple dotted lines), and independent action (IA; orange dotted lines). Observed dose-response curves are represented by solid black lines. Thin lines represent 95% confidence bands.

6 Chapter: General Conclusions

6.1 Summary of Study Outcomes

The overall goal of this thesis was to evaluate the utility and applicability of transcriptomic data to expand current understanding regarding the toxic potential of individual chemicals and complex mixtures. Using PAHs and PAH-containing mixtures as model substances, this goal was achieved via the use of extensive bioinformatics, pathway, and statistical analysis of transcriptome data across different tissues following sub-chronic oral exposures to eight individual PAHs, two defined PAH mixtures, and one complex PAH-containing mixture (coal tar). Additionally, transcriptomic data were used to scrutinise the scientific basis for human health risk assessments of PAHs and PAH-containing mixtures. More specifically, the results from this thesis were used to address key assumptions that are routinely employed for risk assessment of complex PAH mixtures. For example, the analyses presented in Chapters 2 through 4 scrutinise the use of BaP as the representative carcinogenic PAH and the assumption that other carcinogenic PAHs act through the same mode of action. Moreover, Chapter 3 specifically scrutinises the relevance of rodent forestomach tumour data for the determination of PODs for human health risk assessment in light of the fact that humans do not have an analogous organ. Analyses presented in Chapter 5 scrutinise the assumption that carcinogenic PAHs behave in an additive fashion according to CA (concentration addition). In addressing these important issues, this thesis sought to achieve the thesis objectives that were outlined in the Introduction (i.e., Chapter 1). More specifically, to:

- (a) improve the understanding of mechanisms underlying the adverse, toxicological responses induced by individual PAHs in target tissues

(b) evaluate the contention that transcriptional profiles and pathway perturbation information can be used to critically examine interactions between individual PAHs in PAH-containing mixtures, and thus the assumption of additivity.

The following sections provide a summary of the work presented in Chapters 2 through 5, and a synopsis regarding the accomplishment of the thesis objectives presented in Chapter 1. More specifically, the outcomes of each chapter are described in relation to the aforementioned key assumptions, with special attention paid to the utility of the results for assessing the toxicological hazards and human health risks posed by PAHs and PAH-containing mixtures.

Chapters 2 and 3

The goal of Chapters 2 and 3 was to employ transcriptomic profiling to investigate (a) the differences in BaP's tumor potency across target tissues for tumour development and (b) the relevance of rodent forestomach tumours for human health risk assessment.

BaP is commonly accepted as a point of reference in the estimation of excess lifetime cancer risk posed by genotoxic PAHs or PAH-containing mixtures. This is primarily based on the hypothesis that genotoxic PAHs, such as those studied here, act via a similar mode of action. Thus, prior to addressing the question regarding the use of BaP as an appropriate point of reference for other genotoxic PAHs (i.e., Chapter 4), it was deemed important to obtain a more complete understanding regarding the gene expression changes induced by BaP exposure, especially changes in the target tissues for tumour development.

BaP exposure is associated with the formation of tumours in different organs; forestomach tumours are consistently observed following oral exposure to BaP and results regarding the

formation of lung and liver tumours are variable. Many studies have tried to correlate tissue-specific tumour induction with the formation of DNA adducts and the occurrence of mutations (Hakura et al., 1998; Pottenger et al., 2009). However, these efforts have met with limited success, suggesting the involvement of additional events occurring subsequent to DNA adduct formation and mutation induction in determining the tissue-specific carcinogenic potency of BaP. In Chapters 2 and 3 we confirmed that levels of DNA adduct formation did not correlate with published carcinogenic potency information, or with the occurrence of mutations in the three tissues examined. In contrast, the transcriptomic results indicate that changes in gene expression are more closely related to previous reports of tumour incidence in each of the tissues examined (Hakura et al., 1998). More specifically, the results obtained indicate that, in addition to transcriptional changes indicative of a genotoxic mode of action in the lung, and to a lesser extent in the liver, carcinogenic mechanisms related to clear activation of immune and inflammatory responses were noted in the forestomach. These latter observations likely explain BaP's higher carcinogenic potency for forestomach, and indicate that the mechanisms underlying the carcinogenicity of BaP are tissue-dependant. These conclusions were supported by a later study that investigated the BaP-induced transcriptomic changes in target and non-target organs of carcinogenesis in mice orally exposed to BaP (Zuo et al., 2014). More specifically, the study by Zuo et al. (2014) suggested that perturbation of cancer pathways could help distinguish organ-specific responses. Collectively, these studies demonstrate that transcriptomic analyses can be successfully employed to determine the causes underlying BaP's tissue-specific tumour induction.

The transcriptional data from Chapters 2 and 3 were included in a landmark case study on the use of toxicogenomics in the human health risk assessment of BaP in drinking water

(Appendix I). Transcriptomics data from the forestomach, liver, and lung tissues of mice exposed to BaP were used for mode of action development and for deriving potential points of departure (PODs) in two independent risk assessments: one that used only the transcriptomic data and one that used a combination of traditional toxicological data in addition to transcriptomic data. The results showed that transcriptomic data provided valuable insight into BaP's mode of action and transcriptional BMDs were comparable to BMDs calculated from rodent tumour incidence data. Thus, the use of transcriptomic data did not significantly affect the quantitative estimate of risk posed by BaP in drinking water. Collectively, the results from this thesis support the use of mode of action analysis and quantitative dose-response analysis of transcriptomic data to evaluate BaP's carcinogenic potency; and moreover, the risk posed by exposure to BaP.

The mechanistic information assembled from the transcriptomic profiling presented in this thesis can also be used to scrutinise the human relevance of assumptions commonly employed for human health risk assessment. For example, the results obtained can be used to scrutinise the important assumption that chemically-induced responses in rodent and human analogous organs are comparable (NRC, 2007b); however, the human-relevance of tumours in rodent-specific organs (e.g., the forestomach, preputial gland, etc.) is difficult to evaluate. As noted in Chapter 3, and despite the fact that humans lack an analogous organ, the oral, forestomach cancer slope factor for BaP is used for human health risk assessment of PAHs and PAH-containing mixtures. The transcriptomic analysis, and the subsequent meta-analyses presented in Chapter 3, reveal that gene expression changes in BaP-exposed forestomach are strikingly similar to those observed in human gastrointestinal tract cancers. More specifically, the use of the NextBio bioinformatics tool to critically compare the murine forestomach data with published transcriptomic profiles of human gastrointestinal tract tumours revealed significant similarities in

the expression of genes involved in antigen processing and presentation, immune response, chemotaxis, and keratinocyte differentiation. These similarities support the use of forestomach tumour data in assessments of human cancer risk; moreover, the value of transcriptomic profiling to scrutinise the utility and suitability of rodent to human extrapolations.

Chapter 4

The goal of Chapter 4 was to understand if transcriptional profiling can be used to determine whether BaP is an appropriate point of reference for genotoxic PAHs.

The finding that BaP-induced transcriptomic responses are tissue-specific (Chapters 2 and 3), and more complex than anticipated, highlights the seemingly oversimplified assumption that the toxicological activities of PAHs are manifested via a single mode of action. In Chapter 4, using transcriptomics, transcriptional changes elicited by BaP were compared to those elicited by other genotoxic, priority PAHs.

The results presented in Chapter 4, the first cross-tissue transcriptomic study of several genotoxic PAHs, revealed that despite a uniform ability to induce DNA damage (i.e., DNA adducts), mutations, and increases in enzyme activity in the lung (Chapter 4), liver, and stomach (Long et al., 2016), the underlying pathophysiologic pathways, some of which have been implicated in carcinogenesis, and the directions of pathway regulation, differ across PAHs and tissues. For example, a major player in the BaP-induced DNA damage response is the P53 signaling pathway; however, this pathway was not perturbed following exposure to BghiP or BkF, and was only minimally perturbed by CHR and IP. Rather, the transcriptional response for these latter PAHs pointed to a stronger influence of the immune/inflammatory response, and altered cell signaling leading to carcinogenesis. These results were supported by a “Hallmarks of

Cancer” analysis whereby the hallmarks of cancer outlined by Hanahan and Weinberg (2011) were used as a directional tool to classify the wide variety of pathways perturbed by the PAHs into functional groups that highlight the alterations in tissue microenvironment elicited by PAH exposure. Additionally, hierarchical cluster analysis, concordance analysis, and a statistical meta-analysis that explored correlations of gene expression changes between treatment groups, all revealed that the transcriptional patterns induced by each PAH were different, exhibiting separate clustering, few common changes in differentially expressed genes, and few commonalities in the direction of gene expression changes. Furthermore, the tissue-specific transcriptional responses induced by each PAH were distinct, suggesting different tissue-specific rates and capacities for metabolism, clearance, and translocation, which can impact the likelihood of tissue-specific carcinogenesis. Thus, using transcriptome profiling the cumulative works presented in Chapters 2, 3 and 4 have demonstrated that the use of BaP as a representative of PAH carcinogenicity may constitute an over-simplification. Consequently, moving forward, determination of PAH carcinogenicity may require consideration of the effects induced by more than one PAH.

Chapter 5

The goal of Chapter 5 was to understand if transcriptomic profiling can be used to evaluate the assumption of additivity.

The current approach for human health risk assessment of PAH-containing mixtures relies on the assumption that PAHs in a mixture act in an additive fashion according to the CA model of additivity. This assumption is useful since it is not necessary to generate toxicological information for each newly identified mixture of PAHs, thus avoiding issues related to sample

collection/preparation and the lack of standards for each mixture. Additivity among PAHs has been investigated at the level of apical endpoints, such as tumour formation, DNA adducts, and enzyme induction (section 1.2.4); however, the work presented herein demonstrates that these endpoints may not be as predictive of carcinogenicity in comparison with pathway perturbations (Chapters 2, 3, and 4). Consequently, to address the assumption of additivity for PAH-containing mixtures, the work presented in Chapter 5 investigated the utility of pathway perturbation responses, which may be more reflective of cancer initiation and progression, in scrutinizing the assumption of additivity.

More specifically, the work presented in Chapter 5 evaluated whether the differences amongst the PAHs examined, at the level of cancer pathways, would be expected to alter the additive behaviour of PAHs in a mixture. The results showed that the assumption of PAH additivity is valid for all cancer-related pathways, including those related to xenobiotic metabolism, DNA damage response, and altered signaling related to cellular growth and proliferation. However, the additive IA model yielded better estimates compared to CA or GCA. Additionally, the results show that the observed experimental transcriptional BMD values induced by the coal tar mixture and the defined mixture of eight PAHs were 1.1 to 2.4-fold different in comparison with IA-derived estimates, depending on the cancer-pathways investigated. These results were corroborated by another study from our laboratory that analysed induced transgene mutant frequencies in the lungs of animals exposed to the same PAHs and PAH-containing mixture (Long et al., submitted). The authors used a modification of the IA model of additivity (described in Lemieux et al., 2008) whereby the mutagenic potency of each PAH (i.e., slope of the dose-response curve) at the mixture exposure concentrations were summed to estimate the mutagenic potency of each mixture. The results were then compared

with the actual mutagenic potency of each mixture; the authors showed that mutagenic potency estimates assuming additivity were within 1.1-fold of the observed mutagenic potency for coal tar and within 0.5-fold for the defined mixture of eight PAHs. These studies confirmed the work presented in Chapter 4 that showed that eight priority PAHs, which are genotoxic carcinogens, induce compound- and tissue-specific transcriptional responses in three target tissues (i.e., forestomach, liver, and lung). More specifically, differences in the observed responses reflect the unique abilities of each PAH to induce biologically relevant changes related to carcinogenic transformation; these differences would be expected to contribute to non-additive behaviour under the CA model of additivity. Collectively, the results suggest that PAHs behave in an additive fashion according to IA; moreover, that the traditional additive paradigm that employs CA and PEFs may over-estimate the risk posed by PAH-containing mixtures.

In addition to addressing the assumption of additivity, the results presented in Chapter 5 support the assertion made in Chapter 4 that BaP may not be an appropriate representative of carcinogenic PAHs for risk assessment of PAH-containing mixtures. More specifically, hierarchical cluster analysis showed that DBahA and BbF co-clustered on the same branch as the three PAH mixtures, whereas BaP clustered on an adjacent branch, suggesting that DBahA and BbF may play a strong role in the transcriptional response induced by the PAH mixtures compared to the other PAHs, including BaP. Therefore, the collective results from Chapters 4 and 5 suggest that the determination of PAH mixture carcinogenicity may require consideration of effects induced by other PAHs.

Overall, the results outlined and summarized in the preceding sections clearly support the **general thesis hypothesis** that experimental exposure to PAHs and/or PAH-containing mixtures

induce statistically significant changes in transcription, and associated subcellular pathways, that are significantly correlated with an array of disease-related outcomes; and moreover, that this correlation yields mode of action information that can be incorporated into human health risk assessment. Moreover, the thesis results presented in Chapters 2 through 5 achieved the stated thesis objectives outlined in Chapter 1. Each of the thesis objectives is restated below, and the accompanying statements incrementally outline the outcome of each thesis objective evaluated:

Objective (a): To improve the understanding of mechanisms underlying the adverse, toxicological responses induced by individual PAH in target tissues (Chapters 2, 3, and 4).

Outcomes: This objective was realized. Bioinformatic and pathway analysis revealed that the PAH-induced transcriptomes were significantly associated with several pathways and biological processes related to cancer, including those related to DNA damage response, immune/inflammatory response, and altered signaling reflective of enhanced cellular proliferation. Additionally, analyses demonstrate that the PAH-induced changes are tissue-specific with respect to the types of pathways perturbed and the magnitude of change (i.e., the number of genes and the fold changes). Moreover, comparisons of transcriptional profiles of PAH-exposed tissues to publically available transcriptomic profiles from diseased tissues reveal that the PAH-induced transcriptional changes are predictive of adverse toxicological responses in rodents and humans, in particular cancer. The transcriptional information was further used within a formal mode of action framework to improve the understanding of BaP's carcinogenic mode of action for the human health risk assessment of BaP in drinking water. Collectively, the thesis

results demonstrate that transcriptional profiling can be used to improve the understanding of mechanisms underlying tumour initiation and development induced by individual priority PAHs.

Objective (b): To evaluate the contention that transcriptional profiles and pathway perturbation information can be used to critically examine interactions between individual PAHs in PAH-containing mixtures, and thus the assumption of additivity (Chapter 5).

Outcomes: This objective was realized. For the first time, a strategy that employs pathway-level transcriptional data and conventional mathematical models of additivity was developed to scrutinize the assumption of additivity. More specifically, this strategy applied individual PAH-induced pathway perturbation data and pathway BMD values to evaluate three mathematical models of additivity (i.e., CA, GCA, and IA); specifically to generate predicted pathway dose-response curves for each PAH mixture. Comparison of predicted and observed pathway dose-response curves showed that the IA model most accurately predicted the pathway perturbations induced by the PAH mixtures compared to the CA and GCA models. Collectively, the thesis results demonstrate that in contrast to the assumption of dose additivity that is commonly employed for PAH-containing mixtures (i.e., CA-based approach), perturbations of pathways implicated in carcinogenesis are best predicted by an additive model that assumes dissimilar modes of action (i.e., IA-based approach).

6.2 Utility of toxicogenomics in contemporary human health risk assessment

The current toxicity testing paradigm employed to predict the likelihood of adverse outcomes in human populations is at a cross-road; one avenue leads to apical endpoints emphasized in the traditional toxicity-testing paradigm, and the other to high-throughput, mechanism-based

assessment tools (Krewski et al., 2010). Researchers and chemical evaluators are currently collaborating on the development of a strategy that can be universally applied to the range of existing substances that currently require hazard evaluation, as well as the ever-increasing number of novel and newly identified chemicals. With respect to the development of new strategies, the value of toxigenomic technologies for human health risk assessment is increasingly being recognized by international regulatory agencies. Several policies, guidance documents, and reports (USEPA, 2002; USEPA, 2004; Dix et al., 2006; USEPA, 2014a; European Commission, 2015; Cote et al., 2016) have been released, with particular attention paid to using these technologies to understand and predict mechanisms of toxicity and toxicological outcomes. Additionally, international committees have been established to work towards harmonization of approaches in genomics and to advance applications such as those employed here. For example, the Health and Environmental Sciences Institute of the International Life Sciences Institute (ILSI-HESI) has established a committee to critically examine the application of genomics to mechanism-based risk assessment, and the OECD-established Extended Advisory Group on Molecular Screening and Toxicogenomics is using the concept of adverse outcome pathways (AOPs) to internationally harmonize mechanism-based approaches in toxicology (discussed below).

The results presented in this thesis were generated using transcriptional profiling and subsequently bioinformatics and pathway analyses; it is important to evaluate the utility of this type of approach in the broader context of mechanism-based chemical screening. A tiered testing approach for the evaluation of chemicals was proposed; it involves screening and prioritization, and incorporates toxicogenomics in the second tier (Thomas et al., 2013). Moreover, the US National Toxicology Program (NTP) has already implemented a toxicogenomic testing strategy

that measures the transcriptome in a variety of tissues following five days of oral exposure. Thus, regulatory organizations now recognize the value of toxicogenomics for the identification of toxic chemicals that have the capacity to elicit adverse human health effects.

Although the applications of toxicogenomic technologies for human health risk assessment of environmental chemicals have largely focussed on understanding and predicting mechanisms of toxicity and toxic responses, recent advances have critically examined the use of toxicogenomic data for quantitative risk assessment. Approaches for dose-response analysis of toxicogenomic endpoints have been developed, and methods to derive transcriptional BMD values for quantitative risk assessment have been explored (Thomas et al., 2011). Several case studies have been conducted to date, and they collectively demonstrate the potential uses of transcriptomic data in deriving PODs for single chemical risk assessments. Moreover, the results of these studies show a high degree of concordance between apical endpoint PODs and transcriptional PODs (Jackson et al., 2014; Labib et al., 2016; Moffat et al., 2015; Thomas et al., 2011; Thomas et al., 2012; Thomas et al., 2013b). For example, in the case study on the use of toxicogenomics for human health risk assessment of BaP (Appendix I), transcriptional BMDs were comparable to BMDs derived from published 2-year rodent cancer bioassays. Similarly, in Chapter 5, observed and predicted coal-tar induced transcriptional BMDs for cancer-related pathways were comparable to those derived from lung tumour incidence data for mice fed a coal tar-containing diet for two years. Moreover, a recently published framework for the use of transcriptomic data for quantitative risk assessment of manufactured nanomaterials (Appendix II – Labib et al., 2016) noted that regardless of the type of multi-walled carbon nanotube (MWCNT), the transcriptional approach resulted in BMD values comparable to those derived using histopathological diagnosis of exposure-induced lung fibrosis. Thus, this thesis and several

other studies collectively support the use of quantitative dose-response analysis of transcriptomic data to evaluate a chemical's toxic potency.

This thesis has demonstrated that a well-conducted toxicogenomic study can provide valuable information about a chemical's ability to induce a long-term adverse outcome; and moreover, its mechanisms of toxicity. Importantly, toxicogenomic studies are relatively inexpensive, and use small numbers of animals, in comparison with long-term rodent bioassays (e.g. 2-year rodent cancer bioassay). For example, each of the PAH studies conducted for this thesis used only twenty mice exposed to three doses plus a vehicle control for 28 days. In contrast, rodent cancer bioassays commonly employ approximately 800 animals with repeat-dose exposures carried out for up to 2 years. Additionally, with respect to cost, a complete microarray experiment for a single PAH, including the cost of animal handling and microarray analysis for all doses and replicates, costs approximately \$6000 per tissue. The per substance cost for a cancer bioassay is \$2-4 million USD, including substantial labour costs. Nevertheless, despite the relatively low cost of transcriptomic studies, it would not be possible to test all novel and newly identified chemicals via in vivo transcriptional profiling. Therefore, the future of chemical risk assessment must rely on new approaches that build off of the results generated from 'omics data for different classes of chemicals, in particular toxicity-pathway based approaches such as the AOP approach.

The Organization for Economic Cooperation and Development (OECD) launched the AOP program in 2012 as an international collaborative effort involving research scientists, chemical evaluators, and risk assessors. AOPs evolved from the mode of action concept and they emphasize the importance of connecting various events that lead to a toxic (adverse) outcome. Both require the selection and ordering of essential events along the pathway to toxicity, as well

as assessment of empirical data (e.g., temporal and dose concordance) and biological plausibility to support the relationships between events along the pathway (Meek et al., 2013; Villeneuve et al., 2014). AOPs established to date are available through a web-based wiki (aopkb.org/aopwiki), which allows the AOP to exist as a living document that can be edited as new information becomes available. Another benefit of AOPs is that they are not chemical-specific, and therefore provide a way to facilitate the assessment of novel chemicals and mixtures by allowing researchers and risk assessors to use documented AOP relationships to extrapolate from known chemically-induced effects to possible adverse outcomes. Additionally, AOPs require that each key event be readily quantifiable and measurable in order to facilitate targeted key event testing. Lastly, AOPs allow for common key events to be linked forming large networks of possible adverse outcomes (Knapen et al., 2015) that enable visualization of complex responses to chemical exposures, and potential interactions between pathways leading to an adverse outcome. The power behind the AOP initiative lies in its promise to become an invaluable resource for risk assessment as the AOP knowledgebase grows.

Getting the AOP wiki knowledgebase to a state where it can be effectively used for chemical risk assessment will take time. The main reason is that the process of building an AOP requires extensive knowledge of the sequence of events leading to an adverse outcome, and acquiring this knowledge requires exhaustive review of the literature, i.e., the AOP building process requires data and there is currently a paucity of toxicological information. To generate more AOPs, researchers are moving towards the use of next generation technologies, such as high-throughput in vitro assays and toxicogenomics. For example, a recent publication employed transcriptomic data derived from short-term in vivo studies across multiple doses and time-points to construct a hypothetical AOP describing the various key events that likely occur between the initial

exposure to MWCNT and the development of pulmonary fibrosis (Appendix II). Interestingly, the key events identified using transcriptomics data were the same as the key events identified using traditional toxicity testing methods (Vietti et al., 2016), once again supporting the value and applicability of transcriptomics data to support AOP development. As more transcriptional information is generated for different classes of chemicals, hypothetical AOPs can be generated. Subsequently, and rather than traditional toxicity testing methods, novel chemicals and mixtures can then be tested for induction of particular key events using targeted in vitro and in vivo assays. Thus, an approach that uses targeted assays defined by the toxicogenomics-informed hypothetical AOPs would facilitate the screening of novel and newly identified chemicals.

6.3 Concluding remarks

The ultimate goal of regulatory toxicity testing is to assess and manage the risks posed by new and existing chemicals. The availability of sufficient data, and the ability to interpret data with respect to human health outcomes, is a particular challenge to risk assessors. The current approach for assessing the risks posed by PAHs and PAH-containing mixtures employs assumptions that are necessary due to the incomplete toxicological database for most PAHs. This thesis employed transcriptional data to address many of these underlying assumptions, including the use of a single reference chemical for risk assessment, the assumption of similar modes of action among PAHs, the assumption of additivity, and the human relevance of tumours induced in rodent-specific organs (e.g., forestomach). It is anticipated that the approach employed herein, which is currently being incorporated into the evolving toxicity testing approach outlined above, will provide risk assessors with an improved ability to assess the likelihood of adverse outcomes induced by chemicals, as well as improved understanding regarding underlying biological mechanisms. As more toxicogenomic data are generated, communication between bench research scientists and risk assessors will be necessary to facilitate the expected transition from

traditional apical endpoint-driven risk assessments to assessments that are driven by high-content/high-throughput testing and mechanistic data. The implementation of toxicogenomic tools into a standard toxicity testing paradigm promises to meet emerging needs for more rapid and mechanism-based methods to assess the human health risks posed by new and existing chemicals.

7 References

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Appendices

Appendix I. Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo[a]pyrene in drinking water.

REVIEW ARTICLE

Comparison of toxicogenomics and traditional approaches to inform mode of action and points of departure in human health risk assessment of benzo[a]pyrene in drinking water

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Abstract

Toxicogenomics is proposed to be a useful tool in human health risk assessment. However, a systematic comparison of traditional risk assessment approaches with those applying toxicogenomics has never been done. We conducted a case study to evaluate the utility of toxicogenomics in the risk assessment of benzo[a]pyrene (BaP), a well-studied carcinogen, for drinking water exposures. Our study was intended to compare methodologies, not to evaluate drinking water safety. We compared traditional (RA1), genomics-informed (RA2) and genomics-only (RA3) approaches. RA2 and RA3 applied toxicogenomics data from human cell cultures and mice exposed to BaP to determine if these data could provide insight into BaP's mode of action (MOA) and derive tissue-specific points of departure (POD). Our global gene expression analysis supported that BaP is genotoxic in mice and allowed the development of a detailed MOA. Toxicogenomics analysis in human lymphoblastoid TK6 cells demonstrated a high degree of consistency in perturbed pathways with animal tissues. Quantitatively, the PODs for traditional and transcriptional approaches were similar (liver 1.2 vs. 1.0 mg/kg-bw/day; lungs 0.8 vs. 3.7 mg/kg-bw/day; forestomach 0.5 vs. 7.4 mg/kg-bw/day). RA3, which applied toxicogenomics in the absence of apical toxicology data, demonstrates that this approach provides useful information in data-poor situations. Overall, our study supports the use of toxicogenomics as a relatively fast and cost-effective tool for hazard identification, preliminary evaluation of potential carcinogens, and carcinogenic potency, in addition to identifying current limitations and practical questions for future work.

Table of Contents

Abstract..	1
Introduction ..	2
Traditional risk assessment approach (RA1) ..	4
Use, identity, and sources of BaP exposure.	4
Analytic methods and treatment technology.	5
Absorption, distribution, metabolism, excretion, and pharmacokinetics ..	5
Absorption..	6
Distribution.	6
Metabolism.	6
Excretion.	8
Physiologically based pharmacokinetic modeling ..	8
Human health effects ..	8

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History

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Effects on experimental animals, mechanisms, and their human relevance ..	9
Reproductive toxicity ..	9
Developmental toxicity ..	10
Renal and hepatic toxicity ..	10
Cardiovascular toxicity ..	10
Neurotoxicity ..	10
Immunotoxicity ..	10
Genotoxicity ..	11
DNA adducts ..	11
Mutations ..	11
Micronuclei ..	11
Other genotoxicity endpoints ..	11
Mechanisms ..	11
Human relevance ..	12
Summary ..	12
Carcinogenicity ..	12
Skin tumors ..	12
Respiratory tract tumors ..	12
Digestive tract tumors ..	12
Liver tumors ..	12
Other tumors ..	13
Mechanistic information ..	13
Selection of the point of departure for risk assessment 1 (RA1) ..	13

Calculation of BMD/BMDL	13
Selection of the key effect with the lowest biologically relevant BMD/BMDL	14
Genomics approaches (RA2 and RA3)	14
Overview of toxicogenomics data and analyses used in RA2 and RA3	14
Functional annotation	14
Microarray analysis	14
Animal data	15
Human cell culture data	16
Identification of key events and proposal of MOA	16
Calculation of transcriptional PODs	17
Genomics-informed approach (RA2)	19
Proposed mode of action	19
Key event 1: BaP binding to the AHR (molecular initiating event)	19
Key event 2: Induction of cytochrome P450 enzymes (CYPs)	20
Key event 3: BaP metabolism to reactive metabolites	20
Key event 4: DNA adducts and DNA damage	22
Key event 5: Mutations	24
Key event 6: Altered cell signaling, proliferation, and apoptosis	25
Tumorigenesis (adverse outcome)	25
Other potential modes of action and modulating factors	26
Uncertainties, inconsistencies, and data gaps	26
Confidence in the proposed mode of action and selection of the point of departure for risk assessment (RA2)	26
Genomics-only approach (RA3)	26
Proposed mode of action	27
Key event 1: Activation of AHR signaling	27
Key event 2: Nrf2-mediated response to oxidative stress	30
Key event 3: DNA damage, repair, and apoptosis	30
Key event 4: Mutations	31
Other potential modes of action and modulating factors	31
Uncertainties, inconsistencies, and data gaps	31
Quantitative structure–activity relationship	31
Rationale for the proposed mode of action and selection of the point of departure for risk assessment (RA3)	32
Alternative genomics approaches	32
Discussion and conclusions	32
Advantages of a genomics approach	33
Obstacles to incorporation of toxicogenomics into risk assessment	34
Limitations of the data used in this case study	34
Next steps	35
Acknowledgements	37
Declaration of interest	37
References	37

Introduction

Regulatory agencies worldwide suffer from a backlog of chemicals in need of human health risk assessment. Some of this backlog is due to chemicals that have few or no data on which to base evaluations. To address this problem, a paradigm shift has been proposed that moves away from examining every apical endpoint toward broadly examining perturbations of pathways using new approaches, such as genomics and high-throughput assays, followed by testing for specific predicted toxicities (National Research Council [NRC] 2007a, 2007b).

Whole-genome microarrays examine the response of all genes within an organism's genome following a chemical exposure (toxicogenomics). The application of bioinformatics tools allows the categorization of these responses into specific biological functions, thus providing insight into the potential hazards of a toxicant. While the use of toxicogenomics at this time cannot fully replace traditional tests, its use in the prediction of a chemical's effects can offer considerable advantages

over standard practices, including increased throughput, reduced animal use, details on mode of action (MOA; see Box 1 for risk assessment definitions), as well as cost and time savings. Traditional tests are generally both costly and time-consuming; for example, a 2-year rodent cancer assay can cost approximately \$2–4 million and take about 3 years per compound (reviewed in Thomas et al. 2007b); the battery of genotoxicity assays costs approximately \$60,000 and requires several months. In contrast, a short-term *in vivo* toxicogenomics experiment could be done much more cost-effectively in a 1-month period. Thus, the application of toxicogenomics has been proposed to alleviate some of the problems associated with data development in support of risk assessment.

Recently, techniques that allow quantitative dose–response analysis from genomics data have been developed that are expected to facilitate the integration of genomics in human health risk assessment. Specifically, Thomas and colleagues (Thomas et al. 2007a, 2011, 2012) developed an approach to derive transcriptional benchmark dose (BMD) values for quantitative risk assessment. Using this approach, they found that the BMDs for the most sensitive toxicogenomics responses (i.e., the pathways, biological functions, or biological processes with the lowest median gene expression BMD values) correlate well with BMDs for apical cancer and non-cancer endpoints. The rationale for this approach is that these BMD values estimate the doses at which the system begins to be perturbed in response to the toxicant. These approaches are especially useful for chemicals that act via nonselective mechanisms, perturbing multiple different biological pathways (Thomas et al. 2013). In contrast, other chemicals operate through “selective” means (i.e., interacting with specific receptors or signaling pathways), having only one or a few biological processes that they target directly. In these cases, toxicogenomics data can be used to develop detailed MOAs and derive BMDs for key events or molecular initiating events for these toxicants.

In the present study, we incorporate both MOA and BMD approaches to derive points of departure for risk assessment of benzo[*a*]pyrene (BaP), a by-product of the incomplete combustion of organic materials. BaP is an extensively studied polycyclic aromatic hydrocarbon (PAH) and a well-characterized carcinogen with a genotoxic MOA (summarized in detail below). Following metabolism of BaP by cytochrome P450s (CYPs), metabolites of BaP that escape detoxication can bind deoxyribonucleic acid (DNA) to form adducts that cause mutations, which increase the risk of several forms of cancer. Epidemiological studies and experimental animal studies associate exposure to BaP with an increased risk of several forms of cancer, including tumors in forestomach, oral cavity, liver, and lungs (International Agency for Research on Cancer [IARC] 2012). Current guideline values for oral exposure to BaP through drinking water range from 0.007 to 0.7 µg/L (World Health Organization [WHO], California Environmental Protection Agency [Cal/EPA], Australia, the Netherlands, New Zealand, United States Environmental Protection Agency [US EPA] and Health Canada) (Supplementary Table 1 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>). Because of the extensive research that has been conducted on BaP, it provides an excellent model to compare how toxicogenomics may be useful under both data-rich and data-poor circumstances for hazard and risk assessment.

Box 1: Risk assessment definitions

Please note that more details for the concepts defined below are available elsewhere (US EPA 2005, Meek et al. 2014).

Adverse outcome: Broadly defined as impaired functional capacity of an organism or its increased susceptibility to other adverse influences.

Apical endpoint: An observable outcome in a whole organism, such as a clinical sign or pathologic state, that is indicative of a disease state that can result from exposure to a toxicant.

Bench mark dose (BMD): A dose or concentration that produces a predetermined change in response rate of an adverse effect compared to background.

Bench mark dose lower-confidence limit (BMDL): A statistical lower confidence limit on the dose that produces the selected response.

Benchmark response (BMR): A predetermined change in the response rate of an adverse effect (generally, the default BMR is 10% for categorical and one standard deviation for continuous data).

Dose-response assessment: Describes the likelihood and severity of adverse health effects related to the amount and condition of chemical exposure. The first step evaluates how the toxicity is caused using a mode of action/human relevance framework. The second step uses extrapolation to estimate the probability of the adverse effect beyond the lower range of observed data to infer the point of departure.

Genotoxic carcinogen: A compound that causes cancer by inducing DNA damage.

Hazard identification: Determines whether chemical exposure can cause an increase in the incidence of specific adverse health effects (e.g., cancer, birth defects) and whether the adverse health effect is likely to occur in humans.

Human health risk assessment: The process to estimate the nature and probability of adverse health effects in humans who may be exposed to chemicals in environmental media and includes exposure assessment, hazard identification, and dose-response assessment.

Key events: Biological entities that are measurable, repeatable, and necessary, but not necessarily sufficient to produce an adverse outcome.

Low-Adverse-Effect Level (LOAEL): The lowest exposure level at which there are biologically significant increases in the frequency or severity of an adverse effect between the exposed population and its appropriate control.

Mode of action (MOA)/human relevance framework: Composed of a sequence of key events progressing from exposure to an adverse outcome. The framework involves proposing a MOA from animal data and examination of the plausibility of that animal MOA in humans.

No-Adverse-Effect Level (NOAEL): The highest exposure level at which there are no biologically significant increases in the frequency or severity of an adverse effect between the exposed population and its appropriate control.

Point of departure (POD): A dose that can be considered to be in the range of observed responses, without significant extrapolation. A POD can be a data point or an estimated point that is derived from observed dose-response data. A POD is used to mark the beginning of extrapolation to determine risk associated with lower environmentally relevant human exposures.

Toxicogenomics: Measures the response of all genes within an organism's genome following a chemical exposure and includes 'omics techniques (transcriptomics, proteomic, metabolomics, etc.).

The objective of this case study is to evaluate approaches for utilizing toxicogenomics data in risk assessment. Our study was intended to compare methodologies, not to evaluate drinking water safety. The case study focuses specifically on exposure to BaP in drinking water. To accomplish this goal, we compared three risk assessment approaches (summarized in Figure 1) to derive three separate points of departure (PODs; the lower confidence bound on the lowest dose at which an endpoint significantly deviates from normal levels for studies considered critical in exposure risk) in order to compare current standard approaches with toxicogenomics-informed approaches:

1) *Risk assessment 1 (RA1): Traditional approach.* A comprehensive literature review was performed, and toxicity data on all apical endpoints (observable outcomes in a tissue, organ system, or entire organism resulting from a toxicant exposure [Krewski et al. 2010]) were col-

lected. A POD was derived based on the most sensitive apical adverse effect reported that was of relevance to humans.

2) *Risk assessment 2 (RA2): Genomics-informed approach.* Toxicogenomics information from human cell culture and rodent BaP exposures was included in the traditional risk assessment, along with other MOA data, and used to inform the POD selection.

3) *Risk assessment 3 (RA3): Genomics-only approach.* This approach assumed that no data from standard toxicity testing were available for BaP. Genomics data were used exclusively to provide insight into the MOA and for the selection of a POD.

We have focused our risk assessment comparisons on BaP in drinking water, although it should be noted that this work does not represent an evaluation of the safety of BaP in drinking water. The purpose of our work is to compare risk

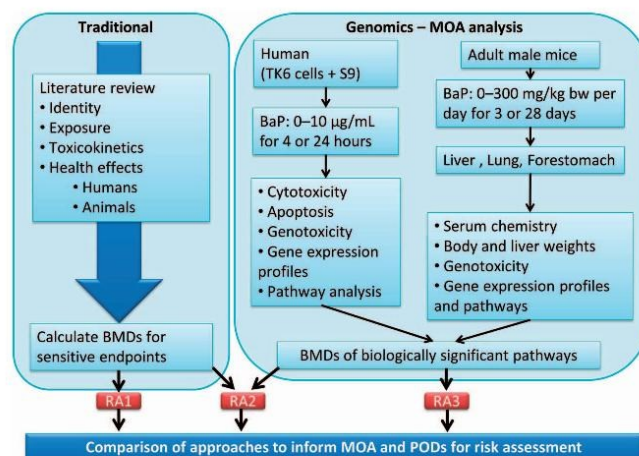


Figure 1. Comparison of traditional and genomics risk assessment approaches for determination of a point of departure (POD) for BaP in drinking water. **RA1: Traditional risk assessment approach.** A comprehensive literature review was performed and a POD was selected based on the most sensitive apical adverse effect reported (Section “Traditional risk assessment approach (RA1)”). **RA2: Genomics-informed approach.** Genomics information was included in the traditional assessment and used to inform the MOA and POD selection (Section “Overview of toxicogenomics data and analyses used in RA2 and RA3” for general methods and Section “Genomics-informed approach (RA2)” for RA2-specific details). **RA3: Genomics-only approach.** This approach assumed a data-poor chemical with little or no information other than genomics information. The definitions of “data-rich” and “data-poor” compounds relate to the amount of toxicity information available for a given compound and may be agency-specific. Only genomics information was used to select a POD for BaP (Section “Overview of toxicogenomics data and analyses used in RA2 and RA3” for general methods and Section “Genomics-only approach (RA3)” for RA3-specific details).

assessment approaches and to provide an example of the potential use of toxicogenomics in risk assessment.

Traditional risk assessment approach (RA1)

The information presented below is consistent with the type of information found in Health Canada’s technical documents for its Canadian drinking water guidelines; some examples can be found at: <http://www.hc-sc.gc.ca/ewh-semt/water-eau/drink-potab/guide/index-eng.php>. A literature review was performed to examine sources of BaP exposure, treatment technologies, kinetics and metabolism, human health effects, and effects on experimental animals. For potential health effects, Scopus and PubMed databases were searched (1 January 1966 to November 2013) using the following search string:

((CASREGNUMBER(50-32-8)ANDLANGUAGE(english)) AND PUBYEAR AFT 1965) AND (TITLE-ABS-KEY(“acceptable daily intake” OR noel OR noel OR “reproductive effect” OR teratogen* OR “developmental effect” OR “neurological effect” OR “endocrine effect” OR “immunological effect” OR “mode of action” OR “mechanism of action” OR pbpk OR toxic OR toxicity OR toxicol* OR neurotox* OR embryotox* OR cytotox* OR hepatotox* OR fetotox* OR genotox* OR maternotox* OR immunotox* OR dermatotox* OR cardiotoxic* OR nephrotoxic* OR ototoxic* OR cancer* OR mutagen* OR carcin* OR tumour* OR tumor* OR malign*))

All of the studies that we evaluated are summarized in Supplementary Table 2 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934> (human health effects) and Supplementary Table 3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934> (animal health effects). The

relevant studies used to determine a POD for human health effects for exposure to BaP in drinking water are discussed below. Relevance was based on potential association with an adverse outcome in humans for animal studies and adequate monitoring of BaP exposure concentrations for epidemiological studies, as well as various other parameters indicating general study quality (e.g., control for confounders, sufficient population size, and appropriate data collection protocols).

Use, identity, and sources of BaP exposure

BaP is a by-product of the incomplete combustion of organic substances. Thus, the vast majority of BaP released in the atmosphere results from anthropogenic activity (e.g., burning of fossil fuels for industrial applications, transportation, waste incineration) and, to a lesser extent, natural processes (e.g., forest fires, volcanic eruptions). Additional sources of BaP affecting water and soil include oil spills, municipal effluents, and urban and agricultural runoff. BaP is not manufactured in Canada. Physicochemical properties of BaP (Table 1), such as low water solubility, low vapor pressure, and elevated octanol–water partition coefficient ($\log K_{ow}$), strongly favor the adsorption of BaP onto particles, soil, and sediment (MacKay and Paterson 1991). As such, approximately 82% of BaP is estimated to partition to soil, 17% to sediment, 1% to water, and < 1% to air, according to a multimedia transport model (Hattermer-Frey and Travis 1991). However, exposures through a variety of the above-mentioned environmental vehicles have been identified as potentially important sources of exposure of humans to BaP (refer to Supplementary File A to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934> for information on exposures through soil, air, as well as, food and consumer products).

Table 1. Physicochemical properties of benzo[a]pyrene.

Property	Value
Chemical Abstracts Service registry number	50-32-8 ^{*†‡}
Chemical formula	C ₂₀ H ₁₂ ^{*†‡}
Molecular weight (g/mol)	252.3 ^{*†‡}
State at ambient temperature	Solid, plates, or needles ^{*†‡}
Vapor pressure (mmHg)	5.6 × 10 ⁻⁹ (20°C) [†] ; 7.47 × 10 ⁻⁷ (25°C) [†] ; 5.49 × 10 ⁻⁹ (25°C) [‡]
Half-life (days)	84.6 (river, calculated) [‡] , 931.3 (lake, calculated) [‡]
Photolysis half-life in water (hours)	0.54 (irradiation in upper layer of clear water, partitioning to sediment will significantly increase half-life) [‡]
Henry's Law constant	0.034 Pa·m ³ /mol (20°C) [†] ; 4.9 × 10 ⁻⁷ atm·m ³ /mol [†] ; 1.13 × 10 ⁻⁶ atm·m ³ /mol [‡] ; 4.5710 ⁻⁷ atm·m ³ /mol (25°C) [‡]
Boiling point	310–312°C (10 mmHg) ^{*†‡} ; 495°C (760 mmHg) ^{*†‡}
Melting point	176.5–179.3°C ^{*†‡}
Specific gravity	1.351 ^{†‡}
<i>n</i> -Octanol–water partition coefficient (log K _{ow})	6.35 [†] ; 6.06 (25°C) [†] ; 5.97 [‡] ; 6.13 (25°C) [‡]
Water solubility	Slightly, 0.001 62 mg/L (25°C) ^{*‡} ; 3.8 × 10 ⁻⁶ g/L (25°C), 2.3 × 10 ⁻³ mg/L [†]
Volatility	Poor [†]
Taste and odor threshold	No data [†]

*IARC (2010).

†Cal/EPA (2010).

‡CHEMFATE (2013): <http://esc.syrres.com/scripts/CASCFcgi.exe?CASNUM=50328>.††HSDB Hazardous Substances Data Bank (HSDB): <http://toxnet.nlm.nih.gov> (search term 50-32-8).†††US EPA. (2013). Estimation Programs Interface Suite[™] for Microsoft[®] Windows, v4.11. United States Environmental Protection Agency, Washington, DC, USA.

BaP primarily enters source waters through atmospheric deposition. Mean concentrations of BaP in the Great Lakes range from 0.03 to 0.7 ng/L (Environment Canada 1991). Given BaP's strong association with soil, it is not expected to leach into groundwater. Nevertheless, concentrations as high as 0.32 µg/L have been reported in groundwater in proximity to a New Brunswick wood preserving plant (Intera Technologies Ltd et al. 1989).

BaP primarily enters drinking water by leaching from asphaltic or bituminous (coal tar) lining of water storage tanks and distribution system pipes. Pipes and tanks installed prior to the 1960s may contain this type of lining and may still be a source of BaP in drinking water. Factors such as disinfectant dose and type, hydraulic disturbances, and increased residence time of the water in the distribution system can contribute to the release of BaP into the water (Maier et al. 2000). Canadian drinking water is estimated to contain BaP at concentrations of <0.005–3 ng/L (Canadian Council of Ministers of the Environment [CCME] 2010), whereas the concentration of BaP in US drinking water is typically 0.55 ng/L (Santodonato et al. 1981). Global concentrations of BaP in drinking water range from <0.04 to 914 ng/L, as reported by WHO (2003). Thus, based on a daily water intake of 1.5 L, a body weight (bw) of 70 kg for the average adult and the global concentrations of BaP in drinking water reported by WHO (2003), daily BaP ingestion is calculated to be 0.000857–19.6 ng/kg-bw.

Analytic methods and treatment technology

A number of analytical methods are available for the routine analysis of BaP in drinking water. These methods include liquid-based extraction followed by gas chromatography/mass spectrometry or high-performance liquid chromatography/ultraviolet/fluorescence detection, with method detection lim-

its as low as 16 ng/L in the methods, approved by the US EPA for routine monitoring (US EPA 1990, 1995, 2003, 2009).

Conventional treatment (i.e., coagulation, sedimentation, and filtration) can reduce the concentration of BaP to <0.001 mg/L in the drinking water at the treatment plant. Adsorption technologies such as granular activated carbon and powdered activated carbon are capable of reducing BaP concentrations in drinking water to below 0.2 mg/L and 10 mg/L, respectively. Point-of-use treatment devices such as activated carbon filters are also capable of removing BaP at the tap (US EPA 2007). The leaching of BaP from coal tar-lined pipes in the distribution system can also be minimized by managing distribution system operations and water quality and minimizing water age (Maier et al. 2000).

Absorption, distribution, metabolism, excretion, and pharmacokinetics

Each section is presented in relation to the route of exposure, which plays an important role in the potential effects induced by BaP exposure. Overall, it is clear that BaP is rapidly metabolized during the first few hours after oral exposure. Uno et al. (2004) analyzed the clearance of BaP in the blood of mice following gavage exposure, and showed that the levels of BaP in the blood increased until 45 min postexposure and were undetectable by 4 h postexposure. Oral BaP is known to be detoxified in the small intestine very efficiently (Uno et al. 2006, Nebert et al. 2013a), where *Cyp1a1* gene expression persists for over 30 days postexposure (Shi et al. 2010), implying that the major contributor to BaP detoxication occurs in the small intestine. While the small intestine plays a major role in detoxifying BaP, BaP distribution to distal organs following oral exposure also occurs. The literature shows that BaP is absorbed in the stomach lining upon ingestion and is absorbed into the circulatory system or into the hepatic portal vein.

Absorption

BaP is rapidly absorbed following administration by oral, inhalation, and dermal routes. The delivery vehicle (the substance in which the BaP is administered, such as the oil/fat content of the gastrointestinal tract) significantly affects BaP absorption by the oral, inhalation, and dermal routes. Based on the broad absorption profile of BaP, it is clear that major tissues and organs involved in its absorption come into direct contact with the compound. Therefore, the widespread distribution of BaP contributes to its toxicity across numerous tissues. Absorption of BaP is described in greater detail in Supplementary File A to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>.

Distribution

Available reports unequivocally confirm the distribution of BaP and its metabolites to major organs and tissues following intravenous, oral, inhalation, and dermal exposure (reviewed in Agency for Toxic Substances and Disease Registry [ATSDR] 1995). BaP is rapidly distributed to the stomach in mice exposed to BaP via oral or topical administration (Carlson et al. 1986). Oral BaP exposure in rats leads to more rapid distribution of BaP to blood plasma compared with inhalation exposure; for example, BaP concentrations in the serum peaked 1 and 8 h following oral and inhalation exposures to BaP, respectively (Ramesh et al. 2002). Lungs and liver were the major organs in which BaP metabolites were detected following oral, inhalation, and intravenous exposures of rats to BaP (Ramesh et al. 2002). The distribution of BaP to various organs following exposure, and its subsequent elimination within 24–72 h, suggests rapid metabolism and excretion of BaP and its metabolites in rodents.

The lipophilicity of absorbed BaP favors its association with lipoproteins in the blood (Busbee et al. 1984), enabling BaP to rapidly access distant organs via the systemic circulation. For example, 5 min following intravenous administration of 15 mg of ^{14}C -labeled BaP/kg-bw in rats, the liver contained

the highest amount of radioactivity, followed by lung, heart, kidney, blood, brain, spleen, testes, and adipose tissue (Moir et al. 1998). Another study (Marie et al. 2010) confirmed the distribution of BaP to various organs and tissues in rats following intravenous administration; 2 h after administration, the largest proportion of the administered BaP was found in the lungs (17.0%), followed by skin (7.6%), adipose tissue (2.5%), liver (2.3%), and kidney (0.5%). These results suggest that BaP is translocated readily to other organs via circulation, potentially leading to systemic effects.

Overall, multiple studies demonstrate that BaP is widely distributed across various organs and tissues following oral, topical, inhalation, and intravenous exposures, such that a considerable fraction of the administered dose is bioavailable. Vehicle is an important factor for BaP distribution. The broad distribution profile of BaP aids in the interpretation of the diverse health effects induced by BaP, described in detail below. Distribution of BaP is described in greater detail in Supplementary File A to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>.

Metabolism

The metabolism of BaP was systematically reviewed recently (IARC 2010) and is briefly summarized here. BaP is subject to phase I (activation) and phase II (conjugation/detoxication) xenobiotic metabolism reactions, driven by its interaction with the aryl hydrocarbon receptor (AHR).

In phase I activation, BaP is converted to an epoxide by CYP enzymes (primarily CYP1A1 and CYP1B1) and other enzymes (e.g., prostaglandin synthase) (Trush et al. 1982). BaP epoxides undergo hydration by epoxide hydrolase (EH) to form diols (Figure 2). Aldo-keto reductases (AKRs) convert BaP diols to catechols. Most notably, the BaP-7,8-dihydrodiol-AKR pathway generates BaP 7,8-catechol that autooxidizes to produce DNA-reactive BaP-7,8-quinone (Lan et al. 2004). Secondary epoxidation by

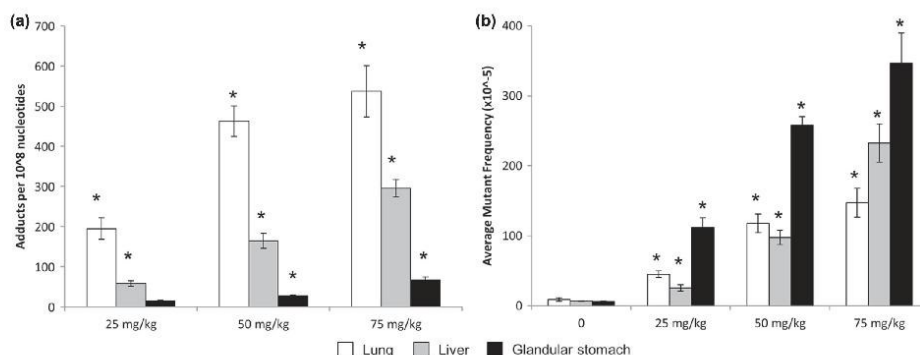


Figure 2. DNA adduct formation (a) and lacZ mutant frequency (b) in the lungs, livers, and glandular stomach from Muta™ Mouse exposed to 25, 50, and 75 mg/kg-bw per day BaP for 28 consecutive days and excised 3 days postexposure. Levels of dG-N2-BPDE adducts were determined using the nuclease P1 enrichment version of the ^{32}P -postlabeling method. Data are represented as average $\pm$ SEM ($n = 5$ mice/group). Average lacZ mutant frequency was determined using the P-Gal positive selection assay. Values shown are average frequencies $\times 10^{-5} \pm$ SEM. Asterisk (*) indicates significance ($p < 0.05$) compared with controls. Please note, no adducts were detected in mice dosed with vehicle control. All data were previously published in Lemieux et al. (2011), Malik et al. (2012) and Labib et al. (2012).

CYPs yields diol epoxides. BaP-7,8-diol-9,10-epoxide (BPDE) is the most mutagenic and well-studied BaP diol epoxide, forming covalent adducts with DNA at the N^2 position of deoxyguanosine (Fang et al. 2001). Alternatively, CYPs, as well as some chemical oxidants and peroxidases, can also catalyze one-electron oxidation of BaP to form a BaP radical cation (Cavalieri and Rogan 1992) with an electrophilic C-3 position (Figure 2). Oxygen transfer to C-3 by CYPs can give rise to 3-hydroxybenzo[a]pyrene (3-OH BaP) (Cavalieri and Rogan 1995), which is proposed as a biomarker of dermal exposure to BaP in humans (Payan et al. 2009). The primary metabolites of BaP *in vivo* as well as the main products of BaP detoxication are thought to be glucuronide conjugates of BaP (Recio and Hsie 1984) and 3-OH BaP (Saunders et al. 2006). In addition to 3-OH BaP, other BaP phenol metabolites have been reported, such as 6-hydroxybenzo[a]pyrene (6-OH BaP) (Cavalieri and Rogan 1995) and 9-hydroxybenzo[a]pyrene (9-OH BaP) (Saunders et al. 2006). Phenols such 6-OH BaP can yield autooxidation products BaP-1,6-, -3,6-, -6,12-diones (Cavalieri and Rogan 1995). BaP radical cations are genotoxic (DNA-reactive) species and form unstable adducts with purinic bases, resulting in apurinic sites (IARC 2010). Genotoxic BaP metabolites can result in mutations in proto-oncogenes, eventually leading to tumorigenesis (IARC 2010). In addition, CYPs can undergo "uncoupling" of their catalytic reactions, during which an electron "leaks out" of the substrate-bound catalytic site of an enzyme into the subcellular milieu and is accepted by molecular oxygen (O_2), creating superoxide ($O_2^{\cdot-}$) and other reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$) (Shertzer et al. 2004). ROS can also be generated during BaP catechol-quinone redox cycling (Figure 2). ROS are genotoxic and can produce mutations (primarily G to T transversions), contributing to the overall mutagenicity and carcinogenicity of BaP (Lan et al. 2004). Thus, both metabolites of BaP and ROS can interact with DNA and cause mutations following BaP exposure.

During phase II xenobiotic metabolism reactions (or detoxication), BaP metabolites are conjugated with hydrophilic moieties (glutathione, glucuronic acid, or sulfate) to enhance their solubility in water, rendering them suitable for urinary and fecal excretion (Bock and Bock-Hennig 2010, Garg et al. 2008, Meil et al. 2008). Phase II metabolism enzymes include glutathione *S*-transferases (GSTs), uridine diphosphate-glucuronosyl transferases (UGTs) and sulfo-transferases (SULTs). Detoxication of BaP is efficiently conducted in the small intestine by CYP1A1 upon oral exposure (Uno et al. 2006, Nebert et al. 2013a). It appears that tight coupling between phase I and phase II metabolism is required to reduce the detrimental effects of BaP exposure, as shown by knockout studies in mice (reviewed in Nebert and Dalton 2006). This coupling is achieved through activation of the AHR and nuclear factor (erythroid-derived 2)-like 2 (Nrf2 or Nfe2l2). These transcription factors regulate the expression of phase I and phase II metabolism enzymes, respectively, with some apparent functional redundancy (i.e., several enzymes are regulated by both factors).

The AHR is the principal transcription factor governing the activation of most phase I and some phase II enzymes and

has been studied extensively (Michaelson et al. 2011). Upon binding of BaP to the AHR, the BaP-AHR complex translocates to the nucleus of the cell, where it dimerizes with the AHR nuclear translocator protein. Once in the nucleus, the complex can then bind to the promoters of genes containing AHR response elements to regulate their expression, including phase I and phase II xenobiotic metabolizing enzymes. Thus, BaP binding to the AHR drives its own metabolism. Genetic polymorphisms in *AHR* that affect the receptor's ability to bind ligands impact the sensitivity of animals to AHR ligands, including the extent of CYP1A1 induction (reviewed in Okey et al. 2005). However, the significance of *AHR* polymorphisms in humans and their influence on AHR ligand binding is still unknown (Okey et al. 2005). In general, humans are typically less affected than mice and rats following exposure to AHR ligands, perhaps due to a lower binding affinity of human AHR to its ligands compared with rodents (Okey et al. 2005). Sequencing of the *AHR* gene of 108 people from 6 ethnic backgrounds revealed 6 single nucleotide polymorphisms primarily outside the ligand binding domain (Rowlands et al. 2010). However, it is possible that differential ligand binding affinity resulting from polymorphisms in the *AHR* gene in humans may contribute to differences in susceptibility to AHR-mediated effects of BaP among individuals, but more research is needed to clarify this hypothesis. In addition, it is conceivable that polymorphisms in other genes that belong to the AHR signaling pathway (e.g., AHR repressor [AHRR]) may also contribute to differential sensitivity to BaP across species and individuals.

Nrf2 is the master regulator of inducible gene expression of phase II enzymes (reviewed by Kaspar et al. 2009). Nrf2 is activated by various pro-oxidants (electrophiles and ROS) and is regulated by subcellular localization, such that it is kept inactive in the cytoplasm under normal homeostatic conditions by interaction with the protein Kelch-like ECH-associated protein 1 (reviewed by Mitsuishi et al. 2012). Nrf2 activation triggers its nuclear translocation and subsequent activation of Nrf2-driven gene expression. Nrf2 deficiency leads to increased formation of DNA adducts and probability of carcinogenesis, whereas increased Nrf2 activity decreases the probability of chemically induced carcinogenesis in mice (reviewed by Mitsuishi et al. 2012). Mutations in *Nrf2* and *Keap1* that affect Keap1-Nrf2 interaction have been identified in various human cancers (reviewed by Mitsuishi et al. 2012).

Finally, male and female rats appear to metabolize BaP differently. For example, significantly lower amounts of metabolites were detected in the feces and urine of females relative to males following oral exposure (Van de Wiel et al. 1993). This sex-specific difference in BaP metabolism (albeit reported only in rats, to the best of our knowledge) may contribute to the greater incidence of BaP-induced liver (Lavoie et al. 1987, Wislocki et al. 1986) and lung (Lavoie et al. 1987) tumors in male rodents. In general, the very short half-life of BaP in rats (about 1, 4, and 6 h for oral, dermal, and inhalation exposures, respectively [Carlson et al. 1986, Ramesh et al. 2002], and approximately 2–5 h in mouse blood after oral administration [Uno et al. 2006]) suggests that BaP is rapidly metabolized and excreted. However, following oral administration a small

amount of BaP escapes detoxication and induces mutations (refer to Section “Mutations”).

Excretion

In general, BaP is eliminated rapidly from the body through urinary and fecal excretion, as evidenced by the detection of its metabolites in urine and feces. BaP clearance from tissues with relatively high lipid content is prolonged (from hours to several days). Only minor traces of BaP were detectable in most tissues in intravenously exposed rats 32 h postinjection, whereas there was some lag time in the elimination of BaP from adipose tissue, brain, and testes (Moir et al. 1998). Inefficient elimination of BaP from adipose tissue could be due to poor circulation to the tissue in combination with the lipophilicity of BaP, which may result in its prolonged residence in the adipose tissue. In addition, BaP metabolites are highly retained in the brain (Saunders et al. 2003), potentially as a result of the high lipophilicity of this tissue. This may provide insight into the manifestation of BaP toxicity in distal organs and tissues, such as brain and testes. Excretion of BaP is described in greater detail in Supplementary File A to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>.

Physiologically based pharmacokinetic modeling

The currently available physiologically based pharmacokinetic (PBPK) models are not adequately developed to describe the series of animal experiments studying the pharmacokinetics of BaP. Only one PBPK model has been developed specifically for BaP (Crowell et al. 2011). This study presents a PBPK model to describe available time series data from the scientific literature on BaP in rats and mice. However,

the model fails to use appropriate physiological values. In particular, the cardiac output does not match the reference values cited by Brown et al. (1997), and the blood flow values applied were incorrect. Even after correcting for errors in blood flow values, the PBPK model predictions are not consistent with the data presented and validated in Crowell et al. (2011). Extrapolation of a PBPK model for the pharmacokinetics of pyrene (Haddad et al. 1998) to BaP yielded inconsistent results. Heredia-Ortiz et al. (2011) presented an alternative toxicokinetic compartmental model to describe the pharmacokinetics of BaP in rats. While the model is consistent with experimental data on rats, the model uses rate constants instead of physiological parameters. These rate constants are consistent with the fitted data only and cannot be extrapolated to other species, such as humans and mice, or to dose conditions beyond those observed in the study. Thus, the available PBPK models are not adequately developed to describe the pharmacokinetics of BaP.

Human health effects

Overall, 74 epidemiological studies of chronic BaP-containing complex mixtures were reviewed in detail (Supplementary Table 2 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>). These include primarily studies on inhalation of BaP-containing complex mixtures in occupational settings and ingestion of BaP-containing complex mixtures from charred and burnt meats. Twenty studies (summarized in Table 2) were selected on the basis of having reported specific exposure concentrations as well as various other parameters that indicated general study quality (e.g., control for other risk factors and/or confounders, sufficient population size, and appropriate data collection protocols). Many potentially adverse health effects of BaP exposure at doses

Table 2. Human health effects potentially associated with benzo[a]pyrene as a component of a complex mixture exposure.

Exposure	LOAEL*	Duration	Response	References
Air	136.6 mg/m ³ ·year	Chronic	Bladder cancer	Armstrong et al. (1986)
	100 µg/m ³ ·year	Chronic	Lung cancer	Armstrong and Thériault (1996)
	35.60 µg/100 m ³	Chronic	Lung cancer mortality	He et al. (1991)
	20 µg/m ³ ·year	Chronic	Lung and bladder cancer	Gibbs and Sevigny (2007)
	0.85 µg/m ³ ·year	Chronic	Lung and stomach cancer	Xu et al. (1996)
	80 µg/m ³ ·year	Chronic	Bladder and stomach cancer	Spinelli et al. (2006)
	273 ng/m ³	Chronic	Ischemic heart disease mortality	Burstyn et al. (2005)
	7.79 µg/m ³ ·year	Chronic	Ischemic heart disease mortality	Friesen et al. (2010)
	78.21 ng/m ³	Chronic	Immunological alterations	Jeng et al. (2011)
	19.5 ng/m ³	Chronic	Neurological function	Niu et al. (2010)
	0.1 µg/m ³	Chronic	Serum TP53	Pan et al. (1998)
	0.05 µg/m ³	Chronic	DNA adducts in white blood cells	Perera et al. (1988)
	42.2 µg/100 g	Chronic	Lung cancer	De Stefani et al. (2009)
	10.4 ng/d	Chronic	Pancreatic cancer	Anderson et al. (2005)
Oral	33.7 ng/d	Chronic	Colon cancer	Butler et al. (2003)
	99.0 ng/d	Chronic	Esophageal cancer	Hakami et al. (2008)
	0.005 µM	Acute	Cell cycle, growth, and apoptosis pathways (correlated with S & G ₂ /M phase cell cycle arrest and cytotoxicity) in human amniotic epithelial cells	Lu et al. (2009)
<i>In vitro</i>	2.5 and 3.0 µM	Acute	Cellular response to xenobiotic exposure and metabolism in human hepatic cell line	Hockley et al. (2006)
	0.017 µM	Acute	Stress response and DNA repair response in human lymphoblastoid cell line	Luo et al. (2005)
Genomics	4 µM	Acute	CYP1B1 expression in normal mammary epithelial cells	Gwinn et al. (2005)

*Estimated values.

that varied considerably between studies were found. Cancer was the most common outcome and was associated with BaP doses as low as 850 ng/m³-year (i.e., for lung and stomach cancer) and 10.4 ng/day (i.e., for pancreatic cancer) for chronic inhalation and oral exposure, respectively. The most sensitive of the endpoints was neurological function; exposure to BaP via inhalation at 19.5 ng/m³ led to a general decrease in learning, memory, and neurotransmitter levels. The most sensitive effect that was correlated with oral exposures was pancreatic cancer (i.e., dose of 10.4 ng/day), as measured by daily meat intake and preparation methods. Thus, there is sufficient evidence that exposure to complex mixtures containing BaP is associated with excess cancer risk in humans. However, due to co-exposure to chemicals other than BaP, as well as the variability in air concentrations and oral doses of BaP at which these effects occur, route of administration (exposure), and subsequent target organ affected, it is not possible to conduct dose-response analysis to determine acceptable exposure levels for risk assessment in humans. In addition, genetic cancer risk prediction in individuals exposed to an environmental toxicant is extremely difficult and defined as a multifactorial trait (Nebert et al. 2013b). For these reasons, our risk assessment of BaP relied more heavily on animal data at the population level.

Effects on experimental animals, mechanisms, and their human relevance

Effects observed in experimental animals were compared to epidemiological data of BaP containing complex mixtures to establish potential relevance to human adverse health outcomes. All studies that were evaluated are presented in Supplementary Table 3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>, and a very brief overview is given below for each toxicity endpoint. Table 3 provides a summary of the effects of BaP on experimental animals that occurred at the lowest

doses. A detailed description of each toxicity endpoint was not possible within this document, but we refer the reader to the supplementary materials (Supplementary Table 3 and Supplementary File B to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>) for further toxicity and mechanistic information. Given the extensive and well-documented evidence demonstrating the link between BaP exposure and genotoxicity/carcinogenicity, we have developed these sections in more detail. Lowest-observed-adverse-effect levels (LOAELs) and no-observed-adverse-effect levels (NOAELs) are presented when available.

Reproductive toxicity

Twenty-seven studies of reproductive toxicity were found (Supplementary Table 3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>). Those pertaining to carbon black-bound BaP (i.e., inhalation studies) were excluded due to the confounding effects of particle inhalation. These papers demonstrate that adverse reproductive effects of BaP, including alterations in gonadal tissues and steroid hormone levels and reduced fertility, are observed in both male and female rodents exposed to BaP as adults or *in utero*. Doses as low as 1 mg/kg-bw per day for 90-day exposures (Zheng et al. 2010) were effective in perturbing reproductive parameters. Major effects include impaired fertility in exposed adults (Arafa et al. 2009, Chen et al. 2011, Xu et al. 2010, Zheng et al. 2010) and developing rodents (Kristensen et al. 1995, Mackenzie and Angevine 1981, Nakamura et al. 2012), which appears to be mediated by an AHR-dependent MOA (Neal et al. 2010, Sadeu and Foster 2011). Overall, it is apparent that BaP affects the reproductive fitness of exposed organisms by impacting germ cell quality and quantity, hormone balance, and gonadal tissue growth and development at exposure doses as low as 1 mg/kg-bw per day in rodents (LOAEL).

Table 3. Summary of the animal studies used to derive PODs for each of the toxicities reviewed in RA1.

Toxic effect	Oral exposure duration (vehicle)	Species	NOAEL/LOAEL (mg/kg-bw per day)	Calculated BMD/BMDL [#] (mg/kg-bw per day)	References
Reproductive toxicity	Subchronic (corn oil)	Male rat	NA/1	No dose-response	Zheng et al. (2010)
Neuro-developmental toxicity	Acute (peanut oil)	Male and female rat	0.02/0.2	0.09/0.05 (0.02) [†]	Chen et al. (2012)
Hepatotoxicity	Subchronic (soybean oil)	Male and female rat	NA/3	No dose-response	Wester et al. (2012)
Renal toxicity	Subchronic (peanut oil)	Male rat	5/50	Data not available	Knuckles et al. (2001)
Cardiovascular toxicity	Chronic (olive oil)	Male mouse	NA/0.36 [‡]	No dose-response	Yang et al. (2009)
Immunotoxicity	Subchronic (soybean oil)	Male rat	3/10	14/8.9 (4.8) [†]	De Jong et al. (1999)
Liver mutations	Subchronic (olive oil)	Male mouse	NA/25	2.2/1.4	Lemieux et al. (2011)
Lung mutations	Subchronic (olive oil)	Male mouse	NA/25	7.2/4.8	Lemieux et al. (2011)
Forestomach mutations	Subchronic (olive oil)	Male mouse	NA/25	0.5/0.3	Lemieux et al. (2011)
Liver tumors	Chronic (soybean oil)	Male and female rat	2.1/7.1*	3.3/2.4 (1.2) ^{†,*}	Wester et al. (2012)
Forestomach tumors	Chronic (soybean oil)	Male and female rat	NA/2.1*	1.5/0.8 (1.1) ^{†,*}	Wester et al. (2012)
Forestomach tumors	Chronic (diet)	Female mouse	0/0.65	0.8/0.5	Culp et al. (1998)

NA, not available.

*Dose adjustment for time (dose × 5/7 dosing days) before modeling.

[†]For comparison of rat and mouse BMDLs, scaled from rat to mouse by multiplying rat values by (0.03/0.35)^{1/4} based on the assumption that the physiological processes scale with body weight to the 3/4 power (allometric scaling).

[#]Benchmark response: BMD₁₀/BMDL₁₀ for quantal data (tumor) and BMD_{1SD}/BMDL_{1SD} continuous data (Neurotoxicity, immunotoxicity and genotoxicity). Refer to supplemental Table 4 for BMD model fit data.

[‡]Scaled from 2.5 mg/kg per week.

There has been only one study that has identified an effect on human reproductive toxicity (Tang et al. 2006); thus, human relevance cannot be clearly established at this time.

Developmental toxicity

Twenty studies pertaining to developmental toxicity effects of BaP were found (Supplementary Table 3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>), all of which reported developmental effects. The studies indicate that there are several detrimental effects of gestational exposure to BaP on the developing fetus and newborns, including decreased fetal viability (Bui et al. 1986, Shum et al. 1979, Wu et al. 2003), reduced birth weight (Mackenzie and Angevine 1981, Perera et al. 2004), increased predisposition to cancer (Holladay 1999, Turusov et al. 1990, Urso and Gengozian 1982, 1984; reviewed by US EPA 2007), and neurodevelopmental effects (Chen et al. 2012). The mechanisms leading to developmental toxicity appear to be governed by both the AHR and genotoxicity (Bolognesi et al. 1985, Lu et al. 1986, Shugart and Matsunami 1985). The most sensitive developmental endpoints are neurodevelopmental, with effects occurring at BaP doses as low as 0.2 mg/kg-bw per day (LOAEL) and no effects occurring at 0.02 mg/kg-bw per day (NOAEL). The use of high doses or single doses in many of these studies impaired our ability to evaluate developmental toxicity.

Human relevance for developmental toxicity is limited. There is some evidence that co-exposure to BaP causes developmental neurotoxicity in children (reduced IQ and cognitive development; Perera et al. 2006, 2008).

Renal and hepatic toxicity

Three studies pertaining to non-cancer effects in the liver and kidney following BaP exposure were found that document increased liver to body weight ratios (De Jong et al. 1999, Knuckles et al. 2001, Wester et al. 2012) and increased tubular casts (Knuckles et al. 2001) in rodents exposed to BaP. The most sensitive endpoint is increased kidney tubular casts, occurring at doses as low as 50 mg/kg-bw per day (NOAEL 5 mg/kg-bw per day).

No studies were identified that could offer information on the mechanisms underlying these effects, nor were there any human data to suggest that these findings are relevant to humans.

Cardiovascular toxicity

Several studies have demonstrated that BaP exposure can lead to cardiovascular toxicity. Of the 23 studies on cardiovascular toxicity reviewed (Supplementary Table 3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>), those that are relevant for dose–response evaluation demonstrate an increase in atherosclerosis (Knaapen et al. 2007, Yang et al. 2009), an increase in heart to body weight ratios and cardiotoxicity biomarkers (Aboutabl et al. 2009, 2011) and impaired cardiovascular function later in life (Jules et al. 2012) following BaP exposure. Mechanisms in cardiovascular toxicity may be related to AHR-mediated responses (Aboutabl et al. 2009, 2011,

Kerley-Hamilton et al. 2012, N'Diaye et al. 2006, 2009, Oesterling et al. 2008, Owens et al. 2009, Podechard et al. 2009). The most sensitive cardiovascular endpoint (atherosclerosis) occurred at doses as low as 2.5 mg/kg-bw per week (LOAEL; 0.36 mg/kg per day, if scaled from once per week to daily exposure).

We found two epidemiological studies to suggest some human relevance of this toxicity: (1) asphalt workers showed a significant positive correlation between BaP exposure and ischemic heart disease (Burstyn et al. 2005); and (2) aluminum smelter workers showed a modest association between BaP exposure and myocardial infarction (Friesen et al. 2010).

Neurotoxicity

We identified a total of 12 reports on BaP-induced neurotoxicity in adult animals. These studies demonstrate that BaP exposure can cause very specific behavioral alterations and span various adversities, including impaired spatial learning and memory (Chen et al. 2012, Grova et al. 2007, Qiu et al. 2011, Xia et al. 2011) and other types of behavioral change (Bouayed et al. 2012, Grova et al. 2008, Saunders et al. 2006). In addition, studies indicate that BaP exposure may also cause neurodevelopmental toxicity (Chen et al. 2012) (Section B5, Supplementary File B to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>). The studies demonstrate that BaP exposure can cause alterations in neurobehavior at doses as low as 0.02 mg/kg-bw per day (LOAEL). The mechanism behind these alterations appears to be associated with changes in *N*-methyl-D-aspartate glutamate receptor (NMDAR) subunit levels.

Human relevance is supported by several studies showing various neurobehavioral effects in humans exposed to BaP mixtures occupationally (Niu et al. 2010) or *in utero* from maternal exposure (e.g., Perera et al. 2004).

Immunotoxicity

The immunosuppressive properties of BaP are well established (reviewed in Holladay 1999). In total, 35 studies of BaP-induced immunotoxicity were found in our literature survey (Supplementary Table 3 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>). Effects of BaP exposure include reduced antibody production (Dean et al. 1983, Urso and Gengozian 1984, White and Holsapple 1984), diminished lymphocyte response (Urso and Gengozian 1984, Wojdani and Alfred 1984) and various other immunotoxicities (De Jong et al. 1999, Fischer et al. 2011). The study by De Jong et al. (1999) was considered to be the most relevant for a detailed evaluation and inclusion in risk assessment because it (1) applied methods from an Organisation for Economic Co-operation and Development (OECD) guideline protocol (No. 407), (2) assayed several well-established markers of immunotoxicity, and (3) employed a wide range of BaP concentrations. These authors demonstrated that BaP-induced immunotoxicity occurs at doses as low as 10 mg/kg-bw per day (LOAEL). BaP-induced immunosuppression is hypothesized to provide a favorable environment for BaP-induced tumors (Urso and Gengozian 1984). Current

clinical immunotherapy aimed at overcoming the immunosuppression that is associated with tumors (Mellman et al. 2011) suggests that weakening the immune system (decreased immunosurveillance) by chemical exposure to AHR agonists favors tumor formation and survival (Ridolfi et al. 2010).

In support of BaP-induced immunotoxicity occurring in humans, Davila et al. (1996) showed that BaP co-exposures affect human peripheral blood T cell mitogenesis, and Allan et al. (2006) showed that BaP exposure inhibits B cell growth.

Genotoxicity

It is well established that BaP is metabolized to reactive metabolites (Section "Metabolism") that are capable of binding to DNA. These DNA adducts can lead to the formation of mutations if they are not properly repaired, and these mutations may subsequently initiate carcinogenesis if they occur in genes that are involved in cancer pathways. A previous detailed review by IARC summarizes the evidence demonstrating that BaP induces DNA adducts, leading to genotoxicity and mutagenicity, as measured in various bacterial and eukaryotic bioassays (including human) *in vivo* and *in vitro* (IARC 2010).

DNA adducts. The 2010 IARC monograph reviews all of the *in vitro* and *in vivo* cases of BaP–DNA adducts in cultured cells exposed to BaP, mouse skin after topical treatment, and internal organs and blood lymphocytes following oral, inhalation, and intraperitoneal administration of BaP (IARC 2010). Of all the *in vivo* studies reviewed, a study by Garner et al. (1985) had the lowest dose administered that caused adducts. In this study, male Wistar rats were given a single dose of BaP (2 mg/kg-bw), and DNA adducts were measured in lungs and liver (Garner et al. 1985) (LOAEL 2 mg/kg-bw).

Mutations. Various studies have demonstrated that BaP induces mutations in proto-oncogenes and tumor suppressor genes. For example, BaP induced three types of Ki-ras codon 12 mutations in lung adenomas in A/J mice: GGT→TGT (56.3%), GGT→GTT (25%), and GGT→GAT (19%) (Mass et al. 1993). This mutation spectrum confirms that deoxyguanosine is a primary target for active metabolites of BaP. BaP also induced codon 13 (DiGiovanni et al. 1993) and codon 61 (Chakravarti et al. 1995) mutations in c-Ha-ras in Sencar mouse skin papillomas. BaP caused mutations in murine embryonic fibroblasts from human TP53 knock-in (Hupki) mice that were similar to those found in smoking-related lung cancers in humans (Liu et al. 2005). In our own experiments, male MutaTM Mouse mice exposed to BaP at 25, 50, or 75 mg/kg-bw per day for 28 days and sacrificed 3 days after the final exposure showed increased lacZ transgene mutant frequencies in the lungs (Labib et al. 2012), liver, glandular stomach, small intestine, and bone marrow (Lemieux et al. 2011) at all doses (Figure 2; LOAEL 25 mg/kg-bw per day). A dose-dependent increase in mutant Pig-a phenotypes was also observed in these mice. A meta-analysis of published *in vivo* dose–response genotoxicity studies used

the BMD approach to calculate the dose representing a specified 10% change in effect in exposed animals versus controls (BMD₁₀) (Hernandez et al. 2011). BMDs represent a more appropriate and advanced measure than NOAELs (described in Section "Selection of the point of departure for risk assessment 1 (RA1)"). The meta-analysis did not provide starting values; as such, no NOAEL or LOAEL could be determined. However, a BMD₁₀ of 1.52 mg/kg-bw per day was observed for mutations (Hernandez et al. 2011).

Micronuclei. Shimada et al. (1990) treated male CD-1 mice orally once, twice or three times with BaP at 0, 250, 500, 1000, or 2000 mg/kg-bw per day at 24-h intervals with a posttreatment sampling time of 24 h. These mice had a significant increase in micronucleus frequency at all doses. In our own work, male MutaTM Mouse mice exposed to BaP at 25, 50, or 75 mg/kg-bw per day for 28 days by oral gavage and sacrificed 3 days after the final exposure also had significant increases in the frequency of micronucleated reticulocytes and normochromatic erythrocytes at all doses (Lemieux et al. 2011) (LOAEL 25 mg/kg-bw per day). A meta-analysis of historical *in vivo* genotoxicity studies using the BMD approach found a BMD_{1SD} of 1.28 mg/kg-bw per day for the dose–response relationship between BaP dose and micronucleus frequency in blood (Hernandez et al. 2011). The meta-analysis did not provide starting values; as such, no NOAEL or LOAEL could be determined.

Other genotoxicity endpoints. In addition to the genotoxicity endpoints described above, BaP also induces single- and double-strand DNA breaks, sister chromatid exchanges and chromosomal aberrations (ATSDR 1995). For example, male DBA/2 mice given two BaP doses of either 10 or 100 mg/kg-bw per day intragastrically experienced significant increases in the frequency of sister chromatid exchanges in bone marrow cells at both doses, whereas male C57BL/6J mice exhibited only a small effect at 100 mg/kg-bw per day (Wielgosz et al. 1991). A single exposure of adult Brown Norway rats to BaP at 62.5 mg/kg-bw intragastrically did not cause the induction of unscheduled DNA synthesis (Mullaart et al. 1989). Intestinal cells from these adult Brown Norway rats had a significant increase in DNA single-strand breaks relative to controls at the 62.5 mg/kg-bw (LOAEL) dose (Mullaart et al. 1989). Thus, the lowest LOAEL established for these genotoxicity endpoints is 10 mg/kg-bw per day (Wielgosz et al. 1991), and a NOAEL was not achieved.

Mechanisms. BaP is biologically inert and requires metabolic activation to exert its genotoxicity. The potential reactive metabolites of BaP and the enzymes involved in producing them are described in detail in Section "Metabolism". Diol epoxides have the ability to form a carbonium ion, which has a high affinity for reacting with DNA and proteins (nucleophiles) in the form of covalent adducts. Owing to their stereoselectivity, the BaP diol epoxides can react with DNA to form *cis* or *trans* adducts, primarily with deoxyguanosine and, to a lesser extent, deoxyadenosine. BaP diol epoxides have the ability to intercalate into DNA and thus

perturb the structure of the DNA at the site of binding (Volk et al. 2003). Improper DNA repair can lead to mutations. While double-strand breaks are not generated directly by BaP diol epoxides, they may be produced as by-products of DNA repair (Zhou and Shephard 2006); bulky DNA adducts are repaired by nucleotide excision repair (Geacintov et al. 2002). Finally, error-prone polymerases such as DNA polymerase kappa (Polκ) can bypass bulky DNA adducts, which can cause mutations during DNA replication.

Human relevance. A large amount of data demonstrates that exposure to BaP in various media can result in DNA adducts in humans. Furthermore, evaluation of genotoxicity in human cells in vitro and in tissue slices demonstrates that genotoxicity is relevant to human health. BPDE–DNA adducts have been detected in human lung samples from smokers and nonsmokers (Lodovici et al. 1998), in lymphocytes from coke oven workers (Rojas et al. 1995) and cigarette smokers (Rojas et al. 1995) and in human mammary epithelial cells exposed to BaP (Moore et al. 1987). BPDE–DNA adducts in human cells in culture are well documented to cause genotoxicity and mutagenicity (ATSDR 1995). A recent study using human skin ex vivo and in vitro models showed that DNA-reactive BaP metabolites were generated in these models following exposure to BaP at 50 nmol/cm² (Brinkmann et al. 2013). Furthermore, these metabolites caused DNA strand breaks in human cultured keratinocytes (NHEK cells) and in human dermal fibroblasts exposed to 3.5 µg BaP. At concentrations of 0.5–50 µM, BaP induced dose-dependent increases in DNA strand breaks (measured using the comet assay) and micronucleus formation in human intestinal Caco-2 cells (Le Hégarat et al. 2012) as well as in human hepatoma HepaRG cells (Le Hégarat et al. 2012). Human liver slices (ex vivo, in vitro) exposed to 10–100 µM BaP for 24 h showed a concentration-dependent increase in DNA strand breaks (comet assay) at all of the concentrations tested (Plazar et al. 2007).

Summary. In summary, a large amount of evidence supports the induction of genotoxicity in human cells exposed to BaP. A review of all of the literature on the genotoxicity of BaP in vivo in rodent models revealed that genotoxicity (specifically DNA adducts and mutations) can occur at doses as low as 2 mg/kg-bw.

Carcinogenicity

BaP is classified as carcinogenic to humans (Group 1 carcinogen; IARC 2012). This classification is based on strong and extensive experimental evidence for the carcinogenicity of BaP in multiple mammalian species, supported by consistent and coherent mechanistic evidence from experimental and human studies that provide biological plausibility. Several routes of exposure, including dermal, oral, and inhalation, lead to tumorigenesis. Below, we describe the data for specific tumor sites.

Skin tumors. Dermal application of BaP to different strains of mice results in benign and malignant skin tumors (mainly squamous cell carcinomas) (reviewed in IARC 2012) at doses as low as 2 µg BaP (LOAEL) per animal twice a week for

63–109 weeks (Habs et al. 1984). The lowest dose at which dermal tumors were observed was in a study of C3H/HeJ mice. Shaved dorsal skin of mice exposed to 0.5 and 5.0 µg in cyclohexane/acetone twice per week developed skin tumors at rates of 20% (5/30) and 90% (27/30), respectively (Sivak et al. 1997).

Respiratory tract tumors. Respiratory tract tumors are formed following both inhalation and oral administration of BaP. For example, the incidence of respiratory tract tumors was measured in male hamsters administered BaP via inhalation at 0, 2.2, 9.5, or 46.5 mg/m³ for 4.5 h per day for 10 weeks and then for 3 h per day for the rest of their lifetimes (total average doses of 0, 29, 127, and 383 mg BaP per animal) (Thyssen et al. 1981). Hamsters treated with the two highest doses showed an increased incidence of papillomas and squamous cell carcinomas in the upper respiratory tract when exposed to as little as 9.5 mg/m³. Female A/J mice fed a diet containing 0, 16, or 98 parts per million (ppm) BaP (total dose 0, 11, and 67 mg) for 260 days developed lung tumors at the highest dose (Weyand et al. 1995). In another study, female B6C3F1 mice fed a diet containing coal tar had an estimated NOAEL/LOAEL for BaP exposure of 0.3 and 0.8 mg/kg-bw per day, respectively (Culp et al. 1998).

Digestive tract tumors. The most sensitive site for tumor formation following oral exposure to BaP is the digestive tract. In a 2-year study, female B6C3F1 mice fed a diet containing 0, 5, 25, or 100 ppm BaP (calculated intake of 0, 0.65, 3.5, or 15.2 mg/kg-bw per day) developed forestomach tumors at the middle and high doses and esophageal tumors at the high dose (Culp et al. 1998) (Table 4). In another study, male and female Wistar rats administered BaP at doses of 0, 3, 10, or 30 mg/kg-bw per day by gavage for 5 days per week for 104 weeks developed forestomach tumors at all doses in males and at the top two doses in females (Wester et al. 2012) (Table 4). Additional studies exist that support BaP-induced forestomach tumors, however, their design limit their use for POD determination. First, male and female CFW mice fed a diet containing BaP at doses up to 32.5 mg/kg-bw per day developed papillomas and squamous cell carcinomas in the forestomach at 5.85 mg/kg-bw per day and higher exposures (Neal and Rigdon 1967). Further, female A/J mice fed a diet containing 0, 16, or 98 ppm BaP (total dose 0, 11, and 67 mg) for 260 days developed forestomach tumors at all exposure doses (Weyand et al. 1995). However, mice were exposed for less than 2 years (not optimal for extrapolating lifetime risk) and the A/J strain is not common for cancer assessment and is prone to spontaneous lung adenomas. In addition, male hamsters exposed to total average BaP doses of 0, 29, 127, or 383 mg per animal via inhalation (exposure to 0, 2.2, 9.5, or 46.5 mg/m³ for 4.5 h per day for 10 weeks, and then 3 h per day for the rest of their lifetimes) (Thyssen et al. 1981) showed an increased incidence of papillomas and squamous cell carcinomas in the upper digestive tract at the two highest doses.

Liver tumors. Exposure of male and female Wistar rats to BaP by gavage at a dose of 0, 3, 10, or 30 mg/kg-bw per day for 5 days per week for 104 weeks caused tumors in the livers of

Table 4. Organs in which benzo[a]pyrene significantly (at least two doses) and dose-dependently increased tumor incidence (number of tumor-bearing animals/number of total animals is presented).

Study	Species, sex	BaP dose			Forestomach			Liver	
		Diet (ppm)	Gavage (mg/kg-bw per day)	Daily intake (mg/kg-bw per day)	Papillomas and/or carcinomas	Papillomas	Carcinomas	Adenomas and/or carcinomas	Adenomas Carcinomas
Culp et al. (1998)	Female mice	0		0*	1/48				
		5		0.65*	3/47				
		25		3.5*	36/46‡				
		100		15.2*	46/47‡				
Wester et al. (2012)	Male rats		0	0‡	0/52	0/52	0/52	0/52	0/52
			3	2.14‡	8/52‡	7/52‡	1/52	4/52	3/52
			10	7.14‡	43/52‡	18/52‡	25/52‡	38/52‡	15/52‡
			30	21.43‡	52/52‡	17/52‡	35/52‡	49/52‡	4/52
	Female rats		0	0‡	1/52	1/52	0/52	0/52	0/52
			3	2.14‡	6/51	3/51	3/51	2/52	0/52
			10	7.14‡	30/51‡	20/51‡	10/51‡	39/52‡	7/52‡
			30	21.43‡	50/52‡	25/52‡	25/52‡	51/52‡	1/52

*Calculated daily intake, corrected for food consumption (Cal/EPA (2010) calculation based on food consumption and body weight).

‡Reported by Schneider et al. (2002).

‡Significantly different from control ($p < 0.05$) as reported in Benford et al. (2010) or in Cal/EPA (2010).§Administered dose of 5 days per week was adjusted to 7 days per week daily dose (dose $\times$ 5/7).

both males and females at the middle and high doses (Wester et al. 2012) (Table 4).

Other tumors. Rats gavaged with 50 μ mol BaP once per week for 8 weeks exhibited increased numbers of mammary gland adenocarcinomas (El-Bayoumy et al. 1995). Female B6C3F1 mice fed a diet providing BaP (0, 0.65, 3.25, or 13 mg/kg-bw per day) for 2 years developed tumors in the tongue and larynx at the high dose (Culp et al. 1998).

Mechanistic information. Well-established mode of action for BaP-induced carcinogenicity is genotoxicity, as described in detail in Section “Genotoxicity”. Unrepaired adducts can lead to mutations, increasing a cell’s susceptibility to carcinogenic transformation.

BaP causes tumorigenesis in tissues directly at the site of contact, as well as in those removed from the site of contact. The most sensitive reported site of tumorigenesis in rodents is the forestomach, with a LOAEL of 3.5 mg/kg-bw per day for chronic exposure (Culp et al. 1998). *In vitro* human cell culture models indicate that human cells are capable of metabolically transforming BaP to reactive metabolites causing genotoxicity (Section “Genotoxicity”). Further, epidemiological work supports the ability for BaP to cause cancer in humans (Section “Human health effects”). The carcinogenicity of BaP may be further modulated by AHR-driven immunosuppression (Section “Immunotoxicity”), which may contribute to tissue specificity.

Selection of the point of departure for risk assessment 1 (RA1)

Only toxicological outcomes with sufficient information to derive probable adverse health outcomes that are applicable to humans (i.e., biologically relevant for humans) were considered. It is advantageous to use BMD modeling in place of the NOAEL and LOAEL approach, as the entire dataset is employed in deriving the POD. By using this approach, our results do not solely rely on the specific doses selected within

each study, and confidence limits can also be established. Moreover, BMDs are necessary in deriving cancer slope factors for genotoxic substances such as BaP and are thus critical to the risk assessment of such chemicals. The NOAEL/LOAEL values, on the other hand, are used for non-cancer effects or non-genotoxic carcinogens and thus cannot be applied to BaP. The US EPA (2012) recommends an extra risk of 10% in the benchmark response for standard reporting of quantal data, to serve as a basis for comparisons across chemicals and endpoints, since the 10% response is near the limit of sensitivity in most cancer and some non-cancer bioassays. For continuous data, the US EPA (2012) recommends that the BMD (and BMDL) corresponding to a change in the mean response equal to one standard deviation from the control mean always be presented for comparison purposes. This value would serve as a standardized basis for comparison, akin to the BMD corresponding to 10% extra risk for quantal data. Thus, BMD modeling was employed when possible (i.e., when a sufficient number of doses were tested, when significance of the effect was established at least at the higher doses and when at least one of the BMD models was an appropriate representation of the data trend) (Table 3). The toxicological outcomes for lungs, liver, and forestomach with the lowest BMDL, and thus the most sensitive endpoint, were selected as the PODs for comparison to RA2 and RA3.

Calculation of BMD/BMDL

Benchmark responses were BMD/BMDL₁₀ for quantal data and BMD/BMDL_{1SD} for continuous data. BMDs and BMDLs (the estimated 95% lower-bound confidence limit on the BMD) were calculated for apical endpoint data using the US EPA’s Benchmark Dose Software (BMDS) version 2.2 (Davis et al. 2011). Only datasets with at least two doses that were statistically significant compared with control levels ($p < 0.05$) were modeled. Prior to modeling, the data were screened for homogeneity of variance. Continuous endpoints (neurotoxicity, immunotoxicity, and genotoxicity) were fit against five dose-response models (Hill,

polynomial, linear, power, and exponential), whereas tumor endpoints were fit against nine dichotomous dose–response models (gamma, logistic, log-logistic, log-probit, multi-stage, multistage-cancer, probit, Weibull, quantal-linear). For continuous transcriptomics data, the Hill model was considered only when the k parameter was more than one third of the lowest positive dose, in order not to artificially minimize BMDs and BMDLs (Black et al. 2012). Curves were visually inspected for a good fit to the data, a goodness of fit > 0.05 , and scaled residuals within ± 2.0 . The lowest Akaike's Information Criterion (AIC) was applied as a cut-off for selection of the appropriate model when more than one model was suitable and the BMDs for these were within 3-fold of each other. In all other instances, the lowest BMD was selected. BMDLs that could be calculated for key effects in experimental animals are presented in Table 3 (refer to Supplementary Table 4 to be found online at <http://informa.healthcare.com/doi/abs/10.3109/10408444.2014.973934> for BMD model fit parameters).

Selection of the key effect with the lowest biologically relevant BMD/BMDL

The estimated human ingestion of BaP through drinking water was calculated to be 0.000857–19.6 ng/kg-bw per day (Section “Use, identity, and sources of BaP exposure”). BaP doses at the high end of this range and above could possibly cause carcinogenicity at multiple tissue sites in humans (Table 2 and Supplementary Table 2 to be found online at <http://informa.healthcare.com/doi/abs/10.3109/10408444.2014.973934>). However, the variability in the estimation of the oral doses of BaP at which these effects can occur makes it difficult to draw firm conclusions regarding acceptable exposure levels for risk assessment. More reliable dose–response data come from studies of laboratory animals.

Tumors in rodent liver and forestomach occur at BMDLs as low as 0.5–1.2 mg/kg-bw per day (Table 3; Section “Effects on experimental animals, mechanisms, and their human relevance”). Even lower doses of BaP are reported to induce neurotoxic effects in developing and adult rodents, with a BMDL as low as 0.05 mg/kg-bw per day, based on diminished learning and memory in BaP-treated rats (Table 3). If the rodent data are scaled to humans using allometric scaling, one would get a human equivalent of 0.076 and 0.013 mg/kg-bw per day for cancer and neurotoxic endpoints, respectively. These two values are approximately 4000 and 700 times greater than the maximal human exposure of 19.6 ng/kg-bw per day. Despite some emerging insights into the neurotoxic MOA of BaP (Chepelev et al. 2014 [manuscript in preparation]), the literature database of the neurotoxic effects of BaP is not as comprehensive as that for carcinogenicity. Therefore, BaP-induced carcinogenicity was selected as the endpoint of concern for POD selection, based on:

- evidence of tumors in humans (Section “Human health effects”);
- strong and extensive evidence of tumor induction in rodents (Section “Carcinogenicity”);
- consistent and coherent mechanistic evidence from rodent and human studies of a genotoxic MOA in cancer (Sections “Metabolism” and “Genotoxicity”); and

- the classification of BaP as “Group 1: Carcinogenic to humans” (IARC 2012).

Rodent forestomach tumors were the carcinogenic endpoint with the lowest BMDL (0.5 mg/kg-bw per day) (Culp et al. 1998). However, as humans do not have forestomachs, the application of rodent forestomach tumor data for predicting cancer risk in humans for a particular chemical should meet specific criteria, including a clear carcinogenic MOA and induction of tumors at multiple sites and in various species (Proctor et al. 2007). BaP meets these criteria, because: (1) BaP acts through a genotoxic MOA and causes tumors at multiple sites by oral exposures in various species and in both sexes; and (2) the MOA of BaP in carcinogenesis is generally understood and considered applicable to humans (Proctor et al. 2007). Thus, the specific criteria outlined by Proctor et al. (2007) are satisfied, and it is appropriate to use BaP-induced forestomach tumor data to predict cancer risk in humans.

Therefore, a BMDL of 0.5 mg/kg-bw per day for rodent forestomach tumors was selected as the POD most relevant for human health risk assessment. Tissue-specific PODs of 1.2 and 0.8 mg/kg-bw per day were also calculated for liver (Wester et al. 2012) and lung (Culp et al. 1998) tumors, respectively.

Genomics approaches (RA2 and RA3)

The aim of this project is to determine if genomics can be helpful in a quantitative risk assessment framework. In RA2 and RA3, we investigate the utility of toxicogenomics for various components of risk assessment, including: (1) hazard identification; (2) dose–response characterization; (3) development of an MOA; (4) supporting human relevance; and (5) deriving potential PODs. In order to accomplish these goals, we employed toxicogenomics data produced in-house, but we note that data available in public repositories could easily serve the same purpose. The methods and data for the toxicogenomics experiments used in the present study are described below.

Overview of toxicogenomics data and analyses used in RA2 and RA3

Functional annotation

Expression profiles were analyzed using Ingenuity® Pathway Analysis (IPA) to determine which functional pathways and processes were enriched within the observed transcriptional changes. Transcriptional BMDs were calculated (as described below in Section “Calculation of transcriptional PODs”) and consolidated with IPA pathways.

Microarray analysis

We analyzed global transcriptional changes in response to BaP exposures over time and across doses in a variety of mouse strains and tissues and in a human cell culture model. All of the data for these studies are publicly available and comply with the minimal information requirements for a microarray experiment (Brazma et al. 2001). All gene expression datasets have been uploaded to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under the following accession numbers: GSE4363, GSE24751, GSE18789, GSE24910, GSE35718, and GSE43438.

Animal data. We relied primarily on data that have recently been produced by coauthors of this manuscript. The experiments were conducted on either whole tissue homogenates or random slices from tissues, and were not selected for any particular cell type. Experimental details from three experiments conducted at different times are described briefly below:

Experiment 1—In the first experiment, 5-week-old male B6C3F1 mice received BaP in corn oil by oral gavage at a concentration of 0 (corn oil alone), 5, 50, 150, or 300 mg/kg-bw per day for 3 consecutive days ($n = 5$ per dose group). Samples were collected 4 or 24 h after the last exposure. Full experimental details were published in Yauk et al. (2011) and Halappanavar et al. (2011). Samples were analyzed using $4 \times 44K$ Agilent gene expression microarrays as per the manufacturer's protocol (Agilent Technologies). Lung microarray data have been published in Halappanavar et al. (2011), and partial results for liver microarrays are found in Yauk et al. (2011); all information regarding array quality control and normalization can be found in these manuscripts. The full liver gene expression dataset (unpublished) has been uploaded to the Gene Expression Omnibus under accession number GSE4363.

Hepatic gene expression analysis in mice exposed to 5, 50, 150, or 300 mg BaP/kg-bw per day for 3 days revealed 4, 36, 373, and 900 genes, respectively, that were altered relative to solvent-treated mice (fold change [FC] ≥ 1.5 and false discovery rate [FDR] adjusted p -value ≤ 0.05) 4 h after exposure and 1, 11, 410, and 579 genes, respectively, 24 h after exposure. In the lungs of these mice, 558 and 1267 genes were differentially expressed (FC ≥ 1.5 and FDR p -value ≤ 0.05) following 150 and 300 mg/kg-bw per day BaP exposure compared with controls, respectively, 4 h after exposure. The overall gene expression response in the liver was consistent with the known physiological function of the organ, including its primary role in the detoxication of xenobiotic compounds. For example, we measured significantly increased transcription of the *Cyp1a1* metabolism gene in liver and lungs (Table 5). The pulmonary gene expression response to BaP included changes in biological pathways involved in B cell receptor signaling, inflammation, and DNA damage response. Additional real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) array analyses were also performed on the lung tissue across all doses and time points. Ribonucleic acid (RNA) samples were processed, and data were analyzed as

described in Labib et al. (2012) on custom PCR arrays that included 162 genes (Supplementary Table 5 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934> includes the 38 genes referred to in the present study).

Experiment 2—Adult (25-week-old) MutaTM Mouse (transgenic mouse strain 40.6) males were exposed to BaP (Sigma Aldrich, Canada) in olive oil by oral gavage at a concentration of 0 (olive oil alone), 25, 50 or 75 mg/kg-bw per day for 28 consecutive days ($n = 5$ per group). Mice were sacrificed 72 h after the final exposure. The right lobe of the lungs, the median lobe of the liver and the forestomach were excised, flash frozen in liquid nitrogen and stored at -80°C until use. All samples were analyzed using Agilent $4 \times 44K$ or $8 \times 60K$ gene expression microarrays. The full DNA microarray and real-time quantitative PCR array analyses are published for liver (Malik et al. 2012), lungs (Labib et al. 2012), and forestomach (Labib et al. 2013); the reader should refer to these publications for details relating to microarray data processing and normalization. Data for liver, lungs, and forestomach are available in the Gene Expression Omnibus (accession numbers GSE24910, GSE35718, and GSE43438, respectively). Microarray analysis of variance (MAANOVA) revealed significant changes in the transcript levels (FDR p -value ≤ 0.05 and FC ≥ 1.5) of 6, 7, and 121 genes in livers, 20, 145, and 373 in lungs and 9, 135, and 408 in forestomachs of mice in the 25, 50, and 75 mg/kg-bw per day exposure groups, respectively. In the liver, the affected genes were primarily associated with biological processes such as xenobiotic metabolism and p53 signaling. In the lungs, there was significant enrichment of p53 signaling and cancer pathways. Although the p53 signaling pathway was also perturbed in the forestomach, the most significantly enriched processes were associated with antigen processing and presentation, immune response, chemotaxis, and keratinocyte differentiation. The results in the lungs and liver are consistent with expected changes in gene expression in response to a genotoxic carcinogen, whereas the forestomach data point to additional modulating factors at work (a pro-immune, inflammatory response) (Hochstenbach et al. 2012). Please note that BPDE-DNA adducts and lacZ transgene mutant frequency were also assessed in the lung, liver, and glandular stomach tissues from the same mice (Lemieux et al. 2011, Malik et al. 2012, Labib et al. 2012) (Figure 2). Adducts were detected via the 32P-postlabeling method and were found in all three dose groups in all tissues, with the highest relative adduct labeling in the lung tissue at 3 days postexposure. LacZ mutant frequency was assessed using the P-Gal positive selection assay and was highest in the glandular stomach at this time point.

Experiment 3—Eight-week-old male C57BL/6J mice were treated four times a week with BaP in corn oil at a dose of 13 mg/kg-bw per day or corn oil vehicle (7.4 mg/kg-bw per day, adjusted for daily exposure). Mice were dosed by oral gavage on days 0, 2, 4, and 6 (autopsy on day 7) and euthanized 24 h after the final exposure. Each treatment group consisted of four mice. Liver, spleen, kidney, bone marrow, and urinary bladder were isolated. Tissues

Table 5. *Cyp1a1* and *Cyp1b1* gene expression in mouse lungs and liver exposed to 5, 50, 150, and 300 mg/kg-bw/day BaP for 3 days and excised 4 or 24 hours postexposure. Those not statistically significant are indicated by ns.

	Dose (mg/kg-bw/day)	Lungs (fold change)		Liver (fold change)	
		4 hours	24 hours	4 hours	24 hours
<i>Cyp1a1</i>	5	↑46.9	↑7.5	↑25.5	ns
	50	↑103.3	↑1.5	↑109.9	ns
	150	↑179.3	↑14.7	↑146.7	ns
	300	↑210.1	↑42.9	↑165.4	↑9.0
<i>Cyp1b1</i>	5	↑10.4	↑1.5	ns	ns
	50	↑23.5	↑1.7	ns	ns
	150	↑161.2	↑21.8	ns	ns
	300	↑208.6	↑28.6	ns	ns

were stored in RNeasy lysis buffer using the manufacturer's protocol (Qiagen, Valencia, CA, USA). Total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA, USA) and the QIAcube (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. All samples passed RNA quality control using capillary gel electrophoresis (RNA integrity number [RIN] > 7.6) (Bioanalyzer 2100, Agilent Technologies, Amstelveen, Netherlands). Messenger RNA (mRNA) was amplified and labeled with the GeneChip Expression 3'-Amplification One-Cycle cDNA Synthesis Kit and GeneChip Expression 3'-Amplification Reagents for IVT Labeling according to the manufacturer's instructions (Eukaryotic Sample and Array Processing 701025 Rev.5; Affymetrix Inc., Santa Clara, CA, USA). Amplified materials were hybridized to Mouse Genome 430 2.0 Array for 16 h at 45°C, subsequently washed and stained with the EukGE-WS2v5_450 protocol and finally scanned using the GeneChip Scanner 3000-7G (Affymetrix Inc., Santa Clara, CA, USA). Image generation and feature extraction were performed using Affymetrix GCOS Software version 1.4.0.036. Quality control and correction of significant hybridization and experimental blocking effects, annotation, RNA normalization, and subsequent data analysis were performed as previously described (Schaap et al. 2012). Gene expression data for the liver are available in the Gene Expression Omnibus (accession number GSE43977). Data for the other tissues will become publicly available (M. Luijten et al., unpublished data). Gene expression profiles for the spleen, kidney, and liver were similar to what we observed at the lowest dose (5 mg/kg-bw per day) in our 3-day experiments described above for the lungs and liver (i.e., only a few genes were significantly affected by the treatment). Unlike the aforementioned tissues, there was no significant change in gene expression profiles in the bone marrow or bladder samples of BaP-treated animals compared with control animals. This suggests that toxicogenomics is useful in identifying the most relevant tissues and organs affected by a chemical treatment.

Human cell culture data. To gain insight into BaP-induced genotoxic hazard and pathway perturbations in human cells, we compared gene expression profiles of BaP-treated human cells in culture to those derived from our rodent studies. Gene expression profiles were generated following in vitro BaP exposure of TK6 cells, a human lymphoblastoid cell line (ATCC No. CRL-8015; Manassas, VA, USA). Detailed methodology and data can be found in Supplementary File C to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934> (Buick et al. submitted). TK6 cells were exposed to BaP in dimethyl sulfoxide (DMSO) (0, 0.45, 1.4, or 10 µg/mL) in the presence of 1% pentobarbital/benzoflavone-induced rat liver S9 with reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system cofactors (for metabolism of BaP) for 4 h. Cells were collected immediately postexposure (4-h time point) or were placed in fresh media and collected 20 h later (24-h time point). Three technical replicates were produced per concentration for both time points. Cisplatin was used as a positive control for genotoxicity. Because cisplatin is direct acting, no metabolic activation system was required. In addition, negative controls (media

only) and vehicle controls were included, in both the presence and absence of the metabolic activation system. The in vitro chemical exposures were performed by Integrated Laboratory Systems Inc. (ILS; Research Triangle Park, NC, USA), in addition to the associated cytotoxicity and genotoxicity testing. The CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure cell viability and proliferation in treated cells by means of a colorimetric assay. In addition, flow cytometry-based cytotoxicity and micronucleus assays were conducted using the In Vitro MicroFlow kit (Litron Laboratories, Rochester, NY, USA) to assess relative survival, percentage of apoptotic/necrotic cells and micronucleus frequency, as described in Avlasevich et al. (2006). RNeasy Mini Kits were used to extract total RNA from treated and control cells following the Purification of Total RNA from Animal Cells Using Spin Technology Protocol (Qiagen, Valencia, CA, USA). RNA samples were labeled with Cyanine 3 and Cyanine 5 using Low Input Quick Amp Labeling kits (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Labeled complementary RNA (cRNA) samples were hybridized to Agilent SurePrint G3 Human 8 × 60K microarrays (n = 3 per dose and time point), following the manufacturer's directions, using a two-color dye-swap design.

The toxicogenomics analysis was performed as described in Goodsaid et al. (2010) and H. Li et al. (unpublished data). The gene expression profiles of BaP-treated cells were measured using Agilent 60K oligo microarrays using a dye-swap model. The presence of a genotoxic signature in profiles of BaP-treated TK6 cells was evaluated utilizing a genotoxic signature/classifier that is capable of differentiating genotoxic and non-genotoxic agents (Goodsaid et al. 2010, Li et al. submitted). The classifier is based on a reference database comprising 28 model agents run on Agilent DNA microarrays. Thus, at this time the approach can only be applied using Agilent whole genome microarray platform. In addition, the classifier is currently limited by the spectrum of chemicals used to train and test the signature. However, the classifier is currently under a formal validation by the Health and Environmental Sciences Institute of the International Life Sciences Institute (ILSI HESI), which has markedly increased the number of agents tested. Finally, the classifier was built using direct acting chemicals and only a few chemicals requiring metabolic activation have been tested and analyzed thus far. In our experience, determining the most effective S9 in terms of concentration and type of induction can also be a challenge.

Exposure of TK6 cells to BaP caused a concentration-dependent increase in cytotoxicity and micronucleus formation and upregulation of known BaP-induced stress response genes (Figure 4; Supplementary Table 6 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>). Expression profiles of BaP-treated TK6 cells clustered with those of DNA-reactive genotoxic toxicants (Figure 5).

Identification of key events and proposal of MOA

To derive a MOA based on the International Programme on Chemical Safety (IPCS)/(ILSI) MOA framework (Meek et al. 2014, Boobis et al. 2006, Meek et al. 2003, Sonich-Mullin et al. 2001), key events were identified by interrogating all

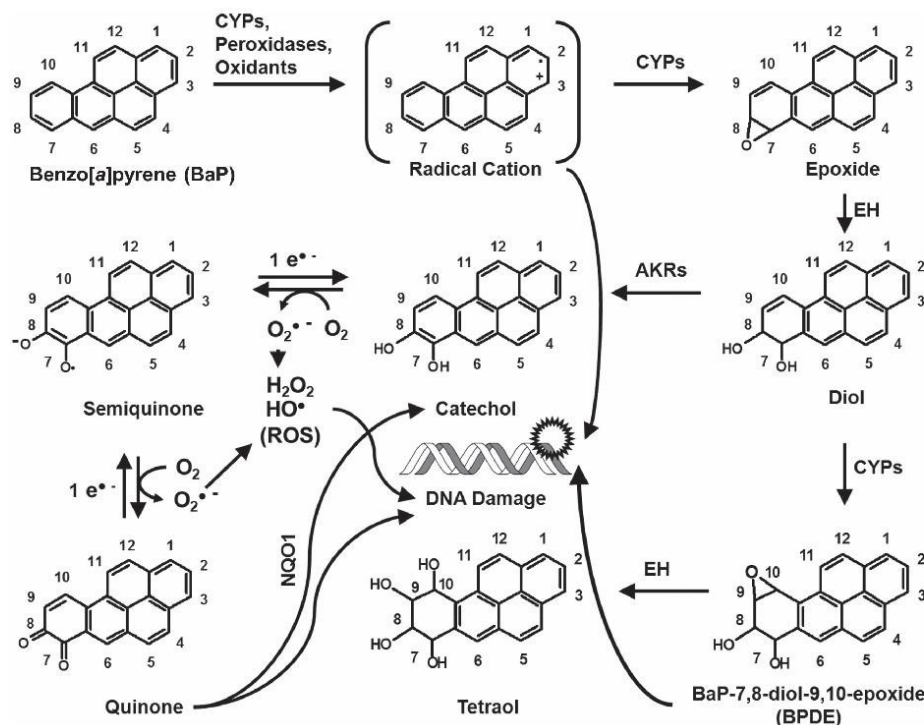


Figure 3. General overview of BaP metabolism. Cytochrome P450s (CYPs) and other enzymes and cellular oxidants can convert BaP to radical cations. These can be further metabolized by CYPs to epoxide and then to diols by epoxide hydrolase (EH). BaP diols are converted to catechols by aldoketo reductases (AKRs) or to BaP-7,8-diol-9,10-epoxide (BPDE) by CYPs. BPDE, BaP quinones, reactive oxygen species (ROS) (generated during BaP catechol-quinone redox cycling or from CYPs' uncoupling), and BaP cations react with DNA (forming adducts) and lead to DNA mutations (predominantly G to T transversions). EH can further metabolize BPDE to tetraols that, along with diols, phenols (not shown) and quinones, are conjugated to glucuronosyl by uridine diphosphate-glucuronosyl transferases (UGTs). Diols, phenols, and quinones can be also converted to sulfate esters by sulfotransferases (SULTs). Glutathione *S*-transferases (GSTs) conjugate BaP epoxides, diol epoxides, and quinones to glutathione (Ramesh et al. 2004). Only representative BaP metabolites (i.e., modified at positions 7, 8, 9, and 10) are shown. In addition, BaP metabolism can yield many other hydroxy-, oxide-, dihydroxy- and quinone-related compounds at each of the 12 carbon atoms and it has been estimated that 709 oxygenated metabolites of BaP exist (reviewed in Nebert et al. 2013a); similar BaP metabolites are formed at other positions (Ramesh et al. 2004). See Section "Metabolism" for additional details.

genomics data based on functional annotation. Key events are empirically observable, necessary precursor events in the MOA or biologically based markers for such events (US EPA 2005). We interrogated our putative key events, comprising

our proposed MOA, based on their temporal and dose-response patterns of gene expression, as well as based on modified Bradford Hill criteria (Hill 1965), in accordance with the MOA/human relevance framework analysis. Once the key events were identified, IPA pathways were assigned to each key event using knowledge acquired from the literature.

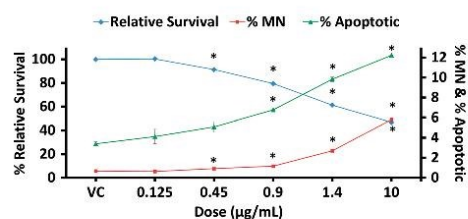


Figure 4. Cytotoxicity and genotoxicity measurements in human TK6 cells following exposure to BaP using a flow cytometry-based assay (*In Vitro* Microflow kit; Litron Laboratories). Relative survival (shown in blue), percentage of apoptotic/necrotic cells (shown in green) and percentage of micronuclei (MN; shown in red) are depicted following 24 h of exposure (4-h exposure + 20-h recovery). *Denotes $p < 0.05$ compared with vehicle control (VC; + S9), and error bars are standard error.

Calculation of transcriptional PODs

Dose-related changes in gene expression were used to calculate transcriptional BMDs using BMDExpress version 1.4.1 (Yang et al. 2007). Briefly, analyses were performed on all genes using four models: Hill, power, linear, and polynomial 2°. The best fit selected was the model that: (1) described the data with the least complexity; (2) had a nested chi-squared test cut-off of 0.05; (3) had the lowest AIC (measure of the relative goodness of fit); and (4) the BMD was lower than the highest dose. Other settings included maximum iterations of 250, confidence level of 0.95, BMR factor of 1.349 (the number of standard deviations defining the BMD, which

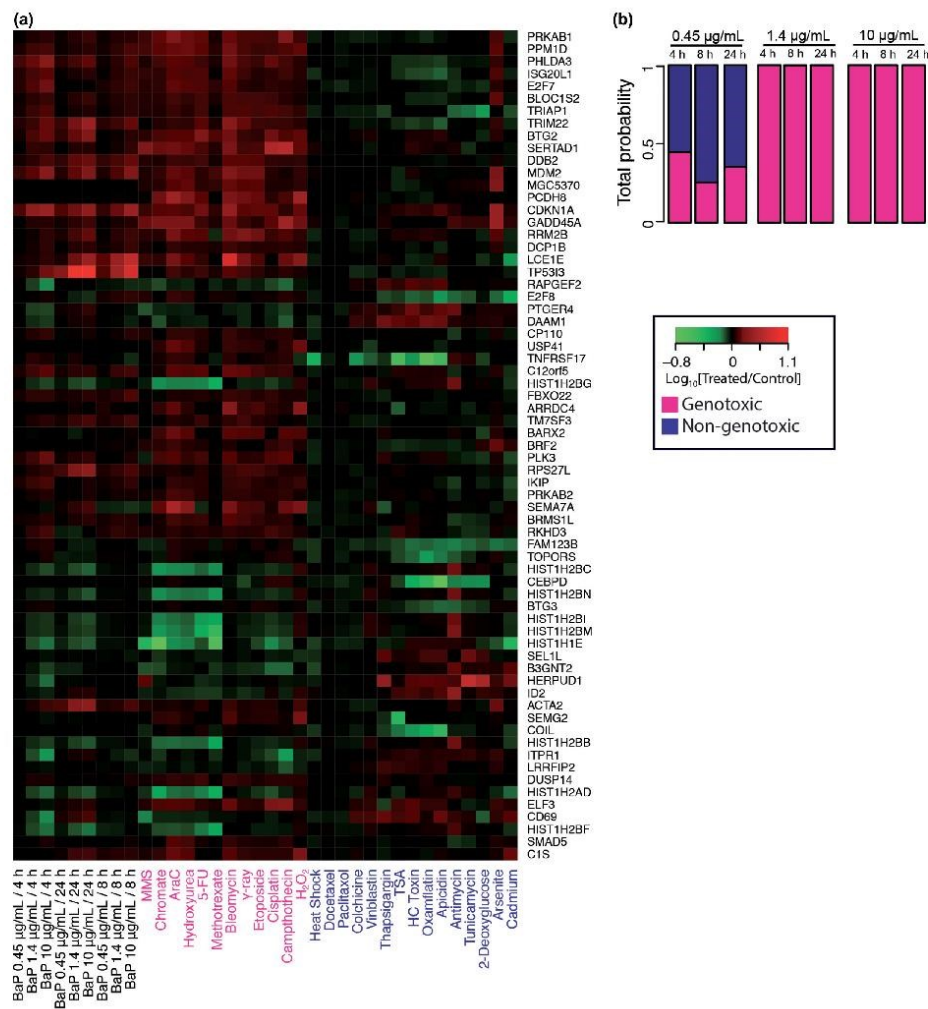


Figure 5. Hazard identification of BaP as a genotoxic compound by comparison of its transcriptome profile with the genotoxicity biomarker following BaP exposure in human TK6 cells in the presence of metabolic activation (1% rat liver S9) at 4, 8, and 24 h. (a) Hierarchical clustering of the expression levels of genes in a genotoxicity signature derived from a training set of 28 genotoxic (pink) and non-genotoxic (blue) agents indicates that BaP clusters with genotoxic agents at the mid- and high concentrations at all three time points. The GenBank accession numbers for the 65 classifier genes contained within the predictive gene signature are indicated on the right hand-side of the heatmap. Gene expression fold-changes relative to control are shown by means of the color scale; upregulated genes are shown in red, downregulated genes are shown in green and genes that are not regulated are shown in black. (b) BaP transcriptome profiles were analyzed using the 65-gene classifier to predict genotoxicity. Nearest shrunken centroids classification probabilities for BaP treatments are shown using the 65-gene classifier. The NSC method was employed to classify BaP transcriptome profiles by examining them for similarities with the transcriptome profiles of the reference chemicals in the database using statistical and bioinformatics tools. Briefly, the standardized centroid (SC) was computed by applying the NSC method for each class of training chemicals, in which SC is the mean expression level for each gene in a class divided by its within-class standard deviation. For each class, the SC is shrunken in the direction of the overall centroid to create the NSC. BaP was then classified through comparison of its gene expression profiles to the class of NSCs for each concentration and time point. Sample classification was achieved by assigning it to a class that is closest to it in squared distance (Tibshirani et al. 2002).

corresponds to a 10% change compared with controls) (Yang et al. 2007) and power restricted to ≥ 1 . The use of the Hill model was restricted such that it was flagged if the k parameter of the model was less than one third of the lowest BaP dose used in an experiment (Black et al. 2012). A flagged Hill model was excluded if at least one of the other models

had a p -value > 0.05 . In such a case, the next model with least complexity and with a p -value > 0.05 was selected. In the case when no model had a p -value > 0.05 , the Hill model BMD was modified to 0.5 of the lowest BMD value. The resulting BMDL datasets were mapped to IPA pathways (probes that mapped to multiple annotated genes were

removed). Supplemental Table 7 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934> displays model fit criteria for key PODs determined in RA2 and RA3. We then calculated the average BMD/BMDLs at the 10th percentile of all genes affected by BaP treatment in the assigned IPA pathway. Given that a standard benchmark response applied to apical endpoints is 10% (akin to 1 standard deviation; U.S. EPA 2005), the 10th percentile is an appropriate threshold for comparative purposes. As pathways can contain many genes that can be expressed at a given time and the gene expression analysis represents a snapshot in time, only a few genes within an enriched pathway may be expected to be differentially expressed at a single time point. Multiple IPA pathways can be representative of a single key event. Thus, the IPA pathway with (1) at least five genes passing the modeling criteria above; (2) BMD/BMDL < 10; and (3) the lowest BMDL aligned with each key event in our MOA was selected as representative of that key event.

The transcriptional data were used to inform the genomics-informed risk assessment (RA2; Section "Genomics-informed approach (RA2)") and the genomics-only risk assessment (RA3; Section "Genomics-only approach (RA3)").

Genomics-informed approach (RA2)

The objective of the genomics-informed approach (RA2) was to evaluate the ability of toxicogenomics to derive relevant PODs and to provide mechanistic information to support the human relevance of BaP-induced effects for human health risk assessment. As carcinogenicity was identified in RA1 as the endpoint of concern for POD selection, RA2 focused on this endpoint as well. Although MOAs are described in detail for many of the toxicity endpoints described in RA1, the availability of genomics data for RA2 provides a means to expand upon the cancer MOA and derive quantitative measures of response for key events for this specific adverse outcome. Thus, we relied on toxicogenomics information (primarily our own data) and all available published BaP information (i.e., RA1) to propose an MOA for BaP-mediated carcinogenicity and to guide the selection of a POD for risk assessment.

We would like to emphasize that our primary goal was to integrate biologically relevant toxicogenomics information into our risk assessment (i.e., focus on pathways and genes that are relevant to existing toxicological knowledge, rather than including all and any gene that responded). Toxicogenomics data were used first to propose an MOA for genotoxicity leading to cancer, as the most notable effect associated with BaP exposure in mouse liver, lungs, and forestomach in our study. We then calculated transcriptional BMDs for genes and pathways comprising the key events in our proposed MOA in order to derive PODs for risk assessment. Our approach contrasts with that of Thomas et al. (2007a, 2011, 2012), who showed that the lowest BMDs for transcription (i.e., the pathways or GO terms with the lowest median BMD values regardless of biological function) correlate well with BMDs for apical cancer and non-cancer endpoints and suggested that these values should be used as PODs. This approach is valuable for chemicals operating as nonspecific toxicants that perturb multiple biological pathways, as such perturbations may be adverse to normal physiological functions. In our example, we aim to

demonstrate how toxicogenomics can be used to obtain information relating to key events to develop more specific MOAs, which has been noted as an important potential application of toxicogenomics in risk assessment (Bercu et al. 2010, Currie 2012, Waters et al. 2010).

Proposed mode of action

Our proposed genotoxic MOA for BaP is schematically represented in Figure 6, summarized in Table 6 (including evidence for human relevance), evaluated based on modified Bradford-Hill criteria (Table 7) and the BMDLs for the key events are presented in Table 8. Based on our data and review of the literature, this MOA is relevant to the three rodent tissues under examination in this risk assessment: liver, lungs, and (fore)stomach. BaP's MOA consists of six key events: (1) AHR binding; (2) induction of cytochrome P450 enzymes; (3) metabolism of BaP to DNA-reactive genotoxic compounds; and (4) formation of DNA adducts and DNA damage, which, if unrepaired, cause (5) mutation via erroneous replication. If these mutations occur in proto-oncogenes or tumor suppressor genes (e.g. *p53*), they can cause the last key event (6) altered cell signaling, proliferation and apoptosis, which leads to the adverse outcome tumorigenesis. Below, we use published information and our own toxicogenomics data to provide evidence supporting this MOA and, where possible, provide insight into the human relevance of each key event.

Key event 1: BaP binding to the AHR (molecular initiating event). Multiple xenobiotic ligands (including BaP) bind to the AHR (Ovesen et al. 2011). Specific binding of BaP to the AHR has been measured in vitro directly using radiolabeled substrate (Monteiro et al. 2008), indirectly utilizing luciferase reporters containing the AHR response element (Malik et al. 2013) and by monitoring the expression of a known AHR target gene, *Cyp1a1* (Ovesen et al. 2011, Table 5). Activation of the AHR by BaP leads to its own metabolism (Section "Metabolism") creating reactive metabolites that produce its subsequent genotoxic MOA. In addition, BaP-AHR activation causes transcriptional changes in genes related to carcinogenesis (Labib et al. 2012, Van Delft et al. 2012) and largely accounts for the non-genotoxic MOAs of BaP. Mice devoid of the AHR do not develop BaP-induced tumors, highlighting the receptor's role in BaP-induced carcinogenicity (Shimizu et al. 2000).

The relevance of this key event to humans has been demonstrated by photoaffinity ligand competition assays of BaP binding to the AHR in human primary hepatocytes (DiNatale et al. 2010). *AHR* polymorphisms can reduce ligand binding to the AHR (thereby reducing sensitivity to AHR ligands as measured by toxicity and the induction of AHR-dependent gene expression), but the effect of human *AHR* polymorphisms on the receptor's ligand binding function is poorly understood (Harper et al. 2002, Okey et al. 2005). The binding affinity of the AHR to its ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and AHR-mediated CYP1A1 inducibility varies greatly in humans (Harper et al. 2002). In addition, human cell lines with impaired CYP1A1 induction have been described, but the underlying mechanisms of these phenomena (e.g., genetic polymorphisms in *AHR* or other AHR signaling

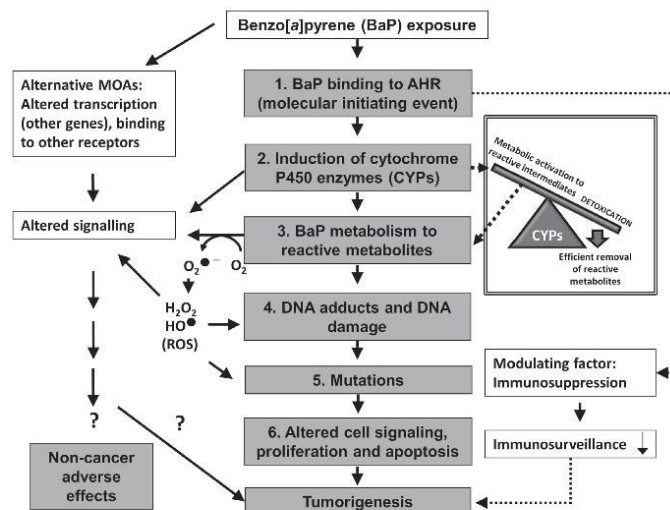


Figure 6. RA2: Postulated genotoxic MOA of BaP in animals (shaded rectangles). BaP binds to the AHR and activates transcription of AHR-controlled genes. These include xenobiotic metabolism enzymes that convert BaP to a variety of products that are subsequently conjugated to water-soluble moieties and excreted. BaP metabolites escaping detoxication (Section “Metabolism”, e.g., BPDE) and reactive oxygen species (ROS) are genotoxic, leading to DNA adducts and oxidative damage. If unrepaired, genotoxic damage may cause mutations, leading to uncontrolled cell proliferation and tumor formation. In addition, BaP-mediated immunosuppression may provide a favorable environment for tumor growth, and activation of other signaling pathways that may favor tumorigenesis (see Section “Tumorigenesis (adverse outcome)” for details). The double lined box illustrates the dual role of BaP metabolism by CYPs: their beneficial role in activating BaP for further conjugation and removal that becomes apparent from CYP knock-out studies of mice, exposed to BaP (e.g., Nebert et al. 2004). Metabolism by CYPs leads to efficient detoxication of BaP metabolites. However, a small portion of genotoxic metabolites escapes the detoxication to lead to DNA damage and mutations (Nebert et al. 2004).

pathway genes) are unknown (Harper et al. 2002). Thus, it is reasonable to expect that this key event is relevant in humans, but that genetic variations in the human population may affect the susceptibility of humans to BaP.

Key event 2: Induction of cytochrome P450 enzymes (CYPs). CYPs are prototypical responders to treatment with AHR ligands (Ovesen et al. 2011). Some of the AHR’s target genes code for the cytochrome P450 enzymes (e.g., CYP1A1 and CYP1B1) that effectively metabolize BaP. Indeed, a large majority of BaP is detoxified at the site of contact in the gastrointestinal tract (Nebert et al. 2013a). However, metabolism can also cause the formation of genotoxic metabolites of BaP that can escape detoxication. Using our mouse data, we noted that the transcription of many P450 genes (e.g., Cyp1a1, Cyp7a1, Cyp1b1, Cyp2b10, Cyp2b13, Cyp3a44, Cyp2b9, Cyp2c38, and Cyp2c40) increased following treatment with BaP treatment in mouse liver and lungs (Halappanavar et al. 2011, Malik et al. 2012, Yauk et al. 2011). Of these, Cyp1a1 responded at the earliest time points and with the highest fold change compared with controls. Table 5 summarizes the Cyp1a1 and Cyp1b1 gene expression changes in the lungs and liver 4 and 24 h postexposure. We did not have access to the forestomach from the 3-day acute studies; however, Uno et al. (2008) have shown that following 5 days of oral BaP exposure, the mRNA and protein levels of CYP1A1 and CYP1B1 are elevated in the forestomach. Increased CYP1A1 activity, as measured by the ethoxresorufin-O-deethylase (EROD) assay, has been shown in the livers of mice exposed to BaP (Bjelogrić et al. 1989)

and in BaP exposed human HepG2 cells (Vakharia et al. 2001). The occurrence of this key event in humans is supported by toxicogenomics work on human cells in vitro. Toxicogenomics analysis of primary human hepatocytes and the hepatoma cell line HepG2 challenged with BaP revealed the induction of P450 enzymes, including CYP1A1, in both systems (Hockley et al. 2006, Wilkening et al. 2003).

Key event 3: BaP metabolism to reactive metabolites. On its own, BaP is chemically inert; however, P450 enzymes metabolize BaP to genotoxic carbon cations or epoxides, such as the ultimate carcinogenic BaP metabolite BPDE (Cavalieri and Rogan 1995). This metabolism depends on CYP1A1 and CYP1B1 enzymes, as demonstrated by knock-out studies in animals. For example, BaP blood levels in Cyp1a1^{-/-} and Cyp1a1^{-/-} Cyp1b1^{-/-} knockout mice are 25- and 75-fold greater than in control animals, respectively, and these mice are less sensitive to many BaP-related toxicities (Uno et al. 2006).

BaP metabolites undergo further transformation by phase II enzymes in order to minimize cellular damage from reactive metabolites (e.g., BPDE) and ROS (Section “Metabolism”). These reactions include BaP conjugation to glutathione, glucuronic acid, or sulfates by GSTs, UGTs, and SULTs, respectively. Many phase II enzymes are activated at the mRNA level by the transcription factor Nrf2 (Nfe2l2) in response to the presence of reactive metabolites and oxidative stress (Kaspar et al. 2009). We observed the transcriptional induction of *Nrf2* and its target genes (*Nqo1*, *Ugdh*, *Srxn1*,

Table 6. RAZ. Key events in the proposed mode of action of benzo[a]pyrene for rodents and their human relevance.

Key event	Evidence in rodents			Evidence in humans		
	Toxicogenomic endpoints	Apical endpoints	References	Toxicogenomic endpoints	Apical endpoints	References
1. BaP binding to AHR (molecular initiating event)	Indirect evidence: upregulation of AHR-responsive genes.	YES: BaP is a well-established AHR ligand.	Malik et al. (2013), Ovesen et al. (2011), Table 5.	<i>CYP1B1</i> gene expression is induced by BaP in normal mammary epithelial cells.	YES: BaP binds to human AHR and activates AHR-dependent gene expression.	DiNatale et al. (2010), Gwinn et al. (2005)
2. Induction of cytochrome P450 enzymes (CYPs)	YES: Multiple P450 genes (e.g., <i>Cyp1a1</i> , <i>Cyp1a7</i> , <i>Cyp1b1</i> , <i>Cyp2b10</i> , <i>Cyp2b13</i> , <i>Cyp3a4</i> , <i>Cyp2b9</i> , <i>Cyp2c38</i> , and <i>Cyp2c40</i>) are induced by BaP in mouse liver, lungs and forestomach.	YES: induction of EROD in diverse tissues following BaP exposure.	Halappanavar et al. (2011), Malik et al. (2012), Yauk et al. (2011), IARC (2010)	YES: BaP induces genes coding for P450 enzymes (<i>CYP1A1</i> , <i>CYP1A2</i> , <i>CYP2B6</i> , <i>CYP2E1</i>) in BaP-treated primary human hepatocytes.	YES: BaP increases the activity of P450 enzymes (EROD, ECOD assays) in BaP-treated primary human hepatocytes.	Wilkinson et al. (2003)
3. BaP metabolism to reactive metabolites	YES: BaP induced phase II enzymes (e.g., <i>Nrf2</i> , <i>Nqo1</i> , <i>Ugt1h</i> , <i>Srxn1</i> , <i>Akr1b15</i> , <i>Ugt2b15</i> , <i>Gstm3</i> , <i>Gstm4</i> , and <i>Gstm7</i>) in mouse liver, lungs and forestomach to remove reactive metabolites.	YES: BaP metabolites have been isolated from animals exposed to BaP and studied in great detail.	Halappanavar et al. (2011), IARC (2010), Malik et al. (2012), Yauk et al. (2011)	YES: BaP induces <i>EPHX1</i> and <i>NQO1</i> genes, coding for the enzymes involved in BaP metabolism, in BaP-treated primary human hepatocytes.	YES: BaP metabolites, including BaP-7,8- diol (a precursor to BPDE), were detected in human liver and lung microsomes.	Shimada et al. (1989), Wilkenning et al. (2003)
4. DNA adducts and DNA damage	YES: Increased expression of genes regulated by p53 in lungs, liver and forestomach from BaP-treated mice. Upregulation of <i>Pdk1</i> and <i>Ddit4</i> in mouse liver and lungs indicates DNA damage repair.	YES: Adducts of BaP metabolites with DNA have been measured directly in several tissues in rats and mice.	Denissenko et al. (1996), Halappanavar et al. (2011), Labib et al. (2012), Malik et al. (2012), Wester et al. (2012)	YES: TP53 pathway is activated by BaP in cultured human TK6 cells; stress response and DNA repair response are activated by BaP in human lymphoblastoid cell line.	YES: BaP metabolites (BPDE) form adducts with DNA, and BPDE-DNA adducts are detected in human white blood cells.	Buick et al. (Submitted); DeMarini et al. (2001), Liu et al. (2005), Perera et al. (1988)
5. Mutations	Indirect evidence: the induction of gene expression changes in DNA damage response pathways is highly correlated with genotoxicity and mutagenicity in rodents.	YES: Mutations were reported in multiple studies in rodents following BaP exposure.	Sakai et al. (2014), Malik et al. (2012), Labib et al. (2012), IARC (2010)	Indirect evidence: the induction of gene expression changes in DNA damage response pathways is highly correlated with genotoxicity and mutagenicity in human TK6 cells.	YES: BPDE-DNA adducts detected at mutational "hotspots" of TP53 (G in codons 157, 248 and 273). Proto-oncogene K-Ras and p53 mutations found in lung tumors of nonsmokers exposed to BaP-containing PAH mixtures. Mutations in tumor suppressor gene p53 in BaP-exposed nonsmokers were detected.	Derks et al. (2014), Waters et al. (2010), Buick et al. (unpublished data), Denissenko et al. (1996), Rojas et al. (2000), DeMarini et al. (2001)
6. Altered cell signaling, proliferation and apoptosis	YES: A number of signaling pathways (including cancer pathways) were perturbed by BaP treatment in mice. <i>Mdm2</i> , an inhibitor of p53, was induced in the lungs and liver of BaP-treated mice at high doses.	YES: Multiple <i>in vitro</i> studies using cultured and animal cells identified increased cell proliferation due to BaP or its metabolites. Mutations in BaP-treated mice were increased compared with controls.	Brandon et al. (2009), Burdick et al. (2003), IARC (2010), Labib et al. (2012), Wester et al. (2012)	YES: Anti-apoptotic signaling gene expression was increased in MCF-7 human breast carcinoma and HepG2 human hepatocarcinoma cells.	Data gap.	Hockley et al. (2006)
Tumorigenesis (adverse outcome)	YES: Gene expression of putative hepatocarcinogenesis markers was altered in liver, lungs, and forestomach of BaP-treated mice (e.g., <i>Cdkn1a</i> , <i>Mdm2</i> , <i>Chrl1</i> , <i>Notch1</i> , <i>Nqo1</i> , <i>Nfe2l2</i> , and <i>Ephr1</i>).	YES: Liver and lung tumors were observed in several other species.	Horikawa et al. (1991), IARC (2010), Lavoie et al. (1987), Park et al. (2011), Wester et al. (2012), Wislocki et al. (1986)	Alteration of CDK, MDM2, NQO1 transcript levels	NO: No direct evidence, but mutational pattern of p53 in human lung cancer is very similar to that of BPDE-treated human HeLa cells and bronchial epithelial cells.	Denissenko et al. (1996), Buick et al. (submitted), Hockley et al. (2006)

ECOD ethoxycoumarin O-deethylase; EROD ethylresorufin O-dealkylase

Table 7. RA2. Evaluation of proposed mode of action by Bradford-Hill criteria. As originally proposed by Hill (1965) and simplified by Meek et al. (2003).

Bradford Hill criteria	Questions asked	Evidence in animals	References
1. Dose–response relationships	Is increasing exposure to the compound associated with increased magnitude of adverse outcome? Are key events observed at doses below or similar to those associated with the adverse outcome?	YES: increased BaP doses were associated with increased tumor incidence in mice and rats. YES: total cumulative doses in the toxicogenomics experiments were below the cumulative doses in the cancer bioassay.	Lavoie et al. (1987), Wester et al. (2012), Wislocki et al. (1986)
2. Temporal association between key events and adverse outcome and reversibility	Is sequence of events logical? Do key events and adverse outcome occur in expected order? Does cessation of exposure (or any interference with the proposed key events) ameliorate adverse outcome?	YES YES	Halappanavar et al. (2011), Labib et al. (2012), Malik et al. (2012), Yauk et al. (2011)
3. Strength, consistency, and specificity of association between key events and adverse outcome	Is the evidence linking exposure to a chemical, key events, and adverse outcome strong? Is it consistent in repeated experiments in the same laboratory and other laboratories? Are the associations between exposure, key events, and adverse outcome specific?	YES YES YES: the specificity of associations has been confirmed by several apical endpoints, including BaP metabolite detection in animal samples, DNA adducts, cell proliferation, immunoblotting, and immunocytochemistry.	Reviewed in IARC (2010)
4. Biological plausibility and coherence of the database	Does hypothesized MOA make sense based on broader knowledge about the chemical and the biology associated with adverse outcome (e.g., chemical properties, disease etiology)?	AHR-mediated metabolism of BaP is consistent with what is known about other PAH ligands of AHR. Genotoxic carcinogens are well known, and DNA–BaP adducts are well documented. BaP is positive in mutagenicity tests.	Reviewed in IARC (2010)

Akr1b15, *Ugt2b15*, *Gsta1*, *Gsta2*, *Gstm3*, *Gstm4*, *Gstm7*, and *Gstol1*) in mouse lungs, liver and forestomach in response to BaP, in at least one time point (3- or 28-day regimens), suggesting that detoxication mechanisms were activated in order to cope with BaP toxicity (Labib et al. 2013, Malik et al. 2012, Yauk et al. 2011).

There is clear evidence that BaP metabolism to reactive metabolites occurs in humans. For example, enzyme assays confirm that BaP treatment causes an increase in the activity of P450 enzymes in primary human hepatocytes (Wilkening et al. 2003). Induction of phase II enzymes in response to BaP also occurs in cultured human cells (Hockley et al. 2006, Wilkening et al. 2003). In addition, the BaP metabolite BaP-7,8-diol, a precursor to the genotoxic metabolite BPDE, is found in the microsomes of human liver and lung donors (Shimada et al. 1989). Similarly, 3-OH BaP, an excreted BaP metabolite discussed in Section “Metabolism”, is readily detectable in the urine of occupationally exposed workers (Forster et al. 2008).

Key event 4: DNA adducts and DNA damage. BaP metabolites, such as BPDE, can form covalent adducts with DNA

and proteins. In addition, ROS produced as by-products of BaP metabolism (from BaP catechol–quinone redox cycling) may oxidize DNA (Lan et al. 2004). BPDE–DNA adducts can be measured directly, and their levels are markedly elevated by BaP treatment in lung, liver and glandular stomach tissues in our studies (Figure 2; Halappanavar et al. 2011, Labib et al. 2012, Lemieux et al. 2011, Malik et al. 2012). Previous studies have established that levels of DNA adducts in the forestomach from mice orally exposed to BaP for 24 h and 5 days are comparable to those formed in the glandular stomach (Arlt et al. 2008). Thus, the forestomach has the metabolic capacity to form DNA reactive BaP metabolites that could influence the extent of DNA damage and the outcome following 28 days of repeated exposure. DNA polymerase kappa (Polκ), which encodes a DNA polymerase involved in the error-free bypass of BPDE adducts, was upregulated in mouse lungs and liver, illustrating the ability of genomic data to identify specific repair mechanisms induced in response to BaP exposure. Gene polymorphisms in Polκ are associated incidence of head and neck cancer in humans (Michiels et al. 2007).

Table 8. BMD_{1SD} and BMDL_{1SD} values (in mg/kg-bw-day) at the 10th percentile of all affected genes in an IPA pathway for each proposed key event.

Key event	Pathway title	Liver						Lung		Forestomach	
		3 day + 4 h		3 day + 24 h		28 day + 3 day		28 day + 3 day		28 day + 3 day	
		BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
1. BaP binding to AHR (molecular initiating event).											
2. Induction of P450.	Aryl hydrocarbon signaling	13.9	10.7	32.9	20.8	24.1	16.8	17.2	7.7	14.6	8.2
3. BaP metabolism to reactive metabolites.	Xenobiotic metabolism signaling	13.6	10.1	20.0	4.3	27.5	14.1	29.8	13.9	13.5	7.9
	Nrf2-mediated oxidative stress response	40.1	18.6	29.7	6.9	31.4	21.3	28.0	18.6	15.4	9.0
	Glutathione-mediated detoxication	70.3	49.6	82.1	54.4	< 5 genes		< 5 genes		15.3	9.0
4. DNA adducts and DNA damage.	p53 signaling	63.4	45.5	22.0	12.5	29.6	13.6	13.5	7.7	11.4	7.4
	Cell cycle: G1/S checkpoint regulation	75.7	43.8	8.1	1.0	24.6	12.4	11.7	8.9	18.5	7.7
	Cell cycle: G2/M DNA damage checkpoint regulation	77.1	29.9	79.7	43.3	< 5 genes		14.8	3.7	22.1	13.1
	Cell cycle control of chromosomal replication	104.1	65.0	70.7	39.7	42.2	17.2	Unperturbed		12.8	8.0
5. Mutations.											
6. Altered cell signaling, proliferation, and apoptosis.	Apoptosis Signaling	68.6	38.9	54.1	34.5	51.5	32.7	27.6	14.6	12.7	7.9
	Induction of apoptosis by HIV1	64.6	36.8	22.0	7.2	< 5 genes		26.5	12.1	15.3	9.0
	LPS-stimulated MAPK signaling	26.9	15.6	23.0	14.9	35.0	20.2	24.3	12.1	15.0	8.8
	Myc mediated apoptosis signaling	26.2	5.2	15.4	1.9	< 5 genes		30.6	7.0	15.0	8.9
	p38 MAPK signaling	53.4	35.3	26.5	19.3	25.3	11.9	23.3	N.A.	19.1	10.1
	PPAR signaling	69.2	37.4	6.5	N.A.	42.9	28.6	27.0	N.A.	18.5	10.1
	Retinoic acid mediated apoptosis signaling	73.8	50.3	58.2	36.3	< 5 genes		< 5 genes		19.6	9.1
	TGF-beta Signaling	73.5	45.9	13.3	1.5	25.3	11.9	9.2	N.A.	12.8	8.0
	UVA-induced MAPK signaling	75.1	58.0	56.5	36.9	29.0	13.4	16.8	N.A.	15.9	8.0
	VEGF signaling	63.4	31.7	9.9	N.A.	26.5	11.6	21.2	7.4	15.1	8.6
	Wnt/beta-catenin signaling	15.8	N.A.	36.0	25.1	23.7	6.7	23.0	7.7	25.1	10.8
7. Tumorigenesis (adverse outcome).	HER-2 signaling in breast cancer	61.5	39.3	31.9	16.6	33.7	19.5	22.9	10.1	16.1	8.2
	Prostate cancer signaling	88.7	47.4	16.4	2.2	37.0	22.7	22.9	N.A.	15.8	9.0
	Bladder cancer signaling	78.4	47.8	6.7	2.3	44.9	29.6	17.2	9.5	21.6	8.6
	Role of tissue factor in cancer	5.6	N.A.	20.3	2.3	24.7	N.A.	20.1	11.1	15.1	7.9

(Continued)

Table 8. (Continued)

Key event	Pathway title	Liver						Lung		Forestomach	
		3 day + 4 h		3 day + 24 h		28 day + 3 day		28 day + 3 day		28 day + 3 day	
		BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL	BMD	BMDL
	Thyroid cancer signaling	60.8	46.5	45.7	28.4	<5 genes		33.4	19.2	15.6	7.6
	Endometrial cancer signaling	26.2	19.1	43.7	27.4	35.0	20.2	30.0	10.0	16.2	9.3
	Estrogen-dependent breast cancer signaling	38.7	24.5	59.7	38.4	37.0	22.0	18.7	7.6	13.0	7.5
	Hereditary breast cancer signaling	75.4	53.2	11.1	2.2	28.7	13.3	26.6	17.3	14.7	8.8
	Molecular mechanisms of cancer	30.4	15.6	16.3	1.8	26.5	11.9	13.4	6.8	16.8	8.9
	Colorectal cancer metastasis signaling	35.0	9.0	16.8	2.2	26.8	13.1	19.0	7.8	16.2	8.1
	Non-small cell lung cancer signaling	62.8	37.8	20.4	5.9	<5 genes		40.5	25.1	15.9	7.8
	Basal cell carcinoma signaling	21.5	13.0	29.0	20.7	43.9	27.1	17.3	10.0	18.2	9.7
	Small cell lung cancer signaling	38.9	24.2	59.2	36.6	<5 genes		23.9	11.0	15.4	7.7
		6.3	0.8	0.3	0.2	25.4	5.3	15.7	2.1	16.1	4.5
Lowest BMDL 10th percentile regardless of MOA	CXCR4 signaling			Notch signaling		Rac Signaling		Cellular Effects of Sildenafil (Viagra)		Phenylalanine Degradation IV (Mammalian, via Side Chain)	

A BMD/BMDL ratio greater than 10 indicates a poor model fit and is indicated as not applicable (N.A.). The number of genes modelled were 8 for lung, 5 for liver and 40 for forestomach. The BMDLs proposed as PODs are bolded.

We also noted increased expression of p53 target genes (a proto-oncogene signaling pathway) in lung, liver, and forestomach samples from BaP-treated mice, indicative of DNA damage, repair, and cell cycle arrest. This key event is also supported by toxicogenomics data from the kidney of BaP-treated mice (M. Luijten et al., unpublished data). For example, BaP exposure led to decreased transcription of the neuronal PAS domain protein 2 (*Npas2*), a transcription factor involved in DNA repair (Hoffman et al. 2008) and circadian rhythm-related processes in the kidney (Zuber et al. 2009). Reduced *Npas2* expression in response to BaP suggests a decrease in the ability of the kidney to repair DNA damaged by BaP metabolites, potentially resulting in renal toxicity (e.g., Knuckles et al. 2001).

Our toxicogenomics analysis of the expression changes in TK6 cells treated with BaP (in the presence of rat liver S9) supports that similar genotoxicity and DNA damage response pathways are invoked in humans as in our rodent models. We observed a gene expression pattern indicative of TP53 activation in TK6 cells treated with BaP, and expression profiles of BaP-treated cells were consistent with genotoxic DNA-reactive chemicals (Figure 5) (Buick et al. submitted). Over 50 genes were expressed (upregulated or

downregulated) that were consistent with TP53 activation. These findings suggest that the genotoxic hazard is likely relevant to human cells following exposure to metabolized BaP. However, there is insufficient information to allow extrapolation of the TK6 cell results to cancer risks in drinking water due to BaP exposure in humans. Please note that the human cell culture work was used to support the MOA derived from the *in vivo* studies and not to extrapolate to human ingestion.

Key event 5: Mutations. Following induction of DNA adducts there are various possible fates of DNA lesions and the cells that contain damage. This can include error-free repair of the DNA lesions and resumption of normal cellular function, or in severely damaged cells, cell death via apoptotic and necrotic processes. Adducts that escape repair can cause mutations upon replication or DNA strand breaks leading to chromosomal aberrations.

There is extensive evidence that BaP causes mutations in animal tissues and that BaP metabolites cause mutations in human cells in culture. BaP metabolites and DNA adducts have been detected in cells isolated from BaP-exposed humans (Rojas et al. 2000). Strikingly, BPDE–DNA adducts were detected at mutational “hotspots” of TP53 (G in codons 157,

248, and 273), which are the sites of mutations in human lung tumors (Denissenko et al. 1996). The association of TP53 gene polymorphisms and cancer in humans has been summarized by Whibley et al. (2009). This, along with mutations in the human proto-oncogene K-RAS and tumor suppressor gene TP53 in BaP-exposed nonsmokers (DeMarini et al. 2001), provides support that the animal MOA is likely operative in humans.

Levels of BPDE adducts reflect the relative contribution of the rates of: (1) BaP metabolism to BPDE; (2) BaP metabolite detoxication by phase II enzymes; (3) adduct repair; and (4) cell turnover (Lemieux et al. 2011). The presence of BPDE–DNA adducts increases errors occurring during DNA replication, providing a mechanism for passing mutations on to the next generations of cells and thus contributing to the onset and progression of cancer (Perlow et al. 2002). Thus, earlier key events (1–3) in this MOA represent adaptive responses, reflecting normal homeostatic responses to xenobiotic exposure that do not necessarily lead to tumorigenesis on their own. Similarly, while the subsequent key event, DNA adducts and DNA damage is undesirable, it is not in itself an adverse effect as certain types of DNA damage may or may not predispose to mutations. However, mutations represent an irreversible “committed” step in the MOA, and mutations are known to increase the likelihood of tumorigenesis. Therefore, we based our POD selection on the key event preceding mutations (i.e., key event 4—DNA adducts and DNA damage) in order to adequately protect against carcinogenic outcome as it precedes the committed step.

Key event 6: Altered cell signaling, proliferation, and apoptosis. In addition to mutations, we found prominent changes in cell signaling pathways, including altered transcription of genes in several “cancer pathways” (Gohlke et al. 2009) in the tissues of rodents treated with BaP. These pathways included TGF- β signaling, p38 mitogen-activated protein kinase (MAPK) signaling, VEGF signaling, and apoptosis signaling in all three tissues and data points (Table 8). Altered intracellular signaling may result from BaP binding to the human β 2-adrenergic receptor and increasing intracellular calcium levels to activate multiple signaling pathways, including protein kinase C (Mayati et al. 2012).

It is difficult to predict which signaling pathways of those noted above drive the underlying mechanisms associated with BaP-induced liver, lung, and forestomach tumors in rodents, although the results suggest that multiple pathways may contribute. Toxicogenomics data can be used to guide future hypothesis-directed/targeted research by revealing the putative signaling pathways that may be associated with tumorigenesis. For example, lipid metabolism was identified as the top function in a network analysis conducted on the gene expression changes occurring in mouse liver following BaP exposure (3-day treatment, animals sacrificed 4 and 24 h later; (Yauk et al. 2011, Halappanavar et al. 2011)). The BaP-altered lipid metabolism network consisted of several genes, including *Hnf4a* (hepatocyte nuclear factor 4 alpha) and *Cyp7a1* (also known as cholesterol 7 α -monooxygenase). *Cyp7a1* affects serum total cholesterol levels in mice (Kojima et al. 2009). Dysregulated cholesterol metabolism may increase cell proliferation through increased mevalonate levels (Nesnow et al. 2011), farnesylation, and activation of Ras leading to tumorigenesis (Murphy et al. 2012).

Several studies have reported increased cellular proliferation in cultured cells (reviewed in Burdick et al. 2003, IARC 2010) and animal models (Brandon et al. 2009, Wester et al. 2012) in response to BaP exposure. Studies in murine and human cells have clearly shown that BaP induces apoptosis in these cell models (reviewed in IARC 2010). Indeed, BPDE–DNA adducts correlate well with TP53 induction in cultured human lymphocytes as measured by immunocytochemical staining (Godschalk et al. 2001). This finding is in agreement with the robust activation of p53 target genes that we observed in the lungs, liver, and forestomach of BaP-treated mice (Labib et al. 2012, 2013, Malik et al. 2012). Similarly, BaP exposure of human TK6 cells increased DNA damage and apoptosis (Figure 4) in parallel with changes in the expression of TP53-regulated genes (e.g., *CDKN1A*, *CCNG1*, and *TP53INP1*) that are involved in DNA damage response, cell cycle arrest, and apoptosis (Supplementary Table 6 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>), in accordance with gene expression data from mouse tissues in our experiments.

Evidence suggests that BaP has similar effects on cellular signaling in humans as it does in rodents. In addition to the robust activation of TP53 target genes by BaP in our TK6 cells, the transcription of genes involved in anti-apoptotic signaling is increased in MCF-7 human breast carcinoma and HepG2 human hepatocarcinoma cells exposed to BaP (Hockley et al. 2006) in a pattern largely consistent with rodents. Thus, we believe that the overall data strongly suggest that alteration of cellular signaling, especially related to p53 signaling and apoptosis is an important part of BaP genotoxicity. More studies are required to verify whether perturbation of single specific signaling pathways, or a group of pathways, leads to deregulation in cellular signaling and tumorigenicity following BaP exposures.

Tumorigenesis (adverse outcome). BaP is a known rodent carcinogen, and various studies have demonstrated liver, lung, and forestomach tumors in rodents following BaP exposure using standard apical tests (IARC 2012, Lavoie et al. 1987, Wislocki et al. 1986). We observed alterations in the expression of various putative markers of chemically induced liver carcinogenesis (Park et al. 2011), including *Cdkn1a*, *Mdm2*, *Ces5*, *Trp53inp1*, *Ccng1*, *Cbr1*, *Notch1*, *Nqo1*, *Nfe2l2*, and *Ephx*, in the livers, lungs, and forestomachs of mice treated with BaP in our 3- and 28-day experiments. Some of these genes (*Cdkn1a*, *Mdm2*, and *Nqo1*) have also been shown to be upregulated by BaP in human cells (Buick et al. submitted; Hockley et al. 2006). Additional global gene expression analyses using *Cyp1a1* and *Cyp1b1* knock-out mice, fed BaP, stress the importance of detoxication enzymes and identify potential cancer biomarker genes (Shi et al. 2010, Gálvez-Peralta et al. 2013). However, more work is required to determine which gene expression changes are cancer biomarkers that can be predictive of tumorigenesis using subchronic bioassays and in vitro testing. We believe that the toxicogenomics data point to strong candidates for hypothesis-based testing. Moreover, the findings suggest that toxicogenomics profiling has the potential to become a valuable predictive tool for hazard identification and risk assessment of chemical carcinogens.

Other potential modes of action and modulating factors. It is widely accepted that toxicants may affect multiple cellular processes and operate via more than one MOA, potentially leading to several adverse outcomes (Woodruff et al. 2008). In keeping with this model, our toxicogenomics data provide insight into other putative MOAs associated with BaP exposure that may be relevant to both cancer and non-cancer adverse outcomes as well as modulating factors that may exacerbate the effects of our proposed MOA. These include perturbation of circadian rhythm, pro-immune-mediated cellular transformation, other receptor-mediated signaling pathways, and immunosuppression. These significantly enriched biological processes may be working independently of our proposed MOA to induce cancer or non-cancer endpoints, or they may be involved in amplifying the effects of our proposed MOA. The toxicogenomics data revealed multiple signaling pathways affected by BaP, which would be tedious and impractical to identify using standard experimental approaches exploring individual apical endpoints. The relevance of these signaling pathways to BaP-mediated tumorigenesis must be examined in more detail in order to determine their relevance in toxicological response.

Uncertainties, inconsistencies, and data gaps. As BaP is a well-known genotoxic carcinogen (IARC group 1), there are relatively few uncertainties relating to the cancer MOA presented in this case study. BaP is genotoxic to a large number of tissues/species and to human cells *in vitro*. There are no significant inconsistencies noted in the literature relating to the required metabolic activation and genotoxicity/mutagenicity of BaP (reviewed in IARC 2010). Thus, the relationship between mutagenicity and cancer is well established. A major limitation leading to uncertainty in RA1 and RA2 is the lack of available human epidemiological data to support genotoxicity and carcinogenicity in humans. This stems from the fact that BaP is generally present in mixtures and thus measured effects cannot be distinguished from the other mixture components and lifestyle factors. In addition, the human AHR is known to have a lower binding affinity for BaP (Harper et al. 2002, Okey et al. 2005); therefore, it is unclear how susceptible humans are to BaP-mediated carcinogenicity. Evaluation of toxicogenomics profiles in combination with genotoxicity/mutagenicity studies in human cell culture models derived from different donors would capture a broad array of potential inter-individual susceptibility and could be useful for addressing some of these data gaps and human relevance.

Confidence in the proposed mode of action and selection of the point of departure for risk assessment (RA2)

Our confidence in the proposed MOA is high because:

- 1) Multiple studies support our proposed key events in the animal MOA, including our three genomics studies *in vivo* on different mouse strains and tissues and a large database of published apical studies.
- 2) In general, all key events in the animal MOA are plausible in humans and are supported by our human cell culture study and published information.
- 3) Apical and toxicogenomics data satisfy the Bradford–Hill criteria (Table 7). Increasing exposure to BaP results in an increased magnitude of genotoxic, toxicogenomic, and

carcinogenic effects. The highest dose used in the toxicogenomics studies (300 mg/kg-bw per day for 3 days) represents a total BaP dose of 900 mg/kg-bw, which is less than the total BaP dose of ~1800 mg/kg-bw that causes tumors in rat livers (2-year cancer bioassay, 5 days per week treatment with 3 mg/kg-bw per day; Wester et al. 2012). The toxicogenomics data support the temporal association between key events and adverse outcome in the proposed MOA. The proposed MOA is consistent with knowledge in the literature about AHR agonists and the well-established effects induced by BaP.

To summarize, the genomics-informed MOA consists of AHR activation, induction of AHR target genes, BaP metabolism, DNA adducts, and DNA damage. Replication of unrepaired DNA damage can yield mutations; if mutations occur in proto-oncogenes or tumor suppressor genes, they can cause tumorigenesis. Thus, earlier key events (1–3) in this MOA that represent adaptive responses reflecting normal homeostatic responses to xenobiotic exposure do not necessarily lead to tumorigenesis on their own. Similarly, DNA adducts and damage are not necessarily adverse effects because of cellular stress response and DNA repair mechanisms that cope with such damage. However, mutations are irreversible and increase the likelihood of tumorigenesis. Therefore, our POD selection was based on DNA adducts and damage because it precedes the committed step in order to adequately protect against carcinogenic outcome. This approach was applied in order to be conservative and protect against subsequent adversity.

Table 8 provides BMD/BMDLs at the 10th percentile for all affected genes in the IPA pathways deemed relevant for each key event. While no formal guidelines exist for considering transcriptional BMDs in risk assessment, the BMD/BMDL ratio for traditional toxicity endpoints is an indication of uncertainty of a BMD estimate (Muri et al. 2009). It has been suggested that BMDL values reflect uncertainty of the data (e.g., due to measurement errors) and that BMD/BMDL ratios of < 2 indicate little uncertainty (Muri et al. 2009). We selected a BMD/BMDL ratio of < 10 as being acceptable to be conservative and also as an attempt to capture the relatively dynamic (and hence, perhaps, more prone to measurement errors) nature of gene expression data, compared to apical data. Further investigations are required to establish appropriate BMD/BMDL ratios for transcriptomics data.

The lowest transcriptional IPA pathway BMDL for key event 4 for the liver was “Cell Cycle: G1/S Checkpoint Regulation” at 1.0 mg/kg-bw per day (Table 8). The lowest BMDLs for key event 4 in the lungs and forestomach were the IPA pathways “Cell Cycle: G2/M DNA Damage Checkpoint Regulation” and “p53 Signaling” at 3.7 and 7.4 mg/kg-bw per day, respectively (Table 8). For the liver, lungs, and forestomach, 18, 5, and 40 genes were modeled (i.e., had adequate BMD fits), respectively (Supplementary Table 7 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>).

Genomics-only approach (RA3)

The goal of RA3 is to determine whether toxicogenomics information (in the absence of any other toxicological data) can be used to develop an MOA and inform a POD for risk

assessment. This approach is particularly useful for data-poor chemicals. To this end, in RA3 we assume that we have no knowledge of BaP other than its structure and solubility. We propose an MOA and select a POD for this uncharacterized chemical using only the available genomics data. We analyzed our toxicogenomics data using a commercially available pathway analysis tool, IPA, but other publicly available data analysis tools (e.g., the freely available DAVID) can also be used. Bioinformatics tools were used to identify the most enriched pathways and biological processes associated with exposure to BaP. The development of our putative MOA was based on the degree of enrichment (i.e., the top affected pathways/processes), published literature on the biology of these pathways, the doses, and time points at which they were affected and their BMDs. Support from other published BaP genomics studies were also used to support the findings.

Proposed mode of action

Our proposed MOA for BaP using exclusively genomics information (Figure 7) was analyzed for dose-response and temporal concordance (Table 9), and BMD/BMDLs at the 10th percentile of all affected genes in an IPA pathway for each proposed key event was determined (Table 9). The genomics information used to construct this MOA includes fold change in transcriptional response of BaP-exposed compared to control rodents or human cells for individual genes, as well as functional pathway analysis of all significantly differentially expressed genes at each dose and time point. Forestomach data were not included in the initial analysis because only samples from the 28 + 3 day regimen were available. However, forestomach data were used to support the MOA generated from the lung and liver data, and genes were modeled to derive BMDLs.

The top canonical pathways in liver and lung tissues (identified using IPA) perturbed in mice exposed to the highest doses of BaP in our experiments (150–300 mg/kg-bw per day) included Nrf2-mediated response to oxidative stress, glutathione metabolism, AHR signaling, and xenobiotic metabolism (Figures 8 and 9). To determine the appropriate order of these pathways to construct a putative MOA, we examined the dose-response and temporal concordances for changes in the transcriptional responses of genes within these pathways. At the lowest dose and time point we found changes in AHR regulated genes, while at the next highest dose we detected changes in genes related to Nrf2-mediated responses to oxidative stress and DNA damage response (occurring at the later time point, 28 days, and at higher doses). Thus, we proposed the four key events described below.

Key event 1: Activation of AHR signaling. At our lower BaP doses (5 and 50 mg/kg-bw per day), significant changes in transcription were found in genes involved in AHR signaling in both the liver (Cyp1a1, Gpr97, Slc46a3, and AHRR) and lungs (Cyp1a1 and Cyp1b1). Activation of AHR signaling plays two roles in response to a toxin that depends on the individual ligands: (1) a transcriptomic response that alters cell signaling; and (2) an adaptive response resulting in metabolism of endogenous and exogenous chemicals. Microarray studies have significantly contributed to the identification of gene products that are regulated by the AHR (reviewed in Denison et al. 2011). The transcriptomic response plays a role in halogenated aromatic hydrocarbon-associated carcinogenesis, whereas metabolism of toxicants to reactive metabolites is implicated in PAH-induced carcinogenesis. CYP1A1 and CYP1B1 show different substrate preferences and can catalyze different stereospecific oxidations (Murray et al. 2001).

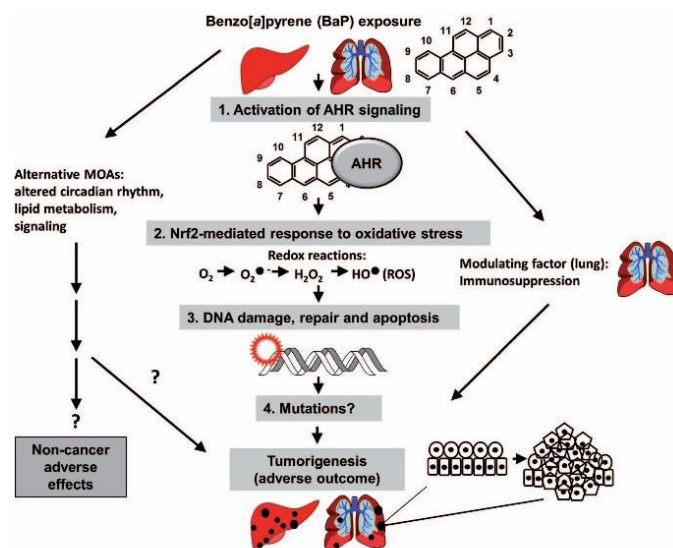


Figure 7. RA3: Carcinogenic MOA of BaP in rodent liver, lungs and forestomach developed using toxicogenomics data exclusively. In addition to the main genotoxic MOA (gray boxes), other factors are plausible based on the data. Redox reactions are unbalanced, showing conversion of molecular oxygen to reactive oxygen species (ROS; Section "Metabolism"). Key events are numbered 1–4 and schematically represented.

Table 9. RA3: Dose-response, temporal concordance and lowest BMD₁₅₀/BMDL₁₅₀^S (mg/kg-bw per day) at the 10th percentile for all affected genes in an IPA pathway for each proposed key event in benzo(a)pyrene's mode of action.

Dose (mg/kg-bw per day)	Key event 1: Activation of AHR signaling				Key event 2: Nrf2-mediated oxidative stress response				Key event 3: DNA damage, repair and apoptosis			
	Liver	Lungs	Fore stomach		Liver	Lungs	Fore stomach		Liver	Lungs	Fore stomach	
5	<i>Cyp1a1</i> 25.5/ns <i>Gpi97</i> 1.8/ns <i>Slc46a3</i> 1.9/ns <i>Cyp1a1</i> 110/ns/ns	<i>Cyp1a1</i> 46.9/–7.5 <i>Cyp1b1</i> 10.4/1.5 <i>Cyp1a1</i> 103/–1.5/ns <i>Cyp1b1</i> 23.5/1.7/ ns <i>Gsta1</i> ns2.4/ns	<i>Gsta1</i> 2.5 <i>Ces5</i> 1.6		<i>Nqo1</i> 1.9/ns/ns <i>Srxn1</i> 1.5/ns/ns <i>Ugt2b15</i> 2.5/ns/ns <i>Ugth</i> 2.0/ns/ns <i>Akr1b15</i> 1.6/ns/ns	<i>Nqo1</i> 5.4/ns <i>Srxn1</i> 1.8/ns <i>Nqo1</i> 27.0/ns/ns <i>Srxn1</i> 7.1/ns/ns			<i>Bax</i> ns/ns/2.5 <i>Ccnd1</i> ns/ns/1.8 <i>Ccng1</i> ns/ns/3.0 <i>Cdkn1a</i> 2.2/ns/8.1 <i>Gadd45g</i> 1.8/ns/ns <i>Polk</i> ns/ns/1.6 <i>Serpine1</i> ns/ns/3.2 <i>Znat3</i> ns/ns/1.7 <i>Pmaip1</i> ns/ns/1.7 <i>Gadd45g</i> 1.5/ns	<i>Bax</i> ns/ns/2.5 <i>Ccnd1</i> ns/ns/1.8 <i>Ccng1</i> ns/ns/3.0 <i>Cdkn1a</i> 2.2/ns/8.1 <i>Gadd45g</i> 1.8/ns/ns <i>Polk</i> ns/ns/1.6 <i>Serpine1</i> ns/ns/3.2 <i>Znat3</i> ns/ns/1.7 <i>Pmaip1</i> ns/ns/1.7 <i>Gadd45g</i> 1.5/ns	<i>Bax</i> ns/ns/2.5 <i>Ccnd1</i> ns/ns/1.8 <i>Ccng1</i> ns/ns/3.0 <i>Cdkn1a</i> 2.2/ns/8.1 <i>Gadd45g</i> 1.8/ns/ns <i>Polk</i> ns/ns/1.6 <i>Serpine1</i> ns/ns/3.2 <i>Znat3</i> ns/ns/1.7 <i>Pmaip1</i> ns/ns/1.7 <i>Gadd45g</i> 1.5/ns	<i>Bax</i> ns/ns/2.5 <i>Ccnd1</i> ns/ns/1.8 <i>Ccng1</i> ns/ns/3.0 <i>Cdkn1a</i> 2.2/ns/8.1 <i>Gadd45g</i> 1.8/ns/ns <i>Polk</i> ns/ns/1.6 <i>Serpine1</i> ns/ns/3.2 <i>Znat3</i> ns/ns/1.7 <i>Pmaip1</i> ns/ns/1.7 <i>Gadd45g</i> 1.5/ns
150	<i>Cyp1a1</i> 146.7/ns <i>Gpi97</i> 1.9/ns <i>Slc46a3</i> 2.5/ns <i>AHRR</i> 2.3/ns	<i>Cyp1a1</i> 179.3/14.7 <i>Cyp1b1</i> 70.9/2.4 <i>Gsta2</i> 1.8/ns <i>Gsta1</i> 1.8/1.6			<i>Nqo1</i> 4.5/2.3 <i>Srxn1</i> 3.2/ns <i>Ugt2b15</i> 3.2/ns <i>Ugth</i> 3.4/ns <i>Akr1b15</i> 2.6/ns	<i>Nqo1</i> 48.2/1.9 <i>Srxn1</i> 12.6/ns <i>Ugt1a9</i> ns/–2.3			<i>Cdkn1a</i> 1.7/ns <i>Ccng1</i> ns/1.5 <i>Gadd45g</i> 4.6/ns	<i>Cdkn1a</i> 7.02.4 <i>Ccng1</i> 1.5/1.6 <i>Mdm2</i> 1.6/ns <i>Polk</i> 1.5/ns <i>Bax</i> 1.8/ns <i>Sesn2</i> 1.6/1.7 <i>Gadd45a</i> 1.5/ns	<i>Cdkn1a</i> 7.02.4 <i>Ccng1</i> 1.5/1.6 <i>Mdm2</i> 1.6/ns <i>Polk</i> 1.5/ns <i>Bax</i> 1.8/ns <i>Sesn2</i> 1.6/1.7 <i>Gadd45a</i> 1.5/ns	<i>Cdkn1a</i> 7.02.4 <i>Ccng1</i> 1.5/1.6 <i>Mdm2</i> 1.6/ns <i>Polk</i> 1.5/ns <i>Bax</i> 1.8/ns <i>Sesn2</i> 1.6/1.7 <i>Gadd45a</i> 1.5/ns
300	<i>Cyp1a1</i> 165.4/9.0 <i>Gpi97</i> ns/ns <i>Slc46a3</i> 2.4/1.7 <i>AHRR</i> 3.0/ns	<i>Cyp1a1</i> 210.1/42.9 <i>Cyp1b1</i> 161.2/21.8 <i>Gsta2</i> 2.2/ns <i>Gsta1</i> 2.5/ns			<i>Nqo1</i> 8.6/4.3 <i>Srxn1</i> 6.3/ns <i>Ugt2b15</i> 3.7/ns <i>Ugth</i> 4.3/ns <i>Akr1b15</i> 3.0/1.5	<i>Nqo1</i> 52.6/6.6 <i>Srxn1</i> 16.2/1.6			<i>Gadd45g</i> 12.6/ns <i>Cdkn1a</i> 5.0/ns <i>Ccng1</i> 2.2/2.3 <i>Ccnd1</i> –1.5/2.2 <i>Mdm2</i> 1.5/ns <i>Ddit4</i> 3.9/ns	<i>Gadd45g</i> 2.9/–1.5 <i>Cdkn1a</i> 15.8/4.5 <i>Ccng1</i> 2.2/2.3 <i>Ccnd1</i> –1.5/2.2 <i>Mdm2</i> 2.0/1.6 <i>Polk</i> 2.1/1.9 <i>Bax</i> 2.6/2.6 <i>Sesn2</i> 2.8/2.3	<i>Gadd45g</i> 2.9/–1.5 <i>Cdkn1a</i> 15.8/4.5 <i>Ccng1</i> 2.2/2.3 <i>Ccnd1</i> –1.5/2.2 <i>Mdm2</i> 2.0/1.6 <i>Polk</i> 2.1/1.9 <i>Bax</i> 2.6/2.6 <i>Sesn2</i> 2.8/2.3	<i>Gadd45g</i> 2.9/–1.5 <i>Cdkn1a</i> 15.8/4.5 <i>Ccng1</i> 2.2/2.3 <i>Ccnd1</i> –1.5/2.2 <i>Mdm2</i> 2.0/1.6 <i>Polk</i> 2.1/1.9 <i>Bax</i> 2.6/2.6 <i>Sesn2</i> 2.8/2.3
Lowest BMD/BMDL pathway	20/4.3 Xenobiotic metabolism signaling	17/27.7 Aryl hydrocarbon signaling	13.5/7.9 Xenobiotic metabolism signaling		29.7/6.9 Nrf2-mediated oxidative stress response	28/18.6 Nrf2-mediated oxidative stress response	15.4/9 Nrf2-mediated oxidative stress response		8.1/1 Cell cycle: G1/S checkpoint regulation	14.8/3.7 Cell cycle: G2/M DNA damage checkpoint regulation	11.4/7.4 p53 signaling	

The values shown with each gene represent fold changes (> 1.5 fold change from control and FDR-adjusted p-value < 0.05 ; ns indicates no statistical significance) at that dose for: (3 days + 4 hours/3 days + 24 hours). An additional timepoint is indicated for the 50 mg/kg-bw/day dose (3 days + 4 hours/3 days + 24 hours/28 days + 3 days). The darkening of the cells indicates the expected progression of gene expression responses from early time points to later time points and with increasing dose as support for progression of proposed key events.

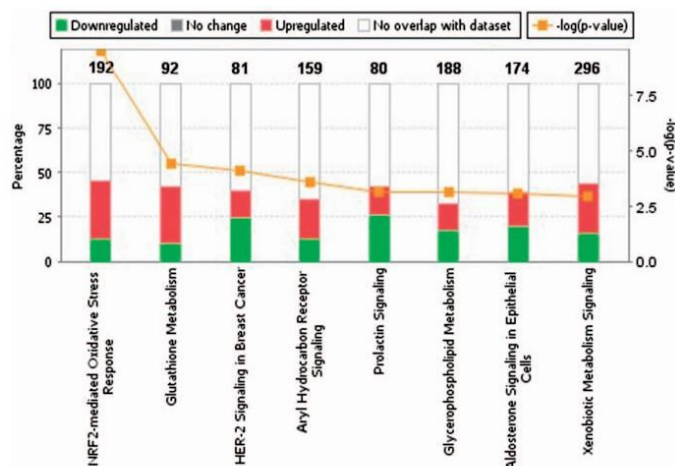


Figure 8. RA3: Top eight canonical pathways affected by BaP in the livers of mice. The data shown are for mice treated with 300 mg/kg-bw per day for 3 days and sacrificed 4 h after the last exposure (Yauk et al. 2011). The left Y-axis represents the percentage of genes in each pathway, the numbers at the top of the graph indicate the number of genes in each pathway and the right Y-axis shows the negative log (p -value) of the Fisher's exact test performed by Ingenuity Systems Analysis software.

These enzymes are known to metabolize certain xenobiotics into reactive metabolites. Upregulation of the AHR-regulated genes Cyp1a1, Cyp1b1, Gpr97, and Slc46a3 at the lowest dose strongly suggests that key event 1 is the activation of

AHR signaling. The function of Slc46a3 (solute carrier family 46, member 3) is unknown, but it appears to be regulated by Cyp1a1 (Dragin et al. 2008). Increased Gpr97 (G protein-coupled receptor 97) has also been observed in mice treated with

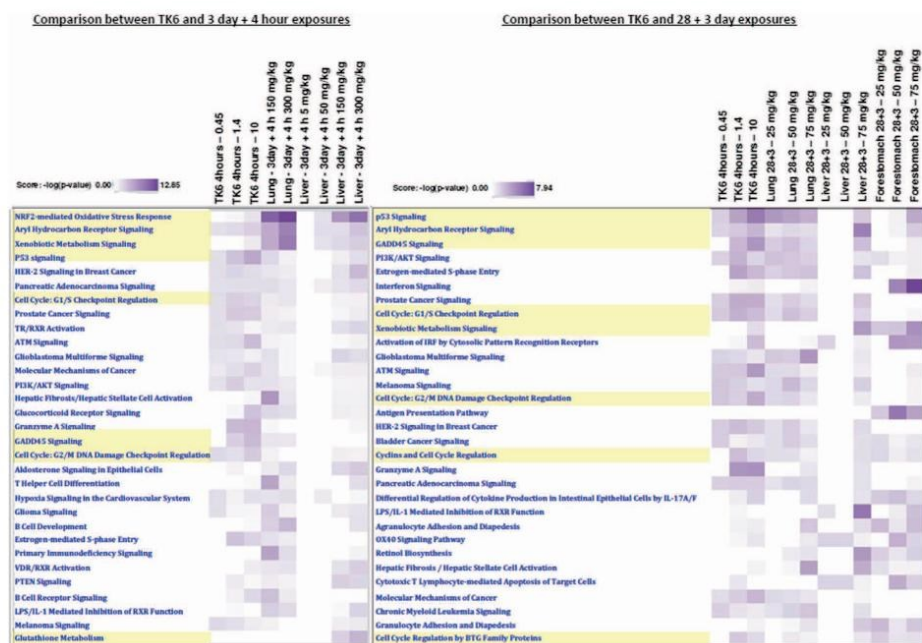


Figure 9. Heatmap analysis showing Ingenuity® Pathway Analysis (IPA) canonical pathways significantly affected in BaP-exposed TK6 cells and livers, lung, and forestomach of mice exposed to BaP. The left panel (A) compares *in vitro* TK6 pathways with lung and liver tissue from acute exposures (3 days + 4 h), whereas the right panel (B) compares the *in vitro* TK6 pathways with lung, liver, and forestomach tissue from the subchronic exposures (28 + 3 days). Pathways highlighted in yellow are consistent with the genotoxic MOA presented in this document. P -value scores (indicated by gradation of purple colors) are a measure of the significance of the pathway's association with the dataset.

the prototypical AHR inducer TCDD (Tijet et al. 2006). Unlike Cyp1a1, the relevance of Gpr97 and Slc46a3 to the MOA of BaP is not obvious, and the expression of these genes lacked a dose–response relationship (Table 9B). The AHR repressor (AHRR) is a negative regulator of the transcriptional activity of AHR. AHRR is also under the regulation of the AHR, and its expression was also increased in a dose-dependent manner in the liver. Therefore, our data suggest that AHR signaling, and the subsequent induction of xenobiotic metabolism, is an early key event in the MOA of BaP-induced toxicity.

There is support for the plausibility of this key event in humans using genomics data. For example, transcriptomic profiles from cultured human HepG2 and MCF-7 cells demonstrate the upregulation of CYP1A1 and CYP1B1 in response to BaP treatment (Hockley et al. 2006, Van Delft et al. 2010).

Thus, activation of the AHR, and subsequent xenobiotic metabolism signaling, is a logical early key event in BaP-mediated toxicity that was identifiable using genomics data in the absence of apical data.

Key event 2: Nrf2-mediated response to oxidative stress. “Glutathione metabolism” and “Nrf2-mediated oxidative stress response” (~50% of the 192 genes in this pathway responded) were the top two pathways affected by BaP treatment in mouse liver and lungs (Figure 8). Expression of high levels of P450 enzymes, such as CYP1A1 and CYP1B1, results in increased metabolism of chemicals and the subsequent generation of ROS and oxidative stress. Nrf2 is a transcription factor that mediates cellular response to oxidative stress and xenobiotics in what is known as the AHR–Nrf2 gene battery (Yeager et al. 2009), including Nqo1 and Ugt (Kaspar et al. 2009, Thimmulappa et al. 2002). Many of the Nrf2-controlled genes aid in the repair of macromolecules damaged by ROS and produced during xenobiotic metabolism, as well as detoxication processes, through the conjugation of water-soluble moieties to xenobiotic metabolites produced by phase I enzymes. Maintenance of the appropriate cellular redox status is crucial, and this is achieved primarily by a high (millimolar) cellular content of glutathione. Downstream targets of Nrf2-dependent gene expression were altered at 50 mg/kg-bw per day in liver and 5 mg/kg-bw per day in lungs, consistent with the idea that AHR-mediated CYP1A1 induction is required to initiate BaP metabolism. At the second lowest dose (50 mg/kg-bw per day), 5 of the 34 genes affected 4 h posttreatment were upregulated Nrf2-dependent genes (Nqo1, Ugdh, Srxn1, Akrib1b15, and Ugt2b15), in addition to upregulation of Nrf2 itself. The transcriptional levels of all five of these genes in the liver, as well as Nqo1 and Srxn1 in the lungs, increased with increasing BaP dose. Nrf2-mediated response to oxidative stress is thus proposed to be key event 2 in both the lungs and liver, based on the mouse genomics data alone.

Toxicogenomics profiling of human cells supports this key event. Indeed, strong upregulation of phase II metabolism genes is observed in cultured human HepG2 and MCF-7 cells (Hockley et al. 2006, Van Delft et al. 2010). Furthermore, the Nrf2 signaling pathway was among the main upregulated gene clusters in BaP-treated HepG2 cells (Van Delft et al. 2010). Thus, genomics data in human cells in culture support the relevance of this key event to humans.

Epidemiological research has shown that certain *Nrf2* gene polymorphisms are associated with an increase in the incidence of breast cancer and decreased survival outcome in breast cancer patients (Hartikainen et al. 2012). This finding suggests that certain perturbations in the Nrf2 signaling pathway are relevant to carcinogenesis in humans.

Overall, in the absence of apical endpoints, the genomics data suggest that the induction of the AHR initiates xenobiotic metabolism of the chemical to a toxic metabolite or produces ROS, which leads to the induction of the Nrf2-mediated signaling. In the absence of data on tissue levels of the chemical and its metabolites and cellular ROS levels, it is not possible to discern between these two mediating factors. Thus, we have assigned the generic name “Nrf2-mediated response to oxidative stress” to this key event. Quantitative structure–activity relationship (QSAR) modeling in parallel with analysis of the genomics data would be beneficial to support the genomics data analysis for data-poor chemicals.

Key event 3: DNA damage, repair, and apoptosis. Following the induction of an oxidative stress response, it is logical to suspect that DNA damage response and apoptosis may ensue. We previously reported a robust response in the p53 signaling pathway in gene expression profiles from the livers (Malik et al. 2012), lungs (Labib et al. 2012), and forestomachs (Labib et al. 2013) of mice treated for 28 days with BaP. Multiple p53-dependent genes (including Cdkn1a, Ccng1, and Ccnd1) were similarly dose-dependently upregulated in mouse liver and lungs at the 3-day time point. In addition, transcript levels of Mdm2 (a known p53 inhibitor), Ddit4, and Gadd45g (growth arrest and DNA damage inducible 45 gamma) were significantly and dose-dependently upregulated in mouse liver and lungs after 3 days of BaP exposure. p53 is a transcription factor that is referred to as “the guardian of genome” and mediates cellular processes, including cell cycle arrest, DNA repair, and apoptosis (reviewed in Meek 2009). Several genes implicated in these three events were differentially expressed in the 28-day lung, liver, and forestomach gene expression profiles (Table 9), such as Cdkn1a and Ccng1 (cell cycle arrest), Polk (DNA repair), Bax, and Pmaip1 (apoptosis). The upregulation of these genes strongly suggests activation of p53 in response to DNA damage. Taken together, these results suggest that BaP exposure causes DNA damage through the generation of DNA-reactive metabolites by AHR-regulated metabolic enzymes. Based on these findings, we would speculate that the chemical operates through a genotoxic MOA and thus has a high probability of being carcinogenic.

Our work in cultured human TK6 cells supports that BaP is genotoxic in these human cells. Our DNA microarray analysis revealed that over 60% of the genes in the TP53 pathway responded to BaP treatment in TK6 cells (Buick et al. submitted). In addition, the toxicogenomics profiles of treated TK6 cells classified BaP as a genotoxic compound that clusters with other known DNA-reactive mutagenic carcinogens (Buick et al. submitted). Toxicogenomics analyses of HepG2 cells have also shown activation of the TP53 signaling pathway and perturbations in apoptosis-related gene sets as a result of exposure to BaP (Van Delft et al. 2010). More recently, activation of oxidative stress and DNA damage response pathways by BaP was confirmed by an independent toxicogenomics RNA-

Seq technology (Van Delft et al. 2012). We note that our toxicogenomics data are consistent with the observed increases in cytotoxicity, apoptosis, and induction of micronuclei that we measured in the TK6 cells treated with BaP (Figure 4).

Key event 4: Mutations. As described in RA2, there are various possible fates of cells with DNA damage including error-free DNA repair or cell death via apoptotic processes. DNA lesions that escape repair may cause mutations or chromosomal aberrations. In contrast to DNA damage, a mutation is a permanent change in the DNA sequence that cannot be repaired. Thus, mutation induction can be considered a committed step in this MOA as described in RA2. Although induction of DNA damage response pathways is not a direct measure of mutation or chromosome aberrations, induction of these pathways is a well-established response to pre-mutagenic lesions and thus provides evidence that mutagenicity is a potential outcome of the exposure. Indeed, many studies have used omics approaches to show that the induction of gene expression changes in DNA damage response pathways is highly informative of genotoxicity (e.g., Sakai et al. 2014, Derks et al. 2014, Waters et al. 2010, our own work in TK6 cells). As noted for the previous proposed key event, our case study data clearly show induction of expression changes in DNA damage response pathways in the tissues of exposed mice and human TK6 cells, inferring that the agent may be mutagenic. Thus, even though published reports of BaP's *in vivo* mutagenicity are not considered here, we argue that our genomics analysis provides evidence that the toxicant is mutagenic *in vivo* in rodents and that this is likely a key event in the MOA.

Other potential modes of action and modulating factors. Other pathways affected by BaP treatment may represent biological responses that result from complex interplays between signaling pathways affected by BaP and its metabolites. Circadian rhythm and lipid metabolism are examples of BaP-inducible pathways (also affected by BaP in other toxicogenomics studies; Hockley et al. 2006, Van Delft et al. 2010) that are difficult to fit into a carcinogenic MOA. In addition, gene signatures related to immunotoxicity were prevalent in the lung and forestomach datasets, indicative of immunosuppression and proinflammatory responses, respectively (Halappanavar et al. 2011, Labib et al. 2012, 2013). Therefore, our genomics data identify immunosuppression as a possible hazard that is a plausible modulating factor in the MOA for BaP-induced carcinogenicity in the lungs and the recognition of excessively accumulated metabolites as potential pathogens in the forestomach. These perturbations may impact a diverse array of toxicities outside of cancer. However, their expression was affected at higher doses compared with those activated in key events 1 and 2.

Although simplistic, our analysis suggests that the genotoxic MOA is the most relevant for health risk assessment of BaP-induced carcinogenicity, especially for extrapolations to low doses.

Uncertainties, inconsistencies, and data gaps. Unlike RA2, there are many uncertainties in the genotoxic MOA that we proposed for BaP based solely on toxicogenomics data. This is due to many reasons including: (a) there were only a handful

of toxicogenomics studies that were conducted on BaP; (b) the studies were conducted in only a few different cell culture and animal models; (c) the studies were focused on different tissues; and (d) the experimental designs were not optimal for risk assessment (e.g., because of suboptimal sampling time and high doses). Moreover, in the complete absence of any ADME, PBPK, or apical data, RA3 is limited. However, we expect that toxicogenomics data alone will be invaluable in prioritization, hazard identification, and determination of other important apical tests, as we discuss in Section "Next steps" below.

For example, although it is clear that the chemical agent induces AHR signaling followed by oxidative stress and a DNA damage response, it is unclear whether the metabolism/detoxification occurs, whether the parent compounds, metabolites, or ROS alone are the genotoxic agents and whether the DNA damage indeed leads to DNA mutations (as DNA damage can be repaired). Given our understanding of the relationship between mutagenicity and carcinogenicity, complementary knowledge on the ability of the chemical to cause mutations will always be critical.

One very general uncertainty lies in the application of mRNA profiling on its own since biological processes are carried out at higher levels of organization. Validation at the phenotype level (anchoring to some apical endpoint(s)) is still required at this time and thus we would recommend targeted testing following a toxicogenomics study. We expect that the predictive capabilities of toxicogenomics will improve as more work is completed in the field to link pathway perturbations measured at the RNA level to apical endpoints and adverse effects. The use of toxicogenomics profiles is promising, as there is a great deal of consistency across toxicogenomics studies in various species. These support the MOA proposed here. Nevertheless, at this time the uncertainties are great.

A major gap in the toxicogenomics approach was the limited number of tissues examined in rodents. Chemicals can and do demonstrate tissue-specificities (and interaction occurs among tissues in MOAs), thus it must be established that the tissues selected are representative of target tissues (see Section "Limitations of the data used in this case study" for further discussion). Given that we observed clear evidence of genotoxicity in every tissue studied in mice, and in cultured human cells, we can fairly confidently say that this chemical induces genotoxicity, and thus most likely tumors, in many tissues. Moreover, our TK6 signatures for the chemical are aligned with highly genotoxic agents, predicting that the chemical is genotoxic. This signature is currently undergoing extensive validation through the Health and Environmental Sciences Institutes.

Overall, there were few toxicogenomics studies and thus it is clear that the gaps and uncertainties will be much larger for RA3. A required next step in a toxicogenomics risk assessment would be targeted testing of the associated adverse effects predicted from the signature, and this was not conducted here.

Quantitative structure–activity relationship

We explored whether *in silico* approaches could provide support to our toxicogenomics analysis for RA3. As noted, exposure of mice to BaP resulted in significantly increased expression of CYP1A1 and a robust DNA damage response

in lungs and liver, indicative of the genotoxicity of BaP. To provide supporting data to demonstrate that BaP is a substrate of CYP1A1 that is metabolized to genotoxic reactive products, an analysis of the correlation between the structural parameters of BaP and other similar compounds with different abilities to be activated by CYP1A1 and inflict DNA damage would be helpful. Indeed, such a correlation analysis was conducted previously for several PAHs, excluding BaP (Lewis 1987, Wan et al. 2006). The models successfully predicted the ability of PAHs to induce CYP1A1 via the AHR, their hydroxylation to reactive intermediates, their carcinogenic potential (Lewis 1987), and their ability to induce apoptosis (Wan et al. 2006). The latter model was validated by a flow cytometry-based apoptosis assay, which yielded positive results for BaP and negative results for 2-methylanthracene, which lacks a bay region. The bay region was demonstrated to be important for the aforementioned biological activities of PAHs. In our case, had we had no other genotoxic endpoints (e.g., DNA adduct data), QSAR could confirm our gene expression data, pointing to BaP-mediated DNA damage. Therefore, QSAR methods could provide strong support for toxicogenomics data and may aid in extending toxicogenomics findings to other related compounds.

Rationale for the proposed mode of action and selection of the point of departure for risk assessment (RA3)

Using toxicogenomics-derived data in combination with supporting evidence from QSAR models, we propose a genotoxic MOA that includes: (1) the induction of xenobiotic metabolism genes via activation of the AHR pathway; (2) metabolic activation leading to reactive metabolites and ROS (Nrf2-mediated response to oxidative stress); and (3) DNA damage, repair, and apoptosis (p53 signaling) and (4) mutations. While this genomics approach did not identify the full mechanism underlying BaP toxicity, we believe that genomics identified the major key events in BaP-induced tumor formation. The proposed MOA is supported by in-house as well as published animal and human genomics data. The key events in the MOA passed the Bradford–Hill criteria (Table 7): (1) dose–response relationship for all key events were consistent with expectations; (2) key events occurred in a logical sequence; (3) the results were consistent across various experiments; (4) the events are biologically plausible; and (5) the key events are supported by human data. Based on the same logic used in RA2 (Section “Confidence in the proposed mode of action and

selection of the point of departure for risk assessment (RA2)”), we chose key event 4 as the “committed step,” increasing the likelihood of achieving an adverse outcome in the genotoxic MOA. Therefore, the preceding key event was selected as the POD. The lowest BMDL values for the key event preceding the committed step in the liver, lungs, and forestomach were 1.0, 3.7, and 7.4 mg/kg-bw per day, respectively (representing the IPA pathways “Cell Cycle: G1/S Checkpoint Regulation”, “Cell Cycle:G2/M DNA Damage Checkpoint Regulation” and “p53 Signaling,” respectively; Table 9).

Alternative genomics approaches

Thomas and colleagues (Thomas et al. 2007a, 2011, 2012) propose that the pathways or biological functions with the lowest median gene expression BMD values correlate well with BMDs for apical cancer and non-cancer endpoints. This approach is especially useful for chemicals that act *via* nonselective mechanisms, perturbing multiple different biological pathways (Thomas et al. 2013). If we use an approach similar to Thomas et al. (2011), whereby the BMDL for the most sensitive pathway or process (i.e., the one with the lowest BMDL value at the 10th percentile) is selected for establishing the POD (after removing pathways in which fewer than five genes were significant), we achieve the following BMDLs: (1) liver IPA pathway “Notch Signaling” BMDL of 0.2 mg/kg-bw/day (seven genes were modeled); (2) lung IPA pathway “Cellular Effects of Sildenafil (Viagra)” BMDL of 2.1 mg/kg-bw/day (nine genes were modeled); and (3) forestomach IPA pathway “Phenylalanine Degradation IV (Mammalian, via Side Chain)” BMDL of 4.5 mg/kg-bw/day (six genes were modelled) (Table 10). Thus, using an approach similar to Thomas et al. (2011) our POD (assuming no knowledge of the MOA) would be 0.2 mg/kg-bw per day.

An additional toxicogenomics approach would select the lowest POD associated with the MOA without defining a committed key event. Using this approach the PODs for liver, lungs, and forestomach would be 1.0, 3.7, and 7.4 mg/kg-bw per day (Table 10). These are the same PODs and pathways identified using the RA2 and RA3 approach (selecting the key event preceding the committed key event as the POD).

Discussion and conclusions

Our objective was to compare toxicogenomics and traditional approaches to inform MOA and POD for the risk assessment of BaP in drinking water. Overall, we demonstrate that the

Table 10. Summary of calculated BMD/BMDLs for local (forestomach) and systemic (liver and lungs) effects using traditional and toxicogenomics approaches. Benchmark response is BMD/BMDL for quantal tumor data and BMD/BMDL for continuous mutations and transcriptional data (mg/kg-bw per day).

Approach	Liver		Lungs		Forestomach	
	BMD/ BMDL	Response	BMD/ BMDL	Response	BMD/ BMDL	Response
RA1: Traditional	1.8/1.2	Tumors	0.8*	Tumors	0.8/0.5	Tumors
RA1: Mutations	7.2/4.8	Mutations	2.2/1.4	Mutations	0.5/0.3	Mutations
RA2/RA3: Toxicogenomics-key event preceding the committed step	8.1/1	DNA damage	14.8/3.7	DNA damage	11.4/7.4	p53 Signaling
Toxicogenomics-lowest MOA-associated pathway	8.1/1	DNA damage	14.8/3.7	DNA damage	11.4/7.4	p53 Signaling
Toxicogenomics-lowest pathway [#]	0.3/0.2	notch signaling	15.7/2.1	cellular effects of sildenafil	16.1/4.5	Phe degradation IV

*Caution, based on a LOAEL from coal tar data (Culp et al. 1998)

[#]Similar to the approach of Thomas et al. (2011) but with examination of BMD/BMDLs at the 10th percentile rather than median.

PODs derived from the three approaches were highly similar (Table 10). However, we note that RA1 was based on the collection of decades of research on BaP and integrated two full 2-year cancer bioassays. In comparison, RA3 used fewer than 100 mice in subchronic and acute exposure scenarios, in parallel with analysis of human cells in culture. The approximate cost for these experiments using the applied technologies was \$100,000. However, we note that with improving technologies (e.g., multiplex RNA-sequencing approaches, similar to what has been described by Li and co-workers (2012)), and with integration of toxicogenomics in acute or sub-chronic repeat-dose studies, tissue profiling could be done much more quickly and cost effectively. Thus, for data-poor chemicals, our RA3 data support that implementing a genomics-based risk assessment approach may provide an effective means to reduce cost, time, and animal use in preliminary human health risk assessment. RA2 demonstrates the utility of integrating toxicogenomics data with data from traditional toxicity testing to support POD selection and MOA development. Below, we compare the RA approaches and describe the existing value-added and limitations of toxicogenomics in human health risk assessment.

As a practical case study, we derived tissue-specific MOA-related transcriptional PODs for RA2 and RA3. RA2 and RA3 PODs added value to the MOA by: (1) comparison of the expression profiles of genotoxic and non-genotoxic compounds, which predicted that BaP is genotoxic in human cell lines; and (2) mining the expression signatures to identify measurable key events that are necessary (but not necessarily sufficient) for induction of apical toxicity (phenotypic anchoring) based on the IPCS/ILSI MOA framework (Boobis et al. 2006, Meek et al. 2003, Sonich-Mullin et al. 2001) and Bradford-Hill test for causation (Hill 1965). Once the key events in the MOA were proposed and their BMDs calculated, the "committed step" was proposed, and the key event immediately preceding it was selected as the most relevant POD for risk assessment. This analysis revealed PODs of 1.0, 3.7, and 7.4 mg/kg-bw per day for transcriptional changes in liver, lungs, and forestomach, respectively, for both RAs. In comparison, a comprehensive literature review (RA1) derived apical PODs of 1.2, 0.8, and 0.5 mg/kg-bw per day for tumors for each tissue, respectively (Table 10). Analysis of tissue-specific mutagenicity (lacZ transgene mutant frequency) also yielded similar PODs of 4.8, 1.4, and 0.3 mg/kg-bw per day for liver, lungs and glandular stomach, respectively (Table 10). Thus, the transcriptional PODs are similar to the tumor and mutation PODs, with the largest discrepancy observed for the stomach. However, PODs for stomach are still within an order of magnitude. We believe that these numbers would be more aligned had the samples been collected immediately following the last exposure rather than following a 3-day break for transcriptional profiling, which is supported by the lower BMDs found in liver (derived 4 and 24 h post-exposure). It is also important to note that the PODs for local effects (forestomach) are within an order of magnitude of systemic effects (liver and lungs). This provides some support for the use of surrogate tissues (liver and lungs) to predict response in other tissues (forestomach). However, we caution that our analysis is on a genotoxic carcinogen and this may not apply to chemicals that have a tissue-specific MOA.

In contrast to expectations, MOA-related PODs for transcriptional changes were higher than apical endpoints. These higher transcriptional BMDs may be due to: (1) doses used in the studies producing apical data were more likely to be within the BMR range, whereas transcriptional study doses were high (only one dose was in the BMR range in one study only); (2) shorter exposure duration of the transcriptional studies may require a higher dose of BaP (resulting in a higher BMD) relative to studies in which exposure doses are accumulated over a longer duration (lower BMD) to achieve the same effect; (3) BMD modeling of apical endpoints is slightly more sensitive (settings more customizable) than the BMDExpress modeling of transcriptional endpoints; and (4) genes are part of dynamic and interconnected networks and a gene expression signature is a single snapshot in time representing the complex response of that network, which may not effectively represent each gene's time-dependent response. It is interesting to note that one hesitation to the use of genomics data in risk assessment has been the concern that genomics endpoints would be too sensitive, leading to extremely low proposed exposure thresholds; The PODs within this case study demonstrate that this is unlikely.

The toxicogenomics data used in this case study revealed that early key events that take place within days of initial BaP exposure and that may contribute to carcinogenesis can be detected and used for MOA development and POD estimation. Nesnow et al. (2009, 2011) used toxicogenomics to identify key events in the carcinogenicity of propiconazole in mice following short-term 4-day and 30-day exposure (Nesnow et al. 2009) and 4-day exposure periods (Nesnow et al. 2011). These experiments demonstrate that when analyzing toxicogenomics data for chemicals that perturb a broad spectrum of pathways, an MOA approach may be used as a filter to reveal the most meaningful and practical information occurring at low doses. In general, these MOA-centric BMD values were similar to those derived using an approach applying the most sensitive pathways associated with the MOA or regardless of the MOA (Section "Genomics-only approach (RA3)"; Table 10) (Thomas et al. 2007a, 2011, 2012). Indeed, the latter approach may be useful in preliminary analyses when the MOA is unknown or unclear. However, we propose that estimating BMD values based on a putative MOA is biologically relevant and provides greater confidence in the use of gene expression data and in selecting a gene expression-based POD.

Advantages of a genomics approach

While all three risk assessment approaches identified similar PODs, the genomics approach offers numerous advantages. These include the following:

- 1) Genomics data can be obtained within a much shorter time frame and at a greatly reduced cost relative to conducting a full battery of standardized tests (a major goal of *Toxicity Testing in the 21st Century*; NRC 2007b).
- 2) The approach is highly effective in decreasing animal use (another major goal of *Toxicity Testing in the 21st Century*; NRC 2007b).
- 3) Potential hazards can be identified using acute and subchronic assays and/or by analyzing toxicogenomics data from cultured human cells. Importantly, the top pathways shared between human *in vitro* and mouse

in vivo data are AHR signaling, Nrf2, and p53 signaling and cell cycle signaling, which is in excellent agreement with the genotoxic MOA proposed here (Figure 9). An important lesson from this is that one can significantly minimize costly animal experiments and rapidly identify genotoxic chemicals (and, perhaps, other health hazards) by obtaining toxicogenomics information from cells cultured *in vitro*. For example, our TK6 study correctly identified BaP's genotoxic MOA rapidly (experiments could be completed within 2 weeks) and did not require any animal use. Such an approach (i.e., screening chemicals for hazards using cultured human cells *in vitro*) would be in excellent agreement with the two-tiered approach to toxicity testing proposed by Thomas and colleagues (2013) and would help streamline hazard identification and MOA development for chemicals with little or no toxicity information. Our findings also support the utility of *in vitro* data as an additional line of evidence.

- 4) Target tissue(s) for specific toxicities can be identified at relatively early time points by assessing global gene expression changes in a small battery of tissues that includes the sites of contact and the most frequent targets for chemically induced cancer, neurotoxicity, developmental toxicity, and reproductive toxicity.
- 5) Since the approach does not target a specific toxicity, inclusion of toxicogenomics tests across an informative set of tissues early in a test battery can relatively rapidly inform a large suite of potential adverse effects. Additional case studies on non-cancer endpoints are needed to confirm this.
- 6) Cross-species extrapolations are problematic in risk assessment in general, as well as in approaches applying toxicogenomics. However, we note that as increased understanding of the degree of conservation in pathways between species is gained, toxicogenomics can be used to provide greater insight into human relevance. This is one of the strengths of mechanistic-based approaches. Indeed, this is the rationale behind the use of the MOA/human relevance framework that we applied as described in Section "Identification of key events and proposal of MOA". In addition, comparison of gene expression and pathway changes in animals and human cells provides plausibility of an animal MOA in humans.
- 7) Toxicogenomics data can be used to fill in gaps in the MOA and minimize uncertainties in the dose–response relationships for intermediary steps, as well as to identify new, previously unknown MOAs for a toxicant.
- 8) Toxicogenomics is unbiased, thus preselection of pathways or genes of interest are not necessary.
- 9) The approach may be applicable to mixtures, other chemicals, and other exposure scenarios.
- 10) The expression signatures can be used to derive preliminary MOAs to identify key events in a chemical's risk assessment and derive PODs based on these, rather than excluding that chemical while waiting for lengthy and costly studies, ultimately increasing the number of published risk assessments.

Obstacles to incorporation of toxicogenomics into risk assessment

While the advantages of genomics approaches are numerous, many concepts in this field need to be refined, and many obstacles stand in the way of the incorporation of toxicogenomics into risk assessment:

- 1) Clear guidelines are necessary for the identification of appropriate toxicogenomics datasets that are suitable for risk assessment purposes.
- 2) Determination of which BMDL should be selected as a POD has not been clearly defined. Selecting the lowest transcriptional BMDL from all pathways and tissues is the most conservative approach, but may not logically relate mechanistically to the adverse effect and may be unnecessarily conservative. We propose that an additional option would be to select the lowest BMDL from a pathway that is known to be associated with a preliminary MOA derived from gene expression data or a known MOA/adverse effect.
- 3) Some toxicants perturb a large number of pathways, and thus it can be difficult to know which to select for POD calculations in the absence of apical data.
- 4) Genes without annotations or assignment to a pathway are excluded from toxicogenomic approaches. In addition, the relationship between specific pathways and disease/apical outcomes is not well defined. As further annotation of gene functions and linkages to apical outcomes are completed this will become less of an obstacle.
- 5) A large number of data must be analyzed, and thus some specialized training and software packages are needed.
- 6) Further biological validation is needed to confirm that expression changes truly reflect adverse phenotypes.
- 7) The technologies and bioinformatics approaches are rapidly evolving.
- 8) There is no OECD guideline in place for any gene expression assay.
- 9) There is a need for additional validation. More exercises such as this one are needed to fully develop a framework for implementing toxicogenomics in risk assessment, especially for chemicals with hazards other than genotoxicity or carcinogenicity.

Limitations of the data used in this case study

Overall, it is clear that further work is required before transcriptional pathway-based risk assessments can be integrated into traditional approaches. In addition to these obstacles, there were several limitations in our experiments, as they were not designed for the purpose of integrating genomics into the risk assessment of BaP, but do reflect real-world data a risk assessor would have access to:

- 1) Our case study reflects one of the major challenges faced by risk assessors—the availability of relevant data. We did not generate any new data and relied on previous experiments that were not specifically designed to address risk assessment issues. Thus, the transcriptomics datapoints were not necessarily collected using an optimal experimental design. Indeed, a gene expression profile is a single snapshot in time from which it

- may be hard to elucidate the dynamic nature of genes operating in networks. This is clearly seen by the fact that transcriptomics PODs for lungs and forestomach (that were sampled 3 days after the last exposure) were several fold higher than their respective cancer PODs, while the transcriptomics POD from liver data (sampled 4 or 24 h or 3 days after the last exposure) was more aligned with the cancer POD (1.0 vs. 1.2 mg/kg-bw per day, respectively). We considered the most appropriate dataset for BMD modeling in our experiment to be the liver 3-day BaP exposure, sampled 4 and 24 h after the last exposure since: (1) samples collected at an early time point would appropriately capture the initial effects of the toxicant; (2) the 3-day dataset included a broader range of doses, including the lower dose range; and (3) the transcriptional responses for the initiating and early key events may be diminished or not captured after the 3-day time-lag in sample collection after our 28-day exposure (instead capturing responses resulting from toxicity). However, toxicogenomics signatures taken after 90 days of exposure (sampling immediately after final dose) are proposed to be more predictive of carcinogenesis than earlier time points (2 or 14 days) (Auerbach et al. 2010, Thomas et al. 2013). Samples collected at the 90-day time point are postulated to express initiating and early key events, as well as early changes involved in the transformation of a tissue to a disease phenotype.
- 2) The number of dose groups was limited. Inclusion of some lower doses within the BMR would have been preferred.
 - 3) The use of a single non-metabolically competent human cell culture model was a limitation and can only be used for hazard identification to support the genotoxic MOA. An improved approach would be to apply tissue-relevant metabolically competent cell types for additional information.
 - 4) The true target tissue, the forestomach, was not assayed at the acute exposure time points. Forestomach tissue was available only from the 28 + 3-day regimen, at which time most of the BaP in the forestomach has been cleared. However, we note that our data support that the evaluation of gene expression changes in key target tissues may also be predictive/protective of potential changes in other tissues.
 - 5) Only two tissues were assayed in both the acute and sub-chronic regimens. However, the toxicogenomics-derived POD is within an order of magnitude of the traditionally derived POD; thus, in the short term, the integration of toxicogenomics into standard risk assessments (such as this one) will continue to drive this field forward and will begin to clarify the precise means by which toxicogenomics data should be used.
 - 6) We acknowledge that because there were only three tissues analyzed in RA3 that this could indicate bias relating to target tissue selection. However, we believe that liver is an obvious first-pass target tissue for preliminary toxicogenomic analyses of a chemical delivered orally as the site of metabolism. Although such an assumption may not hold for chemicals with rapid metabolism, hence the need for two or more time points. For example, CYP1A1

induction by BaP in the mouse liver would be missed by analyzing 24-h data alone (see Table 5). A similar argument can be extended to (fore)stomach as a point of contact for oral exposures, and lungs (exposure *via* circulation). Analysis of a compendium of cancer bioassay data revealed that 92% (354/384) of mouse and 82% (388/471) of rat carcinogens showed positive response in at least one of the eight tissues: liver, lungs, mammary gland, stomach, vascular system kidney, hematopoietic system, and urinary bladder (Gold et al. 2001). Thus, we recommend that these eight tissues comprise the starting point to analyze global gene expression for chemical assessments as has been suggested by others (Thomas et al. 2013). Additional tissues and experimental setups could be considered to address other toxicity endpoints.

- 7) It is worth emphasizing that this proof-of-principle case study illustrates the utility of toxicogenomics in the risk assessment of a genotoxic carcinogen. The selection of a target organ/tissue for genotoxic carcinogens is more straightforward than for the assessment of non-genotoxic or non-cancer effects. This is illustrated by the fact that adducts and mutations were detected in all three tissues that we examined (Figure 2). We note that if the eight tissues proposed above for cancer are profiled exclusively with toxicogenomics, some important toxicity information may be missed. However, collecting all of the target tissues for detailed toxicological evaluations is challenging even for established toxicity endpoints. For example, the OECD guideline 407 for repeat dose 28-day oral toxicity in rodents examines a wide variety of tissues for a diverse array of toxicological effects related to nervous, immune, endocrine, and reproductive systems. We anticipate that examination of various tissues for a variety of toxicological effects may benefit risk assessment by providing rich mechanistic information that could be otherwise missed by traditional testing strategies. Additional case studies like the present one will be required to verify this for non-genotoxic and non-cancer toxicants compounds. It is also worth considering that toxicogenomics approaches are becoming increasingly higher throughput and less expensive (e.g., Li et al. 2012). Thus, it is feasible that in the near future all of the tissues collected in repeat-dose studies can be profiled for gene expression changes. Integration of toxicogenomics into current standard *in vivo* toxicity tests would provide information that could ultimately lead to the appropriate selection of target tissues for toxicogenomics studies in the future to address non-cancer effects. We believe that selection of the appropriate tissues that encompass a large portion of potential toxicities is an important avenue for further research.
- 8) Genomics approaches only capture transcriptional changes; other toxicity mechanisms may not be captured using this approach alone.

Next steps

We believe that there are many take-home lessons from this collaborative project involving both risk assessors and researchers (summarized in greater detail in a separate publi-

follow-up approaches to validate the use of global gene expression profiling in hazard identification, MOA and POD determination. For validation and wider acceptance, toxicogenomics-based MOAs will continue to rely on phenotypic anchoring rather than being used as a stand-alone approach for the time being. Clear pathway annotations, established linkage between expression changes and apical changes, standardized protocols, validated expression signatures that predict a diverse number of toxicities and an approach to deal with interactions or differences among tissues are required before toxicogenomics can be routinely incorporated into risk assessment.

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graph TD; CA[CURRENT APPROACH] --> EC[EXPOSURE CHARACTERIZATION]; EC --> IT["In vitro genetic toxicity & kinetics/metabolism tests, In silico, HTS, QSAR"]; IT --> TT["TRADITIONAL TOXICITY TESTING<br/>Most sensitive toxicity endpoints"]; IT --> TG["TOXICOGENOMICS<br/>Most sensitive perturbed pathways"]; TT --> TI["Targeted testing"]; TI --> TI; TI -- "Refine if needed" --> TI; TI --> HI["HAZARD IDENTIFICATION: Identify appropriate endpoint(s) to determine POD"]; TG --> Q["Obvious toxicity/human relevance?"]; Q -- YES --> HI; Q -- NO --> LP["Lower-priority"]; HI --> POD["POD"]; HI --> TPOD["Toxicogenomic POD"]; POD --> SRM["SCREENING, MOE, RISK ASSESSMENT AND RISK MANAGEMENT"]; TPOD --> SRM;
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The flowchart illustrates two parallel paths starting from 'EXPOSURE CHARACTERIZATION'. The left path follows the 'CURRENT APPROACH' through 'In vitro genetic toxicity & kinetics/metabolism tests, In silico, HTS, QSAR' to 'TRADITIONAL TOXICITY TESTING' (focusing on most sensitive toxicity endpoints). This leads to 'Targeted testing', which has a feedback loop labeled 'Refine if needed'. The right path follows the 'NEW APPROACH' through the same exposure characterization step to 'TOXICOGENOMICS' (focusing on most sensitive perturbed pathways). A decision point asks 'Obvious toxicity/human relevance?'. If 'YES', it proceeds to 'HAZARD IDENTIFICATION'; if 'NO', it results in 'Lower-priority'. Both paths converge at 'HAZARD IDENTIFICATION: Identify appropriate endpoint(s) to determine POD', which then identifies either a standard 'POD' or a 'Toxicogenomic POD'. Both final outcomes lead to the final stage: 'SCREENING, MOE, RISK ASSESSMENT AND RISK MANAGEMENT'.

270

relevant dose does not perturb toxicity-associated pathways, it could be considered of low priority for risk assessment, allowing greater resources for chemicals identified as higher concern), 2) determine if targeted toxicity testing is required and if so, which traditional toxicological assays are relevant for hazard identification and derivation of PODs, and 3) inform hazard and derive a genomic POD (when standard toxicity tests are unavailable) for screening assessments. While it may be desirable to use toxicogenomics changes as an alternative to traditionally obtained toxicity information when standard testing strategies are unavailable, we caution that until the application of toxicogenomics in supporting health risk assessment is fully developed, genomic PODs should be used in the presence of additional lines of evidence supporting the overall hazard potential of the chemical being assessed.

For data-rich chemicals, toxicogenomics data derived from *in vivo* studies should be considered to: 1) help identify the most relevant endpoint (i.e., the most sensitive pathways and processes perturbed by a chemical) for hazard identification; 2) provide mechanistic data to support the development of a detailed MOA; and subsequently to 3) support the risk assessment approach (e.g., linear or threshold). As in the standard approach, the relevance of an animal MOA to humans can then be evaluated based on epidemiological data; alternatively, this can be done with toxicogenomics experiments in cultured human cells, deriving mechanistic information that is rarely achieved in epidemiological studies.

In our case study, we acknowledge that the genotoxicity of BaP would be detected by standard *in vitro* tests. Our work was not done to suggest that standard assays should be discarded in favor of toxicogenomics. Indeed, we believe that these *in vitro* assays are powerful early predictors of potential toxicity and should continue to be used. We note that toxicogenomics does not indicate that a mutation has occurred, but instead indicates that a DNA damage response has been initiated, which is consistent with the presence of genotoxicity. However, toxicogenomics also provides insight into the MOA through which BaP causes genotoxicity (i.e., activation of AHR, metabolism, induction of p53, and DNA damage response, in addition to the effects on cell cycle and other signaling pathways). In addition, the approach can be used to provide insight into the relevance of the MOA in humans and inform the RA approach. This information can be used to support positive results from an Ames test or a standard genetic toxicity assay that address the ability of the compound to reach the tissue, be activated, and interact with DNA *in vivo*. Through the analysis of multiple tissues it offers insight into tissue-specificity, distribution, and metabolism. Moreover, the analysis reveals non-genotoxic carcinogenic (and non-carcinogenic) MOAs that would be missed through application of genetic toxicology assays alone. We have previously published a study on furan, a hepatocarcinogen that operates primarily through the induction of cytotoxicity followed by regenerative proliferation, which demonstrates this point (Jackson et al. 2014). Although detailed genomics information (e.g., the activation of certain DNA-damage response pathways) in the absence of other information is currently not very informative in risk assessment, we believe that development of specific genomic biomarkers will become invaluable in the near future as we move toward MOA-based, mechanistic approaches in toxicity testing.

A tiered testing paradigm whereby first order margins of exposure (MOEs) are calculated using data from *in vitro* high-throughput screening assays has been recently proposed (Thomas et al. 2013). Given the similarity between the PODs derived by the traditional and the toxicogenomics-based approaches, we propose that PODs derived from toxicogenomics data can also be used to inform regulatory guidance on MOEs (historically used for non-genotoxic carcinogens and non-cancer effects) or maximum acceptable concentrations. As per any risk assessment, the specific exposure information and the total uncertainty would be agency-specific.

Overall, our research illustrates the utility of toxicogenomics to inform MOA and PODs in risk assessment and provides two examples (RA2 and RA3) of how toxicogenomics could be applied in human health risk assessment in the future.

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Declaration of interest

The authors' employment affiliations are as shown on the cover page. Health Canada and The Netherlands' National Institute for Public Health and Environment are government agencies in their respective countries with broad missions for protecting and promoting public health. Pfizer is a public corporation engaged in the discovery, development, and manufacture of a range of pharmaceuticals. Utah State University and Georgetown University are educational institutions that conduct research with public and private support. Integrated Laboratory Systems is a private company that conducts research for the U.S. government and private entities. The preparation of the paper was primarily supported by funding from the Health Canada Genomics Research and Development Initiative and the Canadian Regulatory Systems for Biotechnology. The individual authors were compensated by their employers. The conceptual basis for the paper, preparation of the paper, selection of the literature and its review, the conclusions drawn, and the recommendations made, were the professional work product of the authors and may not necessarily represent the views of Pfizer and Integrated Laboratory Systems.

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Supplementary material available online

Supplementary Tables 1–7, Supplementary File A–C to be found online at <http://informahealthcare.com/doi/abs/10.3109/10408444.2014.973934>.

Appendix II Nano-risk science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes.

RESEARCH

Open Access



Nano-risk Science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes

Sarah Labib¹, Andrew Williams¹, Carole L. Yauk¹, Jake K. Nikota¹, Håkan Wallin^{2,3}, Ulla Vogel^{2,4} and Sabina Halappanavar^{1*}

Abstract

Background: A diverse class of engineered nanomaterials (ENMs) exhibiting a wide array of physical-chemical properties that are associated with toxicological effects in experimental animals is in commercial use. However, an integrated framework for human health risk assessment (HHRA) of ENMs has yet to be established. Rodent 2-year cancer bioassays, clinical chemistry, and histopathological endpoints are still considered the 'gold standard' for detecting substance-induced toxicity in animal models. However, the use of data derived from alternative toxicological tools, such as genome-wide expression profiling and in vitro high-throughput assays, are gaining acceptance by the regulatory community for hazard identification and for understanding the underlying mode-of-action. Here, we conducted a case study to evaluate the application of global gene expression data in deriving pathway-based points of departure (PODs) for multi-walled carbon nanotube (MWCNT)-induced lung fibrosis, a non-cancer endpoint of regulatory importance.

Methods: Gene expression profiles from the lungs of mice exposed to three individual MWCNTs with different physical-chemical properties were used within the framework of an adverse outcome pathway (AOP) for lung fibrosis to identify key biological events linking MWCNT exposure to lung fibrosis. Significantly perturbed pathways were categorized along the key events described in the AOP. Benchmark doses (BMDs) were calculated for each perturbed pathway and were used to derive transcriptional BMDs for each MWCNT.

Results: Similar biological pathways were perturbed by the different MWCNT types across the doses and post-exposure time points studied. The pathway BMD values showed a time-dependent trend, with lower BMDs for pathways perturbed at the earlier post-exposure time points (24 h, 3d). The transcriptional BMDs were compared to the apical BMDs derived by the National Institute for Occupational Safety and Health (NIOSH) using alveolar septal thickness and fibrotic lesions endpoints. We found that regardless of the type of MWCNT, the BMD values for pathways associated with fibrosis were 14.0–30.4 µg/mouse, which are comparable to the BMDs derived by NIOSH for MWCNT-induced lung fibrotic lesions (21.0–27.1 µg/mouse).

Conclusions: The results demonstrate that transcriptomic data can be used to as an effective mechanism-based method to derive acceptable levels of exposure to nanomaterials in product development when epidemiological data are unavailable.

Keywords: Nano, Risk assessment, Toxicogenomics, Adverse outcome pathways, Benchmark dose, Case study, Lung fibrosis

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Background

The increased production and use of engineered nanomaterials (ENMs) in industrial and consumer products has raised concerns about potential health and environmental risks posed by exposure to these materials. ENMs exhibit size-associated properties. While the chemical properties such as, solubility and chemical structure are traditionally known to influence the toxicity of bulk chemical substances, the toxicity of ENMs is influenced by their distinct physico-chemical properties, such as being insoluble (as opposed to soluble bulk chemicals), and having a small size, large surface area, and increased surface reactivity. Within the biological microenvironment, these unique properties allow ENMs to interact with surrounding biomolecules and structures in a complex and non-specific manner. As a result, ENMs are expected to be toxicologically distinct from soluble chemicals and more active than larger particles of similar chemical composition. Despite this knowledge, a validated framework for human health risk assessment (HHRA) of ENMs has yet to be established. Sustained development of nanotechnology-enabled products will be hampered by the lack of a pragmatic risk assessment paradigm that can effectively address the unique aspects of nanomaterial risk assessment in a timely manner.

Given the unique properties of ENMs, standardized toxicity testing methods developed originally for bulk chemicals were tested for their suitability for investigating ENM-induced effects [1–6]. Although, the Organization for Economic Co-operation and Development (OECD) has stated that in general the OECD guidelines developed for bulk chemicals may be applicable to ENMs, based on the types of endpoints studied, modifications and adjustments are needed in regards to the sample preparation and analysis. Consequently, significant efforts have been made to develop novel methods, or to modify existing *in vivo* and *in vitro* test methods, to assess ENM toxicity [7]. However, validation of these new methods has been challenging due to the lack of sufficient amount of reference materials and harmonized protocols. An additional challenge is the large number of ENMs requiring immediate human health hazard and risk assessment. Indeed, the generation of toxicological data for each ENM variant using conventional single end-point based methods is not practical. To circumvent this problem, a strategy has been proposed for ENMs that involves the use of high-content omics tools or *in vitro* high-throughput assays to examine the toxicity induced by well characterized ENMs in short-term toxicity models for screening and prioritization [8], a strategy that is in line with the United States National Research Council's vision of a paradigm shift in toxicology [9].

Whole genome gene expression profiling allows for the simultaneous analysis of changes in the expression of all the genes in a tissue or cell type following substance exposure. Numerous studies have employed genomics methods, in combination with bioinformatics tools for ontological and pathway analyses, to investigate the underlying molecular mechanisms of action of toxicants [10–14]. These studies have demonstrated that global changes in gene expression following exposure to a substance are associated with the development of Labib toxicological phenotypes in a dose-dependent manner [15].

In our previous studies, we used whole genome gene expression profiling and bioinformatics analyses of mouse models to study the specific particle properties that influence toxicity and to elucidate the mechanisms of pulmonary response induced by various types of ENMs including: nano-titanium dioxide particles (nanoTiO₂) [16–18], multiwalled carbon nanotubes (MWCNTs) [19, 20], and carbon black (CB) [21–23]. These studies demonstrated that the molecular perturbations at the gene expression and pathway level leading to lung inflammation following exposure to ENMs are the same regardless of the class of ENM studied; however, the magnitude (number of genes and the fold-change response) of the perturbation varies. Subsequently, we conducted a meta-analysis of gene expression data catalogued from 15 individual genomics datasets describing lung responses to disease-inducing chemical substances, pathogens, or ENMs in mouse models, and identified gene expression signatures predictive of disease-causing ENMs [24]. In another study, we used a combination of bi-clustering of gene expression data and gene set enrichment analyses methods to identify gene sets in ENM-induced gene expression changes that are similar to disease conditions [25]. The latter studies demonstrated that gene expression data can be used to predicting disease potential of ENMs before the phenotype is established, and therefore can be used to prioritize ENMs for further toxicological investigation. Thus, the use of genomics tools to characterizing ENM-induced toxic responses is well established.

The use of genomics approaches to provide information on a chemical's hazard and mode-of-action (MOA) is gaining acceptance in the regulatory community; however its utility in a regulatory context and a specific paradigm for genomics-driven quantitative HHRA has yet to be established. Approaches for quantitative dose-response analysis of genomics endpoints have been developed and methods to derive transcriptional benchmark dose (BMD) values that could be applied to quantitative risk assessment have been explored [26–29]. Although several case studies [26, 28, 30] have been conducted to demonstrate the potential use of gene expression data in deriving points of departure

(PODs) for risk assessment, and guidance documents to describe the strategies and methods involved have been developed, much more work is needed before full integration of genomics in routine quantitative HHRA of toxic substances can be realized. In the interim, the value of genomics data as supplementary information contributing to the weight of evidence is recognized [31].

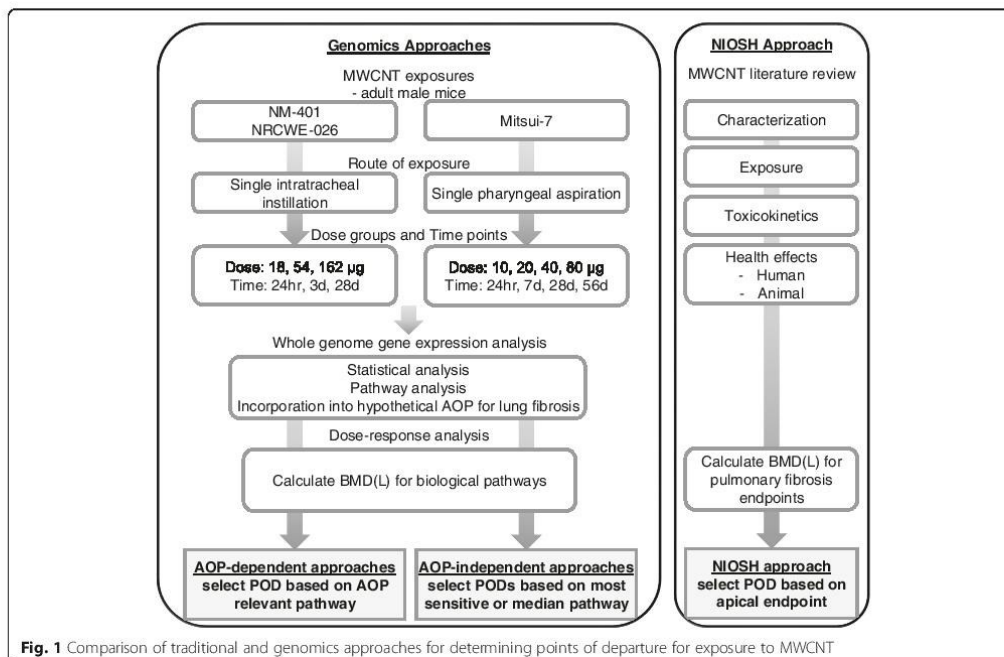
In the present case study, we explored the use of toxicogenomics to establish PODs for MWCNT-induced lung fibrosis, and assessed its relevance for HHRA of MWCNTs. MWCNTs are widely used ENMs in industrial and biomedical sectors. Short-term and sub-chronic studies in rodents have shown that MWCNTs are bio-persistent and induce various effects in lungs, including persistent pulmonary inflammation, granulomas, and lung fibrosis, following exposure via inhalation, instillation, or pharyngeal aspiration [32–35]. Occupational exposure studies have estimated human CNT exposure levels of 10 – 300 $\mu\text{g}/\text{m}^3$ in facilities such as research laboratories and production plants where CNTs are produced, processed, used, disposed of, or recycled [36–42] (reviewed in [43]). The United States National Institute for Occupational Safety and Health (NIOSH) recently completed a HHRA for CNT-induced lung fibrosis that takes into consideration existing toxicological data from animal models [43]. We used this published report to

derive POD values for apical endpoints. For the derivation of genomics driven POD values, first, we used the framework of adverse outcome pathway (AOP) to logically and systematically organise the mechanistic information available in the literature concerning CNT-induced lung fibrosis and the results of global transcriptomic analyses conducted on mouse lungs exposed to three individual types of MWCNTs [19, 34, 44]. The key biological events associated with one potential mechanism linking the MWCNT exposure to the adverse outcome of lung fibrosis were identified. Next, transcriptional BMDs for perturbed biological pathways corresponding to the key events in the hypothetical AOP for fibrosis were calculated using data from dose–response and time-series experiments on rodents exposed to the same three types of MWCNTs [19, 44]. Finally, we compared the PODs derived by NIOSH using traditional risk assessment approaches to transcriptional PODs derived via multiple genomics based approaches (Fig. 1). Our results provide foundational data and guidance to support the use of transcriptional PODs in HHRA of ENMs.

Methods

Study design

The present study employed gene expression data from three studies already published in the literature. No



additional animal experiments or laboratory experiments were conducted. Details on research conducted on animals and ethics approvals obtained are described in Poulsen et al. [19], Guo et al. [44], and Mercer et al. [34]. In order to derive meaningful data for AOP assessment and HHRA, both time-series and dose-response data are required. We searched the literature for studies that describe transcriptional lung responses to multiple doses of MWCNTs at different post-exposure time-points in a mouse model and found two relevant studies [19, 44]. The analyses were restricted to mouse studies to minimize the loss of information during the statistical normalization of cross-species data and included only those datasets that are publicly available and meet the transcriptomic quality checks recommended by Bourdon-Lacombe et al. [31]. Figure 1 shows the different approaches used in the present study to derive PODs. Additional physico-chemical properties of the three MWCNTs are listed in Additional file 1: Table S1. One study that reported mouse lung fibrotic response to MWCNT by histopathological and morphometric analyses was also included for the purposes of comparison of transcriptomic approaches to apical endpoint-driven risk assessment methods [34]. The study design of the three individual studies considered in the analysis is briefly described below.

Study 1. Poulsen et al. [19] investigated the transcriptomic response of mouse lungs exposed to two types of individual MWCNTs of different lengths: NM-401 ($4 \pm 0.4 \mu\text{m}$ in length, European Union Joint Research Centre, Ispra, Italy) and NRCWE-026 ($0.8 \pm 0.1 \mu\text{m}$ in length, Nanocyl NC7000 CNT, Sambreville, Belgium). In this study, female C57BL/6 mice were exposed via single intratracheal instillation to 18, 54, or 162 $\mu\text{g}/\text{mouse}$ of NM-401 or NRCWE-026 dispersed in MilliQ water with 2 % serum. Control mice were exposed to 2 % serum in MilliQ water only. The selected doses reflect 1, 3, and 9 days of exposure at the Danish eight-hour working day occupational exposure limit of $3.5 \text{ mg}/\text{m}^3$ for Printex90 carbon black particles [45]. Mouse lung tissue was collected 24 h, 3 days, and 28 days post-exposure. Total RNA extracted from the left lung lobe ($N = 6$ per treatment group and time point) was hybridized to Agilent mouse $8 \times 60 \text{ K}$ oligonucleotide microarrays. LOWESS normalization and MAANOVA analysis was conducted to identify the significantly differentially expressed genes (DEGs). The DEGs with false discovery rate (FDR) adjusted P value of ≤ 0.05 and fold change of $\geq \pm 1.5$ were used in the downstream analysis by pathway and ontology tools as described in Poulsen et al. [19].

Study 2. Guo et al. [44] investigated the transcriptomic response of male C57BL/6 mouse lungs exposed via

pharyngeal aspiration to XNRI MWCNT-7, commonly referred to as Mitsui-7 ($3.86 \mu\text{m}$ in length, Mitsui & Company). Briefly, mice were exposed to a single dose of 10, 20, 40, or 80 $\mu\text{g}/\text{mouse}$ of Mitsui-7 dispersed in vehicle (Ca^{2+} and Mg^{2+} free phosphate buffered saline supplemented with D-glucose, mouse serum albumin, and 1,2 dipalmitoyl-sn-glycero-3-phosphocholine). Control mice were exposed to dispersion vehicle only. The 10 μg dose used in this study reflects occupational exposure to MWCNTs for a time period between 9 months and 7.5 years based on the average daily MWCNT workplace exposure data for a person performing light work in an environment with MWCNT aerosol of $4\text{--}40 \mu\text{g}/\text{m}^3$ [46]. Lung tissue was collected 24 h, 7, 28, and 56 days post-exposure. Total RNA extracted from the whole lung ($N = 8$ per treatment group and time point) was hybridized to Agilent mouse $4 \times 44 \text{ K}$ oligonucleotide microarrays. This microarray dataset has also been used in other studies by Snyder-Talkington et al. [47, 48] and Dymacek et al. [49, 50]. The microarray dataset was downloaded from the GEO database (www.ncbi.nlm.nih.gov/geo) (GSE29042) and was reanalysed employing the same statistical methods as Poulsen et al. [19] above to derive the list of DEGs. Genes showing FDR P value ≤ 0.05 and fold change $\geq \pm 1.5$ were used in the pathway and ontology analyses.

Study 3. Mercer et al. [34] investigated the pulmonary fibrotic response in male C57BL/6 mice following pharyngeal aspiration of 10, 20, 40, or 80 $\mu\text{g}/\text{mouse}$ Mitsui-7. The exposure regimen was the same as the one by Guo et al. [44]. Lung tissues were fixed by intratracheal perfusion of 10 % formalin. The left lobe of the lung was embedded in paraffin, sectioned, and stained with Sirius Red stain for the histopathological and morphometric measurements. The average thickness of Sirius Red positive connective tissue in the alveolar regions was measured using quantitative morphometric methods and the collagen fiber content of granulomatous lesions in the airspaces was assessed as total Sirius Red staining in the airspaces.

Pathway analysis

Functional pathway analysis for classifying genes significantly affected by the exposure to MWCNTs was conducted using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA). The canonical pathways consisting of at least five DEGs and exhibiting a Fisher's Exact P value ≤ 0.05 were considered for further calculation of pathway BMD values for the AOP-dependent approaches.

Calculation of transcriptional BMDs

Since the purpose of this study was to assess the applicability of toxicogenomic data in quantitative risk

assessment for MWCNT-induced lung fibrosis, BMD modeling was conducted on each microarray dataset using BMDE Express version 1.4.1 [51]. Briefly, the BMD analysis was performed on present genes (defined as those that had signal intensities at least three standard deviations above the background non-murine control spots on the array) that showed ANOVA $P \leq 0.05$ for at least one dose. Four dose-response models; Hill, Power, Linear, and Polynomial (2^0 and 3^0) models were used to assess the best fit. Selection of the best fit model required: (a) a nested chi-squared test cut-off of 0.05, (b) the lowest Akaike Information Criterion (AIC), (c) goodness-of-fit P value > 0.1 , and (d) the BMD was lower than the highest dose. The benchmark response factor was set to 1.349, which corresponds to a 10 % change compared to controls [51]. The maximum number of iterations, the convergence criteria for model fitting, was set to a maximum of 250, and power was restricted to ≥ 1 . If the k parameter of the Hill model was less than one third of the lowest dose used in an experiment [52], the Hill model was flagged and subsequently excluded if at least one other model had a p -value > 0.05 . In the case when no other model had a p value > 0.05 , the Hill model BMD was adjusted to $\frac{1}{2}$ of the lowest BMD value. The resulting BMDs for each gene were mapped to IPA canonical pathways to assess biological relevance. Median BMD and BMDL (lower confidence limit of BMD) values were calculated for each IPA canonical pathway that had at least five modeled DEGs.

Calculation of BMDs for apical endpoints

NIOSH conducted an extensive review of the literature pertaining to the human health effects of occupational exposures to both MWCNT and single walled CNTs (SWCNT). BMD analysis was conducted on CNT-induced alveolar septal thickness and other related endpoints reflective of lung fibrosis using the US EPA's BMD software (BMDS, version 2.1.2) [53]. For dichotomous data, a benchmark response (BMR) of 10 % excess risk was used and for continuous data, a BMR of 1.1 standard deviations above the control mean response was used as an equivalent to a 10 % BMD estimated in dichotomous data. Model fit was considered adequate if goodness of fit P value was greater than 0.05.

Results and discussion

Proposed transcriptomics-driven regulatory testing paradigm

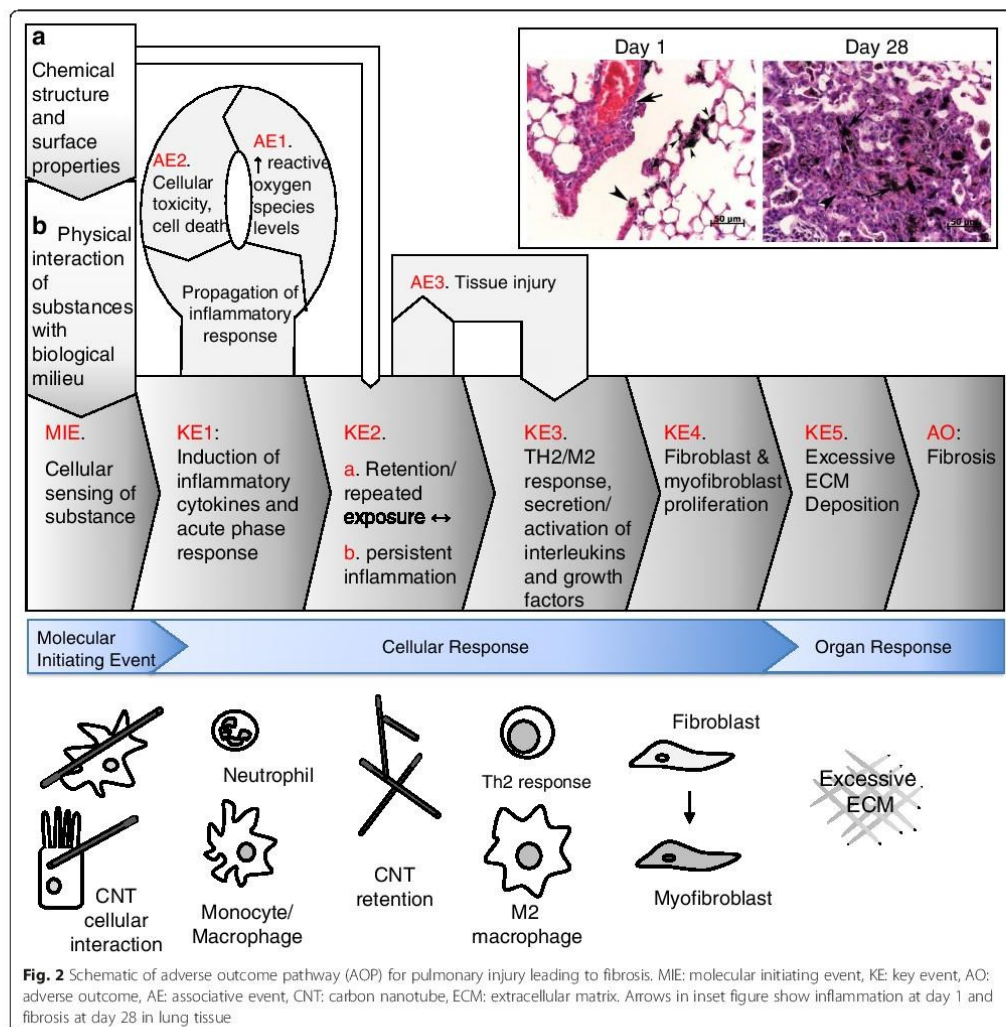
Sustained development of products containing ENMs will be impeded by the lack of a pragmatic risk assessment paradigm that can effectively address the unique aspects of nanomaterial risk assessment in a timely manner. Fundamental understanding of the molecular mechanisms underlying toxicity has become a central component of

proposed changes to human health risk assessment (HHRA) challenges in the 21st century [9]. Global analyses of perturbations in biological pathways, processes, and functions are playing an increasingly important role in dissecting toxicological mechanisms associated with human health risks. In parallel, increasing emphasis has been placed on dose-response modelling of global transcriptional changes to identify the doses at which critical tissue-level effects begin to occur [54, 55]. Here we propose an integrated approach that leverages advances in high-throughput transcriptomics, dose-response modeling, and international initiatives to develop AOPs of toxicological effects be used to address the important risk assessment gaps for nanomaterials.

In a proof of concept study, we obtained publicly available genomics data from rodent pulmonary exposures to MWCNT for BMD modelling of perturbed genes and pathways. A BMD is the dose associated with a predetermined response level. The statistical lower confidence bound on the BMD (the 95 % lower bound on the BMD) is the BMDL, which is equivalent to traditional No Observed Adverse Effect Level (NOAEL) that is used as a POD. Transcriptional BMDs were linked to key events in a hypothetical AOP for pulmonary fibrosis to assess their potential utility in selecting a POD for nanomaterial HHRA.

As part of the first steps in deriving transcriptomics-based BMDs for HHRA of MWCNT-induced lung fibrosis, we organised the vast amount of gene expression data derived from the three studies [19, 44] in a systematic and logical manner using an AOP framework. This enabled us to construct a hypothetical AOP describing the various key events (as derived from gene expression and pathway perturbation data) that may occur between the initial exposure to MWCNTs and the development of fibrotic lesions in the lungs (Fig. 2).

The lung constitutes a barrier between the host and the environment. The alveolar capillary membrane (ACM) plays a critical role in gas exchange and is constantly exposed to inhaled or endogenous challenges. Exposure-induced ACM injury and consequent loss of structural integrity results in injury, inciting a repair process that begins with extravasation of plasma into lung tissue, activation of intrinsic and extrinsic coagulation pathways, fibrin deposition, and establishment of a provisional matrix. These steps involve the release of a number of lipid mediators, cytokines, growth factors, and chemo-attractants required for activation of various immune cell types. If the primary insult leading to ACM injury is removed, the deposited extracellular matrix (ECM) is remodelled or reabsorbed. The epithelial and endothelial cells re-establish their normal spatial orientation on the basement membrane of the ACM, restoring the integrity of the ACM, and complete the repair



process [56]. However, persistent or recurrent injury from repetitive or persistent exposure to an irritant leads to a deregulated tissue repair process that can result in lung fibrosis. As such, there exists no simplified mechanism that encompasses the pathogenesis of fibrosis in all situations. For example, while fibrosis induced by the inhalation of the high aspect ratio particle asbestos is dependent on inflammatory mediators such as $\text{TNF-}\alpha$ [57], cystic fibrosis is driven by a mutation in the CFTR gene and may occur in the absence of inflammation [58]. In idiopathic pulmonary fibrosis (IPF) the specific cause and mechanism is unknown, although exposure to

chemicals and particulate matter is deemed a risk factor for IPF [59]. Mechanistic studies that analyzed the targeted inhibition of specific fibrotic mediators are currently not extensive enough to form a complete picture for in vivo models of particulate-induced fibrosis. In general, what we know of pulmonary fibrosis is drawn from the well-characterized murine model of bleomycin-induced pulmonary fibrosis. Bleomycin exposure models outline a process where there is an initial inflammatory response characterized by macrophage- and epithelial cell-derived cytokine production associated with innate immunity. Disruption of these innate immune

mechanisms, which include IL-1 and IL-6 signaling, significantly attenuates fibrosis in the bleomycin model [60–62]. This initial response progresses to the activation of CD4⁺ T cells, which contribute additional cytokines that are crucial to the development of fibrosis [63, 64]. Specifically the Th2 cytokines IL-4, IL-5, and IL-13 that form the adaptive immune response are up regulated, which are key fibrosis mediators [65–68]. Additionally, production of IL-17 from Th17 cells and down regulation of the Th1 response occurs in the fibrotic processes [63, 64, 69]. This activation of an adaptive immune response is independent of antigen sensitization [64], but traditional antigen presenting cells like dendritic cells may contribute to the inflammatory profile [70]. This signaling leads to the production of growth factors, such as TGF- β , which are key regulators of fibroblast activity, and their increased expression disrupts normal homeostatic collagen metabolism, resulting in the excessive collagen deposition in the extracellular matrix characteristic of fibrotic lesions [66, 71]. Other mechanisms, for example, direct stimulation of fibroblasts and epithelial cells with bleomycin can lead to the release of effector molecules [72], which may also contribute to fibrosis in this model.

MWCNTs are non-biodegradable and persist in lung tissues long after the initial exposure, resulting in prolonged tissue injury and unrestricted tissue repair. In addition to their persistence, the high aspect ratio of MWCNTs, their fibre-like structures, and chemical groups on their surfaces are suggested to contribute to the development of lung fibrosis [73], a potential human health effect associated with occupational exposure to MWCNTs [43]. Although several studies have reported lung fibrosis following exposure to MWCNTs, the exact underlying mechanisms are not completely elucidated. Identifying specific mediators and a single mechanism for MWCNT-induced pulmonary fibrosis is challenging. Though data is more limited with respect to MWCNT exposure models, the studies that are available highlight the similarities between the pulmonary response to MWCNTs and bleomycin [33, 74]. MWCNT-induced inflammation, as indicated by neutrophil influx into the lungs, is dependent on IL-1 signaling through the IL-1 receptor 1 (IL-1R1) [75], which involves the activation of the NLRP3 inflammasome [76]. The characteristics of the inflammatory response induced by MWCNTs parallels the bleomycin model, which is reflected by the induction of Th2-like phenotype, involving the production of cytokines such as IL-13 [77, 78]. The inflammatory response ultimately leads to the alteration of fibroblast activity resulting in enhanced activity of TGF- β , which is also implicated in MWCNT-induced fibrosis [79]; the TGF- β /SMAD pathway is involved in fibroblast differentiation and epithelial mesenchymal

transition; another characteristic of fibrotic lesions [80]. Additional factors such as the generation of reactive oxygen species have been shown to contribute to fibrotic pathogenesis; animals deficient in anti-oxidant responses elaborate exacerbated fibrosis in response to MWCNTs [81]. While results from in vivo models described in the present study suggest a role for pulmonary inflammation in MWCNT-induced lung fibrosis, many in vitro studies have suggested a plausible role for direct action of CNTs (both MWCNTs and SWCNTs) on fibroblast function and viability [77, 82–88] that may lead to fibrosis. Moreover, these studies demonstrate increased fibroblast proliferation, collagen synthesis, and expression of the fibrosis-associated gene *Mmp9*, all of which serve as in vivo markers of lung fibrosis [77, 85–88]. However, these findings have not been confirmed in vivo and additional research as to the relevance of the direct stimulation of fibroblasts in isolation of all of the other resident cells of the lung is necessary. Thus, similar to the bleomycin model, multiple mechanisms likely contribute to MWCNT-induced fibrosis but the focus on the inflammatory response triggered by MWCNTs and the resulting pathology from these pathways is warranted given the data generated from models of MWCNT exposure.

It is important to note that other signaling pathways not directly related to innate and adaptive immune mechanisms may also contribute to MWCNT-induced fibrosis (such as direct stimulation of fibroblasts by CNTs), but the focus on the inflammatory response triggered by MWCNTs and the resulting pathology from these pathways is warranted given the crucial role that inflammation is known to play in other models such as bleomycin-induced or Th2-response triggered fibrosis.

Thus, based on the literature review of the general mechanisms involved in lung fibrosis (described above), and comprehensive analyses of gene expression profiles perturbed following exposures to different doses of MWCNTs at different post-exposure time-points, a hypothetical AOP for pulmonary fibrosis was proposed according to the guidance set by the Organisation for Economic Cooperation and Development (OECD) [89–91]. The AOP that we describe is a purposefully simplified linear pathway constructed for the purposes of organising the mechanistic data derived from the global gene expression studies, and to facilitate selection of key pathways and events to use for POD calculation. A full description of the AOP, with the weight of evidence supporting the key event relationships according to the modified Bradford-Hill criteria described in the AOP Users' Handbook, is beyond the scope of this manuscript and is under development as part of the OECD's AOP program.

Thus, the putative AOP consists of a molecular initiating event (MIE) and key events (KEs) vital to the development

of lung fibrosis (Fig. 2). Although the networks and biology involved in the development of lung fibrosis are very complex, two criteria consistent with the OECD guidelines were used in constructing the AOP: 1) the MIE and KEs were based on their essentiality to the initiation and progression of lung fibrosis (i.e., inhibition of occurrence of these KEs would influence the final fibrosis outcome); and 2) the MIE and KEs had to be measurable/quantifiable. It is important to note that the putative AOP described below is simplistic and reflects only one potential mechanism, by which MWCNTs induce lung fibrosis. We agree that there may be several converging pathways that are involved in lung fibrosis. However, the AOP program itself acknowledges that AOPs are purposefully over-simplified linear pathways to toxicity (http://aopkb.org/common/AOP_Handbook.pdf). The aim here is to develop simple linear pathways that address contained aspects of a pathway to toxicity. The AOPs should contain only key events that are routinely measured (and are essential), and subsequent to that the simplified linear AOPs provide a basis to develop broader networks that can be used in predictive toxicology. Accordingly, the AOP for lung fibrosis induced by MWCNTs consisted of the following steps:

MIE – Cellular sensing of the substance-induced damage resulting in the release of danger signals.

KE1 - Induction of inflammatory cytokines and acute phase response. This KE involves the recruitment and infiltration of various immune cell types into the lung tissue, which will clear the invading substance and initiate the tissue repair process. This step is commonly known as acute inflammation. Although a long list of cytokines and chemokines are induced during the acute inflammatory phase, since MWCNT-induced acute inflammation is predominantly characterised by neutrophil infiltration, we argue that IL-1R1-mediated pathway is primarily involved in this process. Thus, secretion of IL-1R1-regulated cytokines and chemokines including IL1, IL6, CXCL-1, CCL2, CCL5, and SAA3, as well as IL1 β , can be used to measure KE1.

KE2 – a) Retention or repeated exposure and b) persistent inflammation. The inability to clear MWCNTs results in 'frustrated phagocytosis' leading to prolonged interaction of MWCNTs with the lung epithelium, continuous activation of stress signalling, inflammatory response, and unremitting repair process. The ensuing chronicity of inflammation engages the adaptive immune system, which then results in the third KE. Both KE 2a and 2b act in a positive feedback loop and are essential for the activation of KE3. Although 'frustrated phagocytosis' is suggested to occur in response to specific-types of long MWCNTs, other types of short or entangled MWCNTs including

NM-401 and NRCWE-026 also biopersist, which can similarly result in chronicity of inflammation leading to disruptive repair process.

KE3 - TH2/M2 response, secretion/activation of interleukins and growth factors. This KE involves activation of T helper (Th) 2 type cells and the release of specific cytokines necessary for activation of M2 type macrophages. M2 macrophages secrete anti-inflammatory cytokines and tissue inhibitors of metalloproteinases that impair remodelling or reabsorption of the deposited ECM. Several TH2 type cytokines including IL-4, IL-5, IL-13 and growth factors such as transforming growth factor beta, that play an important role in the progression of lung fibrosis are upregulated following exposure to MWCNTs and can be used to quantify the activation of KE3.

KE4 – Fibroblast and myofibroblast proliferation. The late stages of tissue injury involve chronic inflammation, epithelial and endothelial injury, loss of ACM integrity, and activation and proliferation of fibroblasts/myofibroblasts.

KE5 – ECM deposition. The repeated cycles of the preceding KEs result in excessive ECM deposition due to imbalance between the deposition and metabolism of ECM proteins, including collagen.

AO – fibrotic lesions. Excessive ECM deposition results in the development of lung fibrotic lesions (Adverse Outcome - AO).

In addition to the KEs described above, associative events (AEs) occur in parallel and can enhance the probability that the exposure will lead to the AO. In this AOP, the continual release of soluble mediators produced by the activated inflammatory cell types, and subsequent interaction between the cell types, leads to increased reactive oxygen species levels (AE1, oxidative stress) and cellular apoptosis (AE2), which contribute to repeated tissue injury (AE3). The other associative events such as, resistance of fibroblasts to apoptosis, collagen metabolism, or inhibition of collagen-degrading enzymes may play a regulatory role in the progression of lung fibrosis. Thus, these events are not included in the hypothetical AOP presented and will be included in a separate AOP.

The putative AOP as described above was subsequently used to categorize and prioritize the significantly perturbed pathways and to calculate the median BMD(L) values of genes within those pathways to derive pathway-based PODs in an AOP-dependent transcriptomics approach (Fig. 1). The KE-associated BMDs were compared with BMDs derived from analysis of conventional toxicology endpoints (e.g., an apical endpoint associated with fibrosis), and to AOP-independent transcriptional approaches, to explore the best approach to selection of a POD.

In our previous studies, we have characterised transcriptomic changes occurring in lungs of mice exposed to carbon black (CB) or titanium dioxide nanoparticles (TiO₂NPs). In order to determine if the MIE and KEs in the putative AOP described above for MWCNT-induced lung fibrosis are also observed following exposure to other control particles, such as CB or TiO₂NPs, we compared the pathways reported to be significantly perturbed following CB [22, 92] or TiO₂NP [16] exposures to those perturbed following exposure to MWCNTs. The results showed that exposure to CB or TiO₂NP induces gene expression changes associated with lung inflammation, Acute Phase Response Signaling and Chemokine Signaling pathways [16, 22, 92] that are relevant to MIE and KE1. However, it was noted that the number of genes perturbed in each pathway was few and the relative fold-changes were smaller compared to the MWCNT-induced response. Moreover, most of the significantly perturbed genes did not show dose-response (data not shown), as a result they could not be modeled using BMDExpress. These results suggested that although CB and TiO₂NP induce lung inflammation and induce altered expression of genes and pathways associated with MIE and KE1, the pro-fibrotic events described in KE2- 4 and AO are specific to MWCNTs. Thus, control particles were excluded from the rest of the analysis in the present study.

Functional analysis of perturbed genes and dose-response modeling

The BMD(L) values for all of the genes were calculated, the genes were assigned to pathways, and median pathway BMDs were thus calculated for each time-point and for each MWCNT. The 3 days Poulsen et al. [19] and 7 days Guo et al. [44] time-points perturbed similar pathways and since they represent early, acute post-exposure time-points, they were considered together. This also implies that the pathways leading to lung fibrosis are activated as early as 3 days post-exposure; however, the clinical manifestation as measured by alveolar thickness may become apparent on 7 days after the last exposure [93] (reviewed in [94]).

The lists of differentially expressed genes (DEGs) derived from Poulsen et al. [19] and Guo et al. [44] were analyzed for functional significance using IPA. The significantly perturbed pathways ($P < 0.05$) were categorized according to their association with the KEs and AEs in the AOP for lung fibrosis. Pathways for which the biology did not appear to be related to the KEs or AEs in the AOP were not included in the analysis. A few pathways overlapped with more than one KE: Chemokine Signaling (KE1, KE3), Dendritic Cell Maturation (KE1, KE3), Jak/Stat Signaling (KE1, KE3), Leukocyte Extravasation (KE1, KE2), NF- κ B Signaling (KE1, KE2), and

TGF- β Signaling (KE3, KE4). The complete list of perturbed pathways and the associated BMDs can be found in Additional file 2: Table S2. Unique biological pathways were perturbed at different post-exposure time-points with some overlap. For example, Acute Phase Response Signaling and Hepatic Fibrosis/Hepatic Stellate Cell Activation pathways were significantly enriched at the 24 h, 3, and 28 days time-points for NM-401. There is no separately annotated pathway for lung fibrosis in IPA. The "Hepatic Fibrosis/Hepatic Stellate Cell Activation" pathway includes pulmonary fibrosis in the IPA pathway analysis tool. Fibrosis is a wound healing process characterized by accumulation of extracellular matrix and the components of ECM accumulation are similar regardless of the cause or tissue. Thus, for the rest of the discussion, this pathway will be referred to as the 'Fibrosis' pathway. A large number of biological pathways associated with the KEs were perturbed 3 days post-exposure for all three types of MWCNTs investigated. Thus, gene expression profiling supports that the post-exposure time-points at which the KE is observed has a strong influence on whether it is measurably perturbed, which can be used to support the temporal order of events.

In general, analysis of the distribution of pathway BMDs revealed that most pathway BMD(L) values (Fig. 3), including those associated with KEs, were lower at the earlier time-points (24 h, 3 days) for NM-401 and Mitsui-7. In contrast, NRCWE-026 had similar BMDs at 24 h and 3 days, but had very few pathways perturbed at the 28 days time-point and only one KE-associated BMD that could be derived for this time-point (discussed below). Overall, some of the lowest BMD(L) values were for pathways that were associated with more than one KE and that were perturbed across MWCNTs and time-points, including Chemokine Signaling, Dendritic Cell Maturation, and Jak/Stat Signaling. The BMD(L) values were remarkably similar overall across different time-points and MWCNT types. However, a few exceptions were noted where BMD(L) values were higher for the pathways perturbed at the later post-exposure time-points (28 days), suggesting a time-dependent transition in the toxicity pathways and that the pathways implicated in the pathological manifestation of the disease occur at the later time-points. Thus, although different pathways were altered at different post-exposure time-points, the BMD(L) values within a KE were consistent across time-points and particle types.

Interestingly, similar pathways were perturbed following exposure to the three types of MWCNTs across the time-points, despite differences in their physico-chemical properties, suggesting that the underlying mechanisms leading to fibrosis are the same for all three MWCNTs

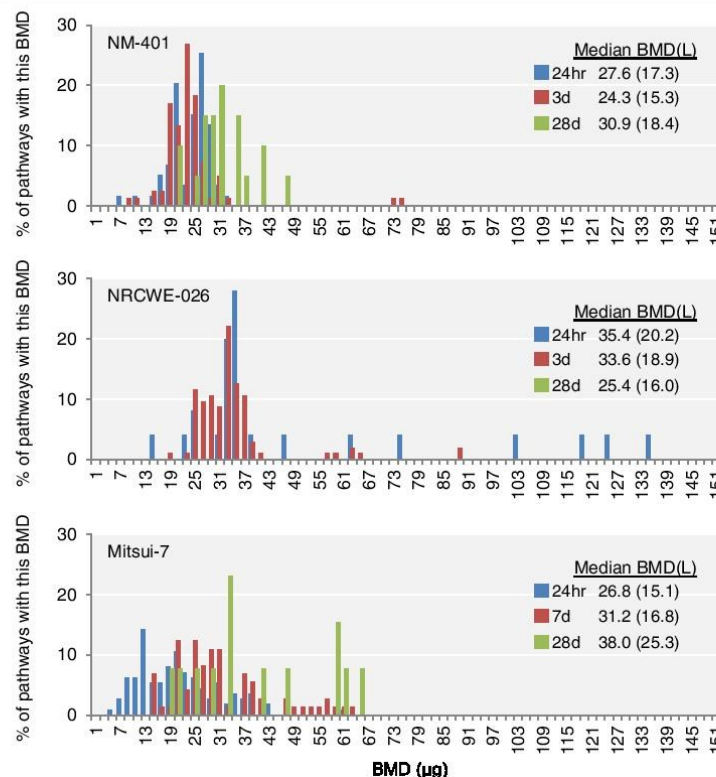
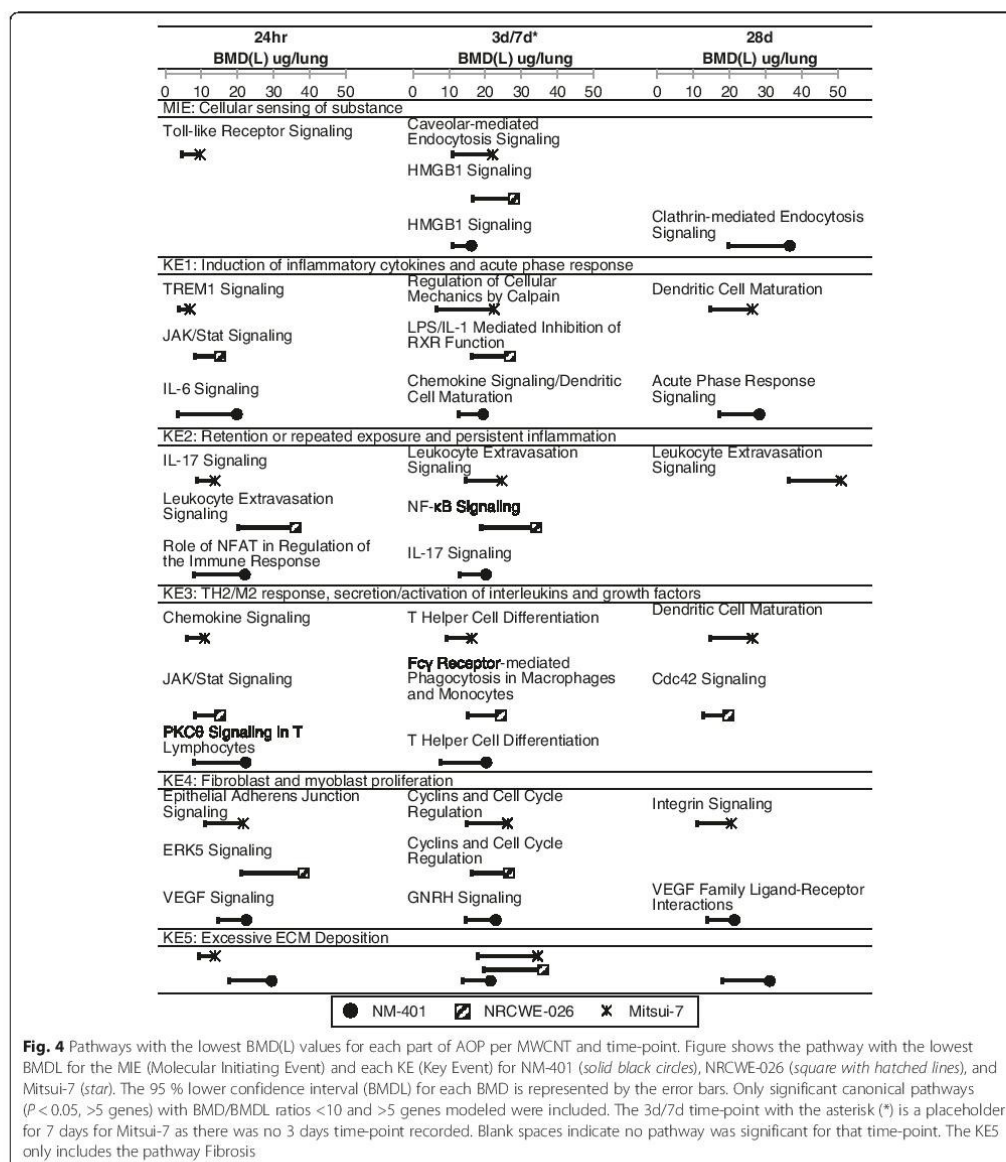


Fig. 3 The distribution of pathway BMD-median values is influenced by post-exposure time. Distributions of pathway BMD-median values for NM-401 (top), NRCWE-026 (center), and Mitsui-7 (bottom). Pathways were only considered in this analysis if they were significant ($P < 0.05$) with five or more DEGs associated with them and if they had five or more molecules with goodness-of-fit P value > 0.1 and BMD/BMDL ratios < 10 . Overlain table indicates the median BMD(L) across all pathways for each time-point

(Additional file 2: Table S2). For example, HMGB1 Signaling, NF- κ B Signaling, and Dendritic Cell Maturation were perturbed at 3 days by NM-401 and NRCWE-026 and at 7 days by Mitsui-7. In addition, Acute Phase Response was perturbed at 24 h, 3, and 28 days by NM-401 and Agranulocyte Adhesion and Diapedesis was perturbed at 24 h, 7, 28, and 56 days by Mitsui-7. Additionally, although the magnitude of changes in the expression levels of the individual genes differed between the MWCNT types [19, 44], which was reflected in the subtle differences between the BMD(L) values for a specific pathway for individual MWCNT types, the BMD(L) confidence intervals across the MWCNT types overlapped (Fig. 4). NRCWE-026 significantly altered a single pathway 28 days post-exposure (CDC42 Signaling). In addition, the pathway BMD(L) values 3 days post-exposure were relatively higher for this MWCNT than BMDL values at

3 days for NM-401 or at 7 days for Mitsui-7. BMDL values ranged from 15.1 to 21.2 μ g/mouse for NRCWE-026, 8.1–18.4 μ g/mouse for NM-401, and 7.1–30.3 μ g/mouse for Mitsui-7. The lack of significant pathways at 28-days post-exposure to NRCWE-026 corroborates histopathological observations in the lungs: qualitative assessment of the lung sections from mice exposed to NRCWE-026 showed fewer incidences of fibrotic lesions 28 days post-exposure than NM-401, as described in Poulsen et al. [19]. Additional file 3: Figure S1 depicts a heat map revealing the dose at which each pathway was significantly perturbed. Given that all three MWCNTs perturbed similar pathways across doses and time-points, the results can be broadly generalized to understand the potential lung responses induced by similar nanomaterials, and the approach can be used as a sensitive alternative for



read across purposes and to identify properties that are important for material toxicity.

Apical endpoint BMD(L)s

NIOSH conducted an extensive review of the literature pertaining to the human health effects arising from occupational exposures to both MWCNT and SWCNT

[43]. BMD(L)s for MWCNTs were derived using fibrosis-related apical endpoint data (e.g., alveolar connective tissue thickening, alveolar septal thickening, granulomatous inflammation, and hydroxyproline amount) presented in four individual studies [32–35] (Additional file 4: Table S3). Only data from Mercer et al. [34] were included in the present study for comparison of apical

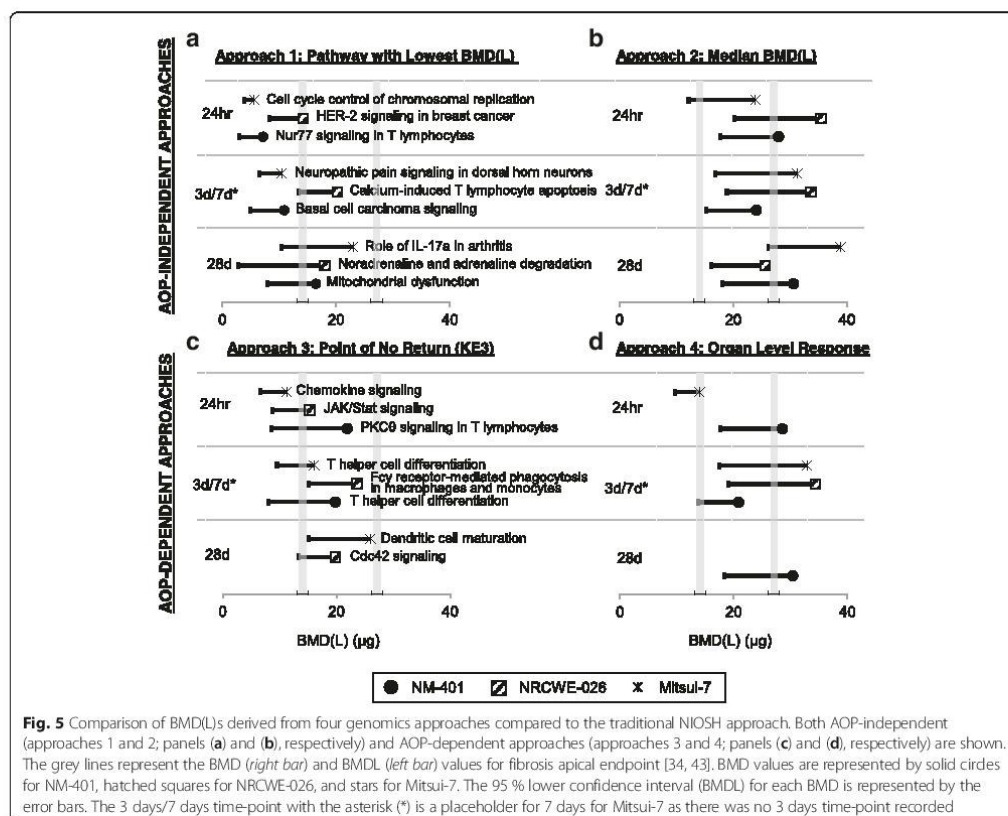
and transcriptomic PODs because: (a) the experimental design and the MWCNT studied was identical to Guo et al. [44], which investigated the pulmonary transcriptomic response; and (b) it is the only study that examined apical endpoints in a mouse model, which minimizes cross-species extrapolation. Morphometric analyses of histological sections for alveolar connective tissue thickening, a biologically relevant event in pulmonary fibrosis, were used in Mercer et al. [34] to calculate apical endpoint BMD(L) values and to derive a POD. This analysis revealed BMD and BMDLs of 27.1 and 14.1 $\mu\text{g}/\text{mouse}$, respectively for lung fibrosis induced by Mitsui-7.

Approaches for deriving transcriptional PODs

Several different approaches were explored to determine the best method to derive a transcriptional POD: 1) the lowest BMD(L) value for any pathway, 2) the median pathway BMD(L) value, 3) the lowest BMD(L) value based on pathways related to the KE determined to be

the 'point of no return' (the initiation/occurrence of a specific key event, beyond which the removal of the toxic substance or inhibition of injury does not result in the reversal of effects or recovery), and 4) the BMD(L) value based on pathways related to lung fibrosis. These approaches include AOP-independent (1 and 2) and AOP-dependent approaches (3 and 4) for deriving a transcriptional POD.

Approach 1, based on the most sensitive pathways (Fig. 5a), yielded BMD(L)s between 7.1 (3.0) and 14.2 (8.2) $\mu\text{g}/\text{mouse}$ for NM-401, 10.9 (4.9) and 20.2 (13.4) $\mu\text{g}/\text{mouse}$ for NRCWE-026, and 18.0 (2.8) and 23.0 (10.43) $\mu\text{g}/\text{mouse}$ for Mitsui-7 across all post-exposure time-points. For the most part, the BMD(L) values for these pathways were below the NIOSH-calculated BMD(L) of 27.1 (14.1) $\mu\text{g}/\text{mouse}$, which was based on measuring alveolar thickness [34, 43]. The most sensitive pathway approach has been recommended for deriving PODs for cancer and non-cancer endpoints [28]. Although the pathways may be reflective of the tissue



injury caused by the initial interaction of MWCNTs with the surrounding lung tissue, they were not consistently observed across the types of MWCNTs, doses, or post-exposure time-points. Moreover, their implication in the final adverse outcome is less understood. Indeed, a POD based on the most sensitive or lowest BMD(L) values may not be based on a biological effect that is connected to the adverse outcome. However, we note that there are very few nanomaterials for which underlying mechanisms of toxicity are characterized or a definite adverse outcome has been identified. Thus, while this AOP-independent approach may be too conservative, it would serve to limit the exposure to particular nanomaterials in the environment until specific hazard and mechanistic information becomes available for them.

The median BMD(L) value approach, which is also AOP-independent (based on BMDs for all pathways with five or more DEGs associated with them and if they had five or more molecules with goodness-of-fit P value > 0.1 and BMD/BMDL ratios < 10), represents a less conservative approach to POD selection (Fig. 5b). For the most part, the BMD(L) values using this approach were above the NIOSH-calculated BMD(L) of 27.1 (14.1) $\mu\text{g}/\text{mouse}$ with the exception of a few values (23.9 (15.1) $\mu\text{g}/\text{mouse}$ for Mitsui-7 at 24 h, 24.1 (15.3) $\mu\text{g}/\text{mouse}$ for NM-401 at 3 days, and 25.6 (16.0) $\mu\text{g}/\text{mouse}$ for NRCWE-026 at 28 days). However, the confidence intervals of the median values (the median is the peak of biological activity and represents the most robust central measure of the response) were very close to the lower confidence intervals of the NIOSH-calculated BMD and BMDL values. Thus, without prior knowledge of the underlying mechanism of toxicity or a definite adverse outcome, as is the case with nanomaterials, this approach provides a point at which the system is likely to be responding to the exposure.

Transcriptional BMDs based on the AOP-dependent approaches (approaches 3 and 4; Fig. 5c-d) resulted in values consistent with the conventional fibrosis endpoint (alveolar wall thickness, represented by grey bars in Fig. 5). Of benefit in these approaches is the fact that these pathway perturbations are biologically linked to the AO, and thus it is biologically plausible that the doses that affect these pathways might approximate the dose at which adversity may arise. Modes of Action (MOAs) have been routinely employed to inform risk assessments for chemicals. However, MOA in the conventional sense provides information concerning molecular and cellular events engaged by the chemicals in eliciting a response that is quantified but may or may not always imply that the response will induce an adverse outcome. AOPs have evolved from the concept of MOAs, and also emphasize the importance of connecting various events that lead to toxicity. Both require

selection and ordering of the essential events that are readily quantifiable along the pathway to toxicity, and require assessment of empirical data (temporal and incidence concordance) and biological plausibility supporting the relationship shared between events along the pathway. In approaches 3 and 4, perturbed pathways were characterized in terms of their association with KEs, which provides a biological rationale for their consideration in POD selection (if apical data are not available). In approach 3, we selected a single KE (KE3: Th2 Signaling activation), the activation of which we argue marks the 'point of no return' in the process of developing lung fibrosis. The various pathways involved in KE3 including Dendritic Cell Maturation, Cdc42 Signalling, T Helper Cell Differentiation, Fc γ Receptor-Mediated Phagocytosis in Macrophages and Monocytes, Chemokine Signalling, JAK/Stat Signalling, and PKC Theta Signalling in T Lymphocytes, showed BMDs that spanned the POD range published by NIOSH for the apical endpoint of concern (alveolar connective tissue thickening) (Fig. 5c). In approach 4, the POD selection was based on a pathway tied directly to the AO, which is the lung fibrosis pathway described under KE5. However, in our case, the BMDs based on the pathways associated with fibrosis (the ultimate result of exposure to MWCNTs) were less consistent for the three MWCNTs and across the different exposure time-points (Fig. 5d) relative to the other three approaches. The inconsistencies may be due to the fact that representative pathways for fibrosis were not significant across the MWCNT types, doses and post-exposure time-points.

Taken together, the four approaches for transcriptional- or pathway-based POD derivation produced comparable results across time-points and across the three types of MWCNTs. The confidence intervals between datasets often overlapped; however, the AOP-centered approach based on the 'point of no return' KE (approach 3) and the median BMD approach (approach 2) more closely reflected the histopathological observations demonstrating alveolar thickness that are critical for the development of fibrosis.

It is important to note that although the individual MWCNTs varied in their physico-chemical properties, all of them bio-persist long after the exposure and exhibit potential to induce lung fibrosis. In addition, at the molecular level, they induced similar pathway perturbations and BMD(L) values suggesting that the mechanisms underlying the fibrotic responses are the same for the three MWCNTs. The fact that all three MWCNT types induce similar response at the pathway level is interesting and suggests that all MWCNTs are inherently capable of inducing lung fibrosis, which may be associated with their fiber-like shape and tissue biopersistence. However, some MWCNTs may be more

potent than others in inducing lung fibrosis, which could be attributed to their additional physico-chemical properties such as chemical impurities, surface functionalization, etc. Previously, we have shown that subtle differences in the degree of pathway perturbation somewhat reflects the extent of fibrosis development [19]. In another study, we have shown that changes in the gene expression levels and pathway perturbations can effectively discriminate between the nanoTiO₂ particles that are weak or strong inducers of lung inflammation [16]. Collectively, these results suggest that transcriptomic responses are a sensitive approach to identifying potentially harmful nanomaterials and that the transcriptomic BMD(L)s can be used to establish acceptable levels of exposure for new nanomaterials. As the public repository of genomics dataset is populated and the underlying mechanisms of toxicity are revealed, these types of data can be used in read across and category approaches for risk assessment.

Conclusions

In conclusion, the results show that transcriptomics-derived PODs accurately reflect the levels of exposure to MWCNTs at which lung fibrosis potentially develops in animals. The significant overlap in pathway responses induced by the different types of MWCNTs and comparable pathway-based POD values suggests that the approach can be generalised to other forms of MWCNTs. This study demonstrates that in the absence of any apical endpoint toxicological data conventionally preferred in HHRA, transcriptomics data can be used to inform the potential hazards of these novel materials and to support interim regulatory decisions. We speculate that this toxicogenomics/AOP approach will be of value for prediction of fibrogenic potency and hazard ranking of different MWCNTs. The case study is limited to the analysis of *in vivo* genomics data and its applicability to HHRA, but we emphasize that this approach does not represent a full risk assessment. While analysis of a well-characterized nanomaterial known to induce adverse effects provides an initial framework for future studies, regulatory validation of these methods will require a large repository of publicly available high quality gene expression data that span several well-characterised reference nanomaterials of diverse properties, multiple doses, a range of post-exposure time-points, and multiple species. Guidance and appropriate criteria for assessing experimental design and quality of data produced, rigorous statistical and computational algorithms to handle the large amount of data, and harmonised guidelines for integrating omics data into HHRA are needed. Finally, given the complexity of data, education and training programs for researchers and risk assessors in the generation, analysis, and application of omics data to different components of the risk assessment paradigm should be developed.

Additional files

Additional file 1: Table S1. Particle physicochemical properties and exposure information. (XLSX 10 kb)

Additional file 2: Table S2. List of all significant pathways associated with the molecular initiating event (MIE), each key event (KE), and each associative event (AE) including the benchmark dose (BMD) value (BMDL values included in parentheses) for each MWCNT investigated at each time-point. n.a. indicates the BMD(L) could not be calculated due to poor model fit. (XLSX 18 kb)

Additional file 3: Figure S1. Heatmap showing the dose at which each pathway was significantly perturbed. Each column represents a post-exposure time point for the denoted MWCNT, and each row represents a significantly perturbed pathway. All colored cells represent the lowest dose at which the pathway is perturbed. Blank cells represent pathways that were not significantly perturbed. (PPTX 76 kb)

Additional file 4: Table S3. Details of rodent studies considered by NIOSH in calculating points of departure (PODs). (DOCX 15 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SL analysed the data and contributed to writing of the manuscript. JN contributed to AOP development. AW contributed analysis tools, CLY contributed to manuscript development. HW, UV contributed data and commented on the manuscript. SH conceptualized the experiments, analysed data, and developed and co-wrote the manuscript. All authors have read and approved the manuscript.

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Appendix III Integrating toxicogenomics into human health risk assessment: lessons learned from the benzo[a]pyrene case study.

REVIEW ARTICLE

Integrating toxicogenomics into human health risk assessment: Lessons learned from the benzo[a]pyrene case study

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Abstract

The use of short-term toxicogenomic tests to predict cancer (or other health effects) offers considerable advantages relative to traditional toxicity testing methods. The advantages include increased throughput, increased mechanistic data, and significantly reduced costs. However, precisely how toxicogenomics data can be used to support human health risk assessment (RA) is unclear. In a companion paper (Moffat et al. 2014), we present a case study evaluating the utility of toxicogenomics in the RA of benzo[a]pyrene (BaP), a known human carcinogen. The case study is meant as a proof-of-principle exercise using a well-established mode of action (MOA) that impacts multiple tissues, which should provide a best case example. We found that toxicogenomics provided rich mechanistic data applicable to hazard identification, dose–response analysis, and quantitative RA of BaP. Based on this work, here we share some useful lessons for both research and RA, and outline our perspective on how toxicogenomics can benefit RA in the short- and long-term. Specifically, we focus on (1) obtaining biologically relevant data that are readily suitable for establishing an MOA for toxicants, (2) examining the human relevance of an MOA from animal testing, and (3) proposing appropriate quantitative values for RA. We describe our envisioned strategy on how toxicogenomics can become a tool in RA, especially when anchored to other short-term toxicity tests (apical endpoints) to increase confidence in the proposed MOA, and emphasize the need for additional studies on other MOAs to define the best practices in the application of toxicogenomics in RA.

Keywords

benchmark dose, carcinogens, dose–response, environmental pollutant, genotoxicity, mode of action, point of departure, polycyclic aromatic hydrocarbon

History

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Table of Contents

Abstract..	44
Background ..	44
Experimental design...	46
Dose selection ..	46
Exposure duration...	47
Timing of sample collection ..	47
Sample size and matched controls..	47
Impact of platform, pre-processing, and normalization of gene expression analysis on BMD estimation .	47
The added advantages of data derived from cultured human cells...	48
Data analysis and interpretation.	48
Pathway and network analysis to develop a MOA...	48
BMD modeling ..	49
Confidence in the MOA...	49
Selecting the appropriate POD values for RA.	50
Conclusions ..	50
Acknowledgments ..	51
Declaration of interest ..	51
References..	51

Background

Significant modifications to existing toxicity testing approaches are required to facilitate the evaluation of thousands of data-poor chemicals awaiting assessment by regulatory bodies worldwide. For example, a standard approach to cancer RA of chemicals is the rodent two-year cancer bioassay. This method is expensive, time- and animal-intensive, and provides very little mechanistic information. Toxicogenomics (the application of “omics” technologies to toxicology) is often touted as a potential alternative to traditional assays, and offers the hope of circumventing some of these limitations. Toxicogenomics may provide toxicological data that are predictive of potentially carcinogenic effects of a chemical in a shorter period of time, and in a cost- and resource-effective manner. In addition, toxicogenomics can provide comprehensive details on the underlying mechanisms of chemical-induced toxicity and thereby identify earlier key events for more refined human health RA that is more relevant to the low environmental doses of chemicals. A key event is defined as “an empirically observable precursor step that is itself a necessary element of the MOA or is a biologically based marker for such an element” (US EPA 2005). Despite these advantages, formal

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guidance for the effective use of toxicogenomics in routine chemical RA is lacking.

In our companion paper (Moffat et al. 2014 [this issue]), we compared a standard RA approach with the approach that uses toxicogenomics data for the evaluation of BaP, a data-rich group 1 human carcinogen (International Agency for Research on Cancer (IARC)). The case study is meant to demonstrate how toxicogenomics might work by providing proof of principle using a very well-established MOA, and a very well-studied chemical. As BaP is a genotoxic carcinogen, our case study did not explore any difficulties associated with tissue selection that is typically required for non-genotoxic and non-cancer toxicants. Therefore, future studies are required for non-genotoxic compounds with wide array of MOAs to demonstrate whether toxicogenomics is also useful in these cases. Such studies assist in investigating the effectiveness of toxicogenomics in identifying relevant hazards, informing MOA, and deriving POD, compared with established toxicity tests. Without such proof-of-principle studies on well-established endpoints/chemicals/MOAs, we will never know how effective is toxicogenomics in predicting apical endpoints and points of departure.

Humans are exposed to BaP in food, water, and air (reviewed in IARC 2012, Moffat et al. 2014 [this issue]). Three RAs were conducted: (RA1) RA based on conventional toxicity testing apical data alone that was taken from the literature; (RA2) RA based on integrating apical and toxicogenomics data; and (RA3) RA using toxicogenomics data alone. While toxicogenomics data are tissue-specific, meta-analysis of cancer bioassay data revealed that eight tissues—liver, lung, mammary gland, stomach, vascular system, kidney, hematopoietic system, and urinary bladder—identified 92% of all carcinogens (354/384) tested in mouse and 82% (388/471) of carcinogens in rats (Gold et al. 2001). Therefore, these eight tissues should comprise the starting point for analyzing global gene expression for chemical assessments as has been suggested by others (Thomas et al. 2013). In our case, there could have been some bias in target tissue selection because of *a priori* knowledge

of BaP in the original experiments. The liver is an obvious first-pass target tissue of a chemical delivered orally as it is the primary site of metabolism, the (fore)stomach is the point of contact for oral exposures, and lung may be exposed to the parent compound or its metabolite(s) via circulation, we believe that our selection of the three tissues is a logical choice for preliminary toxicogenomic analyses. Figure 1 illustrates our proposed approach for the use of toxicogenomics in RA, which we developed in the case study.

Overall, toxicogenomics was able to correctly identify BaP as a genotoxic chemical that operates through interaction with the aryl hydrocarbon receptor and perturbs genes in pathways associated with numerous hallmarks of cancer. Importantly, genotoxicity was independently confirmed by a direct measure of DNA damage and the formation of BaP–DNA adducts following BaP exposure in mice. Data from TK6 cells, a human lymphoblastoid cell line, confirmed that BaP is genotoxic to human cells. These data allowed us to propose a genotoxic MOA for BaP and to derive a point of departure (POD) defined as “the dose-response point that marks the beginning of a low-dose extrapolation. This point can be the lower bound on dose for an estimated incidence or a change in response level from a dose-response model (benchmark dose [BMD]), or a NOAEL or LOAEL for an observed incidence, or change in level of response.” (US EPA 2011). For carcinogens, BMD rather than no(lowest)-observed-adverse level (N(L)OAE) values are normally used in RA (US EPA 2005). The toxicogenomics-derived POD gave information which was quantitatively similar to that obtained from the standard cancer bioassay. Although a method for directly comparing transcriptional (continuous data) and tumor (dichotomous data) PODs is currently unavailable, we felt that this is a fair comparison at this time because both approaches consider a 10% change in response using appropriate models for both types of data.

The case study is meant to demonstrate how toxicogenomics might work by providing proof of principle using a very well-established MOA, a very well-studied chemical and existing data. As BaP is a genotoxic carcinogen, our case study did not

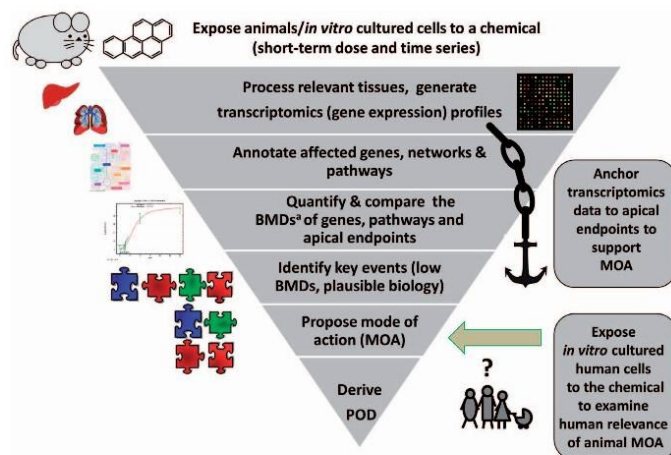


Figure 1. Proposed approach for the integration of toxicogenomics into health RA employed in the case study.

explore any difficulties associated with tissue selection that is typically required for non-genotoxic and non-cancer toxicants. Therefore, future studies are required for non-genotoxic compounds with wide array of MOAs to demonstrate whether toxicogenomics is also useful in these cases. Such studies assist in investigating the effectiveness of toxicogenomics in identifying relevant hazards, informing MOA, and deriving POD, compared with established toxicity tests. The three RAs involved collaborative work among regulators, research scientists, and bioinformaticians. Based on this experience, we provide an overview of the lessons that we learned from the case study that we feel will be valuable in further attempts to harvest the power of toxicogenomics to inform RA. In addition to broad perspectives, advantages, and disadvantages, we describe specific lessons relating to (1) experimental design, (2) data analysis and interpretation, and (3) proposing appropriate POD values for quantitative RA of potential chemical carcinogens. We hope that these lessons (summarized in Table 1) will be useful for both regulators who are trying to apply toxicogenomics in their RAs, and for research scientists who are designing toxicogenomic experiments that they hope may be useful for chemical regulation.

Experimental design

Dose selection

As stated above, POD is the dose–response point that marks the beginning of a low-dose extrapolation; therefore, accurate POD estimation lies at the heart of quantitative health RA. To generate

informative data for dose–response modeling and BMD analysis, PROAST and Benchmark dose software (BMDS) are commonly used. For the latter, at least one dose near the anticipated BMD is preferable (US EPA 2012) such that the doses selected span a range of doses, including the lower doses that should be closer to points of departure for sensitive endpoints. As dose selection to capture the range of anticipated BMDs for proposed key events may be challenging, initial/preliminary experiments, even if they fail to cover the entire range of anticipated BMDs, would aid in the dose selection for future studies. In addition, the BMD approach attempts to extrapolate dose–response to lower doses, outside the study range. A full list of considerations for complying with BMD modeling requirements is offered elsewhere (US EPA 2012). In our review of the literature on BaP and other compounds (papers published before November 2013), we found that many published toxicogenomic studies used excessively high doses that were generally not appropriate for deriving BMDs as they were too distant from the BMD or used only one or two (often very high) doses. The BMD analysis for apical endpoints can be run with a minimum of two doses plus a control (except for the Hill model). Likewise, BMDExpress allows the linear model to be applied for only two doses and a control. However, we encourage researchers to include at least three doses plus a control in their toxicogenomic experiments to get better idea of dose–response using other models available, and to include at least one dose at a level close to the anticipated BMD value. The exact dose selection should reflect the study objectives (e.g., hazard identification vs. evaluating non-linear effects).

Table 1. Ten lessons learned from collaborative work between risk assessors and research scientists for the use of toxicogenomics data in RA.

Lesson learned	Description
1. Communication	Dialogue between research scientists and risk assessors facilitates improved experimental design and appropriate interpretation of the data. Increased communication will expedite the implementation of integrated testing strategies
2. Ease	Toxicogenomic software applications are now readily available and short courses in bioinformatics are available to provide instruction on their application. Several software packages exist to help in data interpretation and are specifically developed for applications in RA
3. Utility of high-content information	Large amounts of data can be generated relatively quickly and inexpensively, providing a wealth of information beyond what is generated from standard assays. The data collected provide a detailed overview of the organismal response at the molecular level
4. Experimental considerations	Research scientists should consider including experimental doses that facilitate dose–response modeling tools (e.g., BMDExpress), as well as at least one dose within the (anticipated) range of the BMD. We urge investigators to ensure that they avoid excessively high doses. The timing of sample collection and the duration of the exposure are also critical considerations and depend on the objectives of the study
5. Statistics	Analyzing thousands of toxicogenomic endpoints requires stringent statistical analysis. Typically, this requires a sample size of at least three (but preferably ≥ 5) for robust analysis
6. Biological significance	Specific genes and pathways that are aligned with key events in the MOA can be used to assign BMDs for toxicologically relevant changes reflecting the known biology and MOA of a compound. See the text for the alternative (NOTEL) approach
7. Human relevance	The plausibility of an animal MOA in humans can be examined by experiments with cultured human cells
8. Support MOA with apical data (targeted testing)	The toxicogenomics-based MOA can be verified by comparing with apical data (phenotypic anchoring or targeted testing). For example, genotoxicity/P53/DNA-damage response can be confirmed by direct measurements of genotoxicity such as DNA adducts, strand breaks, chromosome damage, and mutations
9. Selection of PODs	Current RA science is based on the observation of biologically relevant changes reflecting adverse effect(s) of a chemical. Theoretically, the key event preceding the “committed” step in a proposed MOA may provide the most appropriate POD value that would be protective for the adverse (rather than adaptive) response of an organism exposed to a chemical
10. Great potential	Our case study and other accumulating evidence (Thomas et al. 2011, 2012, Bourdon et al. 2013, Jackson et al. 2014, for example) show that toxicogenomics can provide rich mechanistic data to define the MOA(s) of a given chemical, yielding PODs similar to those derived from traditional assays as our case study suggested. This, together with the relatively lower costs associated with it, compared with some traditional tests makes toxicogenomics a very attractive strategy for immediate and future applications in health RA of chemicals

Selection of high, medium, and low dose(s) for carcinogenicity, chronic toxicity, and combined chronic toxicity/carcinogenicity studies is discussed elsewhere (Organization for Economic Cooperation and Development [OECD] 2012a). In general, these recommendations discuss the need to select (1) a high enough dose to capture toxicity; (2) a medium dose to better characterize the dose–response curve; and (3) low doses (near the anticipated BMD value) in order to provide an assessment of more realistic environmental exposures (OECD 2012a). In addition, it is suggested that available toxicokinetic data Absorption, distribution, metabolism, and excretion (ADME) be always considered during dose selection for a chronic toxicity or carcinogenicity study (OECD 2012a).

Exposure duration

It has been demonstrated that longer exposure durations (e.g., the 90-day sampling time point) may yield more predictive toxicogenomic signatures for hepatocarcinogenesis than shorter ones (e.g., 2- and 14-day exposure) (Auerbach et al. 2010). Indeed, sub-chronic exposure durations where samples are collected shortly after the last exposure would produce expression profiles that encompass genes that span key events in an MOA, including both early and late effects. However, time of sampling post-exposure is equally important in both acute and sub-chronic exposure durations. Our sub-chronic, 28-day exposure yielded genotoxic effects (e.g., 0.3 mg BaP/kg body weight [bw] per day for micronucleus frequency [Lemieux et al. 2011]) that were much closer to cancer endpoints than transcriptomic PODs for the 28 + 3 day experiments in all three examined tissues (liver, lung, and forestomach). In contrast, the POD that could be derived from the liver collected 24h after the 3-day exposure to BaP was much closer to the POD of cancer (1.0 vs. 1.2 mg/kg bw per day). This suggests that, for transcriptomic experiments, timing of sample collection may be of more importance compared with exposure duration, reflecting the dynamic nature of highly interconnected signaling networks. However, both exposure duration and time post-sampling must be carefully considered in experimental design.

Timing of sample collection

The timing of sample collection greatly impacts the information that can be derived from a toxicogenomics experiment. As a gene expression profile is simply a snapshot in time, both exposure duration and time of sample collection following the last exposure are critical considerations. For example, tissues from animals exposed to chemicals that undergo rapid metabolism and excretion must be collected within a short time following the exposure to identify and model the molecular initiating event or early key events in the MOA.

For BaP, we observed altered expression of genes involved in the early key events in our proposed MOA when mice were sacrificed within 4–24 h of the final exposure in a 3-day exposure regimen in the lung and the liver (Moffat et al. 2014 [this issue]). These time points were effective in identifying the induction of the appropriate xenobiotic metabolism pathways and can be highly useful for providing insight into the toxicokinetics/dynamics of the compound. Again, the samples harvested three days following daily exposure of mice to BaP for 28 days, on the other hand, were useful in detecting subsequent

key events involving DNA damage, and altered cell signaling and apoptosis, but provided very little insight into the early key events. Thus, we urge researchers to include a range of both early and late sampling times in order to capture a comprehensive profile of the dynamic time- and dose-dependent nature of the MOA(s).

Sample size and matched controls

It is necessary to use a sufficient (typically, $n \geq 4$ [Thomas et al. 2011]; $n = 4-6$ [Lin et al. 2010]) sample size to ensure that statistical analyses can be undertaken. We encountered toxicogenomic studies with less than three replicates ($n < 3$). This can limit their utility in RA. We recommended that a minimum of three (but preferably more) biological replicates (e.g., individual animals or experimental replicates in cell culture assays) should be used. However, we caution that without sufficient dose groups, a sample size of three would not be enough to justify the asymptotic assumptions that the BMD software is making (e.g., normality). For that reason, we typically do not conduct experiments with less than five animals per biological condition. Importantly, increasing the number of doses rather than increasing sample size usually improves the accuracy in BMD estimation (Allen et al. 1996). The minimum sample size required remains unclear, but it would certainly be experiment-dependent and require consultations with a statistician. We recommend that power analyses be conducted and the objectives of the study be well defined in order to establish sample size. In addition, we note that properly matched time and vehicle controls are essential for all experiments.

Impact of platform, pre-processing, and normalization of gene expression analysis on BMD estimation

Various types of gene expression platforms can be used to evaluate gene expression. Today, the most broadly used platforms are DNA microarrays and quantitative real-time PCR, although RNA sequencing is gaining popularity. Extensive work in this field has demonstrated that although these platforms have varying degrees of sensitivity, the data produced from one platform are generally quite reproducible on another (e.g., Shi et al. 2006, Yauk and Berndt 2007). The type of data processing (i.e., background subtraction, filtering, and normalization) applied for the analysis of gene expression data can impact the genes identified as differentially expressed and the magnitude of the fold change (Steinhoff and Vingron 2006). In addition, the statistical tests applied may also greatly impact what genes are in a gene list (e.g., normal vs. non-parametric tests). It is critical that the methodologies are fully described in order to ensure that the readers are fully aware of any biases that may be introduced by the applied data processing and analytical approaches. Importantly, all data should be publicly available so that new analyses can readily be performed by others in the field.

An Agilent two-color reference design was the primary microarray design used in our case study. The microarray studies were originally developed for “discovery” purposes and not for quantitative analyses. We recommend careful determination of the objectives of the study and the intended use of the data in establishing the utility of any particular microarray

experimental design for RA purposes. The data in our case study were normalized using standard locally weighted scatterplot smoothing (LOWESS) in a similar fashion across all studies (thus all studies were comparable) and genes were identified as differentially expressed using microarray analysis of variance (MAANOVA). As such, we were confident that the considerations above were dealt with in the same fashion and that experiments were directly comparable in the identification of differentially expressed genes in our case study. The BMD analysis of microarray data has been more widely applied to the Affymetrix platform (Thomas et al. 2007, 2011, 2012). It is recognized that a two-color design can compress the fold changes and thus reduce the dynamic range of a microarray experiment (Wang et al. 2006). These differences in dynamic range may have an impact on BMDs, but this has not been specifically tested. In addition, background subtraction may directly impact BMDs. Datasets derived from background-subtracted data would potentially yield higher fold changes for genes in the lower range of signal intensities.

Also, the effect of various types of normalization that are used may also contribute to variances in the BMDs acquired from a specific microarray dataset. The effect of different normalization methods on BMD estimates was studied using DNA microarray (Affymetrix) data generated from the livers of F344/DuCrI adult male rats exposed to five doses of bromobenzene over 13 weeks (Black et al. 2014). A significant effect of the normalization method on the number and identity of the genes that were identified as differentially expressed was found. However, for microarrays, gene-based BMD values exhibited a linear relationship with high (0.843–0.921) correlation coefficients for pairwise comparisons across normalization approaches. Therefore, the normalization approach has a bigger effect on identifying differentially expressed genes than on BMD estimates, which encourages using DNA microarray data for BMD estimation and RA. However, the effect of normalization on BMD estimates for other platforms (e.g., Agilent) is still unknown and is currently being investigated in our group. Finally, we learned that pre-filtering of gene expression data (e.g., analyzing only probes with false discovery rate (FDR) p -value of < 0.05 as defined by MAANOVA or ANOVA) versus not filtering data at all could make a difference for the BMD modeling at the pathway level. We applied the approach of R. Thomas' group and did not filter our data before analysis by BMDExpress. The above considerations are important for the quantitative analysis of gene expression data from microarray platforms that must be addressed in future experiments. Future applications in this field also include higher-throughput real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) approaches and RNA sequencing. All these technologies will be useful for characterizing the dose–response relationship for gene expression data, and analyses should be undertaken to explore the relationship between BMDs derived from various platforms.

The added advantages of data derived from cultured human cells

We found that cultured human cells exposed to BaP provided a tremendous advantage to our RA incorporating genomics. The top pathways perturbed in the toxicogenomic profiles of cultured human BaP-exposed TK6 cells were also among the

main pathways identified in the liver, lung, and forestomach of BaP-treated mice demonstrating the human relevance of these pathway changes. The *in vitro* test system was metabolically competent (TK6 cells were cultured with rat liver S9). The top pathways that are affected in both rodents (*in vivo*) and cultured human cells included Aryl hydrocarbon receptor (AHR), Nrf2, and p53 signaling. A hierarchical cluster analysis of the gene expression data from BaP-exposed TK6 cells using an established set of genes that has been used to differentiate genotoxic and non-genotoxic chemicals (Goodsaid et al. 2010, Li et al. 2014 [manuscript submitted]) clearly showed that the expression profiles were consistent with genotoxic chemicals. These data, along with apical endpoints (micronucleus induction in TK6 cells), support the genotoxic MOA of BaP and its relevance in humans. The finding was also supported by work in another laboratory on a human hepatocellular carcinoma cell line (HepG2 cells) (van Delft et al. 2010). *In vitro* profiling costs substantially less and is much more rapid than animal experiments. That *in vitro* toxicogenomics was effective in identifying the genotoxic MOA and in confirming human relevance supports the idea that *in vitro* toxicogenomics may eventually enable the rapid identification of diverse types of other potential hazards that are relevant to humans. The development of refined gene expression signatures to predict a diverse panel of toxicities will benefit this field. Other such predictive gene sets exist or are in development (e.g., Elferink et al. 2008, Mathijs et al. 2009, Daston and Naciff 2010). However, our case study supports the notion that experimental designs incorporating gene expression profiling in cultured human cells add tremendous value for the preliminary evaluation of chemicals. In addition, toxicogenomics data from cultured cells could be helpful in quantitative *in vitro* to *in vivo* extrapolation (QIVIVE), to examine the relationship between adverse effects seen in *in vitro* toxicity tests, and the possible consequences of target tissue exposures in humans.

Data analysis and interpretation

Pathway and network analysis to develop a MOA

Current software packages are available to facilitate the analysis of genomics data and apply it to RA. For example, various commercial applications (e.g., Ingenuity Pathway Analysis® [IPA®], MetaCore™ [which examines GeneGO pathways], and Pathway Studio) and free web-based applications (e.g., Gene Set Enrichment Analysis and the Database for Annotation, Visualization, and Integrated Discovery, which analyzes Kyoto Encyclopedia of Genes and Genomes' pathways) are readily used to identify genes, pathways, or networks affected by chemical treatments. We note that in the absence of any apical data, and assuming no *a priori* knowledge of BaP, we were able to derive a rough MOA that could be used to identify hazards associated with BaP exposure (hazard identification), and facilitate prioritization of the appropriate follow-up toxicity tests. Our case study demonstrates that toxicogenomics information alone could serve as initial data for proposing MOAs in the RA of data-poor chemicals requiring immediate action. We think that a combined toxicogenomics–quantitative structure–activity relationship (QSAR) analysis would provide the best path forward for chemicals with no toxicity data, especially as the costs associated with genomic technologies in general continue to decline.

BMD modeling

We refer the reader to Thomas et al. (2007, 2011, 2012) for the details of BMD derivation from transcriptomic datasets. BMD values can be analyzed in the context of the established MOA; alternatively, transcriptomics data can be used to identify a no transcriptional effect level (NOTEL) (Lobenhofer et al. 2004). The former is more useful for risk assessors as it seeks to mechanistically describe how a chemical carcinogen achieves its adverse effect. This knowledge helps to reduce uncertainty and provide insight into the human relevance of a MOA operative in animals. While the NOTEL approach could be instrumental for screening and prioritization, it is agnostic in nature and, in terms of mechanistic information provided, is not very different from traditional bioassays. Similar to the traditional approach in RA that considers NOAEL, LOAEL, and BMD, all three descriptors (NOTEL, LOTEL, and transcriptomic BMD values), when available, could be used as part of the POD selection.

Several recent studies have demonstrated that the lowest median BMD values for the most sensitive gene ontology (GO) categories from short-term *in vivo* transcriptomics data in mice correlate well with apical data from standard histopathology and cancer bioassay experiments (Thomas et al. 2011, 2012). In our studies (Moffat et al. 2014 [this issue]; Bourdon et al. 2013), the most sensitive processes (i.e., the IPA pathways or GO terms with the lowest median BMD values) were not obviously related to known biological responses to chemical carcinogens or to what is currently known about cellular responses to BaP. A similar result was noted by Thomas et al. (2012), who showed that the most sensitive GO categories gave BMDs that were highly similar to apical endpoints, but were not obviously associated with the specific apical measures. This may reflect the fact that certain chemical carcinogens affect multiple biological targets (Thomas et al. 2013). In these cases, in the absence of other information related to MOA, applying the lowest overall pathway BMD may be suitable for RA as it may identify the doses at which the perturbations of multiple biological processes commence. Nevertheless, more research is needed to develop effective strategies for the application of transcriptomic BMD modeling to RA.

Unlike an evaluation based exclusively on the lowest BMDs (regardless of the pathway) as described above, we used our pathway-enrichment approach to identify BMDs for perturbations that are related directly to the key events of interest. While many tools (see above) can be used to identify perturbed genes and to develop a MOA from toxicogenomics data, we used IPA as we found that the genes within its pathways were more relevant to AHR-mediated toxicity than some of the other tools. For example, IPA identified "Metabolism of xenobiotics by cytochrome P450" and "Nrf2-mediated oxidative stress response" as the top canonical pathways perturbed by high doses of BaP in the majority of datasets analyzed. This is in excellent agreement with the known genotoxic MOA of BaP and of various polycyclic aromatic hydrocarbons in general. Focusing on the top pathways identified by IPA at the high doses of BaP, we noted that these pathways were perturbed at the low doses of BaP as well, which was confirmed by the BMD modeling. We found that specific genes within these biological pathways were responsive at the lowest doses of

BaP. Our BaP and carbon black nanoparticle studies (Bourdon et al. 2013) demonstrate that such consideration of specific biologically relevant gene expression changes rather than the most sensitive GO categories may also yield POD in-line with apical endpoints and cancer bioassays. Again, in cases where the MOA is unknown or unclear, we found that the pathway with the lowest 10th percentile BMDs would provide POD values that are comparable with those provided by the MOA approach.

Our implementation of a MOA-guided derivation of POD from toxicogenomics data clearly revealed significant gaps in the annotation of genes and pathways in the context of functions and associations with disease. This is an area where our biological knowledge may become the rate-limiting step in developing MOAs. Extensive additional work is needed in order to identify which pathway perturbations are linked to specific apical events and contribute to cancer and other toxicities. This field will only move forward with substantial efforts to conduct more phenotypically anchored toxicogenomic experiments. The database of adverse outcome pathways, which is currently being developed by the OECD, will provide context to toxicogenomics data and help advance the utility of toxicogenomics in RA (OECD 2012b, Yauk et al. 2013). In our case study we used a transgenic rodent mutation reporter assay to provide information on the mutagenicity of BaP, and collected DNA adducts data in parallel. Genotoxicity was also supported through the use of the flow-cytometry micronucleus assay in cultured human cells. These endpoints served as anchors to support the genotoxic MOA of BaP observed in the transcriptomics data. An excellent path forward in this field in the immediate future would be the implementation of toxicogenomics in the two-year cancer bioassay (i.e., at the early time points used for histopathological and clinical chemistry analyses), which would facilitate the linkage of specific pathway perturbations to histopathology and other toxicity measures collected in that assay.

Confidence in the MOA

To build an MOA from toxicogenomics data, we employed the MOA/human relevance (MoA/HR) framework (Sonich-Mullin et al. 2001, Meek et al. 2003, 2014, Boobis et al. 2006, Meek 2008, Meek and Klaunig 2010). This involves scrutinizing the conformance of animal MOA to the Bradford Hill criteria (Hill 1965; simplified in Moffat et al. 2014 [companion paper]) and its plausibility in humans. The examination of individual key events for a given MOA in rodents and their plausibility in humans assists in eliminating instances similar to those of Peroxisome proliferator-activated receptor (PPAR) and Constitutive androstane receptor (CAR)/Pregnane X receptor (PXR) agonists that are relevant to animal, but not human MOAs. We found that apical data (receptor binding studies and enzyme assays in cultured human cells, as well as BaP metabolites and BaP-DNA adducts detection in human samples) helped immensely in demonstrating that the toxicogenomics-based MOA was relevant to humans. The apical data thus supported the validity of toxicogenomics-based MOA and its utility in RA of chemicals. However, our case study suggests that in future studies, if no such data are available, toxicogenomics can be used to identify priorities for targeted testing (and thus be

confirmed by apical endpoints). For example, if there were no apical data available for BaP, our toxicogenomics analysis would suggest that BaP is potentially a genotoxic carcinogen that causes DNA damage and initiates a p53 DNA damage response, in addition to being an AHR agonist. These findings would lead to the recommendation of genotoxicity testing—that is, targeted testing. We suggest that *in vitro* genotoxicity tests be performed in the first tier of toxicity testing. This could be followed by toxicogenomic studies designed to reveal other potential hazards, to identify relevant MOA(s), and derive MOA-based POD(s). This information cannot be derived from *in vitro* genetic toxicity tests alone. Should toxicogenomics suggest other health hazards (e.g., immunotoxicity, as our case study correctly identified for BaP), this information should be used to prioritize further *in vivo* testing. Therefore, the anticipated ability of toxicogenomics to identify relevant health hazard(s) and aid in MOA-based derivation of PODs makes it very promising for addressing RA needs, especially for chemicals with limited toxicity information. Implementing such an approach would significantly reduce the number of expensive standard tests required. This is in-line with the ToxCast™ and Tox21 high-throughput *in vitro* toxicity testing strategies, launched based on the landmark report of the US National Research Council (NRC 2007) and a recently proposed tiered testing strategy for high-throughput and toxicogenomics data (Thomas et al. 2013).

Selecting the appropriate POD values for RA

Toxicologically relevant gene expression changes that are associated with the known or proposed MOA of a potential chemical carcinogen can be used to justify the relevance of specific PODs. This approach facilitates the selection of one value out of potentially hundreds or even thousands. We found that constructing the MOA was helpful in that it enabled (albeit somewhat arbitrarily) discrimination of adaptive and adverse responses (Figure 2). We propose that the pathways most closely associated with the key event “DNA adducts and DNA damage,” be used to determine the POD. While DNA adducts and DNA damage are undesirable, certain types of DNA damage may or may not predispose to mutations (adverse effect). In contrast, mutations represent an irreversible “committed” step in the MOA, and mutations are known to increase the likelihood of tumorigenesis. Therefore, in order to adequately protect against carcinogenic outcome, we based our POD selection on the key event preceding the adverse effect, mutations.

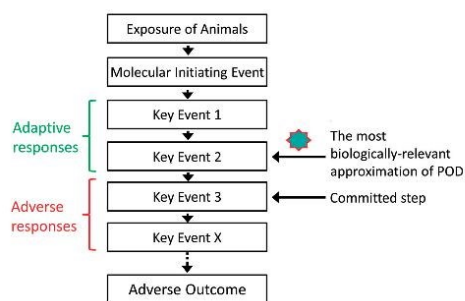


Figure 2. Selection of the most appropriate POD from toxicogenomics data.

We found that the PODs based on the key event preceding the committed step contained the most sensitive pathways within the proposed MOA. Therefore, considering the key event preceding the committed step appears to be conservative, biologically relevant, and protect against subsequent adverse effects. However, if an MOA is unknown, or if multiple genes/pathways are broadly affected by a chemical, selection of the IPA pathway with the lowest BMDL (provided there are at least five genes in the pathway that show statistically significant responses, as proposed elsewhere [Thomas et al. 2011, 2012]) generates PODs comparable with apical endpoints. Although PODs for transcriptional and apical endpoints are generally similar, there is an uncertainty surrounding how the transcriptional POD should be mathematically extrapolated for quantitative RA to express carcinogenic potency. This is because the extrapolation is from continuous (gene expression changes) to dichotomous (tumor incidence) data. Traditional toxicity endpoints, such as cancer incidence, measure the effect at the 10% response rate in the population, which is then extrapolated to calculate the probability of cancer, for example, one in a million in a population. In contrast, transcriptional BMDLs for the pathways related to DNA adducts and DNA damage are clearly not a population response. Therefore, attention is needed to address this issue.

Conclusions

Our case study reveals various places where the design of toxicogenomic experiments could be improved and suggests how toxicogenomics may be integrated to benefit regulatory toxicity testing, in addition to identifying gaps in established approaches/methodologies for integrating new testing strategies. However, with our limited datasets and in a very short period of time, we were able to obtain rich mechanistic information that was easy to process and interpret, and was helpful in deriving a POD that was well received by our RA community at Health Canada. Table 1 highlights ten valuable take-home messages from this work, emphasizing many benefits that toxicogenomics can bring to RA. This exercise emphasizes that successful transition of integrated testing strategies into RA will require close interaction between research scientists and risk assessors, and calls for continued development of toxicogenomic technologies and analytical platforms for applications in RA. While it is encouraging that traditional and toxicogenomic PODs yield quantitatively similar information for a genotoxic carcinogen, the main challenge will be to address the best approaches for assessing non-genotoxic MOAs. Specifically, efforts should be made to determine the tissues that should be characterized and the best strategy for determining the human relevance of the associated expression changes as they relate to non-cancer effects. This is relatively more straightforward for genotoxic chemicals. For example, BaP adducts and mutations were detected in multiple tissues in our case study experiments. If gene expression for the eight tissues that detect 82–92% carcinogens in rats and mice (Gold et al. 2001) is profiled with toxicogenomic tools, it is possible that important non-cancer toxicity information that resides in other tissues may be missed. However, as toxicogenomic approaches are becoming increasingly high throughput and less expensive (Li et al. 2012), it is conceivable that in the

near future all the tissues collected in repeat-dose studies can be profiled for gene expression changes. To this end, the selection of the appropriate tissues that encompass a wide array of toxic effects is an important avenue for further research. More case studies like this one for chemicals with diverse MOAs (including chemicals inducing non-genotoxic and non-cancer effects) will further illustrate the utility of toxicogenomics in many aspects of health RA.

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Declaration of interest

The authors' employment affiliations are as shown in the cover page. Health Canada and The Netherlands National Institute for Public Health and Environment are government agencies in their respective countries with broad missions for protecting and promoting public health. Pfizer is a public corporation engaged in the discovery, development, and manufacture of a range of pharmaceuticals. Utah State University and Georgetown University are educational institutions that conduct research with public and private support. Integrated Laboratory Systems is a private company that conducts research for the U.S. government and private entities. The preparation of the paper was primarily supported by funding from the Health Canada Genomics Research and Development Initiative and the Canadian Regulatory Systems for Biotechnology. The individual authors were compensated by their employers. The conceptual basis for the paper, preparation of the paper, selection of the literature and its review, the conclusions drawn, and the recommendations made were the professional work product of the authors and may not necessarily represent the views of Pfizer and Integrated Laboratory Systems.

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Appendix IV Transcriptomic analysis reveals novel mechanistic insight into murine biological responses to multi-walled carbon nanotubes in lungs and cultured lung epithelial cells.

Transcriptomic Analysis Reveals Novel Mechanistic Insight into Murine Biological Responses to Multi-Walled Carbon Nanotubes in Lungs and Cultured Lung Epithelial Cells

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Abstract

There is great interest in substituting animal work with *in vitro* experimentation in human health risk assessment; however, there are only few comparisons of *in vitro* and *in vivo* biological responses to engineered nanomaterials. We used high-content genomics tools to compare *in vivo* pulmonary responses of multiwalled carbon nanotubes (MWCNT) to those *in vitro* in cultured lung epithelial cells (FE1) at the global transcriptomic level. Primary size, surface area and other properties of MWCNT- XNRI -7 (Mitsui7) were characterized using DLS, SEM and TEM. Mice were exposed via a single intratracheal instillation to 18, 54, or 162 µg of Mitsui7/mouse. FE1 cells were incubated with 12.5, 25 and 100 µg/ml of Mitsui7. Tissue and cell samples were collected at 24 hours post-exposure. DNA microarrays were employed to establish mechanistic differences and similarities between the two models. Microarray results were confirmed using gene-specific RT-qPCR. Bronchoalveolar lavage (BAL) fluid was assessed for indications of inflammation *in vivo*. A strong dose-dependent activation of acute phase and inflammation response was observed in mouse lungs reflective mainly of an inflammatory response as observed in BAL. *In vitro*, a wide variety of core cellular functions were affected including transcription, cell cycle, and cellular growth and proliferation. Oxidative stress, fibrosis and inflammation processes were altered in both models. Although there were similarities observed between the two models at the pathway-level, the specific genes altered under these pathways were different, suggesting that the underlying mechanisms of responses are different in cells in culture and the lung tissue. Our results suggest that careful consideration should be given in selecting relevant endpoints when substituting animal with *in vitro* testing.

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Introduction

It is anticipated that the widespread use of engineered nanomaterials (ENM) in consumer products will lead to increased human exposure as a result of environmental contamination and using consumer goods containing ENM. Because of their nano size and distinct physico-chemical properties, exposure to ENM is suggested to cause different

toxic responses than exposure to their larger counterparts. However, hazard characterization and human health risk assessment of ENM is hampered by a lack of validated toxicological methods for assessing ENM, and a lack of appropriate dose metrics and methodologies for sample preparation (reviewed in 1). Indeed, it has been estimated that it would require billions of dollars and close to 50 years to employ traditional toxicological methods to assess the ENM

currently on the market [2]. Thus, there is an urgent need for the development of rapid alternative testing strategies.

Many efforts have been made to develop inexpensive, rapid, and simple *in vitro* screening assays to assess and predict ENM-induced toxicity. Although limited in number, studies comparing *in vitro* and *in vivo* biological responses following ENM exposure have found generally poor concordance between the two test systems [3,4]; these experiments question the relevance of *in vitro* findings to *in vivo* health effects for ENM. For example, Seagrave et al. [3] found that diesel exhaust particle extracts were less toxic in *in vitro* tests in rat alveolar macrophages, but more toxic in rat lungs [3]. Sayes et al. [4] reported little correlation in the toxicological properties of five different types of fine- and nano-particles following *in vivo* instillation of rats compared to *in vitro* exposures using three different cell culture models (rat lung epithelial cells, rat primary alveolar macrophages and co-culture of lung epithelial and primary alveolar macrophages) [4]. Broadly different responses were also reported for *in vivo* compared to *in vitro* exposures to fine zinc oxide particles, and to fullerene, carbon nanotubes (CNT), gold and silver nanoparticles [5,6], reviewed in 7,8. Differences in *in vitro* and *in vivo* responses may be expected because *in vivo* studies evaluate toxicity in whole organisms or organs with complicated interplay between multiple cell types, whereas *in vitro* studies primarily focus on understanding the response of a single cell type isolated from a specific organ. It should also be noted that the comparisons described above are limited to a small set of biological endpoints that include inflammatory markers, oxidative stress, cytotoxicity and markers of tissue damage.

In contrast to the studies described above, there appears to be agreement between the *in vitro* and *in vivo* models for the evaluation of genotoxic potential of nanomaterials (reviewed in 9). These findings suggest that careful selection of cell types, toxicity endpoints and knowledge of the related tissue physiology is important to derive meaningful comparisons. Moreover, the data demonstrate that the biological responses and pathologies associated with exposure to ENM are complex, and involve perturbations of several pathways and functions. The use of systems biology approaches to gain knowledge of the intricate relationship between the pathways leading to toxicity in these systems is required not only to understand the underlying etiologies of ENM-induced effects, but also to validate the relevance of *in vitro* results, undertaken to predict *in vivo* risk.

CNTs are cylindrical carbon allotropes. Their nano size, high aspect ratio (length/width), and fibre shape render their properties asbestos-like resulting in potentially higher toxicity than larger sized particles [10-12]. CNTs are categorized as either single-walled (SWCNT, single sheet of graphene) or multi-walled (MWCNT, multiple sheets of graphene) based on their wall numbers. MWCNT, which are the focus of the current study, are widely used in many industrial and biomedical applications [11,13,14] and consequently, occupational exposure to MWCNT has increased. *In vivo* animal exposure models clearly demonstrate that inhalation or instillation of MWCNT into the lungs induces inflammation, persistent

interstitial fibrosis, and granuloma formation in rodents [15-20]. Studies on mice given intraperitoneal injections of MWCNT of different shapes and lengths into the mesothelial lining of the peritoneal cavity show that length and diameter are important for the infiltration of inflammatory cells into the lungs and lung fluid [21,22] inciting inflammation. MWCNT are biopersistent, cause chronic tissue injury and at least in some situations, are carcinogenic. Indeed, it was recently shown that p53 heterozygous mice or rats given intraperitoneal or intrascrotal injection of MWCNT develop mesotheliomas in a dose-dependent manner [23,24] (NIOSH bulletin). For these reasons, we believe that at the doses tested, MWCNT should be considered as causing serious health effects and possibly being carcinogenic.

The primary objective of the present study was to delineate the mechanisms underlying the biological responses of an *in vitro* lung epithelial monolayer cell culture model, relevant to the *in vivo* lung tissue response, following exposure to MWCNT to shed light on the utility of *in vitro* models to predict adversity *in vivo*. MWCNT- XNRI -7 (Mitsui7) was used as a model MWCNT particle. To accomplish our objective, we characterized global transcriptional responses in the lungs of C57BL/6 mice exposed to Mitsui7 and compared them to transcriptional profiles from mouse lung epithelial cells exposed to Mitsui7 in culture. C57BL/6 mice were used to study *in vivo* effects because all of our previous and ongoing *in vivo* studies on different particle types have been conducted on this strain. Therefore, the results of this study will also enable consistent and systematic comparisons of biological responses across particle types. The mouse lung epithelial cell line FE1 was used to investigate *in vitro* responses to Mitsui7. FE1 is a spontaneously immortalized lung epithelial cell line derived from a normal healthy Muta™Mouse. This cell line retains key endogenous metabolic capacity and p53 signalling, and presents both a type I and type II alveolar phenotype. Because the Muta™Mouse model contains a *lacZ* mutation reporter transgene, this cell line also enables an assessment of the mutagenic effects of nanomaterials and has been extensively used in our laboratory for studying the genotoxicity of chemicals and nanomaterials [25-31].

In vitro doses were chosen based on their relevance to *in vivo* lung burden and also to reflect the doses reported in the literature. DNA microarrays were used to examine the responsive biological pathways and functions altered in FE1 cells incubated in the presence of 12.5, 25, 100 µg/ml Mitsui7 relative to lung tissues exposed via intratracheal instillation to 18, 54, 162 µg/mouse Mitsui7. Samples were collected at 24 hours post-exposure. Statistical and systems biology tools were employed to provide a global foot-print of Mitsui7-responsive pulmonary transcriptome in the two widely studied models. *In vivo* characterization of lung inflammation and validation of microarray results by RT-qPCR were further used to support the conclusions derived.

Table 1. Known characteristics of MWCNT-Mitsui-7.

References	Dispersion	Tube length	Tube Width $\pm$	
			SD	Impurities
Mitsui, Tokyo, Japan	ND	Segments; 3-5 μ m long	ND	ND
Tagaki et al. 2008	5% Triton X-100	27.5% longer than 5 μ m	100 nm	Fe: 0.35% S: 0.047%
Porter et al. 2010	PBS and dispersion media	Median length: 3.86 μ m	49 $\pm$ 13.4 nm	Fe: 0.32% Na: 0.41%

ND: Not Determined

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Materials and Methods

Material characteristics

The MWCNT used in this study is a MWCNT- XNRI-7; lot 05072001K28, Hadoga Chemical industry (formerly known as Mitsui). In this study it will be referred to as Mitsui7. Chemical composition and length of the same [18] and a different batch [23] have been published previously and results are summarized in Table 1. In brief, the average particle length of Mitsui7, as reported by the manufacturer, is 3–5 μ m. One gram of Mitsui7 corresponds to 3.55×10^{11} particles and consists of particles with an average width of ca. 100 nm. Transmission Electron Microscopy (TEM) revealed that the median length of Mitsui7 is 3.86 μ m with 27.5% of Mitsui7 particles having lengths longer than 5 μ m [23]. The numbers of walls vary from 20 to 50 [18]. Major impurities as determined by the collision type Inductively Coupled Plasma Mass Spectrometry, Combustion Ion Chromatography and trace metal analysis included Fe: (0.3%), Na: 0.4% S: ca. 470 ppm and Cl: ca. 20 ppm [18] [23].

Characterization of Mitsui7 in dispersion medium

Scanning Electron Microscopy. Size and the level of agglomeration was evaluated by Scanning Electron Microscopy (SEM) using a Zeiss Ultra 55 (Carl Zeiss Microscopy GmbH, Germany) SEM equipped with a field emission electron source. High vacuum conditions were applied and a secondary electron detector was used for image acquisition. Samples were prepared on a carbon tape surface and coated with a conductive layer of Au with approximately 20 nm thickness. Mitsui7 as dry pristine powders and dispersed in the exposure media, was analyzed by SEM.

Preparation of Mitsui7 exposure stock

For in vitro exposures. Immediately before the treatment, Mitsui7 suspensions were prepared as follows. A total of 8.9 mg of Mitsui7 was suspended in 2.6 ml of cell culture media to a total stock concentration of 3.4 mg/ml. Particle suspensions were prepared by sonication of Mitsui7 stock concentrations using a S-450D sonifier (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disruptor horn (Model Number:

Table 2. Comparison of *in vivo* and *in vitro* doses.

<i>In vivo</i> exposure doses				<i>In vitro</i> exposure concentrations			
μ g/animal	162	54	18	μ g/ml	100	25	12.5
mg/kg	8.1	2.7	0.9	mg/kg	NR	NR	NR
(assuming an average mouse weighs 20 g)							
mg/kg mouse lung	591	197	65.7	mg/kg	NR	NR	NR
(assuming an average lung weighs 274 mg)							
μ g/cm ² (lung surface: 82 cm ²)				μ g/cm ² (4-wells Petri dish: 21.8 cm ² , 6.8 ml)			
				1.98	0.66	0.22	31.19 7.8 3.9

NR: Not Relevant

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101-147-037) for a total of 4 minutes at amplitude of 10%, with alternating 10 s pulses and pauses on ice.

For in vivo exposures. Particles were suspended by sonication in 1 ml of MilliQ water containing 0.9% NaCl and 10% v/v acellular broncho alveolar lavage (BAL) fluid collected from C57BL/6 mice. Naïve C57BL/6 mice were flushed twice with 0.6 ml of 0.9% NaCl to obtain approximately 1 ml of BAL. BAL was centrifuged at 400 G for 10 min at 4°C to remove cells and the supernatant was used in the dispersant liquid. The Mitsui7 (4.05 mg/ml) suspension was sonicated on ice using a Branson Sonifier S-450D equipped with a disruptor horn (Model number: 101-147-037) for a total of 4 minutes at amplitude of 10%, with alternating 10 s pulses and pauses. This suspension was used for the high dose (162 μ g) and diluted 1:3 in vehicle for the medium (54 μ g) dose and diluted further 1:3 for the low dose (18 μ g). Between the dilutions the suspensions were mixed by pipetting. Vehicle control solutions were prepared with 0.9% NaCl and 10% acellular BAL fluid and sonicated as described above.

Dose selection

Table 2 summarizes the doses chosen for *in vivo* and *in vitro* exposures.

In vivo. Mice were exposed to 18, 54 or 162 μ g of Mitsui7/animal by single intratracheal instillation (these doses are also referred to as low, medium, and high respectively, in the text where appropriate). The doses were selected based on our previous studies [32–36] and reflect 8 h time weighted average (TWA) for carbon black particles (3.5 mg/m³) in an occupational setting in Denmark. The lung of an average female C57BL/6 mouse of 20 g weighs 274 mg with an average surface area of 82 cm². Thus, using surface area of exposure as a parameter, the doses chosen in the study can be expressed as 0.22, 0.66 and 1.98 μ g of Mitsui7/cm² of lung surface area, respectively.

In vitro. Cells were exposed to 12.5, 25.0 and 100 μ g Mitsui7/ml (these doses are also referred to as low, medium, and high respectively in the text where appropriate). These concentrations were selected based on the concentration ranges used in the literature that are demonstrated to induce some degree of cellular response, but that are well below the

concentrations that induce cytotoxicity (data not shown). Monolayers were exposed in a 4-well Petri dish consisting of 21.8 cm² total surface area. The exposure was carried out in a total volume of 6.8 ml. The *in vitro* concentrations amount to 3.9, 7.8 and 31.19 µg/cm² of Petri dish. Thus, the highest *in vivo* dose (1.98 µg/cm²) was approximately half of the lowest *in vitro* concentration used (3.9 µg/cm²), based on µg Mitsui7/cm² area.

Cell culture, exposure and sample collection

The development of the Muta™ Mouse lung epithelial cell line (FE1) and its characterization has been previously described [37]. The FE1 epithelial cells were cultivated in DMEM/F-12 + GlutaMax™-1 (Fisher Scientific Biotech Line A/S, 21765037, Slangerup, Denmark) supplemented with 2% fetal bovine serum (In Vitro AS, B1-04-007-1A, Fredensborg, Denmark), 1% penicillin/streptomycin solution (In Vitro AS, B1-03-031-1B, Fredensborg, Denmark) and 100 ng/µl murine epidermal growth factor (Sigma-Aldrich, E4127-1MG, Broendby, Denmark). Cells were maintained in T150 bottle (Thermo Fisher Scientific, NUN-156502, Roskilde, Denmark) at 37.0°C with 5% CO₂ and 90% humidity. Media was replaced every 2-3 days to ensure proper growth conditions.

FE1 cells were seeded at 10⁵ cells/ml density to a total of 6.81 ml/well in the 4-well plates (NUNC, Thermo Scientific, Hvidovre, Denmark, 167-063) and incubated at 37°C for 24 hours. The next day cells were examined for overall natural appearance and growth. At the time of treatment, cells showed ~80% confluency. Cells were incubated with 0, 12.5, 25, and 100 µg Mitsui7/ml for 24 h. Following the incubation, cells were trypsinized and centrifuged to collect cell pellets. The cell pellets were stored at -80°C until further use. Three individual experiments were conducted (cells were exposed in separate experiments).

Animals

Ethics Statement. All procedure complied with EC Directive 86/609/EEC and Danish laws regulating experiments on animals (permit 2010/561-1779). Mice did not display any signs of respiratory distress, lethargy or any other physical symptoms following exposure.

Female C57BL/6 mice aged 5-7 weeks were obtained from Taconic (Ry, Denmark). The mice were allowed to acclimatize for 1-3 weeks before the experiment. All mice were given food (Altromin no. 1324, Christian Petersen, Denmark) and water *ad libitum* during the whole experiment. Mice were group-housed in polypropylene cages with sawdust bedding at controlled temperature 21±1°C and humidity 50±10% with a 12-h light:12-h dark cycle. Experiments were initiated at 8 weeks of age.

Animal exposure and tissue collection. Mice were intratracheally instilled as previously described for other nanomaterials [33,38]. In brief, mice were anesthetized by subcutaneous injection of 0.2 ml of Hypnom® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen Pharma) and Dormicum® (Midazolam 5 mg/mL, Roche) in sterile water. The mice were kept on their back at a 40-degree angle on a hot plate (37 °C) during the entire procedure. Mice were exposed to 18, 54 and 162 µg of Mitsui7 via a single intratracheal

instillation. A 40 µl suspension was instilled followed by 150 µl air with a 250 µl SGE glass syringe (250F-LT-GT, MicroLab, Aarhus, Denmark). Control animals were instilled with vehicle (0.9% NaCl with 10% BAL). After the instillation catheter was removed, breathing was observed in order to assure that the delivered material did not block the airways. Mice were allowed to recover for 24 hours before sacrifice. For tissue collection, mice were anaesthetized with Hypnorm/Dormicum as described above and killed by exsanguination via intracardiac puncture. The right lung lobes were snap-frozen in cryotubes in liquid N₂ and stored at -80°C.

All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government. The Animal Experiment Inspectorate under the Ministry of Justice approved the study (#2010/561-1779).

Additional animal exposures and tissue collection for validation by RT-qPCR

The doses chosen for both *in vivo* (18, 54 and 162 µg of Mitsui7/animal) and *in vitro* (12.5, 25, and 100 µg of Mitsui7/ml) were high [18], therefore, additional animal exposures to lower doses of Mitsui7 were carried out. Mice were exposed to 2 and 6 µg of Mitsui7 via single intratracheal instillation and samples were collected at 24 hours following the exposure. *In vitro*, cells were incubated with 0.00125, 0.125 and 1.25 µg of Mitsui7/ml and cells were harvested at 24 hours post-exposure time point. Exposure of animals and sample collection is described above. Tissue samples collected from this exposure were used for validation by RT-qPCR.

Transmission Electron Microscopy

For transmission electron microscopy (TEM), mice were euthanized and lungs were fixed for a week in a 2% glutaraldehyde buffered fixative (pH 7.2) via trachea. Fixed lung was cut in small pieces and a standard Electron Microscope embedding procedure was carried out. Samples were rinsed in 0.15 M phosphate buffer followed by a wash with 0.15 M sodium cacodylate. Post-fixation and osmification were carried out in 2% osmium tetroxide in 0.05 M potassium ferricyanide for 2 hours. Following osmification, samples were rinsed in deionized water and placed in 1% uranyl acetate in water overnight at 5 °C. The following day samples were gradually dehydrated in ethanol and lastly in propylene oxide. Embedding was performed in propylene oxide diluted Epon, until 100% Epon 812 was used before polymerisation at 60 °C for 24 hours. Samples were cut into approximately 80 nm sections for TEM using an ultramicrotome with a diamond knife. Sections were stained with uranyl acetate and lead citrate, and imaged using a CM 100 BioTwin instrument from Philips operated at 80 kV accelerating voltage.

BAL cell counts

Infiltration of inflammatory cells into lung lining fluid was determined by BAL done immediately after cardiac puncture and collection of blood. The statistical analyses were performed in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Statistical significance was calculated using a non-

parametric one-way ANOVA with a post-hoc Tukey-type experimental comparison test.

Total RNA extraction

Total RNA was isolated from cell samples ($n = 6$ replicates per concentration) and from lung tissue of 72 mice in total ($n = 6$ mice per dose group). Isolations were done using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy MiniKit (Qiagen, Mississauga, ON, Canada) as described by the manufacturer. An on-column DNase treatment was applied (Qiagen, Mississauga, ON, Canada). All RNA samples showing A260/280 ratios between 2.0 and 2.15 were further analysed for RNA integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). RNA integrity numbers above 7.0 were used in the experiment. If the RNA samples didn't reach the criteria, new RNA extractions from the lung tissue or cell culture were performed. Total RNA was stored at -80°C until analysis.

Microarray Hybridization

Total RNA (200 ng) from each sample ($n=6$ per group) and from universal reference RNA (UMRR, Stratagene, Mississauga, ON, Canada) was used to synthesize double-stranded cDNA and cyanine labeled cRNA using the Agilent Linear Amplification kit (Agilent Technologies, Mississauga, ON, Canada). Reference RNA was labeled with cyanine 3-CTP and experimental samples were labeled with cyanine 5-CTP (Agilent Technologies Inc., Mississauga, ON, Canada). The cyanine-labeled cRNA was *in vitro* transcribed using T7 polymerase and purified using RNeasy mini kits (Qiagen). Sample and reference targets (825 ng) were combined and hybridized to Agilent 8 x 66K oligonucleotide microarrays (Agilent Technologies Inc., Mississauga, ON, Canada) for 17 hours at 65°C . The arrays were washed according to supplier instructions and then scanned on an Agilent G2505B scanner at 5 μm resolution. Data were acquired using Agilent Feature Extraction software version 9.5.3.1

Statistical Analysis of Microarray Data

A reference design [39-41] with the sample labelled with Cy5 and the reference labelled with Cy3 was used to analyze the data. The quantified data for each array was read into R [42,43]. The median signal intensity without background subtraction was used in this analysis and probes with technical replicates were averaged using the median. The background for each array was measured using the (-)3xSLV1 probe. Spots with median signal intensities less than the trimmed mean (trim = 5%) plus three trimmed standard deviations of the (-)3xSLV1 probe were flagged as below detection (i.e., absent). The total number of flagged spots, the median signal intensity and standard deviation for the (-)3xSLV1 probe for each array were recorded. Other microarray level summary statistics included the median signal to noise ratio ($\log_2$ scale) and the median signal intensity for each channel as well as the median absolute deviation. Differentially expressed genes between the control and treated groups were determined using the MAANOVA library [44]. The ANOVA model included treatment and a slide effect. This model was applied to the $\log_2$ of the

relative intensities. The Fs statistic [45], a shrinkage estimator for the gene-specific variance components was used. The p-values for all the statistical tests were estimated using the permutation method (30,000 permutations with residual shuffling). These p-values were then adjusted for multiple comparisons by using the false discovery rate approach (FDR) [46]. Estimated marginal means, also known as least square means [47,48], were estimated for each group. These means are a function of the model parameters and are adjusted for the other factors in the model such as day of hybridization. The least square means were then used to estimate the fold change for each contrast that was tested.

Quantitative Real Time (qRT)-PCR array validation

Fifty-four individual genes from each cells and tissues were selected for further validation by RT-qPCR using custom RT² Profiler PCR Arrays and a BioRad CFX96 Real-Time PCR detection system. The genes selected were statistically significant in the DNA microarray analysis for at least one condition and were associated with three biological functions: oxidative stress, inflammation and fibrosis. A custom RT² Profiler PCR Array plate, the RT² First Strand Kit and RT² SYBR[®] Green qPCR Mastermix (QIAGEN Sciences, Maryland, USA) were used. Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), actin β (*Actb*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were used as reference genes for normalization and were selected based on their stable expression levels in the treated and control samples in the microarray results. A threshold value was set to 10^2 . The final RT-qPCR validation group consisted of a sample size of $n=3$ for both cells (individual passages) and tissues (individual animals).

Results and Discussion

We exposed mice and mouse lung epithelial cells in culture to three different doses of Mitsui7 to evaluate the efficacy of *in vitro* models to predict *in vivo* outcomes following exposure to ENM. Lung tissues and cell lysates were collected 24 hours post-exposure. Global gene expression profiling was employed to characterize the molecular differences and similarities between *in vivo* and *in vitro* models as they relate to the existing mechanistic information available on the MWCNT-induced toxic effects.

Dose and concentration selection

In vivo doses chosen for this study were based on our previous studies and reflect 1, 3, and 9 days of 8 h time weighted exposure limit for carbon black particles (3.5 mg/m^3) in an occupational setting in Denmark. *In vitro* doses were chosen to mimic the total lung surface area available for exposure to particles (Table 2). At present, there are no occupational exposure limits specified for MWCNT in Denmark. Taking into consideration the MWCNT aerosol levels found in an occupational environment, the mass median aerodynamic diameter of MWCNT, and the surface area of human alveolar epithelium, Porter et al. [18] calculated that exposure of mice to $10 \mu\text{g}$ MWCNT is close to one month of human exposure to

aerosol concentrations of 400 $\mu\text{g}/\text{m}^3$ MWCNT in the work environment [18,49]. Mice exposed to 10 μg of MWCNT exhibit transient inflammation, which subsides at 7 days post-exposure. In contrast, mice exposed to 20 and 40 μg MWCNT displayed prolonged inflammation up to 56 days post-exposure, and fibrosis-like conditions are observed following exposure to 80 μg MWCNT [18,50]. The Mitsui7 instillation doses used in the present study span this entire exposure range [18,50].

An aerosol concentration of 440 $\mu\text{g}/\text{m}^3$ is approximately equivalent to human alveolar epithelium exposed to 226 $\mu\text{g}/\text{m}^2$ MWCNT per month, which is less than 1 $\mu\text{g}/\text{ml}$ concentration for cells exposed in culture medium [18]. Thus, according to these calculations, the *in vitro* Mitsui7 concentrations chosen in the present study are approximately an order of magnitude higher than potential human exposures (the lowest concentration being 3.9 $\mu\text{g}/\text{ml}$), as well as approximately an order of magnitude higher than the mouse *in vivo* exposures. However, we note that the lowest *in vitro* dose (12.5 μg) is similar to the highest *in vivo* dose (162 μg) delivered. These very high doses were chosen because they reflect the present literature on MWCNT-induced effects.

Mitsui7 characteristics in the dispersion medium

The particle size of Mitsui7 in the *in vivo* and *in vitro* exposure dispersion medium was determined using dynamic light scattering (DLS; File S1). The DLS technique measures spherical hydrodynamic diameter and since Mitsui7 exhibit rod-like shapes, it is not clear what the size-spectra of Mitsui7 generated by DLS corresponds to. Size scatter may very well include signals arising from more than one MWCNT dimension and metal impurities; however, the sizes may reflect the hydrodynamic equivalent size distributions. The distribution used in this study is number size distribution. The DLS is used only as an indication of the successful dispersion of the MWCNT and caution must be exercised when interpreting DLS data.

The DLS of unfiltered Mitsui7 in the *in vivo* exposure medium (0.9% NaCl with 10% BAL) showed a slightly skewed unimodal size distribution with a peak size at ca. 5000 nm (Figure S1A). The average zeta size was 8719.8 nm. The polydispersity index (PDI) was 1.0, indicating the presence of large Mitsui7 agglomerates of different sizes. After filtration through a 3.1 μm filter, DLS showed a multimodal size distribution with peak sizes at ca. 600 nm, 1000 nm, and 2000 nm, and an average zeta size of 1701.8 nm, thus indicating the presence of smaller agglomerates (Figure S1A). A PDI of 0.658 was found, demonstrating better dispersion after filtration. The *in vivo* exposure medium was analyzed separately and showed a bimodal size distribution with peak sizes at ca. 85 nm and 250 nm, an average zeta size of 389.9 nm (Figure S1B), and a PDI of 0.717, indicating protein agglomerates of various sizes. Filtration of the exposure medium through a 0.2 μm filter revealed the presence of smaller proteins with peak sizes of 25 nm and 55 nm, and an average zeta size of 67.2 nm.

The *in vitro* exposure medium (DMEM-F12 cell culture medium with 2% fetal bovine serum) was also analyzed with and without Mitsui7. The analysis of unfiltered Mitsui7 in the *in vitro* exposure medium showed a unimodal size distribution

with a peak size at ca. 370 nm (Figure S1C). The average zeta size was 368.6 nm and the PDI was 0.406, indicating well-dispersed Mitsui7. A separate analysis of *in vitro* exposure medium alone showed a unimodal size distribution with a peak size of 5.5 and a zeta average of 14.5 (Figure S1D).

SEM was performed on both pristine Mitsui7 and Mitsui7 in the exposure medium used for both *in vivo* and *in vitro* exposures. At all magnifications Mitsui7 appeared intertwined and agglomerated, with both long and straight, and bended tubes (Figures 1A and 1B). Crystalline impurities were observed on the Mitsui7 that may have originated from the dried exposure medium. SEM images of the pristine Mitsui7 are shown in Figure 2A-C. At low quantities, the pristine Mitsui7 appeared long and straight, but at the higher densities agglomerates were observed, with somewhat intertwined and bended tubes. Figures 3A and 3B show light microscopy images of Mitsui7 dispersed by sonication in the *in vitro* exposure medium. Samples appeared well-dispersed at all doses. Cytotoxicity following exposure to the indicated doses of Mitsui7 was assessed *in vitro* previously and revealed no toxicity (NR Jacobsen, unpublished).

TEM was performed on both pristine Mitsui7 tubes and in the lung lining of the mice intratracheally instilled with Mitsui7. As with SEM, the pristine Mitsui7 appeared long and straight, and although agglomeration was observed, the rigid structure remained (Figure 4A). Diameter of the pristine tubes was measured to 74.4 nm (SD 27.2). Images of Mitsui7 found inside a cell three days after exposure are shown in Figures 4B, the MWCNTs have been partially dislodged during ultramicrotomy. An image of Mitsui7 enclosed in an alveolar macrophage three days after exposure are shown in Figures 4C. No tubes are seen penetrating the macrophage, but several tubes appear to have been engulfed and are enclosed in vesicles. This clearly shows interaction between Mitsui7 and inflammatory cells in the alveolar region.

BAL fluid cellular composition

BAL fluid was collected from Mitsui7-instilled mice 24 hours after the exposure to assess the recruitment of inflammatory cells into the lung lumen. The total number of cells, neutrophils, macrophages, eosinophils, and lymphocytes were counted and the results are shown in Table 3. The lung inflammatory response was characterized by an increase in the total number of cells that was predominantly the result of a dose-dependent increase in neutrophils (7.2 - 18.2-fold increase over controls). Although a statistically significant increase in the total number of eosinophils was observed in all the dose groups compared to the vehicle treated controls (129.3, 77 and 7.3-fold increase at 18, 54 and 162 μg doses (0.22, 0.66 and 1.98 $\mu\text{g}/\text{cm}^2$, respectively), an inverted dose response was found, with the highest eosinophil influx found at the lowest dose. Specifically, a 17.6-fold reduction in the number of eosinophils was observed from the lowest dose to the highest dose. A similar trend was observed for the total number of lymphocytes; there was a non-significant increase in the number of lymphocytes in the BAL of the low dose group mice (1.8 fold increase over matched controls) that decreased significantly with increasing dose (0.4 fold compared to matched controls at the high dose).

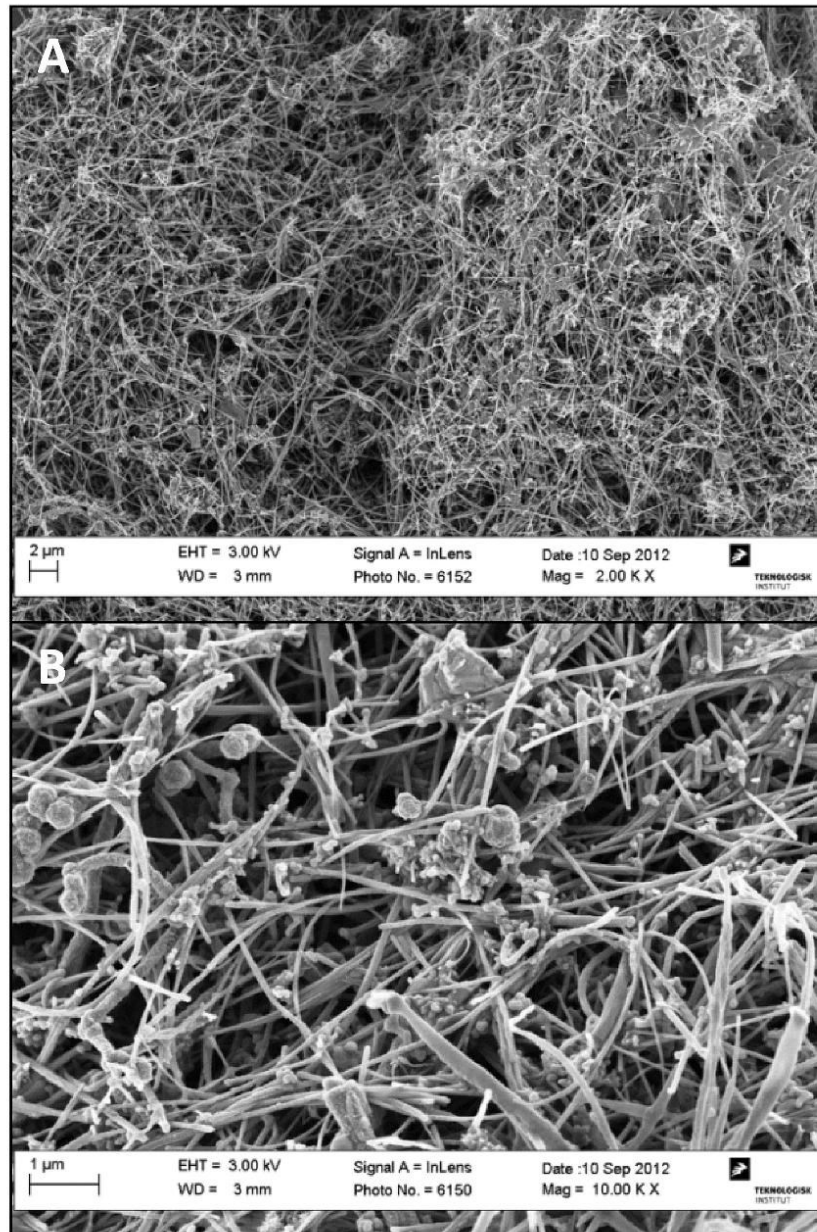


Figure 1. SEM images of Mitsui7 in the *in vivo* exposure medium. (A) High density behavior of Mitsui7 (B) Long, straight, and bended tubes are visible. Impurities seen on the MWCNTs may be dried materials from the exposure medium.

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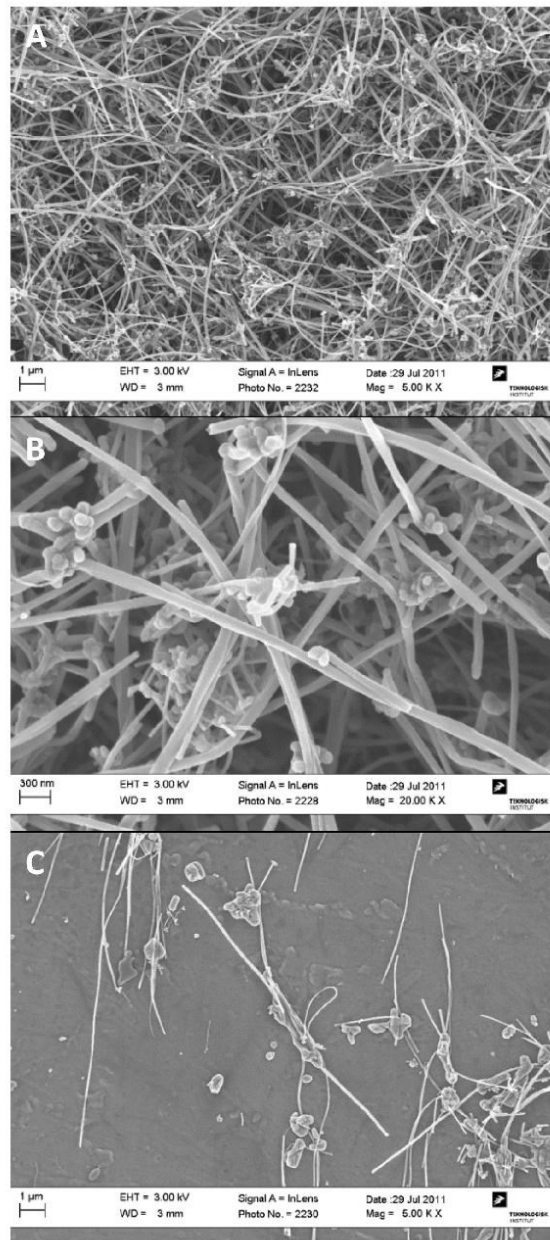


Figure 2. SEM images of dry, pristine Mitsui7. (A) Mitsui7 at high density. (B) A close-up of MWCNT at high density. (C) Mitsui7 behavior at low density.

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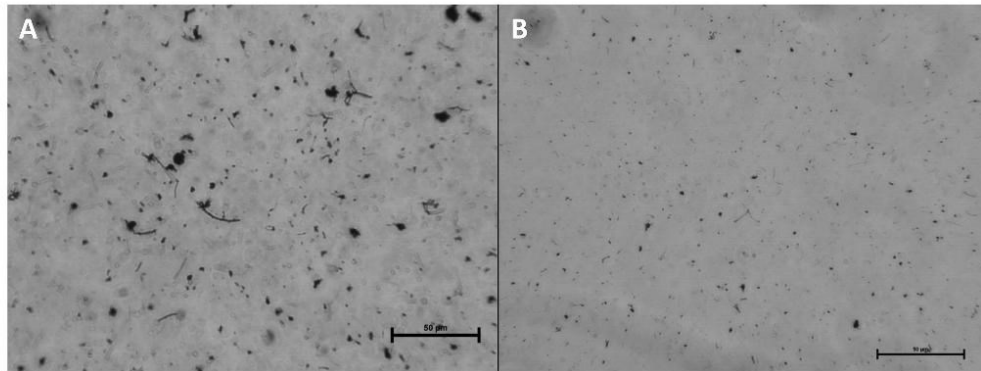


Figure 3. Light microscopy images of Mitsui7 dispersed in the *in vitro* exposure medium after sonication. (A) 100 µg/ml (31.19 µg/cm²) concentration (B) 12.5 µg/ml concentration (3.9 µg/cm²). Bars represent 50 µm.

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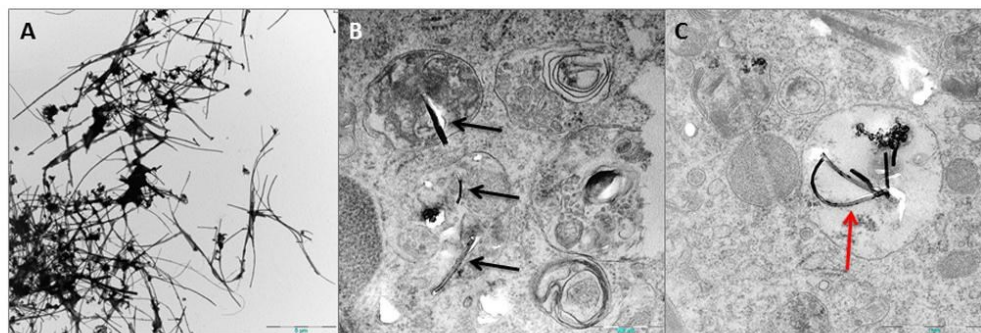


Figure 4. TEM images. (A) Pristine Mitsui7 (B) Mitsui7 MWCNTs interacting with cells in the lung lining fluid (Black arrows). The MWCNTs have been partially dislodged during ultramicrotomy (C) Macrophage containing engulfed Mitsui7 in vesicle (red arrow).

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In contrast, the total number of macrophages decreased in a dose-dependent manner relative to controls with a significant decrease found in the highest dose group (0.6 fold) compared to the vehicle treated matched controls.

Our results showing increased neutrophil and decreased macrophage populations are consistent with Ma-Hock et al. [11]. However, these studies did not show increases in eosinophils. Although a sharp increase in the total number of eosinophils following exposure to low dose of Mitsui7 may be indicative of allergic airway inflammation [51], it is not clear from the observations why eosinophilia decreased with increasing dose. A similar decline in the total number of eosinophils in the lungs of rats exposed to increasing doses of nano-SiO₂ was reported [52]. In a murine model of asthma, polymerized liposome nanoparticles were shown to mimic

physiological ligand 1 and bind to P selectin on activated airway endothelium. Furthermore, competitive binding of nanoparticles to P selectin was shown to inhibit eosinophil activation resulting in reduced eosinophil-mediated inflammatory response, a hallmark of allergic airway disease [53]. These results support the hypothesis that high doses of Mitsui7 could be competing with physiologic- ligand 1 (required for activation of P selectin) to bind to P selectin on the activated endothelium thereby inhibiting the recruitment of eosinophils into the BAL. A similar argument can also be made for the observed decreases in the lymphocyte population at higher doses.

Table 3. Differential cellular counts in the bronchoalveolar lavage fluid.

Cell types	Dose ($\mu\text{g}/\text{animal}$)			
	Control	18	54	162
Neutrophils x 10 ³	7.7 $\pm$ 1.7	55.1 $\pm$ 10.6	60.6 $\pm$ 4.5	140.2 $\pm$ 31.9
(%)	(10.4)	(38.6)*	(44.5)*	(71.8)*
Macrophages x 10 ³	53.2 $\pm$ 2.5	38.2 $\pm$ 5.1	37.4 $\pm$ 7.4	30.3 $\pm$ 8.5
(%)	(72.1)	(26.8)	(27.8)	(15.5)*
Eosinophils x 10 ³	0.3 $\pm$ 0.1	38.8 $\pm$ 10.7	23.1 $\pm$ 11.1	2.2 $\pm$ 0.5
(%)	(0.4)	(27.2)*	(17.1)*	(1.1)*#
Lymphocytes x 10 ³	1.6 $\pm$ 0.5	2.8 $\pm$ 0.5	1.7 $\pm$ 0.5	0.6 $\pm$ 0.3
(%)	(2.2)	(2.0)	(1.3)	(0.3)*#
Total BAL Cell x 10 ³	73.8 $\pm$ 3.6	142.8 $\pm$ 22.3*	134.7 $\pm$ 19.5*	195.3 $\pm$ 40.8*

Statistically significant ($P < 0.05$) difference between (*) treated and controls, and (#) low (18 μg) and high (162 μg) doses.

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Microarray analysis

All microarray data have been deposited in the NCBI Gene Expression Omnibus database and can be accessed through the accession number GSE47000. A total of 4,259 of the 60,000 probes on the array were expressed (where expressed is defined as probes having signal intensities above background in 4 out of 5 samples in at least one experiment condition) in both cells and tissues. Differentially expressed genes were identified using MAANOVA; a probe was considered to be differentially expressed if its normalized signal intensities in treated samples were significantly different from control values following FDR adjustment, with $P \leq 0.05$, and the relative change in expression (fold change) was at least ± 1.5 in either direction.

Overview of *in vivo* microarray results

In general, a high degree of reproducibility was observed within control and treated replicates. A total of 1635 unique genes represented by 2104 probes were significantly differentially expressed in the lungs following exposure to 18, 54, and 162 μg of MWCNT at the 24 hours post-exposure time point. A total of 601 (383 genes down-regulated and 219 up-regulated), 114 (35 down-regulated and 79 up-regulated) and 1353 (754 down-regulated and 601 up-regulated) genes were differentially expressed in the 18, 54, and 162 μg dose groups, respectively. These genes are listed in the Table S1. Only 46 genes were commonly differentially expressed in all three dose groups (Figure 5A). Most of the genes in this list exhibited a dose-response and were mainly associated with acute phase response, immune cell trafficking, lung hypersensitivity, and hematological system functions. Increased expression of acute phase response genes (including *Saa1*, *Saa3* and *Timp1*) has also been reported in the lungs of mice exposed to nano-sized titanium dioxide [54,55] and nano- particles of carbon black [56], suggesting acute phase genes are sensitive markers of exposure to nanoparticles [57]. A list of the significant genes that were responsive in all dose groups is provided in Table 4.

We analyzed the gene ontology (GO) classifications of all of the differentially expressed genes to identify themes in the global pulmonary gene expression patterns following exposure to MWCNT. The processes affected by MWCNT are shown in Figures 5B and 5C, and include 'common' (i.e., occurring in more than one group) and 'unique' (i.e., occurring in one dose group only) processes, respectively. Processes involved in chemotaxis [GO: 6935], immune response [GO: 6955], inflammatory response [GO: 6954], oxidative reduction [GO: 55114] and behavior [GO: 7610] were over-represented among all the dose groups (Figure 5B). In addition, several unique GO processes were over-represented within a dose group (Figure 5C). In the low dose group, the top biological processes altered consisted of 70 genes involved in cell cycle and was associated with five specific GO terms: cell cycle [GO: 7049], cell cycle process [GO: 22402], cell cycle phase [GO: 22403], M phase [GO: 279], and cell division [GO: 51301]. The other over-represented biological processes in the low dose group included: microtubules [GO: 7017], nucleosome assembly [GO: 6334], meiosis [GO: 7126], ear morphogenesis [GO: 42471], regulation of cell substrate adhesion [GO: 10810], and complement activation, alternate pathway [GO: 6957]. In the medium dose group, negative regulation of cell proliferation [GO: 42127] and positive regulation of response to stimulus [GO: 48584] were uniquely enriched with 14 genes in each of the processes. Unique biological processes affected in the high dose group included: cell motion [GO: 6928], vasculature development [GO: 1944], response to hormone stimulus [GO: 9725], regulation of inflammatory response [GO: 50727], and negative regulation of response to stimulus [GO: 48585].

In order to understand the functional significance of these GO changes, Ingenuity Pathway Analysis (IPA Reference) was employed to identify biological networks, functions, and pathways affected in all of the dose groups. Table 5 shows a summary of the top functions and processes altered in the three dose groups 24 hours following the exposure to MWCNT. This analysis revealed that biological functions such as, cellular movement, cellular growth and proliferation, and cell-to-cell signaling were commonly affected across the dose groups. Closer examination of these differentially expressed genes under each of the functional categories in common revealed that they are actually specifically involved in immune and inflammation related responses. Analysis of biological pathways revealed significant enrichment of genes in the pyrimidine ribonucleotides *de novo* synthesis, aryl hydrocarbon receptor signaling, and LPS/IL-1 mediated inhibition of RXR signaling pathways in the low dose group. In the medium dose group, genes involved in the acute phase response signaling, chemokine signaling and IL-17 signaling pathways were significantly enriched. Finally, retinol biosynthesis, neuregulin signaling, and acute phase response signaling were the most affected pathways in the high dose group.

A detailed study of a subset of genes implicated in the processes mentioned above revealed dose-specific responses ranging from imbalances in nucleotide synthesis in the low dose to inflammation, angiogenesis and lipid metabolism related effects in the high dose. For instance, we found significant down-regulation of adenylate kinase 8 (-2.1 fold),

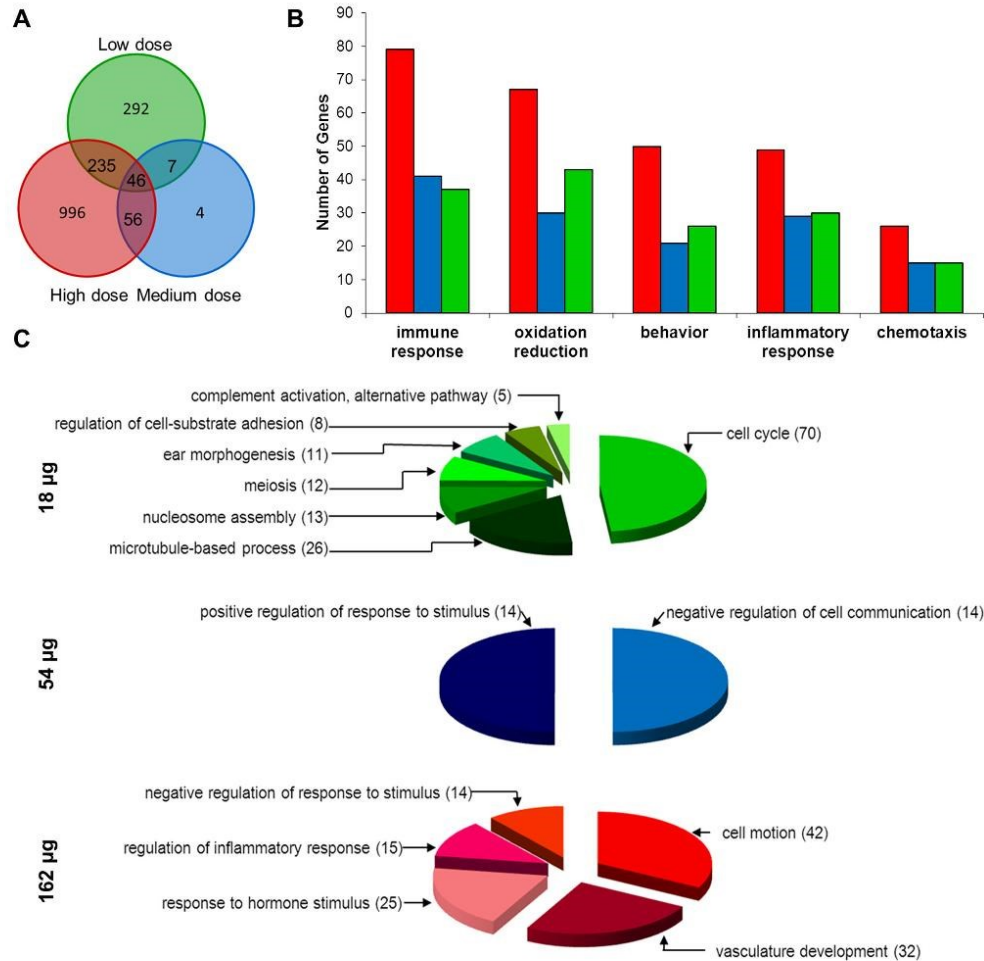


Figure 5. Overview of response in the lung tissue. (A) Venn diagram showing the overlapping differentially expressed genes (FDR $P \leq 0.05$ and fold change ≥ 1.5) in response to low (18 μg , green), medium (54 μg , blue), and high (162 μg , red) doses of Mitsui7 in the lung tissues. (B-C) Gene ontology (GO) analysis of differentially expressed genes. (B) Commonly regulated GO biological processes, and (C) GO biological processes unique to the low, medium, and high dose groups. Numbers in parentheses represent number of genes altered in that process.

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ectonucleoside triphosphate diphosphohydrolase 2 and 8 (-1.9 and -2.0 fold), and nucleoside diphosphate kinase 5 (-2.1) in the low dose. These genes are actively involved in the synthesis and metabolism of uridine-5'-triphosphate (UTP) and

adenosine triphosphate (ATP) nucleotides required for the maintenance of ion homeostasis in the lungs [58,59]. Decreased synthesis and depletion of pyrimidine nucleotides prevents blockade of the epithelial sodium channels, increase

Table 4. Differentially expressed genes common across all three doses.

Gene symbol	Entrez ID	Average fold change compared to matched controls		
		18 µg/mouse	54 µg/mouse	162 µg/mouse
<i>A130040M12Rik</i>	319269	1.8	2.3	2.7
<i>Adams4</i>	240913	1.8	2.4	3.5
<i>Adm</i>	11535	1.9	2.2	3.0
<i>Asgr1</i>	11889	-2.2	-3.1	-2.3
<i>Bcl3</i>	12051	1.6	1.7	1.7
<i>Ccdc129</i>	232016	-3.0	-2.3	-2.7
<i>Ccl11</i>	20292	3.0	5.2	3.4
<i>Ccl17</i>	20295	3.5	3.4	2.8
<i>Ccl2</i>	20296	3.6	5.3	4.0
<i>Ccl7</i>	20306	5.5	6.9	4.3
<i>Ch25h</i>	12642	2.7	3.1	4.0
<i>Cldn8</i>	54420	-2.0	-1.8	-2.0
<i>Ctps</i>	51797	2.0	2.7	2.9
<i>Cxcl1</i>	14825	2.3	3.9	4.7
<i>Entpd8</i>	72090	-2.0	-2.1	-2.4
<i>Gm2045</i>	100039096	2.1	2.5	4.4
<i>Gm2371</i>	100039687	1.7	2.1	2.8
<i>Gm3459</i>	100041663	2.0	2.7	3.9
<i>Gm4148</i>	100042984	2.0	2.1	3.0
<i>Gm4382</i>	100043353	2.1	1.9	2.5
<i>Gm4499</i>	100043524	2.0	2.5	3.6
<i>Gprasp1</i>	67298	-1.6	-1.7	-2.6
<i>Has1</i>	15116	1.9	2.3	1.8
<i>Hbeef</i>	15200	1.8	2.6	2.0
<i>Ilf6</i>	16193	2.6	3.9	6.2
<i>Klhl24</i>	75785	-1.9	-1.9	-1.8
<i>Mt2</i>	17750	2.7	11.7	27.5
<i>Myb</i>	17863	-1.9	-2.1	-2.2
<i>Osbpl6</i>	99031	-1.8	-1.6	-1.9
<i>Rgs16</i>	19734	1.8	2.1	2.5
<i>Rrbp1</i>	81910	1.6	1.6	1.6
<i>Serpina3g</i>	20715	3.2	3.4	3.4
<i>Serpinf1</i>	20317	-1.7	-1.7	-1.7
<i>Six4</i>	20474	-1.7	-1.7	-2.1
<i>Slc26a4</i>	23985	9.0	7.7	5.3
<i>Slc30a2</i>	230810	-2.2	-1.9	-1.9
<i>Slc39a14</i>	213053	1.6	2.3	3.7
<i>Saa1</i>	20208	3.8	7.0	12.3
<i>Saa3</i>	20210	9.9	12.3	45.6
<i>Timp1</i>	21857	7.7	9.7	11.7
<i>Tnfrsf12a</i>	27279	1.6	2.4	2.7
<i>Tnfrsf9</i>	21950	2.0	2.1	2.4
<i>Trib1</i>	211770	1.5	1.7	1.6
<i>Trmt61a</i>	328162	1.8	1.8	1.7
<i>Tubb6</i>	67951	1.8	1.8	1.6
<i>Tyro3</i>	22174	-1.6	-1.6	-2.0

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alveolar fluid clearance and clearance of lung edema in mice infected with respiratory syncytial virus and influenza A virus [58–60]. Metabolism of adenine nucleotides in human airway

epithelial surfaces plays a role in the regulation of mucociliary clearance related epithelial functions. Imbalances in this pathway can lead to inhibition of sodium ion transport leading to accumulation of alveolar fluid and pulmonary edema. Excess ATP in the lung fluids is suggested to contribute to the onset of inflammation in humans suffering from pulmonary pathology including alveolar damage, inflammation and edema (reviewed in 61). Enrichment of *de novo* pyrimidine synthesis pathway in the low dose group may thus reflect a non-specific cellular response to invasion of lungs with particles, possibly an effort leading to their clearance. In the medium dose, a dramatic up-regulation of several acute phase and inflammation associated genes such as *Saa3* (12.3-fold), *Saa1* (7.0 fold), *Saa2* (12.0 fold), *Ilf6* (4-fold), *Ccl7* (7.0 fold), *Ccl11* (5-fold), *Ccl13* (6.0 fold), and *Ccl24* (10.0 fold) was noted. These changes suggest an active recruitment of inflammatory cells to the site of exposure aiding in clearance efforts. These genes are also affected by the high dose. However, in addition to a significant up-regulation of genes implicated in acute phase and inflammation pathway in the high dose, down regulation of carboxylesterases 2C and 2E (-1.7, -2.1 fold), dehydrogenase reductase (-1.6 fold), retinol binding proteins 1 and 7 (-1.7, -2.4 fold), lecithin retinol acyltransferase (-3.0 fold) and up-regulation of esterase D (1.65 fold), lipoprotein lipase (2.5 fold), lipase-hormone sensitive (2.5 fold), amphiregulin (3.0 fold), epiregulin (2.8 fold) were noted. These genes are involved in functions such as angiogenesis, lipid metabolism, and cellular proliferation. Combined analysis of all genes altered under these pathways using the GeneGo Metacore map creator tool revealed that of the 80 genes associated with the pathways described above, 60 were commonly regulated by *E2f1* (a cell cycle regulatory transcription factor), *Ilf6* (an inflammatory modulator) and *C-myc* (an oncogene). We note that these regulators were also significantly differentially expressed in the treated groups (Figure 6).

In general, the above results reveal a clear distinction in tissue response across the doses. The response in the lowest (18 µg or 0.22 µg/cm²) dose was marked by a negative regulation of the cell cycle process, in parallel with changes in the nucleotide metabolism and synthesis pathways. Cell cycle arrest and imbalance of nucleotide metabolism can be viewed as early regulatory signals initiating an organism's defense functions including phagocytic mopping and airway clearance mechanisms in lungs. This was evident in the medium dose (54 µg or 0.66 µg/cm²), which showed positive regulation of acute phase response and inflammatory mediators. In the high dose (162 µg or 1.98 µg/cm²), the overall response was much greater than the other two dose groups and included simultaneous enrichment of several biological processes including inflammation, acute phase response, angiogenesis, pro-and anti-fibrotic factors, and anti-oxidants.

Overview of *in vitro* microarray results

Lung epithelial cell monolayers were exposed to 12.5 (low), 25.0 (medium) and 100 µg (high) Mitsui7/ml (corresponding to 3.9, 7.8 and 31.19 µg/cm², respectively) for 24h as described in methods and materials. MAANOVA analysis was applied to identify differentially expressed genes. This gene list was

Table 5. Significantly enriched canonical pathways and networks in *in vivo* exposure.

Canonical Pathways			Networks	
Dose Group	Name	No. of Genes*	Name	No. of Genes*
18 µg	Pyrimidine ribonucleotides de novo biosynthesis	7	Organismal injury and abnormalities	27
	Aryl hydrocarbon receptor signaling	13	Cell-to-cell signaling and interaction, immune cell trafficking	25
	LPS/IL-1 mediated inhibition of RXR function	16	Cancer, respiratory disease, cell cycle	22
	Glutathione-mediated detoxification	4	Embryonic development, organ development, organismal development	22
	LXR/RXR activation	9	Cell cycle, cancer	19
54 µg	Estrogen-mediated S-phase entry	4	Cellular assembly and organization, cellular function and maintenance, connective tissue development and function	18
	Acute phase response signaling	9	Cellular movement, immune cell trafficking	24
	Chemokine signaling	5	Cancer, organ morphology	16
	IL-17 signaling	5	Organismal injury and abnormalities, inflammatory response	16
	Pyrimidine ribonucleotide de novo biosynthesis	3	Lipid metabolism, molecular transport, small molecule biochemistry	6
162 µg	LXR/RXR activation	5	Cell morphology, cell-mediated immune response	1
	Retinoate biosynthesis I	3	Cell-to-cell signaling and interaction, cellular assembly and organization	1
	Retinol biosynthesis	10	Cellular movement, cellular assembly and organization, cellular function and maintenance	33
	Neuregulin signaling	16	Cellular movement, cell death and survival, drug metabolism	30
	Acute phase response signaling	23	Infectious disease, organismal injury and abnormalities, cell morphology	29
	Role of tissue factor in cancer	16	Lipid metabolism, molecular transport, small molecule biochemistry	29
	Aldosterone signaling in epithelial cells	20	Cellular development, cellular growth and proliferation	28
	Glucocorticoid receptor signaling	29	Cell-mediated immune response, cellular development, cellular function and maintenance	28

*. Number of genes significantly regulated in each pathway or network
doi: 10.1371/journal.pone.0080452.t005

filtered to remove probes that were below our detection threshold (i.e., probes that were not expressed above background levels). This analysis revealed a total of 1931 unique genes represented by 2413 probes that were significantly differentially expressed (FDR adjusted $P < 0.05$ and ± 1.5 fold change) in the cells exposed to 12.5, 25 or 100 µg Mitsui7/ml (3.9, 7.8 and 31.19 µg/cm²). A dose-dependent increase in transcriptional effects was found, with a total of 782 (432 genes down-regulated and 350 up-regulated), 1337 (682 down-regulated and 655 up-regulated), and 1721 (921 down-regulated and 801 up-regulated) genes differentially expressed in the 12.5, 25 and 100 µg Mitsui7/ml (3.9, 7.8 and 31.19 µg/cm²) groups, respectively. The full list of significant genes is available in Table S2. A total of 565 genes were affected by every concentration of Mitsui7 (Figure 7A). Most of the genes that were affected in the medium and high concentration groups were also affected in the low concentration group (Figure 7A) and were mainly associated with Aryl hydrocarbon receptor (AHR) signaling, glutathione mediated detoxification, acute phase response signaling, cholesterol biosynthesis, hepatic fibrosis/hepatic stellate activation, and NRF2 mediated oxidative stress response. A list of genes affected in common for every concentration analyzed is provided in Table S3.

To be consistent with the analysis conducted on *in vitro* microarray results, we employed GO functional classification to assign specific functional or biological processes to the differentially expressed genes following *in vitro* exposure to Mitsui7. The common and unique biological processes significantly enriched in different exposure groups across the

dose groups are shown in Figures 7B and 7C. Processes involved in steroid biosynthetic process [GO: 16126], vasculature development [GO: 1944], regulation of cell proliferation [GO: 42127], response to protein stimulus [GO: 51789], and ncRNA metabolic process [GO: 34660] were the commonly over-represented GO groups (i.e., consistently appearing within two or three exposure groups) and consisted of 96 genes. In the medium and high concentration groups these processes were further represented with GO terms such as blood vessel morphogenesis [GO: 48514], angiogenesis [GO: 1525], and positive regulation of cellular proliferation [GO: 8284]. Several GO processes were uniquely altered in the high concentration group and included oxidation reduction [GO: 55114], response to hypoxia [GO: 1666, GO: 70482], cell cycle arrest [GO: 7050], cell death [GO: 8219, GO: 16265, GO: 12501], organ development [GO: 48568, GO: 60348, GO: 42724, GO: 51216, GO: 1501, GO: 48705], extracellular matrix organization [GO: 30198], regulation of activity [GO: 51338, GO: 45859, GO: 43549, GO: 44092], and lipid biosynthetic process [GO: 8610]. Of particular interest, and in contrast to our *in vivo* findings, analysis of GO processes and functions revealed a high degree of overlap across the exposure groups, with exceptions generally only occurring at the highest concentration. This suggests that although the number of genes and the magnitude of change (i.e., fold change) exhibited a dose-response, there was no accompanying concentration-dependent increment in the nature of toxicity. Thus, the results indicate that the lowest concentration itself

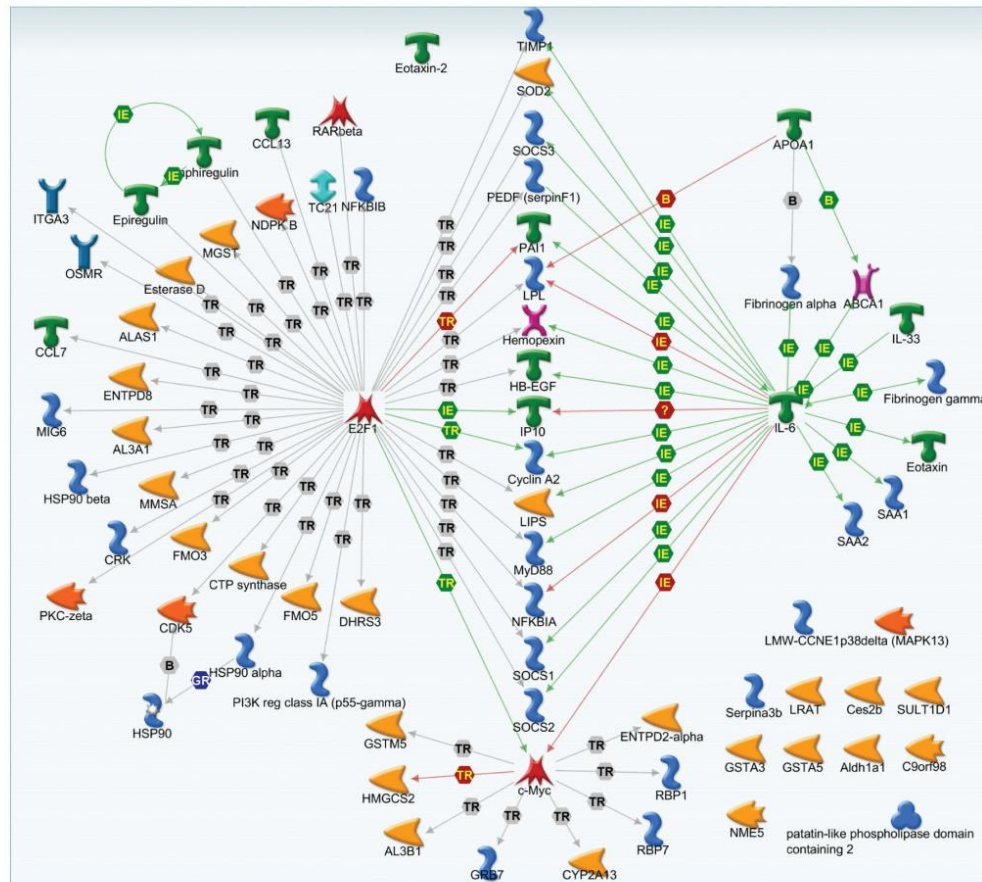


Figure 6. Gene network. Combined analysis of all significantly differentially expressed genes associated with angiogenesis, lipid metabolism, and cellular proliferation pathways in lungs in the high dose group. Analysis was conducted and the network was built using GeneGo Metacore map creator. The figure shows that the majority of the genes are commonly regulated by *E2f1*, *Il6* and *C-myc*. Hexagons represent physical and functional interactions: TR – transcription regulation, IE – influence on expression, B – binding, and GR – group. The green arrows represent positive regulation/activation, the brown arrows represent negative regulation/inhibition and the grey arrows represent unspecified regulation.

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was capable of inducing a similar response/toxicity as what was observed in the medium and high exposure groups.

To identify potential biological pathways affected by MWCNT exposure in lung epithelial cells, the significant gene list (Table S2) was overlaid onto molecular canonical pathways in IPA. Four major canonical pathways were identified as commonly affected in all the three dose groups: hepatic fibrosis/hepatic stellate cell activation, aryl hydrocarbon receptor signaling, super pathway of cholesterol biosynthesis, and glutathione-

mediated detoxification. The majority of the genes implicated in these pathways were down-regulated. Table 6 lists all of the genes involved in these four pathways. Gene interaction networks were then created to visualize the linkages between the different genes and pathways using the IPA network tool. Figure 8 shows the three top scoring networks within each concentration merged into a single concentration-specific meta-network. In the low concentration, organ development, cardiovascular system development and function, and cancer

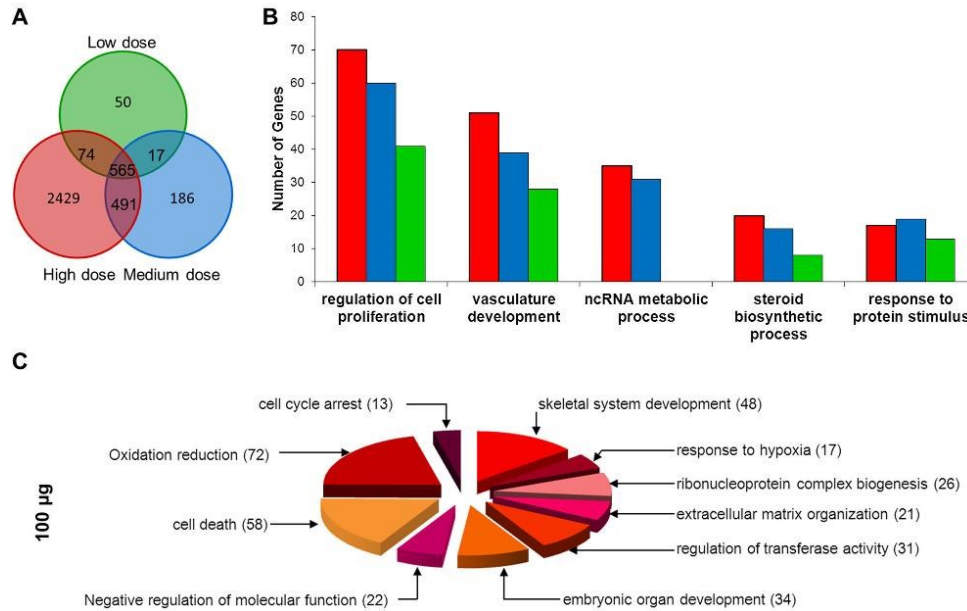


Figure 7. Overview of response in FE1 cells. (A) Venn diagram showing the overlapping differentially expressed genes (FDR $P \leq 0.05$ and fold change ≥ 1.5) in response to low (12.5 μg , green), medium (25 μg , blue), and high (100 μg , red) doses of Mitsui7 *in vitro*. (B-C) Gene ontology (GO) analysis of differentially expressed genes. (B) Commonly regulated GO biological processes (C) GO biological processes unique to the high dose group. Numbers in parentheses represent number of genes altered in that process.

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networks were merged. The network consisted of several nodes including: *C-fos*, *Crem1*, *Ctgf*, *Cebpa*, and *Ahr*. In the medium exposure group, cellular growth and proliferation, organismal development and drug metabolism, and glutathione depletion networks were merged. This network consisted of core nodes including: *alpha catenin*, *Ptgs2*, *Ahr*, *Myc*, and *Nrf2*. In the high concentration, cellular development, lipid metabolism, cell cycle and tissue development networks were merged with *Cebpa* and *Cebpb*, *Hmga1*, *Ptgs2*, and *Fos* as the main nodes.

Overall, *in vitro* lung epithelial cells showed a robust response to Mitsui7 with alterations in numerous critical functions including gene expression (expression of many transcription factors were altered), metabolism, cellular growth and proliferation, and these changes were found in genes involved in numerous potential diseases such as cancer and fibrosis. However, the response was not dose-dependent; major biological processes and functions altered in the high (100 $\mu\text{g}/\text{ml}$ or 31.19 $\mu\text{g}/\text{cm}^2$) concentration were also altered significantly in the lowest (12.5 $\mu\text{g}/\text{ml}$ or 3.9 $\mu\text{g}/\text{cm}^2$) exposure

group, suggesting that the lowest dose chosen was sufficiently high to cause the severe cellular damage.

Comparison of gene expression profiles of *in vivo* lung tissue and *in vitro* lung epithelial cells

In order to determine the relevance of responses observed in cultured lung epithelial cells following exposure to Mitsui7 to *in vivo* lung responses, we analysed all differentially expressed genes (compared to unexposed control) from both cell cultures and tissues using IPA's function analysis tool. Three categories were included in the analysis: Diseases and Disorders, Molecular and Cellular Functions and Physiological System Development and Function. More than 10 individual high-level processes were significantly enriched and in common to both cell cultures and tissues under each of the three categories. We filtered these high level individual functions by 1) removing redundant functions with overlapping genes, and 2) removing functions that were not directly relevant to the present study (e.g. renal diseases, auditory diseases etc.). The final list of the top most significantly affected high-level biological functions are shown in Figures 9A and 9B.

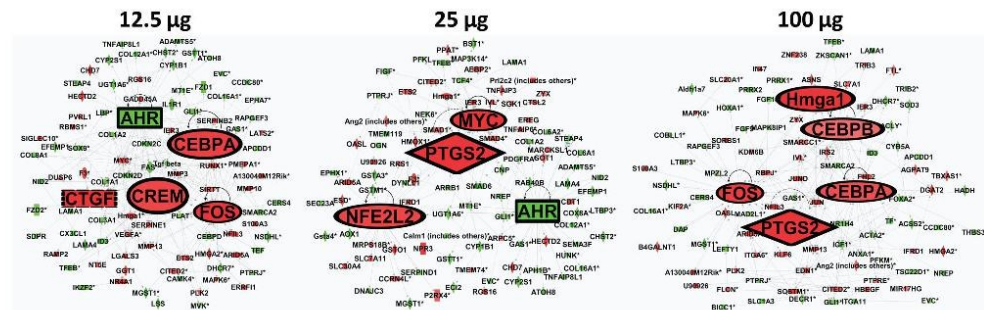


Figure 8. Top three biological networks *in vitro*. The networks were generated by merging the top three networks in each of the dose and were based on the connectivity between each gene and the molecular interaction knowledge base in IPA. Nodes are highlighted in large bold font. Green indicates down-regulation and red indicates up-regulation.

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Table 6. Top four canonical pathways affected in all three doses *in vitro*.

Canonical Pathways	Gene names*
Hepatic Fibrosis/ Hepatic Stellate Cell Activation	<i>Acta2, Ccl5, Col1a1, Col1a2, Col3a1, Ctgf, Cxcr3, Edn1, Fbs, Fgfr1, Fgfr, Igfr1, Il1r1, Il1r1, Il4r, Lama1, Lbp, Met, Mmp13, Myh10, Myl6b, Pdgfra, Pdgfr, Pdgfra, Pdgfrb, Rela, Serpinb2, Smad4, Tgfb2, Tgfb3, Tnfrsf11b, Tnfrsf1a, Vcam1, Vegfa</i>
Aryl Hydrocarbon Receptor Signaling	<i>Ahr, Aldh1a1, Aldh3b1, Aldh4a1, Aldh6a1, Aldh7a1, Aldh9a1, Ccnd1, Ccnd3, Cdkn1A, Cyp1b1, Esr1, Fas, Fos, Gsta3, Gsta5, Gstm1, Gsta3, Gsto1, Gstm2, Gstm1, Hsp90b1, Hspb2, Jun, Mdm2, Mgst1, Myc, Nfe2l2, Nqo2, Rela, Tgfb2, Tgfb3, Tgm2</i>
Superpathway of Cholesterol Biosynthesis	<i>Acat2/Acat3, Dhcr7, Dhcr24, Ebp, Fdft1, Fdps, Hadhb, Hmgcs1, Hsd17b7, Idl1, Lss, Msmo1, Mvd, Mvk, Nsdhl, Pmvk, Sc5dl, Sqle, Tm7sf2</i>
Glutathione-Mediated Detoxification	<i>Gsta3, Gsta4, Gsta5, Gstm1, Gstm3, Gsto1, Gsto2, Gstm1, Gstm3, Gstm1, Gstm1</i>

*. Genes associated with each of the pathways are presented.

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Since the objective was to directly compare the responses in both cells in culture and tissues, we chose to analyze two high-level functions in detail systematically. Cellular growth and proliferation was one of the top high-level functions under the Molecular and Cellular functions category. A total of 155, 214 and 274 genes in the low, medium, and high concentrations *in vitro*, and 32, 36 and 211 genes in the low, medium, and high doses *in vivo* were associated with cellular growth and proliferation. In cell cultures, this function was enriched with annotation terms such as proliferation of cells, muscle cells, fibroblasts, connective tissue cells, and mesenchymal cells (Figure 9C). In the lung tissues however, cellular growth and proliferation function was associated with annotation terms such as proliferation of cells, tumor cells, immune cells, blood

cells and proliferation of mononuclear leukocytes (Figure 9D). We then analysed the high-level function given the name 'cancer' that was significantly enriched in both cell cultures and tissues under the Diseases and Disorders category. A total of 103, 163, and 220 genes in the low, medium, and high concentrations *in vitro*, and 60, 40, and 159 genes in the low, medium, and high concentrations *in vivo* were associated with this function. This function was associated with terms such as cancer, epithelial neoplasia, cell transformation, solid tumor, and metastasis both *in vivo* and *in vitro* (Figures 9E and 9F). In tissues, annotation terms included growth of tumour, neoplasia of cells, carcinoma, metastasis and other related terms for this function. Further systematic analysis of all of the genes associated with cellular growth and proliferation and cancer revealed that *in vitro* these genes primarily include transcription factors and transcription regulators. In contrast, genes affected *in vivo* were grouped primarily into two categories involved with growth factors and inflammatory modulators in addition to other secretory molecules. In keeping with this result we found significant enrichment of the function 'gene expression' in cells in culture, with > 200 genes in this category compared to approximately 10 genes associated with 'gene expression' function in tissues (Figure 10). Our analysis clearly demonstrates that significantly more genes are associated with cell signalling *in vivo* compared to *in vitro* following Mitsui7 exposure. These results indicate that although most of the genes altered in both *in vivo* and *in vitro* belong to the same functional categories, suggesting a similar biological response to Mitsui7, the underlying mechanisms and the outcome of such alteration could potentially be different in the two systems studied.

We explored the *in vitro* and *in vivo* data sets to identify potential commonalities in the two systems that may contribute to the same biological outcome, and thus point to the specific relevance of this cell culture model. We conducted a detailed analysis of the responsive genes in the top five significantly enriched canonical pathways affected in both cells and lung tissues. This downstream analysis was limited to the top five

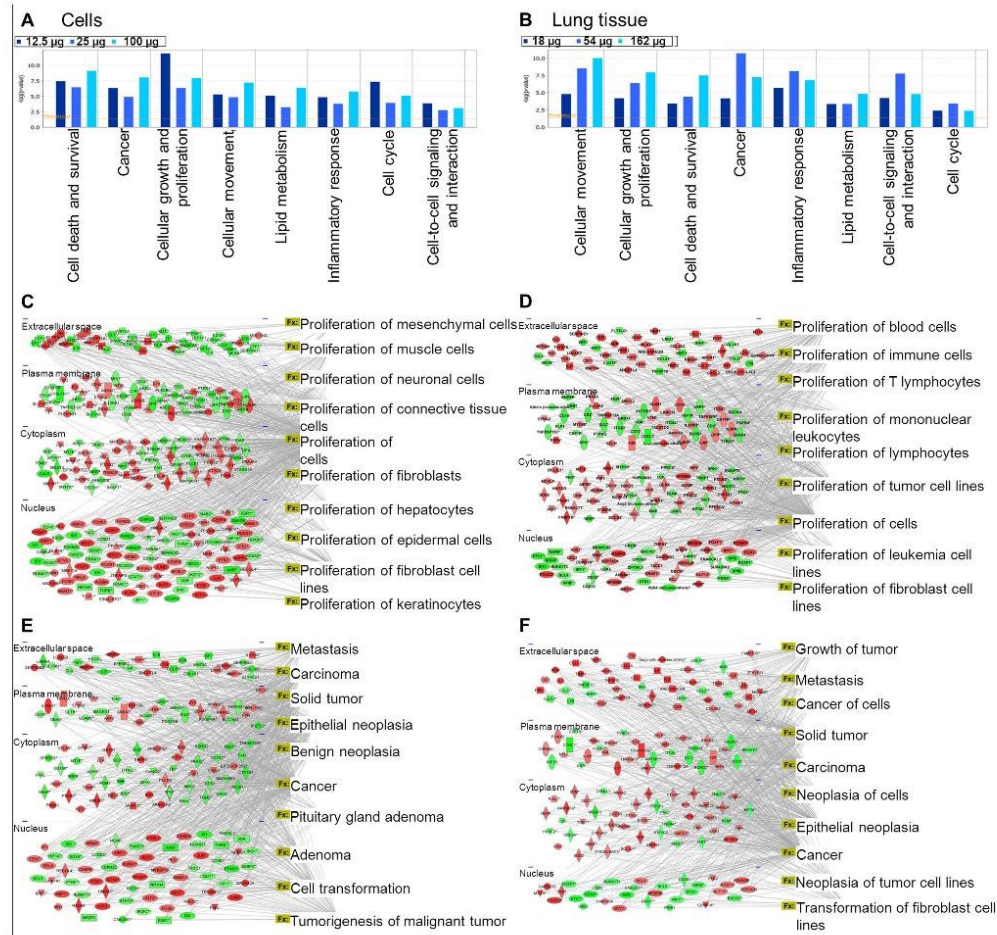


Figure 9. Comparison of biological functions and networks altered *in vitro* and *in vivo*. Top 10 biological functions altered in lung epithelial cells (A, C, E) and in the lung tissue (B, D, F). The spatial visualization of gene network associated with the biological functions cellular growth and proliferation (C-D) and cancer (E-F). Grey lines connect the significantly differentially expressed genes to the annotation terms associated with each biological function.

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affected pathways to minimize the number of interpretations. The top five pathways included: hepatic fibrosis/hepatic stellate activation, AHR signalling, glutathione mediated detoxification, superpathways of cholesterol synthesis, and LPS/IL-1 mediated inhibition of RAR function. Since hepatic fibrosis/hepatic stellate activation and LPS/IL-1 mediated inhibition of RAR function showed a high amount of overlap (i.e., genes functioning in both pathways), we excluded the latter from the

analysis. Our examination of all of the genes grouped under each of these pathways revealed that these genes are all regulated by a common nuclear receptor, AHR, and are associated with four major biological processes regulated by the AHR: cell cycle regulation, xenobiotic metabolism, inflammation, and tissue fibrosis (Figure 11). While the common genes involved in cell cycle regulation were similarly up-regulated in both tissues and cell cultures exposed to

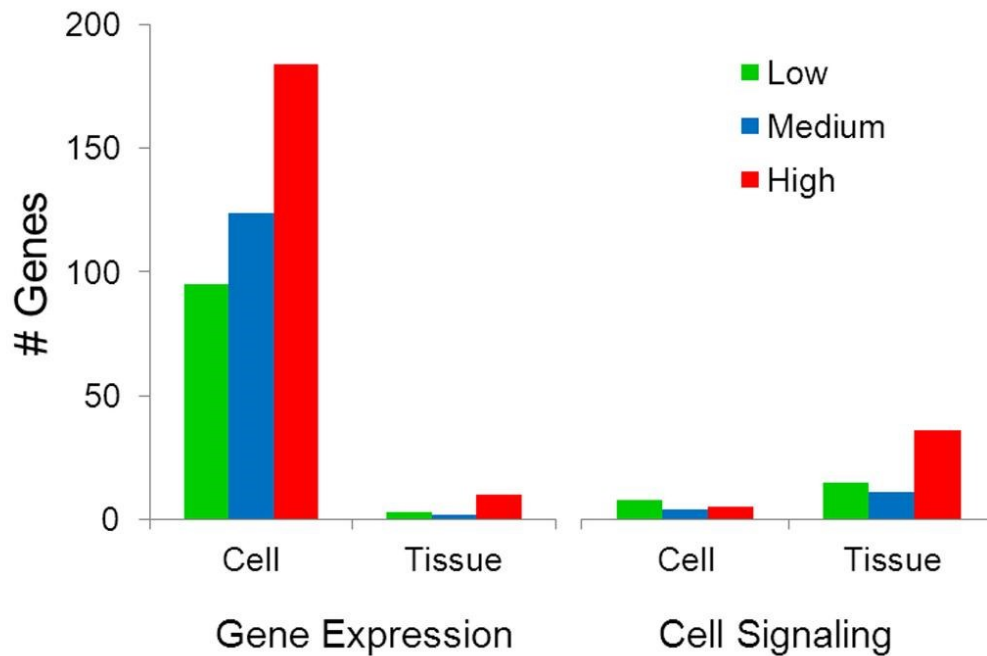


Figure 10. The total number of genes perturbed in the categories of gene expression and cellular signalling functions. Green: low dose (12.5 μg *in vitro*, 18 μg *in vivo*), blue: medium dose (25 μg *in vitro*, 54 μg *in vivo*), and red: high dose (100 μg *in vitro*, 162 μg *in vivo*).
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Mitsui7, genes involved in glutathione mediated detoxification and fibrosis were down-regulated in both systems. Although changes in expression of genes associated with similar functions (cell cycle, inflammation and fibrosis) were observed across all of the concentrations, perturbation of cell cycle and xenobiotic metabolism processes were only observed in the low dose group in the lung tissues, and perturbations in inflammation and fibrosis were only observed at the high dose *in vivo*. These results confirm our earlier interpretation that there was a dose dependent transition in response/toxicity in tissues, but there was no such dose-related transition of biological functions (by IPA) in cell cultures.

In addition to the analysis described above, we also examined our gene lists for markers of inflammation, oxidative stress, and tissue damage (fibrosis) since these effects are the well-characterized endpoints for both tissues and cells in culture following exposure to MWCNT. More than 100 genes that are known to be associated with each of these characteristic markers of MWCNT were used to build a heatmap (Figure 12) to visualize the similarities and differences between the test systems. Only the genes that showed significant expression changes in either cell cultures and/or the

lung tissues treated with Mitsui7 (in at least one exposure group) are shown in color (red up-regulated; green down-regulated; grey no change). Although the total number of genes altered in each of these pathways was somewhat similar between the two models, differentially expressed genes *in vitro* were associated mainly with positive regulation of oxidative stress and fibrosis. In the lung tissues, perturbed genes were associated predominantly with pro-inflammation. Although the lung tissue showed significant alteration in the expression of a number of genes in the fibrosis category, expression profile was indicative of negative regulation of fibrosis. ROS generation and oxidative stress have been proposed to be the primary mode of action of many ENM [1], where a balance between enzyme-mediated ROS generation and ROS detoxification by antioxidant enzymes and the level of intracellular antioxidants such as glutathione regulate intracellular ROS-mediated stress. Up-regulation of antioxidant enzymes including SOD, catalase, GPx and thioredoxin is considered as indication of adaptation to oxidative stress [62]. In the lung tissue, oxidative stress-associated genes, such as *Sod2*, *Ucp2*, and *Ucp3*, were up-regulated. SOD2 is localized to mitochondria and transforms superoxide radicals into

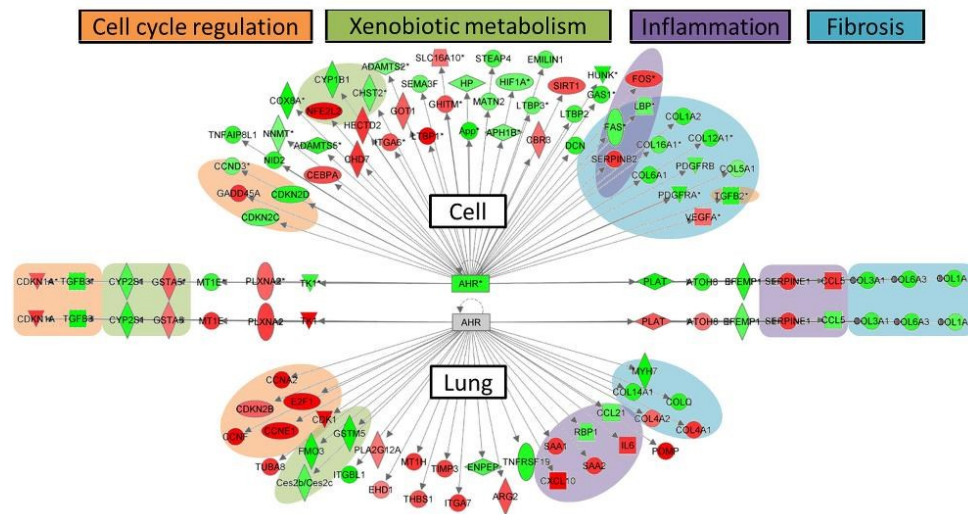


Figure 11. Differentially expressed genes associated with five top canonical pathways derived from IPA analysis. Genes are categorized into four major functions (cell cycle regulation-orange, xenobiotic metabolism-green, inflammation-violet, and tissue fibrosis-blue). All genes in both models are regulated by a common transcription factor, AHR. Genes highlighted with green indicate down-regulation and genes highlighted with red indicate up-regulation. The transcription factor AHR is down-regulated in cells, suggesting negative regulation of the downstream functions in cells.

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hydrogen peroxide that in turn are converted to water and oxygen by catalase or glutathione peroxidase. Uncoupling protein 2 and 3 (UCP-2, -3) are the major components of the electron transport chain. *Ucp* genes act to uncouple the proton gradient in the inner mitochondrial membrane, allowing protons to re-enter the mitochondrial matrix thereby regulating build-up of ROS in cells [63,64]. Similar mitochondrial respiration leakage and up-regulation of mitochondria-specific antioxidants such as SOD2 has been reported in mice intratracheally instilled with SWCNT [65]. The results suggest that the oxidative stress observed *in vivo* was potentially due to mitochondrial respiration leakage. In contrast, *Sod1* and *Sod3* were down-regulated *in vitro* and *Hmox1*, *Sqstm1*, *Srxn1*, *Txnip*, *Txnrd1*, *Txnrd3* were up-regulated. These differences suggest that broadly different mechanisms are driving the oxidative stress *in vitro*. However, it is important to note that the observed differences could also be due to the different time points investigated in the two systems. Further work considering early time points is warranted to rectify these conclusions.

A similar contradiction in the underlying mechanisms involved in the function 'fibrosis' was also observed in the two systems. Matrix metalloproteinases (MMPs) were up-regulated in cells in culture, whereas, the expression of inhibitors of metalloproteinases (TIMP) was increased in the lung tissue. A profound increase in the expression of several acute phase stress genes (*Saa*, *Cxcl5* and *Ccl5*), chemokines, and

cytokines was observed in the lung tissue, mainly reflecting chemotaxis and infiltration of inflammatory cells into the lungs. However, in contrast, increased expression of inflammatory genes was associated with epithelial activation (e.g., *Ccl5*, *Ptgs2*) *in vitro*. Overall, these results suggest different mechanisms, varying degrees of severity in response and potentially different biological outcomes to Mitsui7 exposure in epithelial cells in culture and lung tissue.

In order to understand if the observed discrepancies between the models were due to basal level differences in the expression of genes in the two systems studied, we compared gene expression profiles of vehicle treated controls of lung epithelial cells and mouse lung tissues. We found that 60% of the genes had comparable expression levels (15,972 of 26,409 analyzed), which included genes implicated in the inflammatory and acute phase responses. Thus, differences in expression were observed for 40% of the genes (Table S4) and these genes were mainly associated with biological processes such as Cellular growth and proliferation, Cancer, Cell death and survival, Cell cycle and Cellular movement in lung epithelial cells, which mainly reflect the consequences of cellular immortalization processes. These results imply that altered basal expression levels in thousands of genes involved in Growth and Proliferation, Cell cycle and other important biological functions may play a critical role in how cells in culture respond to external stimuli such as nanomaterials. The high basal levels of expression of several growth and survival



Figure 12. Gene heatmap comparing differentially expressed genes in the categories of fibrosis, inflammation and oxidative stress. Red represents up-regulation, green represents down-regulation and grey represents no change in the expression.

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Table 7. RT-qPCR validation of genes in lung tissue.

Genes	2 µg		6 µg		18 µg		54 µg		162 µg	
	PCR array	PCR array	PCR array	PCR array	PCR array	PCR array	PCR array	PCR array	PCR array	PCR array
<i>Saa3</i>	5.5	-	-	-	9.9	-	215.8	12.3	348.7	45.6
<i>Cxcl5</i>	-	-	66.9	-	-	-	188.8	5.3	81.5	4.1
<i>Cxcl1</i>	-	-	8.0	2.3	-	-	20.5	3.9	22.1	4.7
<i>Ccl2</i>	-	3.6	14.5	3.5	-	-	5.0	-	21.9	3.9
<i>Ccl7</i>	-	8.0	-	5.5	65.9	6.9	20.2	4.3	-	-
<i>Il6</i>	-	2.0	-	2.8	-	-	4.0	-	16.1	6.5
<i>Timp1</i>	-	3.0	-	7.7	-	-	9.7	-	13.1	11.7
<i>Ccl11</i>	-	4.4	-	3.0	15.6	5.2	5.3	3.4	-	-
<i>Saa1</i>	-	-	-	3.8	-	-	7.0	-	5.0	12.3
<i>Ccl17</i>	3.1	5.2	7.8	3.5	7.5	3.4	4.9	2.8	-	-
<i>Ccl3</i>	1.9	-	3.7	1.9	-	-	4.6	2.4	-	-
<i>Lox</i>	-	-	2.1	-	1.8	-	3.8	2.4	-	-
<i>Myc</i>	1.5	-	1.9	-	2.0	-	3.0	1.9	-	-
<i>Cd14</i>	-	-	2.4	-	2.7	-	2.9	2.0	-	-
<i>Ccl19</i>	-	-	-	-	-	-	2.7	2.5	-	-
<i>Il33</i>	-	-	-	2.8	3.8	-	2.6	-	-	-
<i>Cxcl10</i>	-	2.4	-	4.5	13.8	6.0	2.5	-	-	-
<i>Thbs1</i>	-	-	1.9	-	-	2.9	2.4	2.1	-	-
<i>Ctgf</i>	-	-	-	-	-	-	2.4	2.0	-	-
<i>Gclc</i>	-	1.3	2.1	-	-	-	2.2	1.8	-	-
<i>Pth1</i>	-	-	-	-	-	-	2.0	2.3	-	-
<i>Ptgs1</i>	-	-	-	-	-	-	1.9	2.8	-	-
<i>Nox4</i>	-	-	-	-1.8	-3.6	-	-1.7	-	-	-
<i>Ltb</i>	-	-1.5	-	-	-	-	-2.2	-2.1	-	-
<i>Serpinh1</i>	-	-	1.8	-	-	-	-2.6	-2.2	-	-
<i>Tnfrsf10</i>	-	-	-	-	-2.3	-	-4.5	-2.8	-	-
<i>Timp3</i>	-	-1.5	-1.7	-	-2.0	-	-	2.3	-	-
<i>Sod2</i>	-	-	-	-	-	-	-	1.8	-	-
<i>Ucp2</i>	-	-	-1.6	-	-2.0	-	-	2.2	-	-
<i>Cxcl13</i>	-	-	-	-	5.3	-	-	3.8	-	-
<i>Prdx6</i>	-	-1.8	-2.4	-1.5	-3.0	-	-	1.5	-	-
<i>Ucp3</i>	-	-1.7	-2.5	-	-2.0	-	-	2.2	-	-
<i>Edn1</i>	-	-1.7	-	-	-2.5	-	-	1.9	-	-
<i>Itga3</i>	-	-	-	-	-1.7	-	-	2.1	-	-
<i>C3</i>	-	1.8	-	2.3	2.8	-	-	-	-	-

Only significantly differentially expressed genes are shown (FDR $P < 0.05$, fold change > 1.5 in either direction). Data were normalized using *Hprt*, *Gapdh*, and *Actb* as reference genes.

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factors may provide protection against cellular injury caused by exposure to nanomaterials. This hypothesis was supported by the observed lack of response in cells treated with very low concentrations of MWCNT (described below, Table 7 and 8). In contrast, changes in the expression of important acute phase and inflammatory genes were observed in lung tissues even at the very low doses. Our results suggest that data from single cell models must be interpreted with caution.

Validation of microarray results by RT-qPCR

We validated 42 genes altered in cell cultures and 42 genes in tissues (84 in total) categorised under three main established markers of MWCNT-induced effects: oxidative stress, inflammation, and fibrosis. A complete list of genes validated by RT-qPCR is provided in Table S5. Because the exposures used in the DNA microarray analysis are considered to be high compared to the expected exposure levels in the environment, we included a range of lower (lower than 12.5 µg/ml in *in vitro* and lower than 18 µg/mouse *in vivo*) exposure levels for RT-qPCR validation. The final validation by RT-qPCR included 0.0125, 0.125, 1.25, 12.5, 25 and 100 µg/ml in FE1 cells, and 2, 6, 18, 54, 162 µg/mouse *in vivo*. The RT-qPCR results were broadly consistent with the microarray results (Tables 7 and 8). Interestingly, in cell cultures, no significant changes were observed, with the exception of moderate down-regulation of *Mmp3* and up-regulation of *Cx3cl1* at the lowest concentration (0.0125 µg/ml) tested, (we note that the direction of change for *Cx3cl1* was reversed at the higher concentrations). In order to confirm that lack of response in FE1 cells following low concentration exposures was not due to the analysis of only a subset of genes associated with a few biological effects, global analysis of gene expression analysis was performed (as described above) on the lower concentration (0.0125, 0.125, 1.25 µg/ml) groups. The microarray analysis (data not shown) confirmed the subtle response observed by RT-qPCR. In contrast, in the lung tissues, several genes (*Saa3*, *C3*, *Cxcl10*, *Myc*, *Ccl3*, *Ccl17*, *Ccl11*, *Timp1*, *Il6*, *Ccl7*, *Ccl2* and *Cxcl5*) involved in acute phase and inflammation were significantly up-regulated even at the lowest concentrations tested (2 µg/mouse). The data suggest that the lung tissue is generally more sensitive to Mitsui7 exposure than the FE1 lung epithelial cells and that these genes can potentially be used as sensitive markers of exposure to Mitsui7 and like particles.

Perspectives

Despite the urgent need for rapid *in vitro* assays that are predictive of *in vivo* toxicity for ENM testing, few *in vitro* validation exercises have been conducted to comprehensively characterize mechanistic similarities and differences for ENM-exposed cells in culture compared to tissues from live model organisms exposed to ENM. Existing publications on this topic have been limited to a small selection of biological endpoints that include inflammation, oxidative stress, and tissue damage (reviewed in 9,66-68). In these existing studies, the *in vivo* lung toxicity assays applied have generally consisted of analysis of BAL fluid for markers of inflammation (infiltration of inflammatory cells, increased production of cytokines and chemokines), oxidative stress (oxidative stress markers, DNA damage), and cytotoxicity (LDH and alkaline phosphatase). These measurements are then analyzed in parallel in cells exposed *in vitro* to the same substance. For example, Wang et al. [15], measured markers of fibrosis in lung tissues and in different lung cell culture models and found good agreement between the profibrogenic responses *in vitro* and *in vivo* [15]. In another study, Horie et al. [69] measured levels of hydroperoxy octadecadienoate (thODE), an indicator of oxidative stress, in rat lungs exposed to nickel nanoparticles and in cells exposed

Table 8. RT-qPCR validation of genes in cells.

Genes	0.00125	0.125	1.25	12.5	25.0		100.0	
	PCR array	PCR array	PCR array	PCR array	Microarray	PCR array	Microarray	PCR array
<i>Mmp13</i>	-	-	1.6	15.0	9.7	18.2	12.7	27.6
<i>Serpine1</i>	-	-	-	7.3	2.9	5.9	3.9	10.1
<i>Ccl5</i>	-	-	-	2.3	3.0	4.3	2.7	8.0
<i>Ctgf</i>	-	-	-	3.9	3.0	5.4	4.3	7.7
<i>Mmp3</i>	-	-	2.9	33.1	3.5	5.3	3.9	7.0
<i>Sqstm1</i>	-	-	-	4.0	1.8	4.1	2.0	6.2
<i>Grem1</i>	-	-	1.7	2.4	2.5	3.4	2.4	5.5
<i>Tgfb1</i>	-	-	-	5.5	2.0	4.4	2.3	5.0
<i>Hmox1</i>	-	-	-	2.7	-	3.1	2.1	4.7
<i>Myc</i>	-	-	-	4.1	1.8	3.0	2.8	4.6
<i>Tnfrsf11b</i>	-	-	-	-1.7	2.1	3.0	2.2	4.3
<i>Jun</i>	-	-	-	-	2.3	2.8	2.8	3.7
<i>Ptgs2</i>	-	-	-	5.4	2.5	2.6	2.9	3.7
<i>Spp1</i>	-	-	-	3.3	2.0	3.2	-	3.5
<i>Fos</i>	-	-	-1.5	-7.8	-	2.7	-	3.1
<i>Vegfa</i>	-	-	-	-	-	2.2	-	2.7
<i>Edn1</i>	-	-	-	1.5	2.2	2.6	2.5	2.7
<i>Txnip</i>	-	-	-	2.5	-	2.0	-	2.6
<i>Txnrd1</i>	-	-	-	3.1	1.7	2.2	1.9	2.5
<i>Srxn1</i>	-	-	-	-	-	1.7	1.5	2.1
<i>Txnrd3</i>	-	-	-	2.9	-	2.0	1.7	2.1
<i>Cebpb</i>	-	-	-	-	-	-	-	1.8
<i>Cygb</i>	-	-	-	1.7	-	1.6	-1.6	1.8
<i>Cx3cl1</i>	-	-	-	-	-2.4	-1.8	-2.4	-1.5
<i>Plat</i>	-	-	-	-	-1.6	-1.8	-1.6	-1.5
<i>C3</i>	-	-	-	-2.8	-2.0	-1.7	-2.1	-1.7
<i>Il1r1</i>	-	-	-	-2.2	-1.7	-2.1	-1.7	-1.9
<i>Cxcl12</i>	-	-	-	-5.6	-	-1.9	-2.3	-2.1
<i>Col3a1</i>	-	-	-	-8.6	-1.8	-2.1	-2.4	-2.3
<i>Il18</i>	-	-	-	-9.6	-1.8	-2.5	-2.0	-2.4
<i>Smad6</i>	-	-	-	-3.6	-1.8	-2.4	-2.1	-2.5
<i>Tgfb3</i>	-	-	-	-5.0	-	-2.4	-1.9	-2.5
<i>Il15</i>	-	-	-	-3.0	-1.5	-2.6	-1.5	-2.7
<i>Idh1</i>	-	-	-	-4.9	-	-2.3	-1.9	-3.0
<i>Dcn</i>	-	-	-	-4.9	-	-2.7	-3.4	-3.1
<i>Aox1</i>	-	-	-	-4.5	-2.4	-4.1	-3.2	-3.7
<i>Tgfb2</i>	-	-	-	-7.9	-1.7	-3.5	-2.3	-4.6
<i>Il33</i>	-	-	-	-67.3	-3.6	-	-4.4	-13.0
<i>Col1a2</i>	-	-	-	-5.6	-1.7	-	-2.4	-
<i>Gpx7</i>	-	-	-	-2.3	-	-	-	-1.8
<i>Pdgfa</i>	-	-	-	-1.7	-1.5	-	-	-1.5
<i>Cxcr3</i>	-	-	-	1.6	1.5	1.9	-	-

Only significantly differentially expressed genes are shown (FDR $P < 0.05$, fold change > 1.5 in either direction). Data were normalized using *Gapdh* and *Actb* as reference genes.

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in Petri culture dishes. They showed comparable increases in tHODE 24 hours after the exposure to particles *in vivo* and *in vitro* [69]. However, although similar perturbations were described *in vitro* and *in vivo* in the above studies, the magnitude of response between the lung tissue and cells in culture in the above studies was different. These differences in the magnitude of response may occur because of difficulties in

matching the predicted particle-exposed lung surface area to the surface area of Petri dishes in which the cells are incubated. Moreover, *in vivo* studies evaluated toxicity in complex multi-cellular tissues; whereas, *in vitro* studies primarily focus on understanding the response of a single cell type isolated from a specific organ.

In the present study, we exposed mice *via* instillation and incubated mouse lung epithelial cells in culture dishes to three different doses of Mitsui7. Our *in vivo* to *in vitro* dose comparison was based on the total lung surface area and the total surface area of the Petri dish in which cells were exposed (Table 2). Unlike previous studies, we analyzed the global gene expression response to Mitsui7 in lung tissue and cells using comprehensive and high-content DNA microarrays to globally characterize potential mechanistic differences in response. Three major concepts emerged from this analysis: (1) the primary toxic response *in vivo* was an early acute phase and inflammation dose-response which was not activated *in vitro*; (2) the *in vitro* model is less responsive than the *in vivo* at lower doses and does not show a dose-response pattern in the induction of toxicity pathways at increasingly higher concentrations; and (3) both models respond by activating similar core cellular functions (oxidative stress and fibrosis) at the pathway level, however, regulation of most individual genes, the underlying mechanisms and consequent nature of the biological response are different. We suggest that careful consideration should be given to the *in vitro* concentrations selected, the types of cells used, and the endpoints studied when deriving regulatory decisions for ENM such as Mitsui7.

Supporting Information

File S1. Characterization of Mitsui7 in dispersion medium. (PDF)

Figure S1. DLS measurements of Mitsui7 in different dispersion medium. (PDF)

Table S1. Genes differentially expressed *in vivo*.

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(XLS)

Table S2. Genes differentially expressed *in vitro*. (XLS)

Table S3. Genes commonly enriched in the three dose groups *in vitro*. (XLS)

Table S4. List of genes showing differential expression in control lung epithelial cells vs control lung tissue samples. (XLS)

Table S5. List of genes validated by RT-PCR in lung tissue and in lung epithelial cells. (XLS)

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Author Contributions

Conceived and designed the experiments: SSP NRJ HW UV SH. Performed the experiments: SSP NRJ DW MH JPB OA CK KM ZOK ATS. Analyzed the data: SSP SL MH AW SH. Contributed reagents/materials/analysis tools: SH CLY HW UV. Wrote the manuscript: SSP SH. Revisited the manuscript critically for important intellectual content, and approved the final manuscript: SSP NRJ SL DW MH AW JPB OA CK KM ZOK ATS CLY HW UV SH..

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Appendix V Supplemental Materials.

Supplementary tables for each chapter can be accessed online. Legends are included below for reference purposes.

Supplementary Table 2. 1 Significant gene list for BaP exposed lungs

List of all significantly differentially expressed genes in at least 1 treatment group (FDR adjusted P value ≤ 0.05 , fold change ≥ 1.5) in response to sub-chronic oral exposure to 25, 50, and 75mg/kg/day benzo(a)pyrene. The list is sorted from highest to lowest fold change in the 75mg/kg/day treatment group.

Supplementary Table 2. 2 Gene ontology analysis of BaP exposed lungs

List of the most enriched functional annotation clusters using DAVID based on significantly differentially expressed genes in at least one dose with an FDR adjusted $p \leq 0.05$ (a) or FDR adjusted $p \leq 0.1$ (b) and fold change ≥ 1.5 . Each cluster groups similar annotations together to clarify the biological function. The names given to each annotation were arbitrarily chosen based on the constituents of the cluster. The Enrichment Score is the geometric mean of the p values of the cluster constituents transformed by $-\log$. The p value is the EASE score which is a modified Fisher Exact p value.

Supplementary Table 2. 3 KEGG pathway analysis of BaP exposed lungs

Functional annotation into KEGG pathway categories using significantly differentially expressed genes with an FDR adjusted p value ≤ 0.05 (a) or FDR adjusted p value ≤ 0.1 (b) and fold change ≥ 1.5 in at least one dose group. The pathway annotations displayed have an EASE score (one-tail Fisher Exact p value) less than 0.1.

Supplementary Table 2. 4 PCR validation of BaP exposed lung microarray data

All genes validated by qRT PCR using PCR arrays in lungs from BaP exposed mice. Bold genes are validated from microarrays. Genes colored in grey had low expression in both the dose groups and control ($Ct > 30$).

Supplementary Table 2. 5 Relaxed gene list from BaP exposed lungs

List of genes additionally differentially expressed following relaxing of the significance cut-off from FDR $p < 0.05$ to FDR $p < 0.1$. P values shown are FDR adjusted.

Supplementary Table 3. 1 The 59 curated studies in NextBio used in meta-analysis.

Supplementary Table 3.2 Significant probe list

List of all significantly differentially expressed probes in at least 1 treatment group (FDR adjusted p value ≤ 0.05 , Fold Change ≥ 1.5) in response to sub-chronic oral exposure to 25, 50, and 75 mg/kg-bw/day benzo(a)pyrene in the forestomach. The list is sorted from highest to lowest fold change in the 75 mg/kg-bw/day treatment group.

Supplementary Table 3.3 Upstream regulator analysis.

Upstream Regulators according to IPA's Upstream Regulator Analysis showing cytokines, transcriptional regulators, kinases and receptors that may be activated or inhibited according to the gene expression profiles from BaP exposed forestomach.

Supplementary Table 3.4 NextBio disease prediction.

Disease prediction in NextBio of the 75 mg/kg-bw/day dose group showing disease category, top five individual diseases, NextBio score, and the number of studies curated.

Supplementary Table 3.5 Genes validated using real-time PCR arrays in the forestomach of BaP-treated mice.

Supplementary Table 4.1 Collapsed functional groupings for each pathway and process.

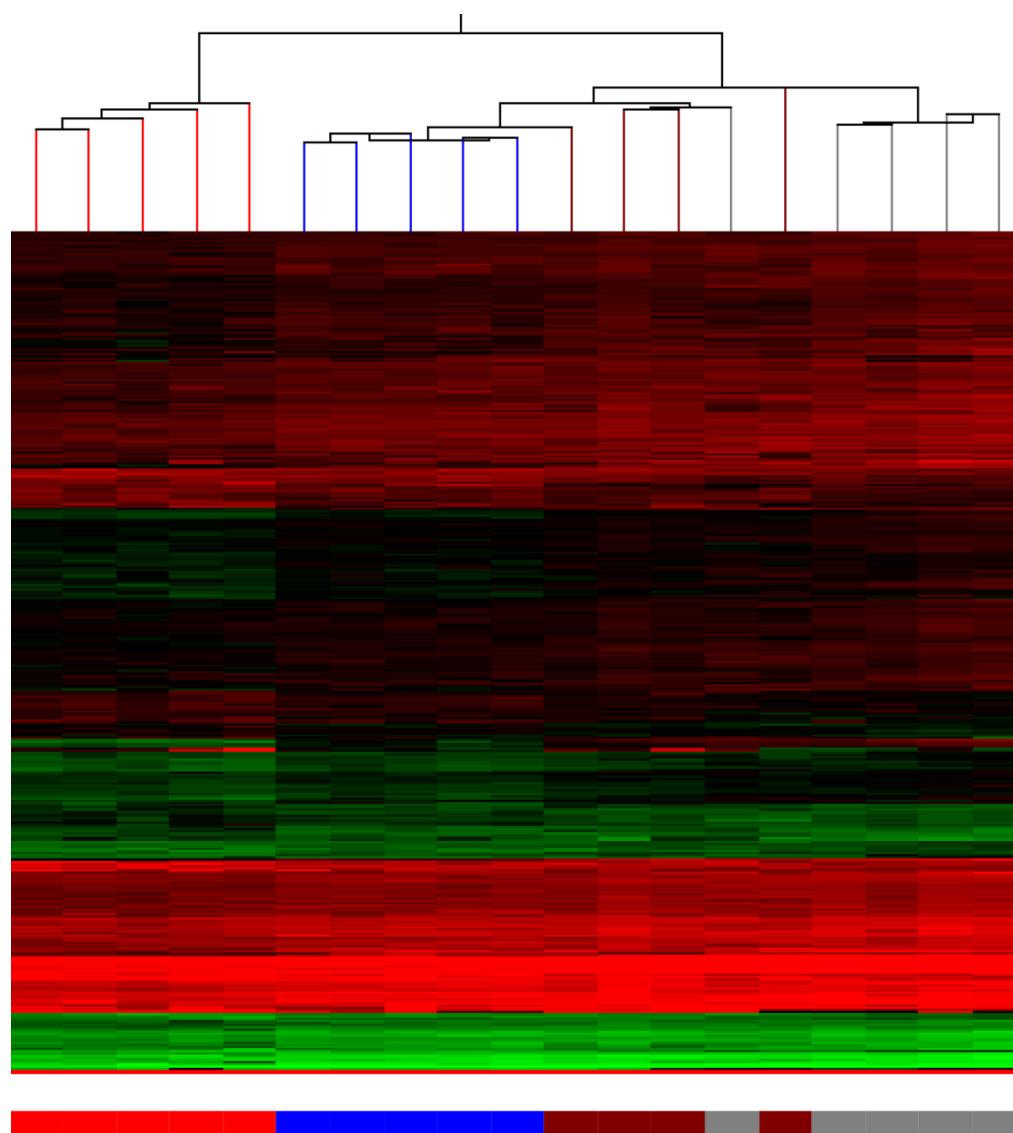
Supplementary Table 4.2 Significant probe and gene lists for each pathway and process.

List of all significantly differentially expressed probes in at least 1 treatment group (FDR $P \leq 0.05$, fold change ± 1.5) in response to sub-chronic oral exposure to the tested PAHs in the forestomach (4.2.1), liver (4.2.2), and lung (4.2.3). The lists are sorted from highest to lowest fold change in the high dose group.

Supplementary Table 5.1 Analytical assessment of purified coal tar.

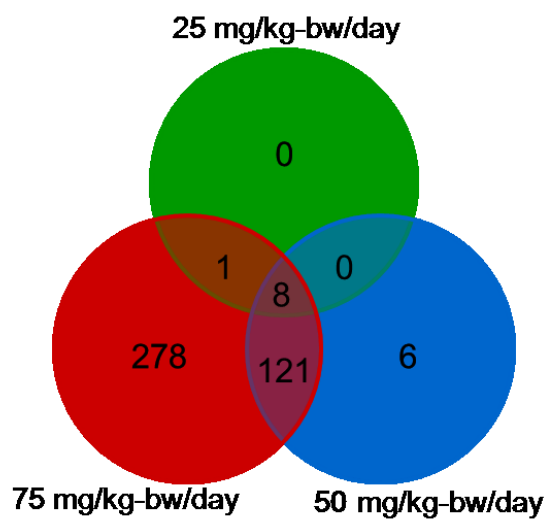
Supplementary Table 5.2 Gene lists for PAH-containing mixtures in lung tissues.

Supplementary Table 5.3 List of median and mean gene expression changes for each pathway in lungs from mice exposed to the 4PAH-Mix, 8PAH-Mix, CT-Mix, and the individual PAHs.



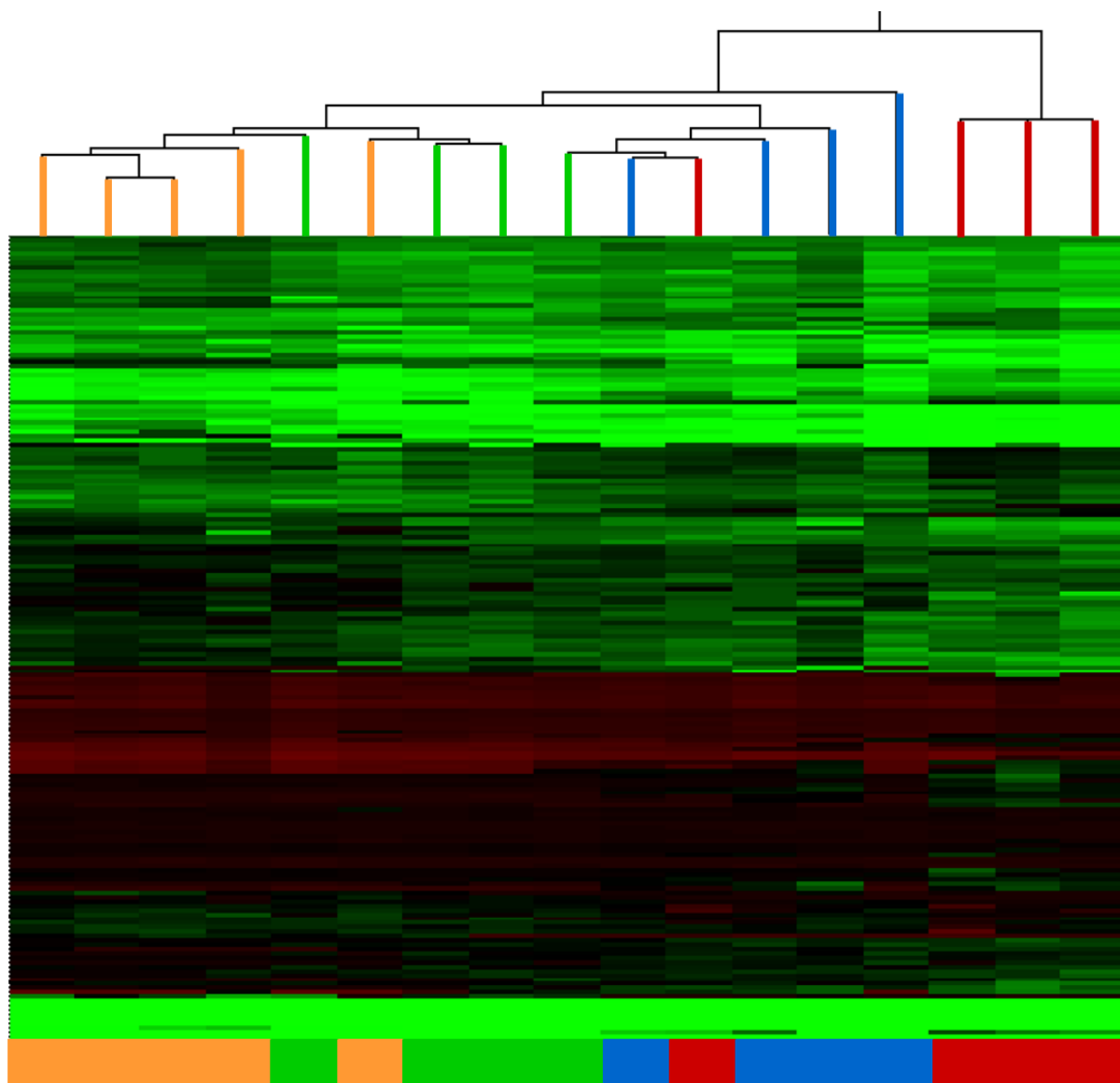
Supplementary Figure 2. 1 Hierarchical cluster analysis of BaP exposed lungs.

Hierarchical cluster analysis of all samples on differentially expressed genes. The heat map represents all genes that were statistically significant and differentially expressed in any of the treatment groups relative to control mice. Green bars represent low expression levels and red represent high expression levels relative to the reference channel. Black bars are similar to the reference channel. The heat map was generated using GeneSpring (Agilent Technologies). Clusters are color-coded: controls red, 25 mg/kg blue, 50 mg/kg brown, 75 mg/kg grey.



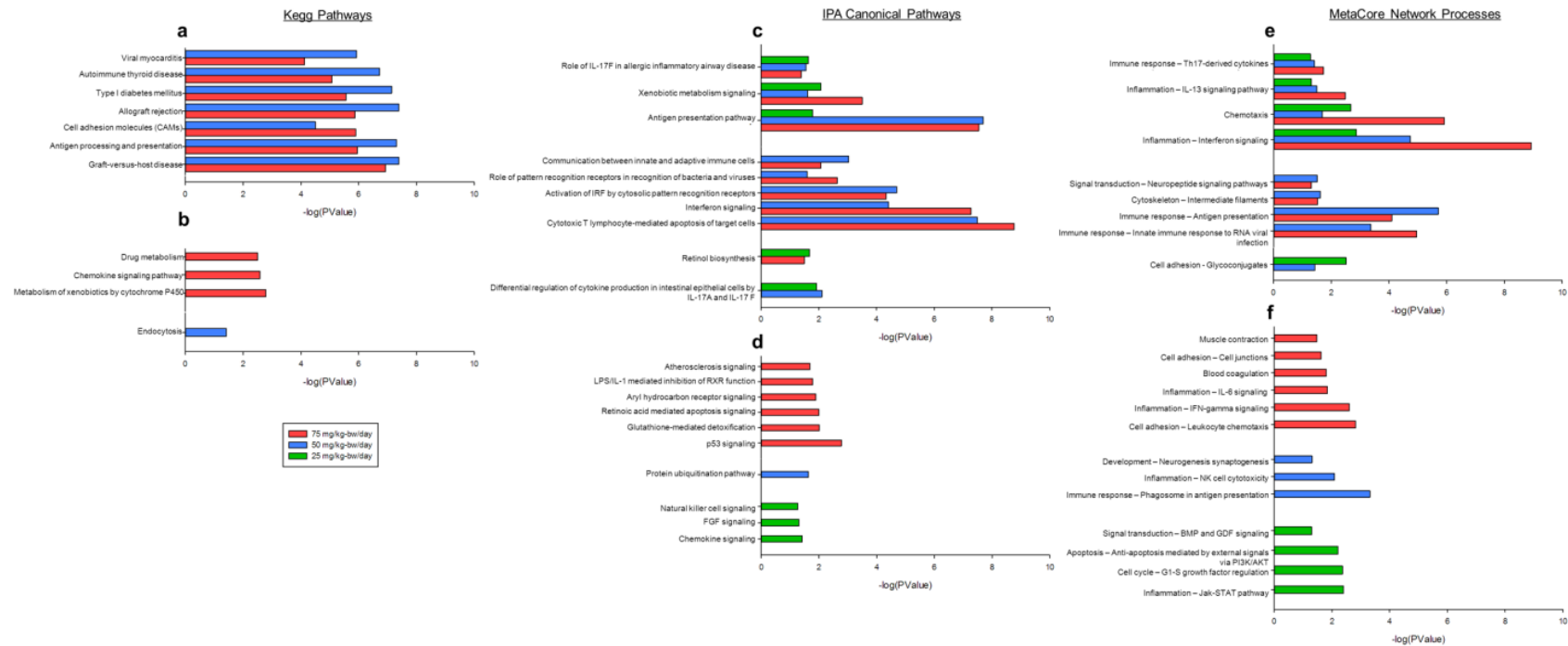
Supplementary Figure 3. 1 VENN diagram of significantly differentially expressed genes.

Venn diagram showing the overlap of genes that were significantly differentially expressed in response to low (25 mg/kg-bw/day), medium (50 mg/kg-bw/day), and high (75 mg/kg-bw/day) doses of BaP *via* oral gavage.



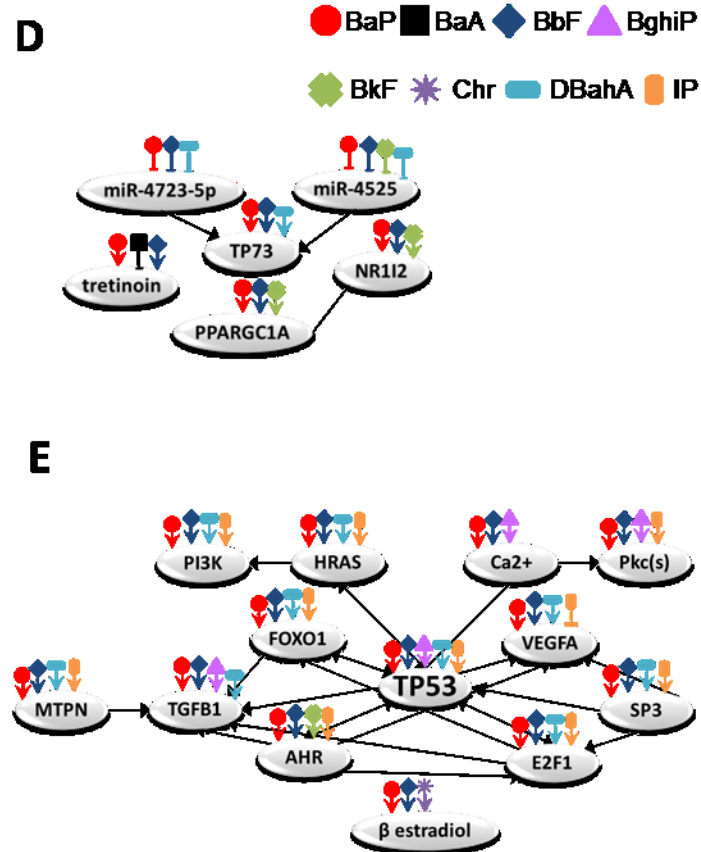
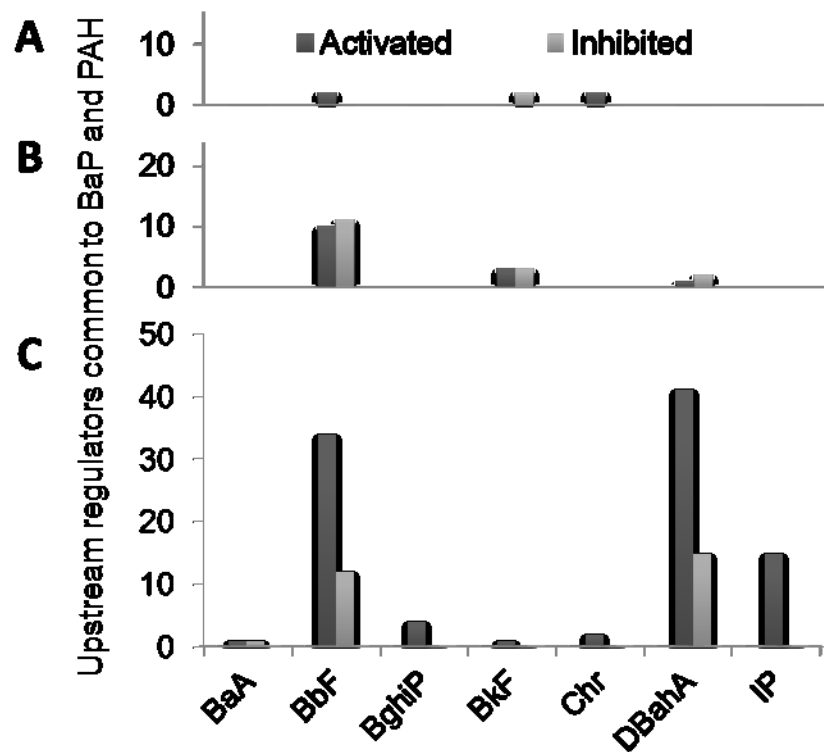
Supplementary Figure 3. 2Hierarchical cluster analysis.

Hierarchical cluster analysis of all samples on differentially expressed genes. The heat map represents all genes that were statistically significant and differentially expressed in any of the treatment groups relative to control mice. Green bars represent low expression levels and red represent high expression levels relative to the reference channel. Black bars are similar to the reference channel. The heat map was generated using GeneSpring (Agilent Technologies). Clusters are color-coded: controls orange, 25 mg/kg-bw/day green, 50 mg/kg-bw/day blue, 75 mg/kg-bw/day red.



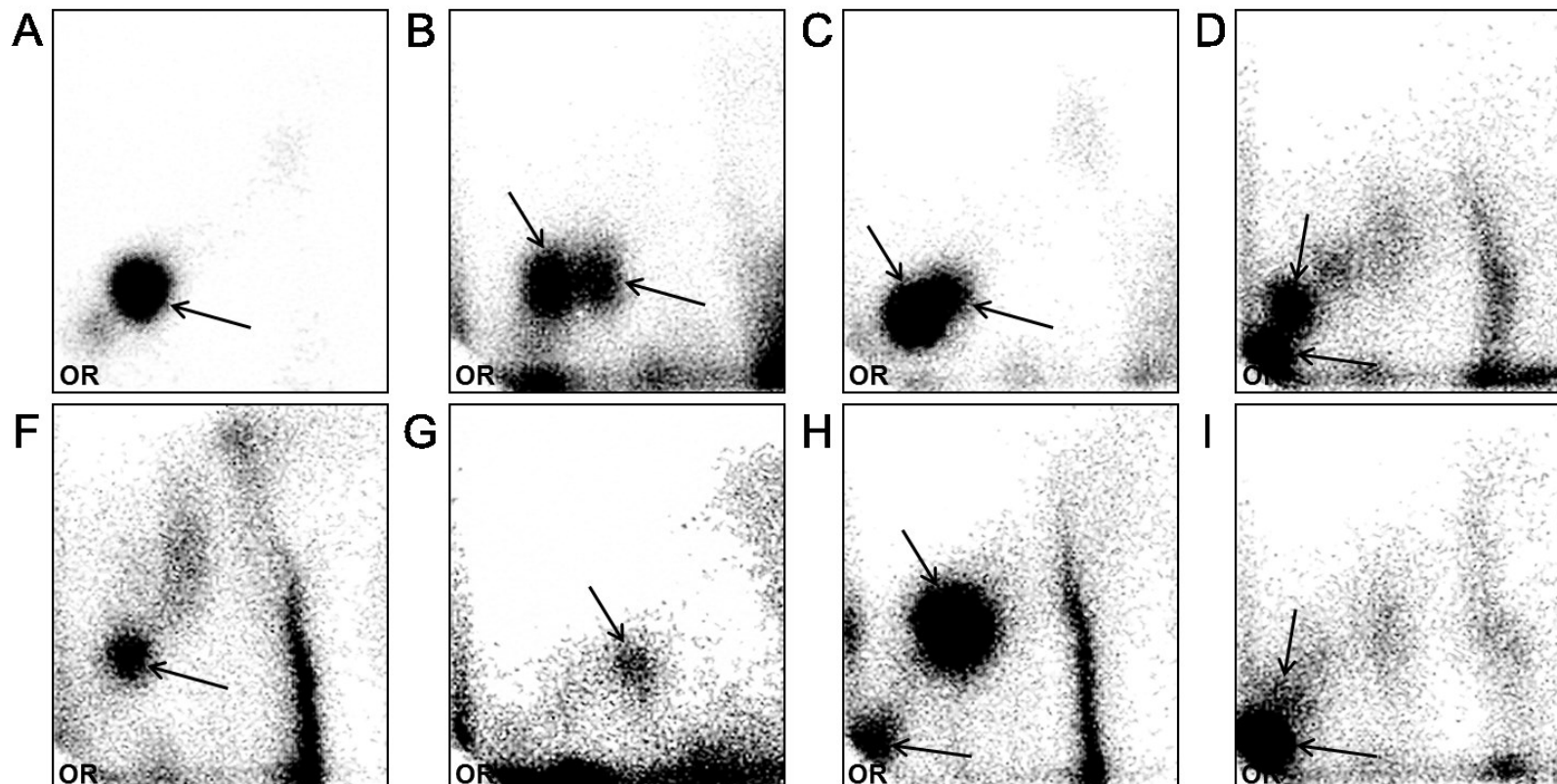
Supplementary Figure 3. 3 Pathway analysis of differentially expressed genes

The upper panels (a,c,e) show pathways common to at least two dose groups. The lower panels (b,d,f) indicate pathways that were uniquely enriched in the 25, 50, and 75 mg/kg-bw/day dose groups. The left hand column (a,b) shows the significant (Benjamini Corrected $P < 0.05$) KEGG pathways, the middle column (c,d) shows the significant ($P < 0.05$) IPA canonical pathways and the right hand column (e,f) shows the significant ($P < 0.05$) MetaCore network processes. 25, 50, and 75 mg/kg-bw/day dose groups are in green, blue, and red columns, respectively.



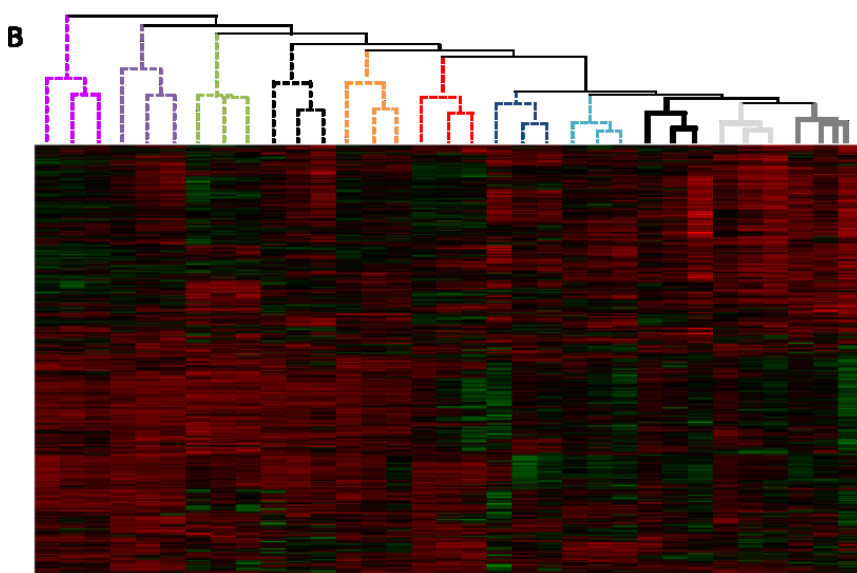
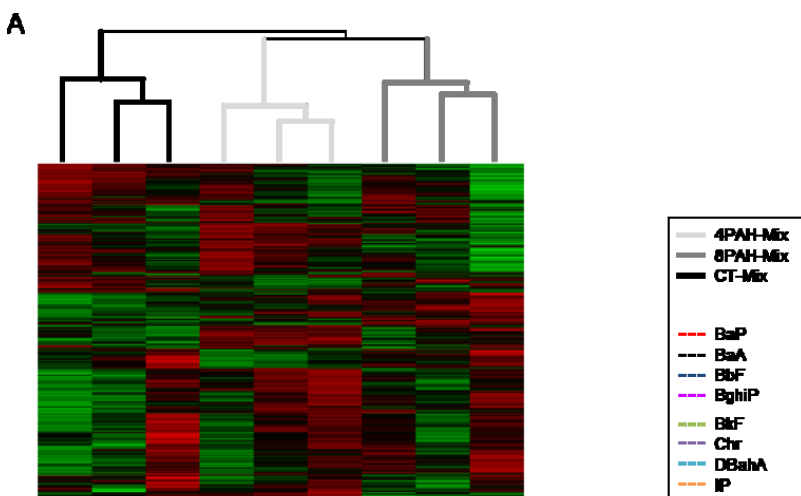
Supplementary Figure 4. 1 IPA upstream regulator analyses

Graphs show number of upstream regulatory molecules common to each PAH and BaP in the (A) forestomach, (B) liver , and (C) lung. (D-E) Network of upstream regulators common to the PAHs and BaP in the (D) liver and (E) lung.



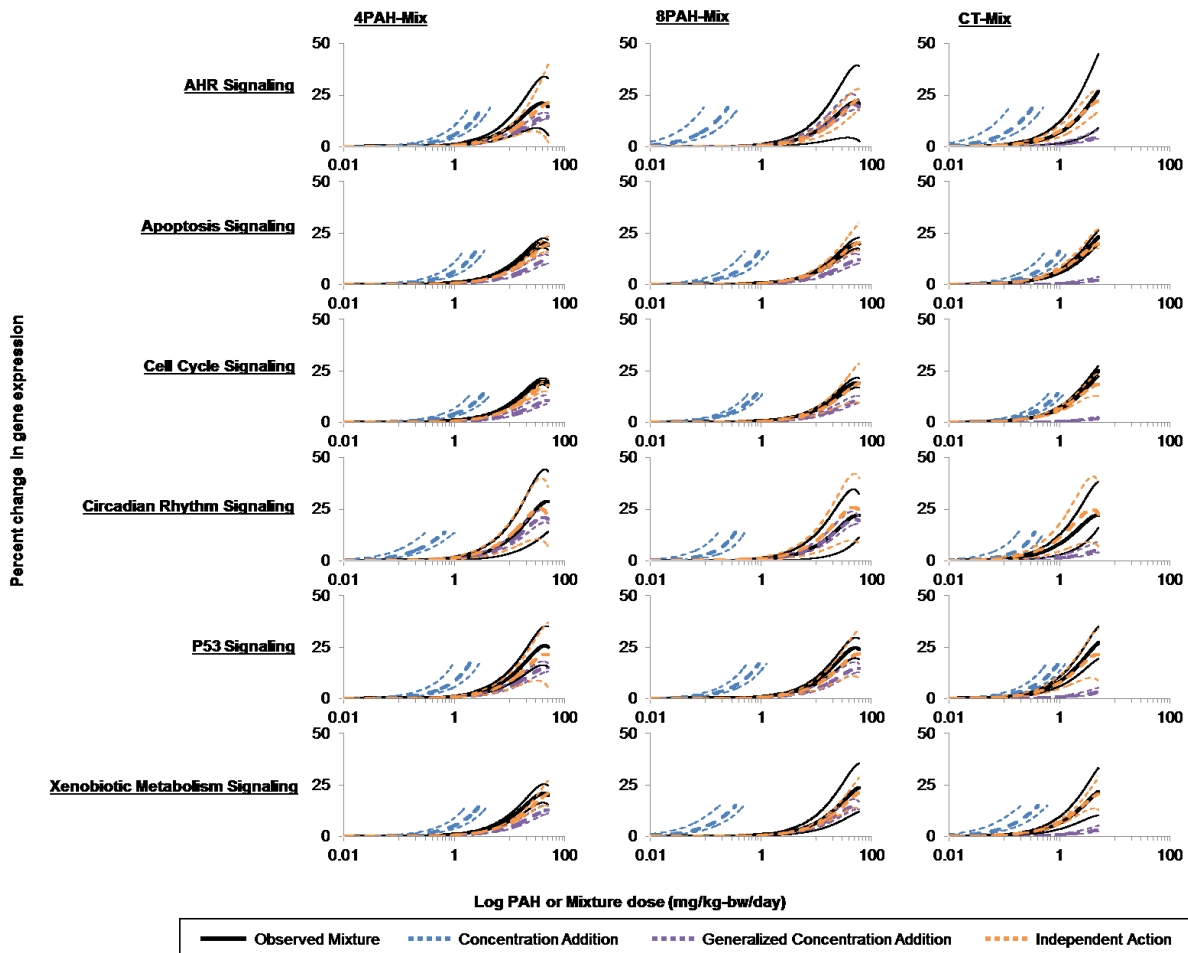
Supplementary Figure 4. 2 Sample autoradiographs ^{32}P -post-labeling of PAHs in lung.

Representative autoradiographic profiles of DNA adducts measured by ^{32}P -post-labeling, in lungs from MutaTM Mouse subchronically exposed to BaP (A), BaA (B), BbF (C), BghiP (D), BkF (F), CHR (G), DBahA (H) and IP (I). 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. Arrow(s) indicate major adduct spot(s) used to calculate DNA adduct level(s).



Supplementary Figure 5. 1 Hierarchical cluster analyses.

(A) Hierarchical cluster analysis of 3 mixtures. (B) Hierarchical cluster analysis comparing mixtures to individual PAHs.



Supplementary Figure 5. 2 Impact of no statistical filtering on mixture predictions using mathematical models of additivity

Supplementary File 4. 1 Detailed methodology

A - Tissue RNA extraction and purification

Total RNA was isolated for gene expression analysis as described previously (Halappanavar et al. 2011; Labib et al. 2013; Labib et al. 2012). Briefly, total RNA was extracted from a random slice of the forestomach, liver, and lung using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) following manufacturer's recommendations. All samples showed high quality RNA: with a A260/A280 ratios between 2.0 and 2.2 as determined by the NanoDrop Spectrophotometer (ThermoFisher Scientific, Mississauga, ON, Canada) and RNA integrity numbers above 7 as determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Mississauga, ON, Canada).

B - Microarray hybridization and analysis

Detailed protocols of the microarray hybridization and statistical analysis of the gene expression data are described in Labib et al. (2013). A minimum of four individual animals were used from each treatment group from each PAH except for BkF low dose group, which had only 3 mice. Sample sizes of 5 were used for each PAH and treatment group, except for the BkF and IP exposures (Table 1). In brief, 200 ng of total RNA from each individual sample and 200 ng of Universal Mouse Reference RNA (Stratagene, Mississauga, ON, Canada) was used to synthesize cDNA and cyanine-labeled cRNA using the Agilent Linear Amplification Kit (Agilent Technologies Inc., Mississauga, ON, Canada). The labeled cRNA (Cyanine-5 for experimental samples and Cyanine-3 for reference RNA) was purified using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). 300 ng of labeled cRNA from each experimental sample was hybridized with the same amount of labeled reference RNA to Agilent Sureprint G3 Mouse GE 8x60K microarrays (Agilent Technologies Inc., Mississauga, ON, Canada) at 65°C for 17 hours in the Agilent SureHyb hybridization chamber. The arrays were washed and scanned on the Agilent G2505B Scanner according to the manufacturer's recommendations. Data were extracted using Feature Extraction 10.7.3.1 (Agilent Technologies Inc., Mississauga, ON, Canada).

Details of the statistical normalization methods of the gene expression data are described in Labib et al. (2013). Briefly, a reference design (Kerr and Churchill 2001; Kerr and Churchill

2007) was used to analyze microarray data. Non-background median signal intensities were normalized using LOWESS (Yang et al. 2002) using the R (R Development Core Team 2010) software. A gene was considered differentially expressed using the F_s statistic (Cui et al. 2005) in the MAANOVA R library (Wu et al. 2003). The P values for all statistical tests for each probe ID were estimated by the permutation method using residual shuffling, followed by adjustment for multiple comparisons using the false discovery rate (FDR) approach (Benjamini and Hochberg 1995). The fold change calculations were based on the least-square means (Goodnight and Harvey 1978; Searle et al. 1980). All microarray results are deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE51321.

C - Hierarchical clustering analysis

Biological replicates of each experimental condition were collapsed to an average expression value for each gene and were normalized to the median of the control samples. The expression data relative to controls for all samples treated with the eight PAHs were then merged together using the Agilent probe IDs. This dataset was then filtered using the differentially expressed genes (DEGs) from the eight independent MAANOVA analyses. Hierarchical clustering was then applied to the filtered data using the one minus correlation dissimilarity metric using the spearman correlation with average linkage. Data was visualized using a heatmap.

D - Bioinformatics and pathway analysis

List of DEGs ($\text{FDR } P \leq 0.05$, fold change ± 1.5) from each PAH congener were independently analyzed to identify biological functions or processes perturbed in response to the treatment. DAVID (Huang da et al. 2009) Functional Annotation Charts were used to identify gene ontology (GO) terms (biological processes and cellular compartments) associated with the significant genes, and to classify the DEGs into biological pathways using KEGG pathways (Kanehisa and Goto 2000). MetaCore (Thomson Reuters, <http://www.genego.com/metacore.php>) pathway analysis and network process analysis were used for functional classification. Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) Canonical Pathway analysis, Biological Function analysis, and Network analysis were used to identify biological pathways and functions associated with the DEGs. Based on the tool used, different criteria were

used to determine the significance of pathways, functions or biological processes perturbed. Any pathway or process that was associated with more than 3 DEGs was included in the interpretation of the results. In addition, an EASE score cut-off of $P \leq 0.05$ was applied to DAVID ontologies and KEGG pathways, and $P \leq 0.05$ to IPA canonical pathways and MetaCore process networks. Redundancy in pathways and processes were reduced by collapsing multiple pathways or processes implying perturbation of the same biological function (Supplementary File 2).

IPA's upstream regulator analysis was performed to determine the common regulatory mechanisms (transcription factors and receptors) operating in response to individual PAHs compared to BaP. This analysis constructs a network of all possible relationships between upstream regulators and the genes in the data. The activation Z-score makes predictions about the activation state of the regulators (activated or inhibited) by using information about the direction of gene regulation. All upstream regulators with ≥ 3 genes, $P \leq 0.05$, and activation Z-score $\geq |2.0|$ were included in the interpretation. Upstream regulators categorized as chemical drugs, chemical toxicants, biologic drugs, chemical kinase inhibitor, chemical protease inhibitor, chemical endogenous (non-mammalian), chemical reagent, and chemical toxicant were not included in the analysis.

The NextBio meta-analysis function was used to compare the transcriptomic responses following exposure to individual PAHs and following exposure to BaP. A less stringent non-FDR p-value cut-off of less than 0.05 and fold change of ± 1.2 was applied. A pairwise comparison was made for each gene between the two datasets (for example, BaP vs BbF). A rank-based enrichment statistic was applied to determine the final correlation score. If the direction of change in the expression of a gene following a PAH is the same as the direction of change in expression of that gene following exposure to BaP, then the correlation is positive.

Since the focus of the study was to compare the carcinogenic potential of PAHs, significantly perturbed pathways and processes were reorganized according to their association with the 6 hallmarks of cancer (activating invasion and metastasis, enabling replicative immortality, evading growth suppressors, inducing angiogenesis, resisting cell death, sustained proliferative signaling), 2 emerging hallmarks (deregulating cellular energetics, avoiding immune destruction), and 2 enabling characteristics (genome instability and mutation, tumour promoting

inflammation), as described in Hanahan and Weinberg (2011). The pathways or processes that could not be classified as cancer related were not used in the analysis and interpretation of the results.

E - DNA extraction from lung tissue

The frozen lung tissues were sliced randomly for isolation of genomic DNA as described in Labib et al. (2012). In brief, lung tissue was minced and degassed to remove all traces of air in the alveoli and washed in ice cold Phosphate Buffered Saline (PBS) twice. The minced tissue was lysed in 10 mM Tris, pH 7.6, 10 μ M EDTA, 100 μ M NaCl, and 1% SDS and digested with proteinase K (1 mg/ml) overnight on a rotating platform at 37°C. Genomic DNA was isolated on the following day using a phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) extraction procedure (Renault et al. 1997). The DNA was precipitated in ethanol and dissolved in TE buffer and stored at 4°C until used.

F - LacZ mutant frequency in lung tissue

The lacZ mutant frequency analysis in lung tissues using the phenyl- β -D-galactopyranoside (P-Gal) positive selection assay was conducted as described previously (Labib et al. 2012; Lemieux et al. 2011). Briefly, the λ gt10lacZ DNA was removed from the mouse genomic DNA and packaged using the Transpack lambda packaging system (Stratagene, La Jolla, CA, USA). The packaged DNA was mixed with bacteria (*Escherichia coli* lacZ-, galE-, recA-, pAA19 with galT and galK), plated on minimal medium (0.3% (w/v) P-Gal), and incubated overnight at 37°C. At the same time, total plaque forming units were measured on titers that did not contain P-Gal. Mutant frequency is expressed as a ratio of the number of mutant plaque forming units to total plaque forming units.

G - DNA adduct analysis in lung tissue

DNA adduct formation in each sample was determined using the nuclease P1 digestion enrichment version of ³²P-post-labeling assay as described previously (Phillips and Arlt 2014; Phillips and Arlt 2007) with minor modifications as described in Labib et al. (2012). Briefly, 4 μ g of total DNA was digested with micrococcal nuclease (288 mUnits, Sigma) and calf spleen phosphodiesterase (1.2 mUnits, MP Biomedicals), enriched, and was radiolabelled (Phillips and

Arlt 2014; Phillips and Arlt 2007). The labeled adducted nucleotide biphosphates were separated by thin-layer chromatography (TLC) on polyethyleneimine-cellulose plates (Macherey-Nagel, Düren, Germany) with chromatographic conditions described in (Arlt et al. 2008).

Chromatographs were scanned using a Packard Instant Imager (Canberra Packard, Downers Grove, USA) and DNA adduct levels (relative adduct labeling) were calculated from the adduct counts per minute (cpm), the specific activity of [γ - 32]PATP, and the amount of DNA (pmol of DNA-P) used. Results are expressed as DNA adducts per 10⁸ nucleotides.

H - Enzymatic analysis of lung Cyp1a activity

For the enzymatic analysis, since the available amount of tissue from each treatment group was small, samples from the same treatment group were pooled (Supplementary File 1).

Approximately 30 mg of lung tissue from each individual sample from specific treatment group were pooled and homogenized using a serrated Teflon pestle and glass grinding vessel in 2.5 volumes of ice cold 0.05M Tris-1.15% KCl buffer pH 7.4. The homogenate was centrifuged at 10,200 g for 20 minutes at 4°C (Sorval Legend Micro 21R) and the supernatant (S9 fraction) was separated for protein and EROD analyses. EROD activity, a measure of CYP1A1 and CYP1A2 enzyme activity, was determined according to the method of Burke et al. (1985), with modifications. Reactions were carried out at 37°C with triplicate technical replicates in 96-well plates. The final incubation consisted of Tris buffer (0.05 M, pH 7.4), 0.5 μ M 7-ethoxyresorufin substrate, 15 μ M dicumerol, 11 mM MgCl₂, 20 μ l of lung S9 supernatant, and 280 μ M NADPH (Sigma Aldrich Canada Ltd, Oakville, ON, Canada), with a final well volume of 220 μ l. The fluorescence of resorufin was measured every minute for 8 minutes with a SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA), with excitation and emission wavelengths of 530 nm and 585 nm. Fluorescence values were converted to nanomoles using standard resorufin fluorescence. Total protein content of the S9 fraction was measured using the Quick StartTM Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), using a standard curve of bovine serum albumin.

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