

Weight of Evidence Analysis in Framework Approaches to Cancer Hazard Identification: A Case Study with Phenobarbital

Thesis Submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Ehi Idahosa

2011

**Imperial College London
Department of Medicine
Division of Experimental Medicine
Centre for Pharmacology and Therapeutics
Hammersmith Hospital Campus
Du Cane Road
London, W12 0NN
United Kingdom**

DECLARATION OF ORIGINALITY

I declare that this thesis:

**Weight of Evidence Analysis in Framework Approaches to Cancer Hazard
Identification: A Case Study with Phenobarbital**

Is entirely my own work and that where any material could be construed as the work of others, it is fully cited and referenced, and/or with appropriate acknowledgement given.

ACKNOWLEDGMENTS

I would like to thank the Department of Health for providing me with the funding to complete this PhD.

I would like to express my sincere gratitude to my supervisor, Professor Alan Boobis for providing me with the opportunity to do this PhD. I am grateful for the opportunities that I have had to attend conferences, courses and seminars within my subject area. I am thankful for his help in providing me with the opportunity to meet with additional experts that proved to be essential for my project. His depth of knowledge has been invaluable to for my work and I am extremely grateful for his help in not only the interpretation of my results but also for his help in getting me to view problems in a much more global context.

I am deeply grateful to my second supervisor, Dr Lesley Rushton for her encouragement and personal guidance throughout my PhD. Her expertise in specific aspects of my project has also been invaluable. I am also grateful for her support in helping me view my project within broader contexts and her constant appraisal of my work. I would like to thank both my supervisors for their detailed comments and suggestions in the write up of this thesis and for their continued support in my professional development. I am particularly grateful for the support that I received from both my supervisors during the period of my interrupted studies.

I am extremely grateful to Dr Susan Hester from the United States Environmental Protection Agency, for her advice and guidance on the development of an analytical strategy for the toxicogenomics aspects of my PhD. I thank her for making the trip to the UK to provide me with training and for taking me under her wing as one of her students. I am grateful to her for being so patient and approachable and for all the time she took discuss my work over the phone or via email.

While it is not possible to mention everyone in my department, I would like to thank all my colleagues here at the Centre for Pharmacology and Therapeutics for all their support during my PhD. I am especially grateful to Mr Ken Okona-Mensah for taking the time to proofread my thesis and for his unwavering support throughout the period of my PhD. I would like to thank Dr Ranti Fayokun for all her words of encouragement and for her incredible kindness. Finally I would also like to thank Drs Halina Garavini, Nabil Hajji, Karen O'Leary, Karin Burnett, Vahitha Abdul Salam, Odu Okoturo, Sven van Eijl, and Ms Bernadette Edinborough for all their support during my PhD.

I would not be where I am today without the love and support of my family. I wish to thank my parents who have taught me the importance of education and who encourage and support me in everything that I have embarked on. I am especially grateful to my Mum who has sacrificed so much to get me to where I am today and who encourages me in all that I do and never ceases to tell me how proud she is of me, she is truly my inspiration. I would like to thank my sister Adesuwa, my Aunts Jain and Ebuwa, my cousins Eghe, Osahon and Iyayi, and my sister-in-law Joanna for all their love and support. I wish to thank my parents-in-law, Sheri and John for all their love and encouragement and also for their help in looking after Kai. Finally I wish to say a huge thank you to all my wonderful friends for their words of encouragement and for helping me keep my sanity over these past four and a half years.

Last but not least I would like to thank my husband Anthony and my son Kai for bringing so much joy and happiness into my life and for giving me the motivation to keep going even during the hardest periods. I so grateful to Anthony for the love, support and encouragement he has shown me during this very challenging part of my life. I owe the achievement of completing this thesis to these two wonderful men in my life!

Abstract

This thesis addresses the transparency of weight of evidence-based analysis in the application of the IPCS Framework for Assessing the Human Relevance of a carcinogenic response to a chemical. This thesis also explores how, and to what extent, available toxicogenomics data can be incorporated into a cancer mode of action analysis.

A systematic review was carried out on epidemiological data available for phenobarbital (PB). A positive association between liver cancer and PB was reported among a large Finnish cohort however the study was assessed to be unreliable on the basis of key limitations. However owing to the size of cohort investigated, the study could not be disregarded and the conclusions reached by the authors were considered to be associated with a high level of uncertainty. Studies of two smaller cohorts of patients, assessed to be of a high quality, reported no findings of such an association. The methods applied in this thesis illustrated the value of systematic review techniques in increasing the transparency of decisions made when applying a WOE based approach to assessing the human relevance of cancer mode of action and in helping to identify important data gaps.

Comparative analysis of publicly available toxicogenomics data for PB using Pathway Analysis software led to the identification of perturbed pathways that were interpreted within the context of the key events for PB MOA for hepatocarcinogenesis. The analysis demonstrated that prior mechanistic knowledge about a compound under investigation is currently necessary before efficient interpretation of the biological relevance of perturbed pathways is possible. To realise the true potential for the application of toxicogenomics data in risk assessment, future toxicogenomics study designs will need to be more targeted (e.g. in their considerations of dose-response, time-response and species differences) before they can be applied to help fill the current gaps in mechanistic knowledge. The experience from the comparative analysis reported here suggests that at the present time, information on perturbed pathways obtained in this way is not sufficiently robust to be used as stand alone evidence in MOA analysis. The potential to read across toxicogenomics evidence for PB MOA to other compounds, which may act via the same MOA however deserves further exploration.

TABLE OF CONTENTS

TABLE OF CONTENTS	Page
DECLARATION OF ORIGINALITY	2
ACKNOWLEDGEMENTS	3
ABSTRACT	4
LIST OF FIGURES	15-16
LIST OF TABLES	17
LIST OF ABBREVIATIONS	18-19
CHAPTERS	
Chapter 1	Introduction
	20
1.1	Background to Chemical Risk Assessment
	20-22
1.2	Historical Approach to Risk Assessment
	22-25
1.3	Harmonisation of Approaches to Risk Assessment
	25-26
1.4	IPCS Mode of Action Framework
	27-28
1.4.1	Mode of Action Approach to Cancer Risk Assessment
	28-29
1.4.2	Weight of Evidence Evaluation Based on the Bradford Hill Criteria
	29-30
1.5	The IPCS Human Relevance Framework
	30-32
1.5.1	Application of the Mode of Action Approach by Different Organisations: United States Environmental Protection Agency
	32-34
1.5.2	The European Union: European Food Standards Agency
	34-35
1.5.3	International Agency for Research on Cancer (IARC)
	35-37
1.5.4	United Kingdom
	37-38
1.5.5	Joint FAO/WHO Meeting on Pesticide Residues
	38-39

1.6	Weight of Evidence considerations for the application of the IPCS MOA/HRF: transparency and data incorporation.	39-43
1.7	Aims and Objectives	43-45
Chapter 2	Phenobarbital	46
2.1	Key Events for PB Mode of Action For Liver Tumour Formation in Rodents	46-53
2.2	Activation of the Nuclear Receptor CAR	47-49
2.2.1	Increased Liver Weight Characterised by Hypertrophy and Hyperplasia	49-50
2.3	Cell Proliferation	50
2.4	Inhibition of Apoptosis	51
2.5	Development of Altered Hepatic Foci	51-52
2.6	Toxicological Events Associated with the Observed Key Events	52
2.6.1	Oxidative Stress	52-53
2.6.2	Inhibition of Gap junction Intercellular Communication	53
2.7	Concordance of Dose Response	54
2.7.1	Temporal Association	54
2.7.2	Species Differences	54-55
2.8	Summary	55
Chapter 3	Systematic Review of Epidemiological Data for Phenobarbital	56
3.1	Methodology for Epidemiology Review	57
3.1.1	Development of Search Strategy for the Identification of Epidemiological Studies	57-58
3.1.2	Study Quality Assessment Scoring	58-59
3.2	Results	60-80

3.2.1	Study Quality Assessment	65
3.2.2	The Danish Cohort of epileptic patients	72-76
3.2.3	The English cohort of epileptic patients	77-78
3.2.4	The American Cohort of Epileptic Patients	78
3.2.5	The Finnish Cohort of Epileptic Patients	78-80
3.3	Discussion	81
3.3.1	Methodological Considerations: Methods of Exposure Assessment	82
3.3.2	Measures of Effects	83
3.3.3	Identification of Cohort of Patients Treated with PB	83-84
3.3.4	Methods of Dealing with Confounding	84-85
3.3.5	Findings for the Association Between PB and Liver Cancer	85-86
3.3.6	The Finnish cohort: Lamminpaa et al, 2002	86-87
3.3.7	The Danish Cohort: Olsen et al, 1995	88
3.4	Conclusions	89-91
Chapter 4	Toxicogenomics: Methodology for the Transcriptional Analysis of the Hepatocarcinogenic effects of PB	92
4.1	Brief Background to Toxicogenomics	92-94
4.1.1	Microarray Study Design	95
4.1.2	Identification of Differentially Expressed Genes	95-97
4.1.3	Biological Relevance of Differentially Expressed Genes	97-99
4.2	Development of Methodology for Transcriptomics Analysis	99-101
4.2.1	Search Strategy to Identify Relevant Microarray Studies for PB	99
4.2.2	Pre-processing of Microarray data (Statistical Analysis of Microarray Data)	100-103

4.2.3	Selection of Pathway Analysis Tools	103-105
4.3	Pathway and Network Analysis of DEGs Using Ingenuity Pathway Analysis (IPA™)	105
4.3.1	Core Analysis in IPA	105-107
4.3.2	Levels of Significance/ P-value Calculated for Functions and Pathways in IPA	108
4.3.3	IPA Result Output: Functional Analysis of dataset	108-109
4.3.4	IPA Result Output: Toxicology Lists (Tox List)	109-110
4.3.5	IPA Result Output: Canonical Pathways	110-111
4.3.6	Graphical representation of Networks	111-112
4.3.7	Biomarkers for Non-genotoxic Carcinogenicity and Reference Genes for Hepatocellular Carcinoma	112
Chapter 5	Results of Transcriptional Analysis to Investigate the Hepatocarcinogenic Effects of Phenobarbital	113
5.1	Analysis of Differentially Expressed Genes (DEGs) using Ingenuity Pathway Analysis (IPA™)	113
5.2	Toxicological lists (Tox Lists)	114-116
5.2.1.1	CAR/ RXR Activation	117
5.2.1.2	PXR/ RXR Activation	117-118
5.2.1.3	PPAR α / RXR Activation	118
5.2.1.4	Mechanism of gene regulation by Peroxisome Proliferators via PPAR α	119
5.2.1.5	Oxidative Stress & Oxidative Stress Response Mediated by Nrf2	120-121
5.2.1.6	Aryl Hydrocarbon Receptor Signalling	121
5.2.1.7	P53 signalling	121-122
5.2.1.8	G2/M Transition of Cell Cycle	122
5.2.1.9	G1/S Transition of the Cell Cycle	123
5.2.2	Mitochondrial Dysfunction	123
5.2.2.1	Pro-Apoptosis	124

5.2.2.2	Anti-Apoptosis	124
5.2.2.3	Hepatic Cholestasis	124-125
5.2.2.4	LPS/IL-1 Mediated Inhibition of RXR Function	125-126
5.2.2.5	Negative Acute Phase Response	126-127
5.2.2.6	Positive Acute Phase Response Proteins	127
5.2.2.7	TGF- β -Signalling	128
5.2.2.8	Hepatic Fibrosis	128-129
5.2.2.9	<i>Hepatic Stellate Cells Activation</i>	129
5.3	Toxicological Function (Tox Function)	130
5.3.1	Liver Proliferation	133
5.3.2	Liver Hyperplasia/ Hyperproliferation and Liver Hypertrophy	134
5.3.3	Liver Necrosis/ Cell Death	135
5.3.4	Liver Cholestasis	135
5.3.5	Liver Steatosis	136
5.3.6	Hepatocellular Carcinoma	136
5.4	Comparison of all Datasets with Published Multi-gene Signatures for the Identification of Non-genotoxic Hepatocarcinogens and Reference Genes for Hepatocellular Carcinoma as Identified by the Literature	137
5.5	Canonical Pathways	138
5.5.1	Cell Growth, Proliferation and Development	138
5.5.2	Apoptosis	138
5.5.3	Cellular Stress and Injury	139
5.5.4	Tox Function: Rat	140
5.5.5	Tox Function: Mouse	140
5.5.7	Tox Function: Human	141
5.6	Discussion	146-147
5.6.1.1	Evidence for CAR activation and CAR Related Effects on Liver Function	147-149

5.6.1.2	Evidence for Cross Talk between CAR and PXR: Glucose and Lipid Metabolism	149-153
5.6.1.3	Inhibition of RXR	153-155
5.6.1.4	Evidence for Cell Proliferation and Growth	155-156
5.6.1.5	Evidence for the Inhibition of Apoptosis	156-159
5.6.1.6	Evidence for Cellular Stress (Oxidative Stress)	159-161
5.6.2	Evidence for Hepatocellular carcinoma – Comparison with Reference Gene Lists	162-166
5.6.3	Discussion of Applied Methodology: Application of Evidence from Transcriptomics Analysis to MOA Analysis – Learning Points and Recommendations	167
5.6.4	Identification of Toxicologically Informative Pathways	168-169
5.6.5	Identification of Key Genes within a Pathway that are Indicative of Key Biological Processes for a MOA	169-170
5.6.6	Biological relevance of Transcriptional Changes observed for Key Genes; Consideration of Experimental Evidence	170-172
5.6.7	Study Limitations	172-173
5.6.8	Problems Encountered Using IPA	173-176
5.6.9	Summary of Toxicogenomics Evidence for PB Key Events	176-180
5.7	Conclusions	181-183
Chapter 6	General Discussion and Conclusions	184-185
6.1	Techniques to Improve the Transparency of the use of a WOE-Based Approach in MOA and Human Relevance Analysis: Systematic Review	186-189
6.2	Consideration of Toxicogenomics Data as Part of the WOE for MOA analysis	189-191
6.2.1	Generalisation from the Case-study	192-197
6.2.2	Challenges for the Application of Toxicogenomics Data in Regulatory Risk Assessment	197-199

6.2.3	Practical considerations for use of Toxicogenomics Data in Risk Assessment	200-202
6.2.4	General Considerations and Identified Areas for Future Work	202-203
6.2.4.1	Future Work	203
6.3	Final Conclusions	204-206
	REFERENCES	207-240
	APPENDICES	
	The Appendices detailed have been submitted as supplementary data. The data is provided on a CD attached to the back of this thesis.	
APPENDIX III	Data Associated With Information Detailed In Chapter 3	II
A3.1	Search strategy for the identification of epidemiological studies for PB	III-VI
APPENDIX IV	Data Associated With Information Detailed In Chapter 4	VII
A4.1	Summary of experiments from which the microarray data were obtained. The microarray data generated from the studies were exported from the public microarray repository, Gene Expression Omnibus.	VIII-XII
A4.2	Gene Biomarkers Identified By Fielden et al. (2007) For Non-Genotoxic Carcinogenicity	XIII-XV
A4.3	Signature Genes Identified By Nie et al (2006) To Predict Non-genotoxic Carcinogenicity	XVI
A4.4	Reference Genes: Genes Identified By Pei et al. (2009) To Have Been Highlighted In Hepatocellular Carcinoma Microarray Studies	XVII-XVIII
APPENDIX V	Data Associated With Information Detailed In Chapter 5	XIX
A5.1	Toxicity Lists	XX
A5.1.1	CAR/RXR Activation	XX-XXI
A5.1.2	PXR/RXR Activation	XXII-XXIII

A5.1.3	PPAR α /RXR Activation	XXIV- XXVI
A5.1.4	Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR α	XXVI- XXVII
A5.1.5	Oxidative Stress Mediated by NrF2	XXVIII- XXXI
A5.1.6	Oxidative Stress	XXXII- XXXIII
A5.1.7	Aryl Hydrocarbon Receptor Signalling	XXXIII- XXXV
A5.1.8	P53 Signalling	XXXVII
A5.1.9	G1/S Transition of the Cell Cycle	XXXVIII
A5.1.10	G2/M Transition of the Cell Cycle	XXXIX
A5.1.11	Mitochondrial Dysfunction	XL-XLI
A5.1.12	Pro-Apoptosis	XLII
A5.1.13	Anti-Apoptosis	XLIII
A5.1.14	Hepatic Cholestasis	XLIV- XLV
A5.1.15	LPS/IL-1 Mediated Inhibition of RXR Function	XLVI- XLIX
A5.1.16	Negative Acute Phase Response Proteins	XLIX
A5.1.17	Positive Acute Phase Response Proteins	L
A5.1.18	TGF- β Signalling	LI
A5.1.19	Hepatic Fibrosis	LII
A5.1.20	Hepatic Stellate Cell Activation	LIII
A5.2	Toxicity Functions (Networks Generated using Data Obtained from the Tox Function Analysis.	LIV
A5.2.1	Toxicity Function (Mouse Data): Liver Proliferation	LV
A5.2.2	Toxicity Function (Human Data): Liver Proliferation	LVI
A5.2.3	Toxicity Function (Data): Liver Hyperplasia/ Hyperproliferation	LVII
A5.2.4	Toxicity Function (Mouse Data): Liver Hypertrophy	LVIII
A5.2.5	Toxicity Function (Mouse Data): Liver Necrosis/ Cell Death	LIX

A5.2.6	Toxicity Function (Mouse Data): Liver Cholestasis	LX
A5.2.7	Toxicity Function (Rat Data): Liver Cholestasis	LXI
A5.2.8	Toxicity Function (Human Data): Liver Cholestasis	LXII
A5.2.9	Toxicity Function (Mouse Data): Liver Steatosis	LXIII
A5.2.10	Toxicity Function (Mouse Data): Hepatocellular Carcinoma	LXIV
A5.2.11	Toxicity Function (Rat Data): Hepatocellular Carcinoma	LXV
A5.3	Comparison of selected Tox Function genes with genes from IPA Knowledgebase Known to be associated with Key Events for Hepatocarcinogenesis.	LXVI
A5.3.1	Genes From IPA Knowledgebase Associated with Liver Proliferation	LXVI
A5.3.2	Genes From IPA Knowledgebase Associated with Hyperplasia/ Hyperproliferation	LXVII
A5.3.3	Genes From IPA Knowledgebase Associated with Liver Hypertrophy	LXVIII
A5.3.4	Genes From IPA Knowledgebase Associated with Cell Death	LXIX
A5.3.5	Genes From IPA Knowledgebase Associated with Liver Cholestasis	LXX
A5.3.6	Genes From IPA Knowledgebase Associated with Hepatocellular Carcinoma	LXXI
A5.4	MOA Related Signalling Pathways for Cellular Growth, Proliferation and Development, Apoptosis, Cellular Stress and Injury.	LXXII
A5.4.1	Signalling pathway: Cellular growth, proliferation and development (represented as a bar chart)-Rat Day 1	LXXII
A5.4.2	Signalling pathway: Cellular growth, proliferation and development (represented as a stacked bar chart)- Rat Day 1	LXXIII
A5.4.3	Signalling pathway: Cellular growth, proliferation and development (represented as a bar chart)- Mouse Day 2	LXXIX

A5.4.4	Signalling pathway: Cellular growth, proliferation and development (represented as a stacked bar chart)- Mouse Day 2	LXXV
A5.4.5	Signalling pathway: Cellular growth, proliferation and development (represented as a bar chart)- Human Day 3	LXXVI
A5.4.6	Signalling pathway: Cellular growth, proliferation and development (represented as a stacked bar chart)- Human Day 3	LXXVII
A5.4.7	Signalling pathway: Apoptosis (represented as a bar chart) - Rat Day 1	LXXVIII
A5.4.8	Signalling pathway: Apoptosis (represented as a stacked bar chart) - Rat Day 1	LXXIX
A5.4.9	Signalling pathway: Apoptosis (represented as a bar chart) - Mouse Day 2	LXXX
A5.4.10	Signalling pathway: Apoptosis (represented as a stacked bar chart) - Mouse Day 2	LXXXI
A5.4.11	Signalling pathway: Apoptosis (represented as a bar chart) - Human Day 3	LXXXII
A5.4.12	Signalling pathway: Apoptosis (represented as a stacked bar chart)- Human Day 3	LXXXIII
A5.4.13	Signalling pathway: Cellular Stress and Injury (represented as a bar chart)- Rat Day 1	LXXXIV
A5.4.14	Signalling pathway: Cellular Stress and Injury (represented as a stacked bar chart)- Rat Day 1	LXXXV
A5.4.15	Signalling pathway: Cellular Stress and Injury (represented as a bar chart)- Mouse Day 2	LXXXVI
A5.4.16	Signalling pathway: Cellular Stress and Injury (represented as a stacked bar chart)- Mouse Day 2	LXXXVII
A5.4.17	Signalling pathway: Cellular Stress and Injury (represented as a bar chart)- Human Day 3	LXXXVIII
A5.4.18	Signalling pathway: Cellular Stress and Injury (represented as a stacked bar chart)- Human Day 3	LXXXIX
A5.5	Genes Found to Be Common across the Different Mouse Datasets Investigated (Mouse datasets: 10hrs and Days 2, 4 and 30).	XC-XCI

LIST OF FIGURES

- Figure 3.1 Flowchart detailing the number of studies identified for the review
- Figure 3.2 The relationship between the different epidemiological studies carried out using data on patients admitted to the Filadelfia Centre for epilepsy treatment in Denmark between 1933 and 1962
- Figure 4.1 Steps of Microarray Analysis
- Figure 4.2 Screen shot showing the analysis page in IPA which allowed the different criteria for the analysis for each dataset
- Figure 4.3 An example of the results output for the functional analysis in IPA. The bar chart produced shows the different toxicological functions into which the datasets were grouped.
- Figure 4.4 An example of the results output for the Toxicity List in IPA. The bar chart produced shows the different types of toxicities into which the datasets were grouped.
- Figure 4.5 An example of the results output for the Canonical Pathways produced in IPA. The bar chart produced shows the different pathways into which the datasets were grouped.
- Figure 5.1 Selected signalling pathways for Rat 6 hours
- Figure 5.1.2 Selected signalling pathways for Rat 1 day
- Figure 5.1.3 Selected signalling pathways for Rat 3 days
- Figure 5.1.4 Selected signalling pathways for Rat 5 days
- Figure 5.1.5 Selected signalling pathways for mouse 10 hours
- Figure 5.1.6 Selected signalling pathways for mouse 2 days
- Figure 5.1.7 Selected signalling pathways for mouse 4 days
- Figure 5.1.8 Selected signalling pathways for mouse 30 days
- Figure 5.1.9 Selected signalling pathways for human 3 days
- Figure 5.2 Energy Metabolism in the Fasting Liver
- Figure 5.3 Tox List Analysis of genes common between all mouse datasets
- Figure 5.4 Canonical Pathway Analysis of genes common between all mouse datasets
- Figure 5.5 Tox Function Analysis of genes common between all mouse datasets

- Figure 5.6 Strategy for Pathway and Network Analysis of Transcriptomics Data for MOA Analysis.
- Figure 6.1 The vision for the future of toxicity testing put forward by the National Research Council

LIST OF TABLES

Table 3.1	Quality assessment criteria and scoring system applied to the individual studies
Table 3.2	Summary of included epidemiological studies
Table 3.3	Quality assessment based on the Quality Criteria
Table 3.4	Summary of the main findings from the individual studies
Table 3.5	Primary Liver cancers among the 6 Danish epileptic patients exposed to PB with no evidence of Thorotrast exposure by histopathological examination and X-ray analysis for thorium residuals (Source: Olsen et al., 1995)
Table 3.6	Summary of overall results reported in the studies evaluated
Table 4.1	Summary of experimental data exported from GEO
Table 5.1	Statistically significant DEGs obtained from Rosetta Resolver Analysis
Table 5.2	Table showing IPA toxicity lists produced from all datasets
Table 5.3	Toxicity Functions Related to Hepatotoxicity
Table 5.4	Summary of Tox Functions According to the Different Hepatotoxicity Categories
Table 5.5	Summary of results from the comparison of all datasets with genes identified in the literature to be associated with HCC
Table 5.6	Summary of results from the comparison of all datasets with literature derived Biomarkers for nongenotoxic chemicals

LIST OF ABBREVIATIONS

ALARA	As low as reasonably achievable
ADI	Acceptable daily intake
AHR	Altered hepatic foci
BMD	Benchmark dose
BMDL	95% lower confidence limit on the benchmark dose
CAR	Constitutive androstane receptor
COC	Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment
COM	Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CI	Confidence interval
DAVID	Database for Annotation, Visualization and Integrated
DEGs	Differentially Expressed Genes
DMSO	Dimethyl sulfoxide
EFSA	European Food Safety Authority
ERCC	External RNA Controls Consortium
FDR	False discovery rate
FAO	Food and Agriculture Organization
GEO	Gene Expression Omnibus
GO	Gene Ontology
HCC	Hepatocellular carcinoma
HESI	Health and Environmental Sciences Institute
HRF	Human Relevance Framework
IARC	International Agency for Research on Cancer
IFCS	Intergovernmental Forum on Chemical Safety
ILO	International Labour Organization
ILSI	International Life Sciences Institute
IPA	Ingenuity Pathway Analysis™
IPCS	International Programme on Chemical Safety
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KOBAS	KEGG Orthology-Based Annotation System
LOAEL	Lowest observed adverse effect level
MAQC	Microarray Quality Control Project
MIAME	Minimum Information About a Microarray Experiment
MOA	Mode of action
MOE	Margin of Exposure
NOAEL	No observed adverse effect level
NRC	National Research Council
OR	Odds ratio
PB	Phenobarbital
PXR	Pregane X receptor
POD	Point of departure
ROS	Reactive oxygen species
RXR	Retenoid X receptor

SIR	Standardised incidence ratio
SMR	Standardised mortality ratio
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
T25	Chronic dose rate in mg/kg bw/day which will give 25% of the animal's tumours at a specific tissue.
TD50	The dose rate in mg/kg bw/day which, if administered chronically for the life-span of the species, will half the probability of remaining tumourless throughout that period.
UNCED	United Nations Conference on Environment and Development
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
WHO	World Health Organization
WOE	Weight of Evidence

Chapter 1

Introduction

Humans are all exposed to chemicals on a daily basis from many different sources and at varying levels of exposure. The sources of these chemicals include, but are not limited to, drugs, agricultural chemicals such as pesticides, environmental pollutants, industrial chemicals and food additives. The regulation of these chemicals is often required to mitigate any potential risks to human health or the environment as a result of voluntary or involuntary exposure to these chemicals. The process of regulating these chemicals relies on the effective use of available but sometimes limited resources to characterise potential hazards as part of the chemical risk assessment process.

In risk assessment, the overall evidence evaluated by a risk assessor is commonly referred to as the “weight of evidence” (WOE) [1]. Linkov et al [2] define WOE as “*a framework for synthesizing individual lines of evidence, using methods that are either qualitative (examining distinguishing attributes) or quantitative (measuring aspects in terms of magnitude) to develop conclusions regarding questions concerned with the degree of impairment or risk*”.

1.1 Background to Chemical Risk Assessment

Risk assessment is part of a risk analysis paradigm developed by the Codex Alimentarius Commission (CAC). The CAC was formed in 1963 by the Food and Agriculture Organization (FAO) and World Health Organization (WHO) to develop food standards, guidelines and codes of practice under the programme of the Joint FAO/WHO Food Standards Programme [3]. Risk analysis comprises of risk assessment, risk management and risk communication. Although the paradigm is split into three separate components, the process requires the interaction and communication between those responsible for each of the separate component [4]. Risk assessment describes the scientific process that consists of hazard identification, hazard characterisation, exposure assessment and risk characterisation. Risk

management describes the process of considering various policy alternatives in consultation with relevant stakeholders. The process also involves the consideration of issues identified from the risk assessment that are relevant for human health protection and factors that affect the promotion of fair trade practices. Finally, risk communication, requires the exchange of information and opinions among relevant stakeholders (risk managers, risk assessors, consumers, industry, members of the academic community etc) on issues that relate to the different risk related factors and/or perceptions of risk. This process also requires the adequate communication of the findings from the risk assessment and justification for the basis for any risk management decisions made [4].

This thesis focuses on aspects that are considered as part of the risk assessment part of the risk analysis paradigm. The International Programme on Chemical Safety (IPCS) defines risk assessment as a *"conceptual framework that provides the mechanism for a structured review of information relevant to estimating health or environmental outcomes"* [5]. A major aspect of the risk assessment paradigm which consists of hazard identification, hazard characterisation, exposure assessment and risk characterisation, is the consideration of the hazard and the risk. The IPCS defines hazard as *"an inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub)population is exposed to that agent"*, while risk is defined as *"the probability of an adverse effect in an organism, system or (sub)population cause under specific circumstances by exposure to an agent"* [4].

Evaluation of the qualitative and quantitative evidence at the different stages of the risk assessment process allows for the consideration of all relevant information required to fully assess any potential risks associated with a chemical/substance, thereby facilitating the identification of any adverse health effects that may occur in humans [4]. The different aspects of the risk assessment paradigm are briefly discussed below.

Hazard identification: during this first stage of the risk assessment process, the WOE available for the substance under review is evaluated in order to determine whether the substance has the potential to produce adverse health effects, the nature of those

effects and also to determine the conditions under which the substance might exhibit any hazards [6].

Hazard characterisation: the second stage of the risk assessment process involves the qualitative and quantitative assessment of the varying properties of the compound under evaluation and considerations for the circumstances where the compound has the potential to cause adverse health effects. This stage normally includes a dose response assessment and consideration of any underlying uncertainties [6].

Exposure assessment: the third stage of the risk assessment process aims to define the nature of exposure and evaluate the different exposure levels for the compound (and its derivatives) to which humans are exposed or are likely to be exposed. The process also requires the consideration of the duration of exposure to the relevant chemical/substance [4].

Risk characterisation: in the final step of the risk assessment process, the WOE from the previous steps are summarised in a clear and transparent format that is aimed specifically at risk managers - to aid decision-making processes. The result of this process is the qualitative and quantitative determination of the risks associated with exposure to this chemical after full consideration of the different scenarios to which humans might be exposed. At all stages of the risk assessment, the underlying uncertainties are considered thoroughly. Subsequently, it is the role of the risk manager to weigh the output of the risk assessment, together with all other relevant considerations to determine whether and what action should be taken to mitigate the risk [4].

1.2 Historical Approach to Risk Assessment

An important consideration during the hazard characterisation stage of the risk assessment process is the dose response relationship. The conventional approach is to characterise the shape of the dose response curve to determine the location of a putative threshold for a toxicological effect. To establish whether a compound

exhibits a threshold effect requires consideration of the relevant underlying biological factors.

For compounds that exhibit a threshold mechanism of effect it is assumed that a level of exposure exists below which no adverse health effects are likely to occur. Often, the no observed adverse effect level (NOAEL) is used as basis for estimating such a level in humans. Toxicological effects thought to act in this way include most non-cancer, organ-specific effects, neurological/behavioural effects, immunological effects and reproductive or developmental effects. Non-DNA reactive carcinogenic compounds are also considered to have a threshold for toxicological effect [6-8].

Fundamentally, the differences relating to the evaluation of compounds that exhibit either a threshold or non-threshold toxicological effect, relate to the determination of the ability of a compound to interact with DNA. The risk characterisation stage of the risk assessment process requires the use of different methods to estimate the risks associated with either DNA reactive or non-DNA reactive compounds. The traditional approach to dealing with non-DNA reactive compounds has been to derive a health based guidance value such as an acceptable daily intake (ADI) or acute reference dose (ARfD), by using a reference point (or point of departure), such as the no observed adverse effect level (NOAEL), and an uncertainty factor [8].

DNA-reactive carcinogens have generally been considered to exhibit no threshold in their effects, and the assumption is made that any level of exposure to these chemicals might result in an adverse health effect [6, 9, 10]. However, recent studies suggest that some DNA-reactive compounds display apparent thresholds at some low doses investigated [11]. Doak et al [11] investigated the low-dose-response relationship of four different DNA alkylating agents (methylnitrosourea (MNU), ethylnitrosourea (ENU), methy methane sulfonate (MMS) and ethyl methane sulfonate (EMS)) and assessed the induction of chromosomal damage and gene mutation. The authors demonstrated that both MMS and EMS displayed nonlinear dose response curves and observed the lowest observed effect level for the induction of chromosomal aberration at 0.85µg/mL and 1.40µg/mL for MMS and EMS respectively. Similarly, the lowest observed effect level for point mutations required 1.25µg/mL and 1.40µg/mL for MMS and EMS respectively before a mutagenic effect was observed. Such data

provide evidence that contradict the hypothesis previously for all DNA reactive genotoxic carcinogens that any level of exposure could result in an adverse health effect [4].

Qualitative and quantitative methods have previously been used to characterise the risks associated with DNA reactive compounds (compounds that act via a non-threshold effect). For genotoxic carcinogens where the risk assessment can lead to the conclusion that there is no safe exposure level, risk management strategies have included the application of a qualitative approach such as the as low as reasonably achievable (ALARA) principle to minimise the risk levels by ensuring that exposure levels to these compounds are kept as low as reasonable/ achievable practicably. Quantitative methods of risk characterisation rely on the use of data obtained from the hazard characterisation stage of the risk assessment, particularly data from cancer bioassay, epidemiological data or estimates of exposure compiled to assess the dose response relationship. Three different types of quantitative/semi-quantitative methods applied to the risk characterisation of DNA reacting chemicals are: the application of the threshold of toxicological concern (TTC); low dose extrapolation of data from rodent carcinogenicity bioassay (also referred to as linear extrapolation); and calculation of the margin of exposure (MOE) [7, 8].

The TTC approach is applied for the risk characterisation of substances where biological data are lacking but the chemical structure of the substance being considered is known and there are also good exposure data for the substance [12]. If the substance of concern is known to be genotoxic or has a structural alert for genotoxicity (after exclusion of a small number of very potent structures), it is compared to a large database of carcinogens for which the distribution of doses estimated to be associated with a 1 in 10^{-6} risk of cancer has been determined. If exposure is below the 5% value for this distribution (0.15µg/person/day), it is concluded that there is a low probability of harm to humans.

The linear extrapolation approach involves the mathematical modelling of high dose carcinogenicity data obtained from animal experiments, to produce quantitative estimates of the risks of the cancer effects for humans who are normally exposed at doses several orders of magnitude lower than the lowest experimental dose

administered in an animal study [13, 14]. Extrapolation from the high doses observed in animals to the low doses to which humans are exposed can be carried out through the selection of reference points (RP) or so called “points of departure” (POD) on the modelled dose-response curve. The RPs/PoD normally selected for this approach include the benchmark dose (BMD10) or the statistical lower bound estimate of the 95% confidence interval (BMDL10) for a 10% response, the lowest observed adverse effect level (LOAEL) or carcinogenic potency estimates such as TD50 or T25 (simplistically, the effect dose for a 50% and 25% response, respectively, of the exposed population) [14-17]. This method of risk characterisation which can be applied to the evaluation of both threshold and non threshold substances provides an estimation of the level of exposure that is associated with an upper bound, lifetime risk of cancer of 1 in a million (1×10^{-6}) or 1 in a hundred thousand (1×10^{-5}). However one of the major limitations of this approach is that the models are highly conservative and there is sometimes the wrong assumption that the response observed in animals will also be seen in humans.

The MOE approach for characterising the risks associated with genotoxic carcinogens is defined as the ratio between the dose leading to a specified tumour response in experimental animals and the human intake [18]. The MOE approach aims to inform risk managers on the relative risks associated with exposure from genotoxic carcinogens and also helps to prioritise risk management action for dealing with such compounds. The European Food Safety Authority (EFSA) and the World Health Organization (WHO) recommend the use of the BMDL10 as the POD when calculating a MOE from animal bioassay data [13, 14, 18].

1.3 *Harmonisation of Approaches to Risk Assessment*

Different approaches to chemical risk assessment can lead to different conclusions using the same data and the same underlying assumptions. In addition, assumptions have varied among risk assessment groups and there has been a lack of transparency such that the basis of a risk assessment sometimes cannot be determined or repeated. This highlighted the need for global harmonisation of basic scientific principles employed in risk assessment. In 1992, this issue was raised at the United Nations

Conference on Environment and Development (UNCED) in which the eco-friendly management of chemicals was addressed specifically in Chapter 19 of Agenda 21. The chapter highlighted the role of governments for developing and implementing national programmes for chemical management within the principles of sustainable development. In highlighting the need for collaboration between international organisations such as the United Nations Environment Programme (UNEP), the International Labour Organisation (ILO), and the World Health Organisation (WHO) (represented by the International Programme on Chemical Safety, IPCS), IPCS was identified as the lead organisation to take forward the action plan to address issues relating to co-operation on the environmentally sound management of toxic chemicals. The role of IPCS was identified to be that of increasing co-operation between member states in an attempt to improve scientific knowledge in the area of chemical management [19, 20].

In 1993, IPCS initiated a project entitled *Harmonisation of Approaches to the Assessment of Risk from Exposure to Chemicals* ("Harmonization Project"), following the recommendations of UNCED [20]. In 1994, the intergovernmental forum on chemical safety (IFCS) provided further support to IPCS in their lead role of promoting increased global harmonisation of scientific principles in chemical risk assessment. The Harmonisation Project sought to "improve chemical risk assessment globally, through the pursuit of common principles and approaches and hence, strengthen national and international management practices which deliver better protection of human health and the environment within the framework of sustainability". It is the ongoing aim of the Harmonisation Project to consider all issues related to the qualitative and quantitative stages of the risk assessment paradigms [21]. The Harmonisation Project aims to harmonize global approaches to risk assessment by:

- Increasing understanding and agreement on basic risk assessment principles
- Developing international guidance documents on specific issues [22].

1.4 *IPCS Mode of Action Framework*

Workshops organised by IPCS led to the identification of key areas of risk assessment that were in need of harmonization. These included: transparency, terminology, weight of evidence, flexibility and accessibility/ communication. The consideration of the WOE for mode of action (MOA) was identified as a key area for harmonization. Increased understanding of the carcinogenic process in both laboratory animals and humans has prompted a more evidence-based approach to the chemical risk assessment process in contrast to the previously simplistic and somewhat conservative techniques applied by different organisations, particularly in the field of cancer risk assessment. The increased availability of data relating to the pharmacokinetics and pharmacodynamics for specific agents has promoted the case for a chemical-specific approach [23, 24]. Efforts to address the types of criteria that should be considered when evaluating a MOA led to the development of a conceptual framework for the evaluation of an animal MOA. One of the focus areas of the *Harmonisation Project*, where significant progress has been made and work is ongoing, relates to cancer risk assessment.

In collaboration with the United States Environment Protection Agency (US EPA), Health Canada, and the International Life Sciences Institute Risk Science Institute (ILSI/RSI), IPCS published a conceptual framework for evaluating a mode of action for chemical carcinogenesis [24]. The term "Mode of Action" was introduced by the US EPA in acknowledgement that often only the key events in the carcinogenic process were known as opposed to the exact biochemical/molecular processes involved in the induction of cancer, as implied by the term "Mechanism of Action". The term MOA was, therefore, adopted for the IPCS framework in recognition of the fact that it was possible to reach conclusions about the key events that lead to specific carcinogenic endpoints without detailed mechanistic information [25]. A MOA is therefore defined as "a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data". It describes in a logical manner, the key cytological and biochemical events i.e. those that are both measurable and necessary to the observed carcinogenicity" [9]. The *IPCS Conceptual Framework for Evaluating a Mode of Action for Chemical Carcinogenesis* was published in 2001. The MOA framework is defined as "a tool

which provides a structured approach to the assessment of the overall weight of evidence for a postulated mode of action" [9, 24].

1.4.1 Mode of Action Approach to Cancer Risk Assessment

The previously held default assumption for chemical risk assessment was that animal cancer data were always relevant to human risk. A move towards the use of the MOA approach for the evaluation of chemicals as illustrated through the evaluation of case study compounds, led to the acceptance that certain cancer MOAs are not relevant in humans. A noteworthy example of this is the MOA by which the chemical d-limonene causes renal tubular tumours in male rats. Evaluation of the evidence for d-limonene has shown that the accumulation of the protein α_2 u-globulin leading to induced toxicity and neoplasia is species- and sex-specific, i.e. to male rats and therefore not relevant to humans. As part of the effort to move away from applying default assumptions when using animal data in risk assessment, the MOA approach requires a thorough evaluation of the weight of evidence allowing for the provision of a biologically plausible explanation for the occurrence of a cancer [25, 26].

The concept of using a MOA approach is not new to the field of chemical risk assessment. In the past, although not formally incorporated into risk assessment guidelines, the MOA approach consisted of evaluating available data relating to the biological events of observed toxicological endpoints from animal data to determine the relevance to human health. In attempting to harmonise scientific principles in the field of risk assessment, particularly in carcinogenic risk assessment, it was therefore essential to promote the incorporation of formal and systematic applications of the mode of action concept into risk assessment procedures.

A significant development in the past few decades has been a recognition that carcinogens can act via a genotoxic or non-genotoxic (also referred to as epigenetic) mechanism. The process of mutagenesis as part of the carcinogenic process observed in experimental animals has also been useful in gaining wide acceptance by the scientific community that some chemicals can exhibit carcinogenic properties without direct interaction with DNA i.e. via a non-genotoxic mechanism. It is now accepted

that for most compounds acting via a non-genotoxic a threshold dose exists below which no adverse effects are observed [7]. The induction of tumours by non-genotoxic carcinogens are considered to be a secondary adverse effect caused by an initial toxicological effect (which would exhibit a threshold) [7, 27]. Contrary to previously held default assumptions reserved for all carcinogenic chemicals, it is now clear that thresholds do exist for non-genotoxic carcinogens as tumours are not induced at doses below the levels that do not cause the initial toxicological effect [28]. More importantly, it has been realised that cancer and other toxicological effects result from a series of key events that reflect the structural changes occurring in the process of the final observed toxicological outcome. The need to thoroughly consider the effect that a foreign compound can have on disturbing the biological balance of key processes has become more pertinent. In the case of cancer, genotoxicity may be another key event that drives the mode of action and dictates the nature of the dose response curve [9].

1.4.2 *Weight of Evidence Evaluation Based on the Bradford Hill Criteria*

The mode of action conceptual framework allows for the thorough assessment of the weight of evidence (WOE) in a systematic format. The MOA framework provides, in the form of a stepwise approach, guidelines detailing the manner in which all WOE should be compiled and evaluated. This stepwise approach is intended to facilitate transparent decision making in the process of postulating the MOA in animals. The various points to be addressed in this format have been based on the criteria proposed by Bradford Hill [29] now commonly referred to as the "Bradford Hill criteria for causality". Hill identified nine different points to address when trying to determine whether a disease outcome was the result of an incidental association or, was in fact, causally related to a certain environmental exposure. While Hill was cautious to not refer to these points as criteria, the different points identified were intended to explore the idea of association before automatically assuming causality. The criteria for the evaluation of causality therefore included: strength of association, consistency of association, specificity of association, temporal relationship of the association, evaluation of the biological gradient or dose response curve, evaluation of the biological plausibility, the coherence of the observed effect, evaluation of

experimental evidence (e.g. the effectiveness of preventive steps towards observed associations) and the use of analogies in circumstances where evidence may be insufficient.

In keeping with the Bradford Hill criteria for causality, the MOA framework suggests consideration of the weight of evidence in the following stepwise format:

- A. Postulated MOA.
- B. Key events; associated critical parameters.
- C. Dose Response Relationships.
- D. Temporal Association.
- E. Strength, Consistency and Specificity of Association of Key Events and Tumour Response.
- F. Biological Plausibility and Coherence.
- G. Possible Alternative MOAs.
- H. Uncertainties, Inconsistencies, and Data Gaps.
- I. Conclusion about the MOA.

1.5 *IPCS Human Relevance Framework*

In order to address the issue of how to evaluate the human relevance of the MOA(s) determined in animals, a working group comprising of representatives from ILSI/RSI, US EPA and Health Canada was assembled. The working group discussed the issue of expanding the 2001 IPCS MOA framework to incorporate information relating to the determination of human relevance of a postulated animal MOA. This collaborative work led to the development of the ILSI/RSI Human Relevance Framework (HRF) that provides guidance on how MOA information can be used to determine the relevance of animal tumours when carrying out human risk assessment. In 2003, the findings of the working group were published and provided details of the case studies evaluated, and also guidance on how to apply the ILSI/RSI HRF in the risk assessment process [26, 30, 31].

Between 2004 and 2005, meetings and workshops were co-ordinated by the IPCS cancer working group to update the 2001 IPCS MOA framework. The opportunity was also taken to refine the proposed ILSI/RSI HRF and, incorporate this information into the updated IPCS MOA framework. This work has led to the most recent publication, *IPCS Framework for Analysing the Relevance of a Cancer Mode of Action for Humans* (IPCS HRF) [9].

The IPCS HRF provides a structured approach to determining the human relevance of an animal mode of action. It provides a stepwise approach for determining whether the WOE is sufficient to form judgements regarding a postulated carcinogenic mode of action for a chemical and the relevance to humans. The framework is intended to further the understanding of the carcinogenic process, improve the transparency of the risk assessment process and improve the identification of data gaps, inherently making it easier to identify areas where study designs can be improved. It provides a stepwise approach to answering a series of three questions in the form of a narrative review on the basis of the weight of evidence, allowing for the documentation of all logical conclusions regarding the human relevance of the carcinogenic animal MOA. Question one of the framework requires the application of the IPCS MOA framework discussed above [9].

Question 1) Is the weight of evidence sufficient to establish the mode of action in animals?

Question 2) Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?

Question 3) Can human relevance of the MOA be reasonably excluded on the basis of qualitative differences in either kinetic or dynamic factors between experimental animals and humans?

The IPCS HRF is being promoted to the wider scientific community who are encouraged to apply this framework approach when carrying out cancer risk assessment. The most recent publication that considers the human relevance of animal mode of action has been undertaken in partnership with various international

organisations. The involvement of representatives from different international organisations has added substantially to the credibility of this framework. The following sections discuss the extent to which some regulatory organisations have implemented the MOA framework within their own regulatory guidelines

1.5.1 *Application of the Mode of Action Approach by Different Organisations: United States Environmental Protection Agency*

The USEPA has been very active in workshops organised by IPCS to discuss issues related to cancer risk assessment and they have played a key role in the development of the IPCS MOA Framework. The timely revision of their “Guidelines for Carcinogen Risk Assessment” [32] led to incorporation of the MOA Framework approach to address carcinogen risk assessment. Previous US EPA cancer risk assessment guidelines promoted methods that relied heavily on the use of mathematical models to propose upper bound estimates of potential risks. Although these methods were intended to be protective of human health, they incorporated no chemical specific data and the methods were considered by many to be very conservative.

The move towards a MOA approach when attempting to characterise the hazard and consider the dose response relationships, incorporates recent advances in the biology of cancer. However, in the absence of chemical specific information, default assumptions are still applied. The US EPA recommends the MOA approach to help determine when to use linear or non-linear extrapolation methods and also to provide guidance on the considerations of MOA changes associated with the doses investigated [32].

The 2005 final draft of the US EPA guideline document has incorporated same concepts as in the 2001 IPCS MOA framework for animal carcinogenesis [24] and the ILSI/ RSI Human Relevance framework document [26]. The resulting EPA guidelines consider the overall hazard characterisation in animals and the relevance and cancer risks to humans. These guidelines incorporate the use of a MOA framework approach, and additionally, analysis of the human relevance for observed animal carcinogenicity.

Data compiled for the MOA analysis are intended to form part of the WOE evaluated to enable reasonable conclusions to be drawn about the carcinogenicity of an agent and, therefore, characterise any potential carcinogenic hazards.

A similar MOA analysis format to IPCS has been adopted in the EPA cancer guidance document. Whilst a “check list” approach to compiling the information used for the MOA analysis is not endorsed, the guidance provided encourages a stepwise approach to cover specific points that are intended to facilitate a transparent and overall analysis of the evidence used to hypothesise a MOA. Similar to the IPCS framework, the format proposed below is provided in a stepwise manner and is based on the Bradford Hill criteria for causality. It provides a clear structure for laying out the scientific facts and the reasoning behind any conclusions drawn.

1. Description of the hypothesised mode of action
 - Summary description of the hypothesised mode of action
 - Identification of key events
2. Discussion of the experimental support for the hypothesised mode of action
 - Strength, consistency, specificity of association
 - Dose-response concordance
 - Temporal relationship
 - Biological plausibility and coherence
3. Consideration of the possibility of other modes of action
4. Conclusions about the hypothesised mode of action
 - Is the hypothesised mode of action sufficiently supported in the test animals?
 - Is the hypothesised mode of action relevant to humans?
 - Which populations or life stages can be particularly susceptible to the hypothesised mode of action?

The first three steps of the US EPA MOA analysis are very similar to that proposed by IPCS as it encourages the risk assessor to carry out the analysis in a stepwise approach based on the Bradford Hill criteria for causality. The final step allows for conclusions to be drawn with regard to any hypothesised MOAs. A narrative review is

also required to address the issue of human relevance of observed animal carcinogenic MOAs. For observed key precursor events, it is recommended that an evaluation be carried out to critically examine any similarities and differences between laboratory animals and humans. It is recommended that analysis of the chemical specific data be undertaken to consider specific differences in toxicokinetics or toxicodynamics between animals and humans.

The US EPA promotes the flexibility of its guidelines because it recommends the consideration of different MOAs for different routes/ patterns of exposure that include for example the intensity of exposures. The guidelines also go a step further by calling for the consideration of the potential susceptibility of different populations and life stages to the postulated MOAs. This is consistent with previous US EPA policy approaches to protect susceptible groups of the population and in fact a supplementary document, entitled: *Supplemental Guidance Assessing Susceptibility for Early Life Exposure to Carcinogens*, [32] has been produced by the agency to address this specific issue, although these are apply only to genotoxic carcinogens.

1.5.2 *European Union: European Food Safety Authority*

Within the European Union, the IPCS Mode of Action (MOA) Framework approach is currently being applied by the European Food *Safety Authority* (EFSA) scientific panel on Plant protection products and their Residues (PPR).

Explicitly between May 2003 and May 2006, the EFSA PPR panel published opinions based on questions posed to them by the European Commission on issues relating to the human toxicology of six different pesticides: methamidophos, dinocap, daminozide, mepanipyrim, dichlorvos and alachor. A non-default approach was applied to evaluate the carcinogenicity of these compounds. In cases where the genotoxic potential of a compound was not sufficiently demonstrated in experimental studies carried out *in vivo* or *in vitro*, the panel concluded that the compounds were acting via a threshold mode of action regardless of whether there was sufficient evidence to actually demonstrate the specific non-genotoxic MOA for the observed tumour [33].

The mode of carcinogenic action was considered for daminozide, alachor, mepanipyrim and dichlorvos. The focus of the MOA considerations was to confirm whether, on the basis of the evidence considered, the compounds acted via a threshold mechanism. Although opinions were formed about the MOA of the chemicals on the basis of the WOE evaluated, for the majority of the compounds a structured approach was not applied in the evaluation of this evidence. The discussion of the WOE examined for alachor was however based on the IPCS Mode of Action Framework [33].

Although the IPCS mode of action approach was not applied to the evaluation of all the pesticides evaluated, the value of using the framework was noted by the PPR panel, including the extension of the framework to include addressing non-cancer endpoints [33].

1.5.3 *International Agency for Research on Cancer (IARC)*

The International Agency for Research on Cancer (IARC), with the help of international working groups of experts, publish in the form of *Monographs*, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures.

Information provided in the monographs relates to that required for the hazard identification stage of the risk assessment. In addition, information regarding the dose-response assessment may also be addressed. The monographs make no attempt to carry out complete risk assessments and information relating to characterising an agent as a hazard is used mostly by national and international organisations as the starting point to carry out risk assessments.

A preamble is produced at the beginning of each IARC monograph. It describes the scientific principles and procedures applied in the development of the monograph. It also provides an overview of the types of scientific evidence considered and it lays down the criteria applied by scientists carrying out the evaluation. The following

section reviews the methods used by IARC and attempts to make comparison with the IPCS MOA framework.

When evaluating an agent, IARC review both animal and human studies and pay particular attention to the narrative review of the qualitative aspects of the following:

1. Exposure data
2. Studies of cancer in humans
3. Studies of cancer in experimental animals
4. Mechanistic and other relevant data
5. Summary
6. Evaluation and rationale

The general stepwise approach of the IPCS MOA framework has not been adopted in the most recent revision of the preamble (January 2006). Rather, aspects of the MOA framework have been incorporated into the general structure addressed by the preamble in the format listed above.

The IARC has built up an international reputation for producing strong evaluations of mechanistic data. Although not applying the format promoted by IPCS within the IPCS MOA framework under the definition of MOA, the IARC has previously produced internationally accepted criteria for some MOAs e.g. species differences in thyroid, kidney and urinary bladder carcinogenesis [34] and peroxisome proliferation and its role in carcinogenesis [35].

The terminology utilised by IARC also differs somewhat from that of IPCS. The term *mechanism of action* is used by IARC and it is assumed that the use of term *mechanism* in this context refers to the biological sequence of events that leads to cancer, inferred by MOA in the IPCS framework. In all IARC monographs and technical reports, the working group evaluates the findings of cancer observed in animals and humans. Scientific data reviewed by the working group include those on toxicokinetics, mechanisms of carcinogenesis, susceptible individuals, populations and life stages, and other relevant data including adverse effects. In attempting to describe the mode(s) of action/mechanism(s) of action by which an agent may

increase the risk of cancer, the working group's objective is to summarise key mechanistic data obtained from studies carried out in animals and humans.

Similar to the IPCS mode of action framework, IARC highlight the need for identifying the different mechanisms associated with the carcinogenic action of a single agent. IARC recommend that for each identified mechanism of action the focus should be on identifying gaps in the data, reviewing and summarising key data that suggest that more than one mechanism may be operating and evaluating the relevance of postulated mechanisms to humans. In doing so, the aim is to identify the different mechanisms by which the agent might operate.

Similar to the IPCS framework, IARC provide guidelines on how the working group carry out the narrative reviews of studies of cancer in humans and animals within the context of the Bradford Hill criteria for causality. Guidance is also provided for the interpretation of studies of cancer carried out in experimental animals and the determination of biological plausibility with relevance to observed carcinogenic effects in humans.

1.5.4 *United Kingdom*

Risk assessment in the UK is carried out by different governmental departments and agencies with the aim of informing policy makers, usually government ministers, tasked with the role of developing policies that are geared towards regulating individual chemicals, or clusters of chemicals, that have the potential to cause undesired health risks to humans. Evaluation of scientific evidence is usually carried out by scientists within government departments and agencies who also prepare risk assessment dossiers for consideration by the relevant UK advisory committees. The advisory committees include the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC), the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) and the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). The latter committees provide advice to the Department of

Health, the Food Standards Agency (FSA) and other government departments and agencies involved in dealing with issues relating to chemical safety [36].

The traditional UK approach to carrying out chemical risk assessment has been to evaluate scientific data relating to chemicals on a case-by-case basis. Such an approach requires a thorough evaluation of the body of evidence. The most recent guidance document produced by the COC entitled, *Guidance on a Strategy for the Risk Assessment of chemical Carcinogens* [7], includes the consideration of the mechanisms of the potential carcinogens and whether a compound is acting via a genotoxic mechanism. The COC recommend that the latter be confirmed through the use of short-term mutagenicity tests which help to determine whether a compound is an *in vivo* mammalian mutagen, an approach endorsed by the COM [37]. Carcinogenic compounds found to be positive *in vivo* mutagens, should be regarded as potential genotoxic carcinogens.

Compounds that cause cancer in laboratory animals and exhibit no mutagenic activity *in-vivo* are, therefore, assumed to be non-genotoxic carcinogens and thorough consideration of their modes of action is recommended by the COC. The committee also endorses the use of the IPCS MOA framework and the ILSI/ RSI Human Relevance Framework as tools for considering the weight of evidence for carcinogenic chemicals, in particular non-genotoxic chemicals, their mode of action and subsequent relevance to humans [7].

1.5.5 Joint FAO/WHO Meeting on Pesticide Residues

The Joint FAO/WHO Meeting on Pesticide Residues (JMPR) has applied the MOA framework to their evaluation of certain pesticides. The framework was applied by the 2006 JMPR to provide a structured approach to the assessment of the WOE or the MOA analysis. The framework was applied specifically in their evaluation of pyrethrin extract and the framework was subsequently incorporated as part of the published monograph [38]. More recently, the IPCS HRF was incorporated into the WHO Environmental Health Criteria document which updates risk assessment methodologies for chemicals in food. The Environmental Health Criteria 240:

Principles and Methods for the Risk Assessment of Chemicals in Food included the IPCS HRF as part of its methodologies for the consideration of hazard identification and characterization in risk assessment [39].

1.6 Weight of Evidence considerations for the application of the IPCS MOA/HRF: transparency and data incorporation.

The first part of the IPCS MOA/HRF which requires the risk assessor to address the question: “is the weight of evidence sufficient to establish a mode of action in animals?”, requires the consideration of different steps that are based on the considerations of causality proposed by Hill [29]. In this context, the use of the WOE approach for the consideration of the MOA is as an analytical tool required for the qualitative assessment of overall evidence to take into account the strength and specificity of association, dose-response concordance, temporal relationships and the biological plausibility and coherence amongst other aspects deemed relevant for consideration. Information relating to the WOE is then provided in the literature review to describe and summarise the extent to which the evidence reviewed supports a decision about a carcinogenic endpoint [2, 9].

One of the main drivers of the use of a WOE approach in the IPCS HRF and indeed risk assessment in general, pertains to the need for greater transparency in the risk assessment process [9, 40, 41]. The EFSA Scientific Committee recently produced a guidance document to address the procedural issues that are considered beneficial to improve the transparency of the scientific aspects of risk assessment carried out by EFSA. The resulting document consisted of general principles for the identification and evaluation of data and issues relating to dealing with assumptions and uncertainties [42]. Similarly, the issue of transparency was also addressed by a committee of the ILSI Health and Environmental Sciences Institute (ILSI/HESI) who examined existing guidance and recommendations related to transparency in risk assessment as identified by different regulatory and advisory bodies [40]. The ILSI/HESI committee highlighted the different criteria cited for improved transparency in risk assessment and concluded that the transparency and documentation of the risk

assessment process were fundamental to credible risk assessment. Transparency was also identified as an essential component of a WOE based approach to risk assessment.

Scientific data considered as part of the weight of evidence for the hazard characterisation of chemicals have traditionally relied on mutagenic and carcinogenic studies carried out in animals and where available, epidemiological evidence on carcinogenicity [43].

Over the past decade however, technological advances in the field of genomics has led to the generation of vast amounts of data within the public domain and increasingly regulatory bodies are starting to address the question of what to do with the vast amounts of data being generated and how such data can be utilised as part of the risk assessment process [44, 45]. The incentive for the pharmaceutical industry who have invested significant amounts of money in the field of toxicogenomics is the hope of identifying candidate drugs more quickly and economically [46, 47].

There have been many promises made about the potential benefits of toxicogenomics to chemical risk assessment. Most significantly, has been the promise that it will aid the screening and prioritisation of chemicals and drug candidates that require further testing and development [46]. Alongside this, it is hoped that signature patterns of gene expression, so called biomarkers, can be developed to assess the similarities between chemicals to facilitate the prediction of adverse health outcomes [48]. This process involves the comparison of toxicogenomics profiles to databases of already known toxicants, whereby biomarkers of exposure and toxicity can be identified and used in the high-throughput screening [46].

It is envisaged that toxicogenomics will have a significant impact on improving the mechanistic insight for chemicals under consideration. The prospect of identifying characteristic preneoplastic changes using gene-expression profiles that can be detected within hours or days (compared to conventional toxicity studies which can take months or years to observe evidence of toxicity) to predict a disease outcome is considered to be extremely advantageous. It is this identification of potential preneoplastic changes (which would be indicative of key events that lead to a final

toxicological outcome) that may enable the application of toxicogenomics data to the MOA analysis component of risk assessment [48].

Even more important is the prospect for toxicogenomics data to be used in cross species extrapolation. It is thought that since genes are conserved between species, experiments carried out in human and animal cells can facilitate cross species comparisons of the level of response at the same exposure levels. Similarly, it is hoped that the generation of species specific data will enable the identification of the most appropriate animal models to predict human response.

While supporters and investors within the field of toxicogenomics pledge that the data being generated in this field will revolutionise mechanistically based quantitative risk assessment and improve predictions of environmental and human safety [46, 49], there are still many challenges to be overcome. A survey carried out by the Health and Environmental Sciences Institute (HESI, part of the International Life Sciences Institute) Committee on the Application of Genomics to Mechanism-based Risk Assessment [50] to identify key issues and perspectives on the use of toxicogenomics for decision making within organisations and by policy makers, showed that the interpretation of toxicogenomics data and confidence in the results were considered to be a major hurdle for the increased impact of such data. The survey conducted by the HESI committee received a 30% response rate and participants included scientists from government, academia and industry, representing the chemical, consumer products and pharmaceutical industry.

Respondents of the HESI survey considered the greatest impact of toxicogenomics would be the contribution to the understanding of biological mechanisms, identification of biomarker candidates and identifying species differences. However it was the opinion of all respondents of the survey that the biological interpretation of toxicogenomics data was at present a major hurdle in achieving a larger impact in these areas. It was felt that this result was an indication that more remains to be gained from toxicogenomics data in order to achieve a greater impact [50]. When questioned about the use of toxicogenomics data as supportive or supplementary data for decision making, it was therefore unsurprising that respondents from the pharmaceutical industry considered that the impact would be low. In contrast,

respondents from academia considered this to be a high impact area for the use of toxicogenomics data.

The inclusion of toxicogenomics data in regulatory submissions was considered to be the benchmark by which to evaluate the successful impact of the use of such data in chemical safety assessment [50]. One of the main outcomes of the HESI survey was the identification of the need to improve both data quality and the quality of interpretation of the data.

In response to the significant contribution anticipated to be gained in risk assessment from data generated in the field of toxicogenomics, regulatory bodies such as the United States Food and Drug Administration (USFDA) and Environmental Protection Agency (USEPA) actively encourage the use and submission of such data as part of their effort to establish formal guidelines and protocols for the inclusion of such data in submitted applications [46]. Most significantly in 2002, the USEPA issued its Interim Policy on Genomics [51] which outlined its intention to consider genomics data as part of their decision making process and as part of the WOE on a case by case basis. The US EPA defined genomics as the study of all the genes of a cell or tissue at the DNA, mRNA or protein level. Crucially however, the USEPA noted that such data could not be used in isolation as the basis for a risk assessment decisions as there were still research needs to improve the interpretation of genomic responses to adverse effects [44, 45, 51]. In 2004, the USEPA produced a White Paper entitled, *“Potential Implications of Genomics for Regulatory and Risk Assessment at EPA”* [52].

A workshop was organised by IPCS to address the issue of the use of toxicogenomics data in chemical risk assessment [53]. Participants of the workshop discussed and acknowledged the generally held views about the contributions expected to be made from data generated using toxicogenomics technologies and identified areas in which they felt more collaborative work was necessary to facilitate successful application of toxicogenomics data in risk assessment. The workshop participants identified the need for greater collaboration among those generating the data and traditional toxicologists and risk assessors who would be responsible for interpreting the data within the context of risk assessment. In addition it was felt that more involvement was

necessary from academic institutions to improve the understanding of the broader applications for toxicogenomics data. The involvement of experts from a range of backgrounds that included epidemiology, molecular and clinical genetics and bioinformatics was considered to be essential. One of the recommendations of the workshop was that a collaborative effort should be undertaken to develop case studies for chemicals with known MOAs and available toxicogenomics data.

1.7 Aims and objectives

Fundamental aspects of the application of a WOE-based approach to risk assessment, relate to the collection and presentation of evidence that forms the basis for conclusions about a chemical of concern or conclusions about an animal MOA and subsequent relevance to humans, in the case of the IPCS HRF [9, 54]. This research thesis aims to address both these aspects as part of the general consideration of the use of WOE approach in MOA analysis within the context of human relevance as part of the more general consideration of how such approaches could be refined.

1. Improved Transparency of WOE-based risk assessment

An important part of the WOE-based approach to applying the IPCS framework is the compilation of the scientific data that informs the narrative review for each section of the MOA/HRF framework. While the IPCS framework goes a long way to formalising the process of transparent decision-making, an objective approach to evaluating the weight of evidence would greatly improve the transparency of different stages of the risk assessment process that currently relies solely on the provision of a narrative review.

To address the issue of the improvement of transparency in the application of a WOE-based approach to the IPCS HRF, the use of systematic review techniques will be explored to illustrate how such techniques can improve the transparency of evidence evaluated as part of the application of a MOA/HRF framework.

Objective:

- Demonstrate the use of systematic review techniques for the evaluation of epidemiological evidence for a chosen case study compound, as a means of improving the transparency of evidence that will form part of the MOA/ HRF analysis.

2. Incorporation of non conventional data into MOA/HRF analysis

An IPCS workshop organised to discuss the potential application of toxicogenomics data in risk assessment recommended that more case studies should be developed for chemicals with known MOAs and available toxicogenomics data [53].

To address the current disconnect between the large amounts of toxicogenomics data being generated and the contribution of such data to framework approaches to hazard characterisation, a data based approach will be applied to investigate the extent to which toxicogenomics data can be used to inform the MOA/HRF analysis.

Objectives:

- Conduct a comparative analysis of publicly available toxicogenomics data to investigate the extent to which the observed changes can provide evidence for key events of the MOA for the hepatocarcinogenic effects of the case study compound.
- Conduct a critical appraisal of the state of the science for the use of published toxicogenomics data in MOA analysis.
- Provide recommendations for the effective utilisation of toxicogenomics data as part of a MOA/HRF analysis.

Phenobarbital (PB) was selected as the case study compound to carry out the objectives outlined above. PB has been administered to patients for long term treatment of epilepsy. Long-term experiments carried out in rodents in the 1970s showed that liver tumours were induced after administration of PB. In order to determine whether this same effect were observed in humans, epidemiological studies of groups of patients that were administered PB were carried out to investigate whether these patients were more likely to develop liver cancer than similar groups of

individuals within the general population. The use of this drug in a clinical setting allowed for ease in identifying a cohort of patients that could be followed up, in order to investigate whether there was any association between the occurrences of liver cancer among patients exposed to this drug [55].

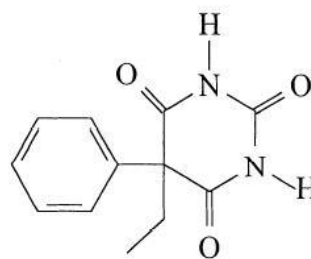
PB was considered to be an ideal case study compound for this investigation owing to the fact that the same outcome of disease could be investigated in both experimental animals and in humans. Evidence obtained from human studies, depending on its reliability and robustness, are fundamental to the overall evaluation of the human relevance of the toxicological effect. Irrespective of the information obtained from animal studies, good quality epidemiological evidence is generally preferred over animal data in a risk assessment. In the case of carrying out the human relevance analysis, PB provides a unique example of a compound where the cumulative dose observed in humans after long-term administration of PB for epilepsy treatment is similar to the doses required to induce a carcinogenic effect in long-term animal studies [9, 56].

PB is considered to be the prototype of several rodent hepatocarcinogens e.g. oxazepam and DDT [56]. Over the past few decades there has been a considerable amount of data generated on PB to investigate its mechanisms of action for hepatocarcinogenesis in rodents. The vast amount of knowledge accumulated for PB means that it is readily employed as a positive control in mechanistic studies. It is therefore not surprising that PB has also been employed in toxicogenomics investigations. With the increased promotion of genomics technologies, toxicogenomics data on PB is becoming more readily available within the public domain.

The next chapter provides a brief summary about mechanistic evidence supporting the postulated mode of action for PB induced hepatocarcinogenesis. The remaining chapters in this thesis describe the efforts and methods applied to compile and evaluate epidemiological and toxicogenomics evidence for PB obtained from the public domain.

Chapter 2

Phenobarbital



Introduced in 1912, phenobarbital (PB) has been used as a sedative or hypnotic and for the long term treatment of epilepsy [57]. Its use has decreased since the 1960s but it is still used extensively worldwide.

The International Agency for Research on Cancer (IARC) evaluated PB in 1977, 1987 and again in 2001. IARC concluded that there was sufficient evidence for the carcinogenicity of PB in animals but inadequate evidence for the carcinogenicity of PB in humans. Based on evidence evaluated by IARC, PB was assessed to be *possibly carcinogenic to humans* (Group 2B) [58].

In a battery of genotoxicity tests, PB has been observed to be generally negative overall. It has also been observed to be negative in tests for DNA damage, leading to conclusions that PB is not genotoxic. In addition it has been shown to act as a liver tumour promoter when administered after different initiating carcinogens [59].

PB is thought to induce tumours via its tumour promoting effects. Its ability to induce tumours in rats when administered alone has been demonstrated only in aged animals, which are thought to already possess considerable numbers of spontaneously occurring initiated cells [60, 61]. This evidence in addition to the lack of observed mutagenicity, have led to the assumption that PB's promoting activity is the result of epigenetic interactions as opposed to genomic interactions [60].

The non-genotoxic effects of PB have been studied extensively for decades. PB is considered to be the prototype of several rodent hepatocarcinogens. Compounds like oxazepam and DDT are referred to as "PB-like carcinogens" in reference to their characteristic effect on hepatocytes [56, 58]. As a prototype of many compounds, PB provided excellent case study for this research. PB is one of a few compounds for

which the human exposure (for patients administered PB) results in circulating levels similar to those observed after doses producing carcinogenic effects in study animals. Phenobarbital is reported to have been administered to epileptic patients at doses of 3-4 mg/kg/day with the target plasma PB concentration of 10-25 µg/mL [59, 62]. An epidemiological study carried out by Clemmesen and Hjalgrim-Jensen [63] reported that PB was routinely administered at doses of 100-300 mg/day to prevent seizures in epileptic patients. Studies reviewed by Whysner et al [59] show that doses of between 2 to 150 mg/kg were administered to rodents (rats and mice).

2.1 *Postulated Key Events for PB Mode of Action for Liver Tumour Formation in Rodents*

Mechanistic information accumulated for PB has enabled the identification of key toxicological responses that are postulated to be crucial to PB induced tumourigenesis. These include increased cell proliferation, inhibition of apoptosis, hypertrophy and hyperplasia and development of altered hepatic foci (resulting in advanced liver tumours). It has also been suggested that factors such as oxidative stress and the inhibition of gap junction intercellular communication may also be associated with PB's MOA for liver tumour formation [56, 59, 64, 65]. Exposure to PB elicits a wide range of biological responses and processes in rodents. The key events for PB are discussed in more detail below.

2.2 *Activation of the Nuclear Receptor CAR*

One of the first key events for the PB MOA is the activation of the nuclear receptor, constitutive androstane receptor (CAR) [56].

A characteristic effect of PB is its ability to induce members of cytochrome (CYP) P450 family *in vivo*. Members of the CYP family shown to be induced in rats by PB include 2A1, 2C6, 2C7, 2C11, 3A1 and 3A2, however the CYP forms most affected by PB are members of the 2B sub-CYP family, (CYP2B1 and CYP2B2 in the rat [59,

66]. Selective induction of CYP2B forms is considered diagnostic of CAR activation in rodents [56].

Evidence for the regulation of CYP2B10 gene by PB in mouse has been obtained in experiments carried out using CAR knockout mice. These have show that the mRNA levels of CYPB10 is greatly increased in CAR wild-type mice after treatment with PB, whereas the gene was not expressed in the CAR-null mice [67].

PB has also been shown to induce other enzymes that include aldehyde dehydrogenase, epoxide hydrolase, NADPH:P450 reductase, some forms of UDP-glucuronyl transferase and several members of the glutathione-S-transferase family [59]. Microarray studies carried out by Ueda et al [67] have shown that CAR is involved in the upregulation of many of these genes particularly NADPH-cytochrome P450 reductase, sulfotransferases, glucuronosyltransferases and glutathione S-transferases, as well as genes encoding various enzymes and transporter proteins that are not only involved in metabolism but also in the secretion of steroid and thyroid hormones, bilirubin and bile acids.

Studies carried out in rodents showed that upon exposure to PB, CAR translocates from the cytoplasm into the nucleus where it forms a heterodimer with the retinoid X receptor alpha (RXR α) and binds to a DNA sequence identified as an enhancer region upstream of the targets gene. This enhancer region, termed the phenobarbital responsive enhancer module (PBREM), is present in a number of genes that include cytochrome P-450s, UDP-glucuronosyltransferases, sulfotransferases and drug transporters. Indeed studies in CAR knockout mice confirm its role in the up regulation of genes that encode the latter group of proteins [64, 67-71].

The activation of CAR has been demonstrated to be essential for the induction of CYP2B10 in the mouse (and CYP2B6 in humans and CYP2B2 in rats), the development of cell hypertrophy and observed increased liver weight. These effects have been clearly demonstrated to be associated with the activation of CAR through the use of CAR knockout mice models. Wei et al [64] observed that the liver weight in wild type mice administered PB was significantly increased compared to mice lacking the CAR gene (CAR^{-/-}) that were also administered PB. Yamamoto et al [72]

also investigated the effects of PB in CAR knockout mice and demonstrated that CAR was essential for PB induced liver tumour development. The authors administered CAR^{-/-} and CAR^{+/+} mice with the initiator diethylnitrosamine (DEN) followed by chronic treatment with PB. After 32 weeks the authors observed that only CAR^{+/+} mice had developed hepatocellular carcinoma and similar to Wei et al [64] they also observed increases in liver weight in the wild type mice compared to CAR^{-/-} mice. By 52 weeks, PB treated CAR^{+/+} mice had all died and had developed tumours. The authors did not observe any tumours or deaths among the CAR^{-/-} mice.

2.2.1 Increased Liver Weight Characterised by Hypertrophy and Hyperplasia

PB exposure has been shown to result in the increase of rodent liver size (hepatomegaly) after a few days to weeks of continued exposure. Chronic exposure of PB in mice resulted in 40-70% increase in liver size thought to be the result of both hypertrophy¹ and hyperplasia² [59, 73-76]. The effects were observed to a lesser extent in rats and shown to be reversible following withdrawal of PB [77].

Increased hypertrophy or hyperplasia resulting in an increase in liver size can occur independently of each other. Carthew et al. [76] observed that rats treated with PB led to significant increases in hypertrophy at both 2 and 12 weeks of treatment compared with controls. In contrast, the authors observed that PB induced hyperplasia was not significantly increased until after 12 weeks of PB exposure. Similarly, Ross et al [78] carried out experiments using humanised knockout models for CAR and PXR and demonstrated that mice treated for 4 days with PB developed liver hypertrophy without hyperplasia in the huPXR/huCAR models. This was in contrast to the CAR and PXR knockout mice in which no increases in liver weight were observed and there was no induction of the target genes for both CAR and PXR (CYP2B10 and CYP3A11 respectively).

Evidence for hyperplasia was also reported by Jupundzic et al [79] who observed that the mitotic activity of parenchymal liver cells from rats treated with PB were 4.9

¹Increase in liver caused by an increase in the size of cells.

² Increase in liver size caused by the increase in the number of cells as a result of increased cell division.

times higher than control groups. Plant et al [80] observed similar findings in rat hepatocytes but reported mitogenic activity 2-3 fold above control levels.

2.3 *Cell proliferation*

Exposure to PB results in the stimulation of cell proliferation [59]. Evidence reviewed by Whysner et al. [59] showed that the observed increase in cell proliferation occurs in both normal hepatocytes and cells of altered foci.

Cell proliferation has been observed to be much higher in foci compared to normal cells even without PB treatment [81]. The data suggests that PB stimulates cell proliferation in normal cells but this stimulation only lasts a few days and may even be inhibited. Upon the development of hepatic foci after continuous exposure to PB, the proliferation in altered hepatic foci have been observed to be sustained at a much higher rate for a prolonged period of time compared to the proliferation in normal cells. The enhanced cell proliferation in foci compared with normal hepatocytes is thought to contribute to the mechanism by which PB promotes tumour formation [59].

Plant et al [80] reported that PB could induce DNA synthesis in cultured rat hepatocytes and observed a peak of PB induced DNA synthesis after 36 hours of dosing. Similar observations were reported by Peraino et al [82]. It has been suggested that the observed foci growth is not solely the result of stimulated cell proliferation but also a result of the stronger expression of phenotypic changes that render the foci more detectable [83]. Schulte-Hermann et al [83] observed an increase in the amount of detectable foci during PB treatment and concluded that these results implied that the foci populations studied in control and PB treated animals were not identical. The body of evidence reviewed by Whysner et al [59] showed that PB induced cell proliferation was caused by direct mitogenic effect rather than regeneration due to cell loss.

2.4 ***Inhibition of apoptosis***

Inhibition of apoptosis has been observed to be an important part of PB's ability to promote liver tumours. Evidence reviewed by Whysner et al [59] showed that a marked reduction in the level of cell death as measured and demonstrated by the observed decrease in the numbers of apoptotic bodies (AB) was observed in different strains of rat and mouse following the administration of an initiator. Plant et al [80] treated primary rat hepatocytes with PB over a period of 14 days and observed that the inhibition of apoptosis occurred in a dose dependent manner.

The inhibition of apoptosis has been observed to be reversible following the withdrawal of PB administration [59]. Bursch et al [77] observed a 10-fold increased excess of ABs within the focal cells compared to normal tissues after withdrawal of PB. This led the authors to conclude that the focal cells appeared to have a shorter life span than normal cells and appeared to be more sensitive than normal cells to apoptosis.

The resumption of apoptosis following withdrawal of PB suggests that the inhibition of apoptosis is a key aspect of PB tumour promotion. The inhibition of the cell death of initiated cells by PB exposure enables cell survival and continued proliferation until further alterations in the genome occur [59].

2.5 ***Development of Altered Hepatic Foci***

PB is a known tumour promoter and enhances the development of liver tumours particularly if it is administered following initiating agents such as 2-acetylaminofluorene (AAF) or N-nitrosodiethylamine (NDEA). It is therefore used extensively as a tumour promoter in initiation-promotion protocols [59]. Following the administration of an initiator, PB has been shown to lead to the increased numbers and size of altered foci.

Altered hepatic foci (AHF) are morphologically and histochemically distinguishable from normal liver parenchyma [84]. Differences have been observed in the genetic

expression of specific enzymes such as adenosine triphosphate (ATP), glucose-6-phosphatase (G6P) and γ -glutamyltranspeptidase (GGT) and these are used as histochemical markers to determine the cellular populations of hepatocytes that are involved in carcinogenesis. Determination of enzyme expression levels for GGT(+), G6P(-) and ATP(-) and histochemical staining for these enzymes are used to monitor the number and growth of hepatic foci during neoplastic development [85]

Altered foci are the preneoplastic lesions that precede tumour formation [59]. Peraino et al [82] observed significant increases in the incidence of hepatomas following the administration of 0.05% PB to rats in the diet either simultaneously or after administration of AAF. Mechanistic studies carried out in rodents have shown that chronic exposure to PB led to the rapid increase in both the number and size of liver foci which subsequently developed into adenomas and hepatocellular carcinomas in different strains of both the rat and mouse [59, 81].

2.6 *Toxicological Events Associated with the Observed Key Events*

2.6.1 *Oxidative stress*

It has been suggested that reactive oxygen species (ROS) and other free radicals that are generated during oxygen activation might play a part in the epigenetic effects of the observed promoting activity of PB [60, 86, 87].

Reactive oxygen species can be generated by both endogenous and exogenous sources and have been shown to produce single or double stranded DNA breaks. Where DNA damage persists, the resulting effects can be cell arrest, induction of transcription, induction of signal transduction pathways, replication errors and genomic instability; all of which are observed in carcinogenesis [87, 88].

ROS can be generated during normal metabolic processes and many forms of ROS are able to form oxidised bases [87]. The role of cytochrome P450 enzymes in the production of ROS was proposed by Parke et al [89] and this has since been

demonstrated in different studies. Dostalek et al [90] showed that some cytochrome P450 enzymes contributed to the process of oxidative stress as a result of exposure to PB *in vivo* in mice [90] and in rats [91]. Hydroxy radicals have also been shown to produce oxidized DNA lesions. An *in vivo* mouse study carried out by Kinoshita et al [92] demonstrated the formation of oxidative stress after exposure to PB. The authors measured the levels of hydroxyl radicals generated in liver microsomal fraction and attributed the observed DNA damage to the increased protein and activity levels of CYP2B1/2 and CYP3A2. Similar findings were obtained from a study in the rat after PB exposure [93].

2.6.2 *Inhibition of Gap junction Intercellular Communication*

The regulation of cellular homeostasis is accomplished by the passage of low molecular weight molecules such as neurotransmitters, calcium ions and hormones through cell transmembrane channels or so called gap junctions that consist of aggregated channels that link to the interior of neighbouring cells enabling cell to cell communication in a process known as gap junction intercellular communication (GJIC) [94, 95]. The gap junctional proteins, connexins play a prominent role in the process by allowing the intercellular passage of negative and positive homeostatic elements. GJIC therefore plays an important role in biological processes that include; cell growth, differentiation, embryogenesis and neoplastic development [94-96].

GJIC has been shown to be modulated during the carcinogenic process [96] and the inhibition of GJIC can lead to a disruption of cell homeostasis resulting in an increase in cell proliferation [94, 96, 97].

PB has been demonstrated to inhibit the intercellular communication in rat [98] and mouse [97] hepatocytes. Similar findings were observed by Kolaja et al [94] who observed inhibition of GJIC in intact rat liver following exposure to PB. The inhibition of hepatic GJIC occurred at the same time as the observed decrease in apoptosis and an increase in hepatocyte proliferation.

2.7 *Concordance of Dose response*

Some of postulated key events described above have been observed to occur in a dose dependent manner. Jones et al [99] demonstrated that exposure to PB led to an increase in liver weight and replicative DNA synthesis. Male and female C57BL/10 J strains of mice treated with 200 and 1000 ppm PB showed increased liver weight and a transient increase in replicative DNA synthesis. These effects were also observed after 99 weeks of exposure and the effects occurred in a dose dependent manner. In addition Jones et al [99] observed that the male mice treated with PB for up to one month led to increases in relative liver weight while replicative DNA synthesis was increased only after 3, 8 and 15 days of treatment at 1000 ppm PB [99]. The authors only observed altered hepatic foci and liver tumours after 1000 ppm PB.

2.7.1 *Temporal Association*

The key events are an essential element for carcinogenesis and their occurrence must precede the appearance of tumours [9]. As already discussed above CAR activation, liver hypertrophy and increased cell proliferation are all early events while key events like altered hepatic foci occur only after chronic treatment with PB as demonstrated by Jones et al [99].

2.7.2 *Species differences*

PB has been studied extensively in rodents. Studies reviewed by Whysner et al [59] showed that PB was investigated using different strains of rat (wister rats, inbred Fischer rats, aged and young F344) and mice (CF-1, B6C3F₁ hybrid mice, C57, C3H and (C3H X VY)F-1 hybrid mice).

The investigations of the effects of PB showed that chronic oral exposure to PB resulted in the increased incidence of liver foci in several strains of rat but no increased incidences of adenomas were observed in any of the strains of rat [59]. Mice were observed to respond more sensitively to the exposure to PB. PB administered to the different strains of mice via chronic oral exposure or by dietary

exposure resulted in an increase in liver foci and subsequent increases in hepatocellular adenomas and carcinomas [59]. The C57 mice were reported to be more resistant to tumours compared to the other strains of mice. Investigations have also been carried out using syrian hamsters. Diwan et al [100] administered PB in drinking water (500 ppm) to Syrian hamsters and observed that exposure of the hamsters to PB did not lead to the induction of liver tumours.

The effects of PB in humans have also been investigated in various epidemiological studies owing to the fact PB was administered for decades within a clinical setting. These studies will be reviewed in detail in the following chapter.

2.8 Summary

In summary the postulated key events for the MOA for PB induced hepatocarcinogenesis includes:

- Activation of CAR and associated effects
 - Induction of CYP2B
 - Cell hypertrophy
 - Increased liver weight.
- Increased cell proliferation
- Inhibition of apoptosis
- Development of altered hepatic foci

Other associated events include:

- Oxidative stress
- Inhibition of Gap junction Intercellular Communication

Chapter 3

Systematic Review of Epidemiological Data for PB

The need for a more systematic review of the evidence used in risk assessment processes and the incorporation of diverse evidence is widely acknowledged [31, 36, 101, 102]. The application of systematic methods to evaluate scientific evidence is common in the field of medicine and epidemiology where systematic review or meta-analysis are employed to provide a more transparent and objective appraisal of the scientific evidence [103, 104]. Meta-analysis provides a means of systematically appraising scientific evidence and where relevant, it enables different results to be combined so that conclusions can be reached more objectively [105].

A systematic evaluation of the weight of evidence using a conceptual framework approach will inevitably identify key data gaps in the information available to postulate a mode of action in animals. While experimental data required to identify key events in humans may be lacking, well conducted epidemiological studies have the potential to bridge data gaps between an animal mode of action and the key events in humans.

This chapter describes the systematic review undertaken to evaluate epidemiological evidence available for PB. The aim was to review whether there is any association between the administration of PB for long term treatment of epilepsy and the occurrence of liver cancer in patients taking this drug.

A systematic evaluation of the evidence available was carried out broadly applying guidelines provided by the NHS Centre for Reviews and Dissemination [43, 101].

3.1 Methodology for Epidemiology Review

3.1.1 *Development of Search Strategy for the Identification of Epidemiological Studies*

Epidemiological studies were identified by searching electronic databases that included: PubMed, EMBASE, Cochrane library, Toxline and Web of Science. In order to develop a robust search strategy, an initial broad search was carried out in PubMed using keywords associated with PB and disease outcome of interest (liver tumours). Keywords used to describe the compound of interest and the disease outcome by the individual publications were used as the starting point to determine other synonyms under which these keywords may have been indexed or catalogued in the search databases. Using facilities available in PubMed and Web of Science (MeSH terminology, term exploding facilities or subheading trees) the different terminology associated with the keywords of interest was compiled and used to generate the search strategy. Rather than using Medial Subject Headings (MeSH) terminology alone to search for papers in the relevant databases, where applicable, efforts were made to consider other terminology not included in the MeSH database, for example where the nomenclature had changed for certain keywords. This was carried out with the aim of capturing all relevant papers necessary for a complete evaluation of the weight of evidence for a compound under investigation. Other relevant publications were identified by citation searching (searching the reference provided in of all relevant publications identified from the initial search using the different electronic databases). A full list of the search terms applied is provided in Appendix III.

Criteria were developed to enable the selection of relevant publications. The searches were limited to studies published in English and published at any time or at least from 1970³ to the present date

Inclusion criteria:

- studies carried out in humans

³ Experimental studies evaluating the hepatotoxicity effects of PB in animals were first published in the 1970s. It was therefore reasonable to assume that studies to evaluate the effects of liver cancer in humans would not have been published prior to this period.

- Observational studies (these included: case series, cross sectional, cohort, case-control or nested case control studies)
- publications that mentioned a combination or all of the keywords⁴ in the title or abstract: "PB" /"liver cancer"/"epileptic patients"

Exclusion criteria:

- studies carried out on human cells
- *in vivo* and *in vitro* animal studies

3.1.2 Study Quality Assessment Scoring

Lists of criteria were developed to evaluate the quality of the studies reviewed. A study was considered to be of good quality on the basis of how well the quality criteria identified were addressed by authors of the individual studies. Criteria was developed using key guidelines available from the public literature to identify various aspects that were essential for the qualitative evaluation of the relevant studies. Using guidelines provided by the NHS Centre for Reviews and Dissemination [43, 101] and the principles of a qualitative scoring method applied by Key et al [106], a qualitative scoring system was developed and scores of good, adequate or poor were applied to the rating of each study. Quality considerations for each study related to: how well issues of bias and confounding were considered and adjusted for by the authors, mechanisms used to identify and select patients treated with PB, methods used to define the exposure or exposure assessment and methods used to confirm the disease outcomes (Table 3.1). The scores were assigned on the basis of the strengths and weaknesses reported from each study. A score of 1, 2, or 3 corresponding to the qualitative rating of poor, adequate or good respectively were then assigned to give an overall qualitative score. The total of the assigned scores for the individual paper was then obtained. The score weightings assigned to the individual studies were used as part of the overall assessment of the quality of the published study and as part of the critique of and evaluation of the findings and conclusions reached by the individual authors of the papers evaluated.

⁴ Includes any other associated synonyms identified in the search strategy using the search methodology. Details of the full search strategy for the identification of epidemiological evidence for PB are detailed in Appendix 3.

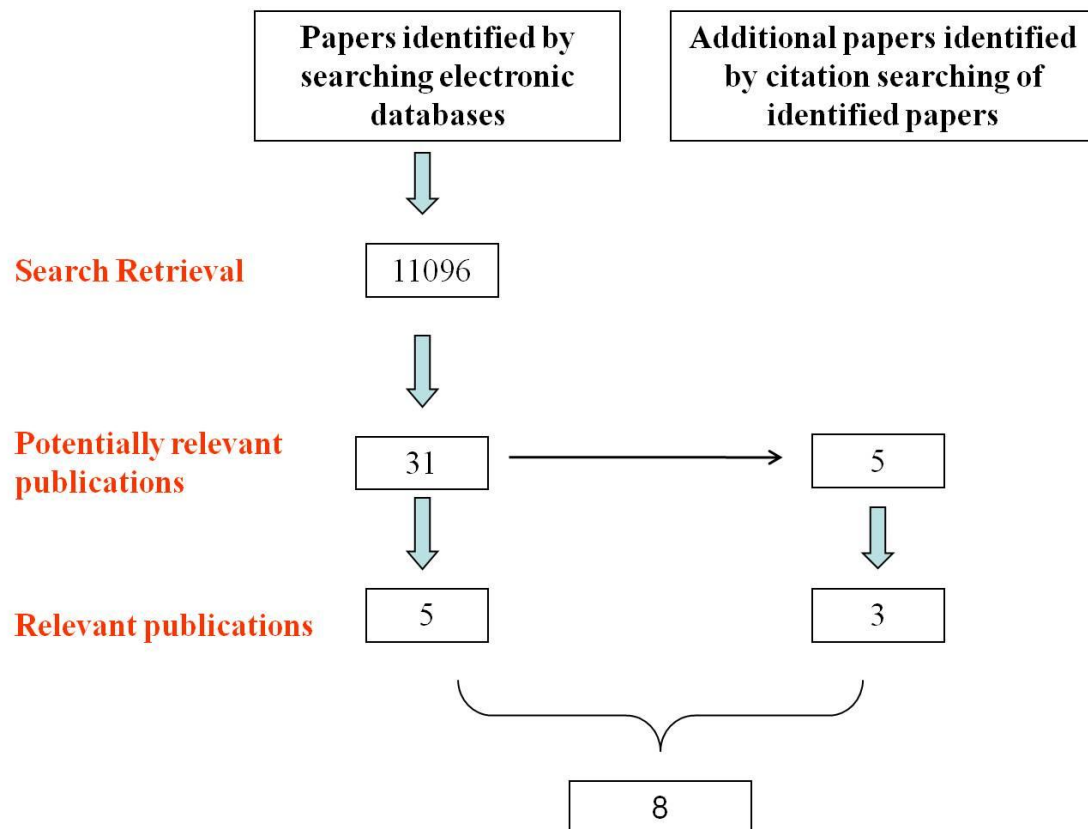
Table 3.1: Quality assessment criteria and scoring system applied to the individual studies

Quality Criteria	Quality score		
	Good (Score = 3)	Adequate (Score = 2)	Poor (Score = 1)
Consideration of bias and confounding in the study	The authors addressed any limitations to the study design by identifying any sources of bias and how these were managed. Any confounding factors were also highlighted by the authors and efforts were made to adjust the results to account for any confounders.	The authors identified any limitations and bias inherent in the study design. Potential confounding factors that may have influenced the results observed were also highlighted by the authors however no effort was made to adjust for any of these factors.	No effort was made by the authors to address any issues of bias, confounding or limitations to the study.
Identification of patients treated with PB	Authors were able to use medical records to identify epileptic patients within the cohort that were predominately treated with PB.	Authors were able to identify epileptic patients that were treated with antiepileptic drugs which included PB although not exclusively.	Authors were unable to identify specifically or failed to mention which drugs were administered to epileptic patients within the cohort.
Methods used to estimate dose levels of PB exposure	Authors applied well defined quantitative methods to estimate cumulative dose exposure to PB using patient drug information obtained from medical records or other appropriate patient records.	Where no quantitative methods of exposure assessment were used, any surrogate methods used to estimate drug exposure was clearly defined by the authors.	No clear definition of methods used to estimate levels of patient exposure to PB.
Confirmation of disease status of liver cancer in epileptic patients.	Authors carried out histopathological examinations on available tissue samples to confirm diagnosis of liver cancer as indicated by medical records or Cancer Registry records.	Authors used only medical records or Cancer Registry records to confirm patient diagnosis of liver cancer.	The diagnosis of liver cancer could not be adequately confirmed as a result of grouped recording of diseases into medical records or cancer registry records.

3.2 Results

Application of the search strategy and selection of the relevant studies using the predefined inclusion criteria identified eight studies for the review. Figure 3.1 below details the number of studies that were identified for the review.

Figure 3.1: Flowchart detailing the number of studies identified for the review



The initial search from the electronic databases using the derived search strategy identified 11,096 publications. Studies were selected on the basis of the inclusion and exclusion criteria leading to the identification of 31 potentially relevant studies. Only five of these were actually observational studies and therefore considered to be relevant for the investigation of the association between liver cancer and PB. Citation searching of these potentially relevant studies identified a further three relevant studies. The search therefore identified eight relevant studies to be included in the review.

There were eight epidemiological studies investigating the association between the exposure of epileptic patients to the drug PB/and or any other antiepileptic drug (in cases when it was not clear what drug was administered to the patients) and the occurrence of liver cancer. Table 3.2 gives details of the characteristics of the identified studies, Table 3.3 gives the quality scores and Table 3.4 summarises the main findings for liver cancer. All studies reviewed were observational studies using a cohort or a nested case-control study design. Four of the eight studies were based on a cohort of patients identified from the Neuropsychiatric Hospital of Philadelphia, Centre for Epilepsy Treatment in Denmark that had been followed up over time [63, 107-109]. The remaining four studies reviewed were cohorts of epileptic patients identified from institutional treatment centres for epileptic patients [110, 111] or were epileptic patients identified from within the general community [112, 113].

Table 3.2: Summary of included epidemiological studies

First Author	Date Published	Period of Study	Country	Study Design	Study Subjects	Controls / Reference Population	Antiepileptic Drugs Administered	Methods used to Collect Data	Other Risk Factors Investigated	Methods of Measure of Exposure to PB
Clemmesen [114]	1974	1933 to 1962	Denmark	Cohort study	Epileptic patients admitted to the Neuropsychiatric hospital of Filadelfia, centre epilepsy treatment (n= 9136) Men= 4201 Women = 3957	Morbidity rates for all of Denmark	1920s: PB 1940s: phenytoin from 1954: primidone	Danish Cancer Registry and death certificates.	Exposure to thiorotrast.	Period of therapy defined as the remaining lifetime of the patients. Deaths verified by checking against national register for deaths for the years up to and including 1967.
Clemmesen [63]	1978 Follow up study to Clemmesen 1974	1933 to December 1972	Denmark	Cohort study	Epileptic patients admitted to the Neuropsychiatric hospital of Filadelfia, centre epilepsy treatment n= 8078 patients Men= 4268 Women = 3810	Cancer incidence rates for all of Denmark	PB, phenylhydantoin, primidone	Death files and Danish Cancer Registry	Effect of epileptic patient exposure to thiorotrast	Not clearly defined although this has been assumed to be the period of follow up; i.e. the follow-up period until the last month of contact, the period up until death or until December 1972 when the follow-up period ended. Doses administered to patients were noted. PB: 100 - 300 mg; phenylhydantoin: 100 - 400 mg; primidone: 1.5 - 1.5 g
Olsen J. H. [108]	1989 The follow-up to Clemmesen 1978	1933 to 1962; 1976 to 1984	Denmark	Cohort study	Epileptic patients admitted to the Neuropsychiatric hospital of Filadelfia, centre epilepsy treatment n=8004 Men=4246 Women= 3758	The general population	1920s: PB 1940s: phenytoin 1950s: primidone 1960s: carbamazepine **	medical records, Danish Cancer Registry, Central Population Registry	The effect of thiorotrast	Time since hospitalisation was taken as a surrogate for a cumulative drug exposure .

White SJ [111]	1979	1931 to 1971	Chalfont, Buckinghamshire, England	Cohort study	2099 patients admitted to the Chalfont Centre for epilepsy and taking anticonvulsants only patients, medically diagnosed as having epilepsy were included in the study. 1448 men and 651 women	Comparison of mortality with that in the general population in England and Wales in the same period.	Most of the epileptic patients in this study were treated with PB, phenytoin and primidone.	Medical records for all patients living in the centre in 1931. Death certificates,. Follow-up through the national health service central register (NHS CR)	None.	This was not made clear although it was noted that the type and duration of drug therapy was noted. It was assumed that this was taken as the period of follow up for each patient.
Shirts [113]	1986	1935 to 1979	Rochester MN, America	Cohort study	All patients with seizure disorders in the Rochester area. 959 patients were included in the cohort of 730 had epilepsy (classified as having two seizures or more).	The general population in Rochester. Comparison was made on the basis of age and sex and specific incidence rates for Rochester by means of SMR.	No mention of exactly what anticonvulsants medications the cohort was under.	Medical records, all records with the diagnosis.	None.	The number of person-years of the cohort while on anticonvulsants medication. The dates of initiation and discontinuation of prescribed drugs were obtained from the medical records.

First Author	Date Published	Period of Study	Country	Study Design	Study Subjects	Controls / Reference Population	Antiepileptic Drugs Administered	Methods used to Collect Data	Other Risk Factors Investigated	Methods of Measure of Exposure to PB
McLean AEM [110]	1992 Study one: follow up from White et al 1979.	1951 to 1977	Chalfont, Buckinghamshire, England	Cohort studies	Study one: patients with severe epilepsy at the National Centre for epilepsy, Chalfont (636 deaths observed). Study two: people with well-controlled epilepsy (4488 persons with epilepsy; car driving licence holders).	The general population in England and Wales.	PB	UK system of registration of deaths and diagnosis of cause of death in the death certificates as the office of population Census and survey (OPCS, formerly the registrar General) and the central National Health Service records.	None addressed.	Study one: not stated although it is assumed that this would be have been the same as in the White et al 1979 paper since the information was based on the same dataset. Study two: none stated studies has been assumed to be the period of follow up.
Lamminpaa [112]	2002	1979 to 1997	Finland	Cohort study	All patients seeking reimbursement of antiepileptic drugs from the Finnish social insurance institution in 1979 to 1981 14,487 men and 13,932 women.	Corresponding cancer incidence in Finland	Enzyme inducing epileptic drugs used in the 1980s, these included the carbamazepine, phenytoin, PB and primidone	Finnish Cancer Registry	The effect of alcohol as a liver enzyme inducer was discussed by the authors.	Not stated. This was therefore assumed to be the number of years of reimbursement for each drug.

** Individual exposures to anticonvulsants drugs could not be quantified, time since hospitalisation was taken as a surrogate cumulative drug exposure.

3.2.1 Study Quality Assessment

Table 3.3: Quality assessment based on the Quality Criteria

Quality criteria	Author and year of publication				
	Lamminpaa et al [112]	McLean et al [110]	Olsen et al [109]	Shirts et al [113]	White et al [111]
Consideration of bias and confounding in the study	Adequate	Poor	Good	Poor	Poor
Identification of patients administered PB	Adequate	Adequate	Good	Poor	Good
Methods used estimate cumulative dose levels of PB exposure	Adequate	Poor	Good	Adequate	Adequate
Confirmation of disease status of liver cancer in epileptic patients.	Adequate	Poor	Good	Adequate	Adequate
*Quality Score (scores are additive)	8	5	12	6	8

*Good =3; Adequate = 2; Poor = 1 for each criterion, summed

Table 3.4: Summary of the main findings from the individual studies

First Author	Date published	Major findings for liver cancer	Strengths/weaknesses	Conclusion	Additional comments
Clemmesen [114]	1974	Slight excess for liver cancer was not considered to be statistically significant. When the values were combined the total number of liver cancers observed among patients treated with anticonvulsants for less than 10 years was 2 (Exp.= 1.5; RR= 1.33). Total number of liver cancers observed among those treated for more than 10 years was 8 (Exp = 3.5; RR= 2.3). One of the cases of liver cancer observed in 1964 and diagnosed with cholangiocarcinoma of a cirrhotic liver was confirmed to have received thorotrast.	Large study of patients evaluated using complete dataset compiled from the Cancer Registries and based on notifications from hospitals and supplemental information from death certificates. No adjustments were made for exposure to alcohol and tobacco because the patients were considered to have had a sheltered life and obtained relative protection against exposure to alcohol and tobacco at the treatment centre. The study evaluated the associations between the cumulative exposure from combined anticonvulsants drugs rather than PB exposure only.	The observed cases of liver cancer was not considered to be statistically significant as some of these results may have been confounded by exposure of the patients to thorotrast a known liver carcinogen. The authors concluded that the observed neoplasms were not causally linked to anticonvulsants therapy.	Community-based study. Institutionalised epileptic patients.
Clemmesen & Hjalgrim-Jensen [63]	1978 Follow up of original cohort compiled by Clemmesen et al, 1974	A significant excess of liver cancer was observed among the study group (Obs = 11;Exp= 2.8, p<0.001; RR = 3.9). 8 of the 11 cases of liver cancers had received injections of thorotrast. Exclusion of the thorotrast cases lead to the observed cases of liver cancer being very similar to the numbers expected (Obs. =3; Exp. =2.8; RR = 1.1).	This was a large study with a further five-year follow-up compared to the original 1974 study by Clemessen et al. All observed cases of liver cancer were verified using histological techniques. Analysis was not carried out on the basis of the different types of drugs taken in comparison with the observed neoplasms.	Histological evaluation of 8 thorotrast cases confirmed that the neoplasms were histologically similar to the type of liver neoplasms observed in thorotrast related liver cancer cases, leading the authors to concluded that the observed liver cancer cases was therefore likely to have being associated with exposure to thorotrast rather than PB. Owing to the lack of difference between the observed in the first of non-thorotrast cases and the expected numbers for occurrence of liver cancers, the authors noted that the latter results provided a good basis for argument against any measurable effects of PB on the occurrence of liver cancer.	Although the title suggests that the study examined on the effects of PB among follow-up of 8078 epileptic patients, it is impossible to determine with any certainty that the 11 observed cases of liver cancers were only exposed to PB. Although PB was a primary drug introduced in the 1920s, its administration may have been substituted by other anticonvulsants drugs or administered in combination with another antiepileptic drug.

First Author	Date published	Major findings for liver cancer	Strengths/weaknesses	Conclusion	Additional Comments
Olsen J. H. [108]	1989 Follow up front cohort compiled by Clemmesen & Hjalgrim-Jensen (1977) and based on original cohort compiled by Clemmessen et al, 1974	140 patients known to have received thorotrast were excluded from the study (n= 7864 non thorotrast patients with epilepsy). Eleven cases of liver cancer were observed compared to the expected seven. Statistical analysis was carried out on observed cases of liver cancers and other cancers. Non-statistically significant elevations were observed for liver cancers (RR or O/E=1.9; n= 9; expected = 4.7; 95% CI= 0.9 - 3.6). Elevations were observed for patients 10 years after hospitalisation (RR= 2.4) and the risks were significantly increased after 30 years of the first admittance (RR= 2.9*; *P< 0.05). The latter however only marginally exceeded the expected value for the same period (O=6; E= 2.1).	The study had an over 90% follow-up success rate and a long follow up over 30 years. The authors attempted to identify epileptic patients exposed to thorotrast in an effort to consider any potential confounding factors. This was done by linking available data from Cancer Registry with the Danish thorotrast study. Inherent errors were highlighted by the author such as the use of patient hospitalisation as a surrogate to drug exposure. Although PB was the primary drug administered to patients the observed cancers could not solely be attributed to the effects from PB. No detailed drug and alcohol histories were compiled for the individual patients. No information was provided on smoking and alcohol intake for the epileptic patients at Filadelfia. Although it was noted by the authors that the physicians and nurses at the Centre for Epilepsy , previously a religious home, discouraged smoking and drinking alcohol.	The authors concluded that the non-significant elevations of liver cancer observed may have been related to undocumented exposure to thorotrast. It was noted that there were some limitations in the Danish thorotrast study which would have meant an incomplete data set on the compilation of patients administered thorotrast. Overall the site-specific analysis did not appear to suggest causal associations with anticonvulsants drugs.	

First Author	Date published	Major findings for liver cancer	Strengths/weaknesses	Conclusion	Additional Comments
Olsen [109]	1995 Nested case-control study into the cohort compiled by Olsen et al, (1989).	26 cases of primary liver cancer were observed among a cohort of 8004 epileptic patients. Only nine of these patients however were known not to have received thorotrast and only 6 of these 9 non-thorotrast exposed patients were actually treated with PB. 19 cases of patients with liver cancers who had received PB were identified and compared to 29 controls (OR = 2.0; 95% CI = 0.5- 7.2). The latter values were further analysed to exclude any patients that had also received thorotrast. Six cases fitting this criteria were identified and matched with 11 controls (cases: controls = 6: 11; OR= 1*; 95% CI= 0.1- 8.0). The restriction of the analysis to patients not exposed to thorotrast significantly reduces the number of the cases available for analysis thereby reducing the stability of the risk estimation. In the absence of thorotrast, overall administration of PB was associated with non significant increased risks for liver cancers. * matched analysis unadjusted for other anticonvulsants therapy due to small numbers	The study specifically identified cases of epileptic patients with liver cancers who had received long-term treatment of PB. Where tissue samples were available, the authors carried out histopathological examinations and x-ray analysis for thorium residuals. This analysis was carried out on the tissue samples of the non-thorotrast cases, receiving PB and diagnosed with primary liver cancer. Potential confounding factors were efficiently addressed by the authors. Thorotrast: separate analysis was carried out to consider the effects of thorotrast on the patient's with liver cancers. The authors attempted to identify cases of liver cancer specifically associated with PB exposure on the basis of biological relevance as observed in experimental rodent studies. The small numbers of cases investigated were identified by the authors as a limitation to their study. To address issues of bias, medical records were abstracted without knowledge of case control status of study subjects. All assumptions made by the authors were discussed in the study and this included the reasoning behind the use of cumulative exposure as a surrogate estimation of the actual dose of PB exposure.	Although histopathological examinations were carried out on the tissue samples where available, only one of the histopathologically examined tissue samples (in cases receiving PB and with no exposure to thorotrast) confirmed that the cancer was the result of hepatocellular carcinoma, a subtype of neoplasm associated with PB administration in experimental rodents. The results obtained in the latter were on the basis of a tissue sample from a metastasis in the lung, therefore it was not possible for the authors to determine whether there was underlying liver disease. The authors found no significant association between the treatment with PB and human liver cancer. It was therefore concluded that the increased risk for cancers of the liver observed among Danish epileptic patients were due to other factors other than anticonvulsant treatment, including thorotrast administration and conditions relating to cirrhosis of the liver.	This was a well-designed study that sought to identify any issues of bias and confounding as well as clearly stating the reasoning behind any assumptions made. Efforts were made to confirm all diagnosis using histopathological examinations. The study also specifically identified cases of patients with liver cancer that had been treated with PB. To calculate the cumulative exposure, the assumption was made that the epileptic patients were exposed to the daily doses until the date of the cancer diagnosis (or equivalent equivalents date for matched controls) or at the end of 1960 whichever came first. The median cumulative dose of 750 g was chosen to differentiate from high exposure.

First Author	Date published	Major findings for liver cancer	Strengths/weaknesses	Conclusion	Additional Comments
White SJ [111]	1979	There were no observed cases of primary liver cancer with the observed frequency of 0, the underlying value was estimated to live between 0 - 3.7 (95% confidence limit). The expected mortality rate for liver cancer was estimated by the authors to be 0.3. Although there were no observed deaths from liver cancers in the study, the range for the mortality ratio was estimated to be between approximately 0 and 12.	The authors could only estimate the mortality rates expected in the general population because the number of deaths caused by primary liver cancer was not separately recorded from gallbladder cancer before 1958.	The only conclusion that could be reached by the authors was that the limits for the mortality ratios were approximately 0 to 12, signalling that the risk of death from primary liver cancer for epileptic patients could be less than that for the general population or up to 12 times greater.	
Shirts [113]	1986	65 primary cancers were diagnosed among patients followed up. No cases of liver cancers were observed although the expected number for the area of study was 0.2. (Obs. = 1; Exp. = 0.2)	One of the positive aspects of the study was that the authors followed up a cohort of patients from initial seizure of diagnosis rather than only including patients with established seizure disorders prior to entry into the cohort. One of the main limitations of this study was the lack of discussion of the actual antiepileptic drugs taken by the epileptic patients within the cohort study. Owing to the lack of observed cases of liver cancer, there was no discussion about the results.	There were no observed cases of liver cancer among the cohort investigated.	

First Author	Date published	Major findings for liver cancer	Strengths/weaknesses	Conclusion	Additional Comments
McLean AEM [110]	1992	Study 1: statistically significant results were obtained in the first study seeking to examine the number of deaths in patients with epilepsy in Chalfont treatment centre for epileptic patients (Obs. = 1; Exp. = 0.6; RR = 1.5; 95% CI = 0.0 - 8.5, for liver and gall bladder cancer). Study 2: two deaths were observed from liver or bile tract cancer were observed among 4488 epileptics holding driving licences (Obs. =2; Exp. = 1; RR= 2.0).	The authors noted that one of the disadvantages of carrying out a community-based study was the fact that people living within these institutions were likely to be exposed to very different environmental factors when compared to the general population. They therefore identified a group of well-controlled epileptics; people within the UK diagnosed as having epilepsy but also have a driving licence for a motor vehicle. The latter group of individuals were only permitted to have a driver's licence if they could prove they had not had an epileptic fit for over two years, such documentation could only be provided by a family practitioner or consultant physician who would normally state that the patient's epilepsy was under control and also state what medication the patient was under. Both study designs included a well-defined population of people taking PB as long-term treatment for epilepsy. These cohorts were then followed up using the UK system of registration of deaths and diagnosis of cause of death. There was no distinction in the reporting of results in the second study between the observed mortality rates from cancer of liver or bile tract. It was therefore impossible to determine whether the death (s) was attributed to liver cancer or bile tract cancer. Although the disease classifications were also grouped in the first study, owing to the fact that the data was the same as that reported in the White et al study, the observed case was confirmed to be gall bladder cancer and not liver cancer.	Study 1: on the basis of the results obtained the authors concluded that the risk of liver cancer to people from taking PB must be very small though it could still be up to eight times the risk found in the general population in the UK, without having shown up in the studies. As the authors observed no increase in liver carcinoma in people taking PB, the overall conclusion reached was that there was no significant cancer risk to man as a result of taking anticonvulsants drugs especially in comparison with the other causes of mortality.	This study had a clearly defined group of people taking PB for long term treatment of epilepsy. The study also attempted to follow up a cohort of individuals that were more comparable to the general population in terms of exposure to similar environmental factors.

First Author	Date published	Major findings for liver cancer	Strengths/weaknesses	Conclusion	Additional Comments
Lamminpaa [112]	2002	An overall significant increased risk of liver cancer (1.71) was reported by the authors (Obs.= 37; Exp.= 22; SIR= 1.71; 95% CI= 1.20 - 2 .35). Similar increased risks were also reported for patients aged 60 years or above as well as in younger patients. It should be noted however that the increased relative risk was on a statistically significant in patients above 60 years (Age <60 years: Obs.=9; SIR= 2.2; 95% CI= 0.97- 4 .02. Age > 60 years: Obs.= 28; SIR= 1.6; 95% CI= 1.0 6 - 3.31) . No cases of liver cancer were observed in patients less than 30 years of age.	A large cohort of over 28,000 people taking antiepileptic drugs. The study allowed for a long follow-up period in order to study the risk of cancer among patients taking AEDs. Each patient was identified using a personal identification number, therefore followed up until death or until the study was completed. The authors noted that the cancer registry system in Finland was also complete therefore linking the datasets allowed for a precise follow-up of all such patients within the cohort in order to establish any disease outcomes. The authors assumed compliance of regular use of the antiepileptic drugs by patients within the cohort since treatment of epilepsy in Finland also resulted in the regular monitoring of blood concentrations of the drugs. It was noted that lack of compliance resulting in under use of the antiepileptic drugs would if anything, result in an underestimation of the cancer risks.	Alcohol was identified as a major risk factor for liver cancer. The authors noted that more than 20% of newly diagnosed epilepsy cases in adults are alcoholic epilepsies, therefore it was acknowledged that alcohol might be a contributing factor for increased risk of liver cancer in epileptic patients. The authors concluded that the study showed that there was an increased risk of some cancers among users of enzyme inducing antiepileptic drugs. It was noted however that the excesses observed for liver cancers may be associated with lifestyle but the role of enzyme inducing antiepileptic drugs could not be ruled out.	Thorotrast was used in Finland in one hospital as the beginning of the 1950s although but was not considered by the authors to have contributed to their findings. The exposure assessment was very poorly defined in this study.

3.2.2 The Danish Cohort of epileptic patients

Figure 3.2: The relationship between the different epidemiological studies carried out using data on patients admitted to the Filadelfia

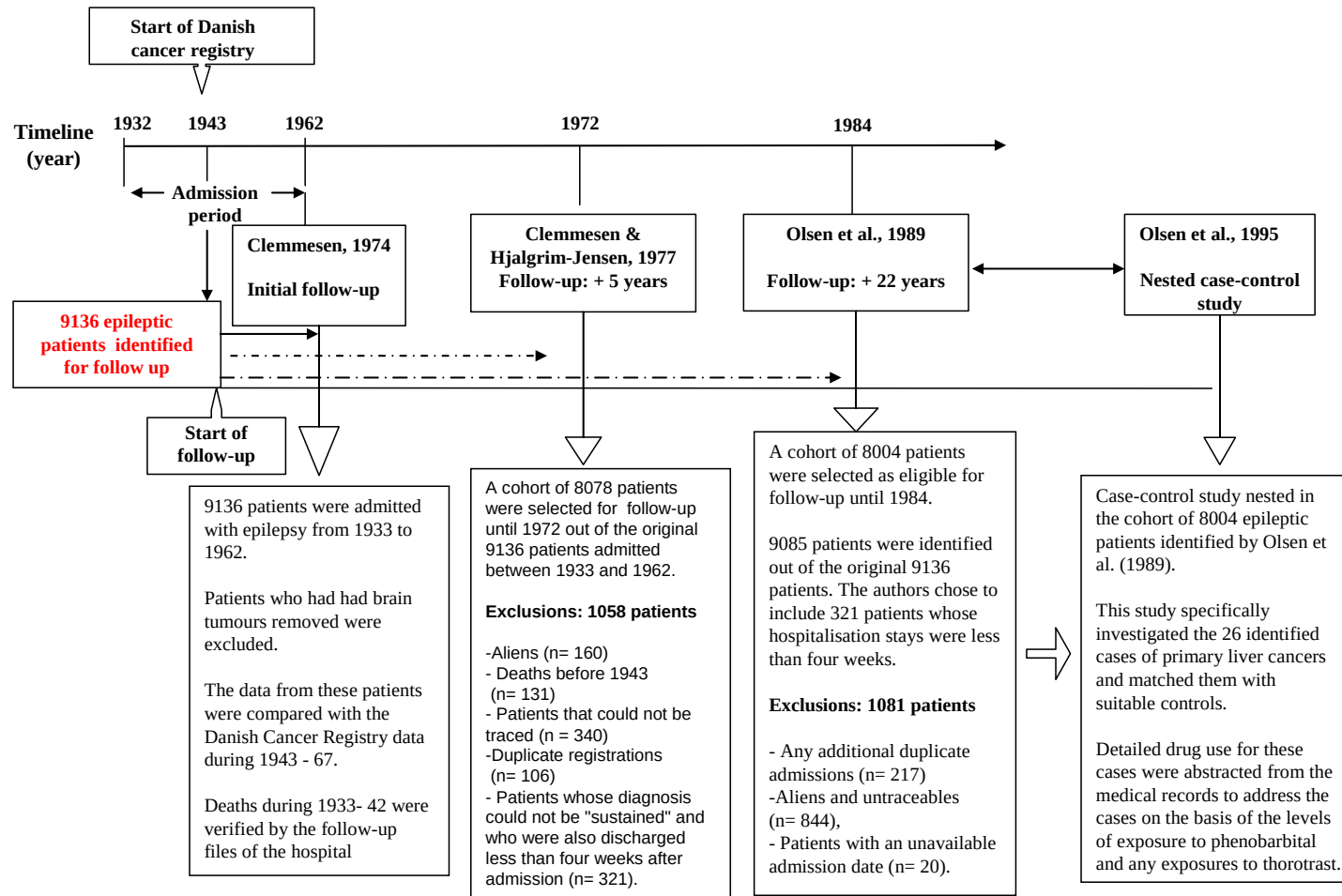


Figure 3.2 summarises the relationship between the four Danish papers reporting results from studies of a cohort of epileptic patients identified from the Neuropsychiatric Hospital of Filadelfia, Centre for Epilepsy Treatment. In the initial follow up study by Clemmensen [107, 114], the occurrence of cancer (ascertained from the cancer registry for 1943-67) was determined among 9136 patients admitted to hospital with epilepsy. Five cases of liver cancer were observed among the patients identified compared to 3.5 expected to occur among the general population. The slight excess reported for liver cancer was not statistically significant. In addition Clemmensen reported that one of the cases, a man of 61 who died in 1964 with cholangiocarcinoma of a cirrhotic liver had received thorotrast [114].

Clemmensen and Hajalgrim-Jensen [63] followed up 8078 out of the initial 9136 patients identified by Clemmensen [114] for an additional five years to combined with a reassessment of the data to provide strength and clarity to conclusions drawn from the initial follow up study. The study led to the exclusion of 1058 patients and the exclusion of a further three cases (patients treated with thorotrast) from the original five observed cases of liver cancer. The authors concluded that the remaining three cases were close to the expected number.

Olsen et al [108] identified a cohort of 8004 epileptic patients. As the majority of the patients identified were from the initial 9136 patients identified by Clemmensen [114], the follow up of the initial cohort was therefore extended by a further 22 years. The author observed liver cancer to be elevated ten years after hospitalisation and was significantly increased among 30 year survivors. The authors reported a relative risk of 2.9 (n=6). Following this study a case control study was nested in the cohort of the 8004 patients identified in the previous study. The study carried out by Olsen et al [109], the most recent publication from this Danish cohort, was chosen for evaluation in this review in order to avoid duplication of results documented in previous studies published by these authors.

Olsen and colleagues identified 26 cases of primary liver cancers among the Danish cohort of 8004 epileptic patients and these cases were matched with suitable controls. In order to identify the number of patients with liver cancers among the cohort who had also received thorotrast (identified as a potential confounder by the authors) for

other treatment, the authors linked this dataset with the Danish thiorast study to confirm that 17 of the 26 epileptic patients had been administered thiorast. Patients known to have received thiorast were therefore evaluated separately from the other patients with liver cancers that were known to have not received thiorast. Out of the latter nine remaining cases of liver cancers, only six were in fact receiving PB treatment for epilepsy. Analysis of these observed six cases when compared to the 11 matched controls showed no increased risk for liver cancer among epileptic patients (OR: 1.0, matched analysis unadjusted for other anticonvulsant therapy; 95% CI: 0.1-8.0). Further histopathological examinations were carried out on 4 available tissue samples from the observed cases of patients defined as having being exposed to PB. Epileptic patients were defined as having been "exposed" if they had received more than 5g (50 tablets) of PB or more than 10g (40 tablets) of primidone⁵ lifelong. The authors chose a median cumulative dose of 750g to separate low from high doses of exposure to PB, see table 3.5.

⁵ Primidone is extensively metabolised to PB, which is responsible for much of its therapeutic effect

Table 3.5: Primary Liver cancers among the 6 Danish epileptic patients exposed to PB with no evidence of Thorotrast exposure by histopathological examination and X-ray analysis for thorium residuals (Source: Olsen et al [109])

Gender and Year of birth	Age at diagnosis (Year)	Source of verification	Original diagnosis	Results of re-evaluation		Cumulative dose* of PB (g)
				Histocytological review	Comments	
F, 1899	65	Death certificate	Liver cancer	No tissue available		824
M, 1904	81	Death certificate	Liver cancer	No tissue available		647
M, 1905	61	Autopsy	Cholangiocarcinoma	Cholangiocarcinoma		1079
M, 1908	67	Autopsy	Hepatocellular carcinoma	Cholangiocarcinoma		1589
M, 1913	65	Autopsy	Hepatocellular carcinoma	Hepatocellular carcinoma	Tissue from a metastasis to the lung	2200
F, 1920	59	Autopsy	Cholangiocarcinoma	Possibly secondary tumour		1065

* Estimated as a cumulative dose from date of initial treatment till end of 1964

The original diagnosis of the four liver cancer cases among the Danish cohort subject to histopathological assessment included two cases of hepatocellular carcinoma (the neoplasm subtype confirmed in animal experiments to be linked with exposure to PB) and two cases of cholangiocarcinoma. Further histopathological examination by the authors confirmed that one of the cases of hepatocellular carcinoma was in fact a case of cholangiocarcinoma. Since the latter was not biologically consistent with exposure to PB, the authors concluded that these three observed cases could not be biologically associated with exposure to PB and that the observed disease outcome was the result of some other risk factor.

It should be noted that, for the two remaining cases of liver cancer for which no tissue samples were available, the doses to which these two patients were exposed was almost three times less than that received by the patient with the histopathologically confirmed diagnosis of hepatocellular carcinoma. By application of the authors' classification of low and high exposure, the exposure of patient M; 1904 was assessed to be low. In addition, the only tissue sample available for analysis in the latter case was a metastasis to the lung; the authors therefore concluded it was not possible to determine whether there was any other underlying liver disease. Without tissue samples to confirm the diagnosis in the remaining two epileptic patients, one can only interpret the findings by taking into account the levels of uncertainty associated with the observed results. The actual number of PB receiving patients that actually developed hepatocellular carcinoma could be a minimum of one or a maximum of three.

3.2.3 *The English cohort of epileptic patients*

Two papers were published on cohort studies carried out on epileptic patients identified from the Chalfont Centre for Epilepsy, Buckinghamshire, England. The cohort was originally assembled by White and colleagues in 1979 and the dataset later re-evaluated by McLean et al [110]. The paper published by McLean et al [110] also sought to define a population of free-living people taking PB for long-term treatment of epilepsy. The cohort of institutionalised patients studied by McLean and colleagues was the same as that defined by White et al [111]. Although both papers reported the same result of no observed cases of primary liver cancer, a decision was made to use the results reported in the 1979 paper for this review because unlike the results provided by McLean et al, White et al, provided an estimation of the confidence limits and of the standard mortality ratio based on the estimated expected number at general mortality rates. These results could therefore be used for comparison with the results reported from the other studies.

McLean et al [110] also sought to define a non-institutionalised epilepsy cohort. Their aim was to examine the mortality rates from liver cancer among a defined "well-controlled" group of epileptic patients taking PB. The results obtained from this new cohort were also included in this review as this newly defined group was separate from the group of patients previously identified from the Chalfont Centre for Epilepsy.

About 1980 epileptic patients admitted to the Chalfont Centre for epilepsy were followed up for a total of 32,873 person-years between 1951 and 1977 by White et al [111]. During this period there were no observed deaths from primary liver cancer among the epileptic patients. The authors estimated that the frequency of death from liver cancer was between 0 and 3.7 and the 95% confidence limits for the mortality ratio was estimated to be between 0 - 12. The authors concluded that the risk of death from liver cancer among epileptic patients could be less than that observed among the general population or 12 times higher.

McLean et al [110] identified 4488 persons suffering from epilepsy and permitted to hold a driving licence due to the fact that their epilepsy was well-controlled (i.e. people who had not had a fit for more than two years and were motivated to continue

taking their treatment with PB for their epilepsy). No significant increases in liver tumours were observed among this identified group of epileptic patients taking PB (observed: 2; expected: 1; O/E: 2.0). The liver cancers were grouped with those from bile tract cancer so it was difficult to determine whether the two cases observed were actually true cases of liver cancer.

3.2.4 *The American cohort of epileptic patients*

Shirts et al [113] identified a cohort of 959 residents of Rochester, MN suffering from seizure disorders, between 1935 and 1979. This cohort was followed up until death or to the end of the study period in 1982. There were no observed cases of primary liver cancer among this group of people suffering from seizure disorder; this was in line with the expected number of cases for liver cancer estimated to be 0.2. Although there was no direct mention of which anticonvulsant medications were actually prescribed to the patients, the dates of initiation and discontinuation of prescribed anticonvulsant medications were used to calculate standard morbidity rates for the observed cancer incidences. While the cohort of patients was identified using medical records similar to the Danish and English cohorts, this study differed in that the identification of patients was not restricted to that of hospitalised or institutionalised patients. Instead, authors followed up the cohort of epileptic patients within the community from the onset of their diagnosis until the end of the study.

3.2.5 *The Finnish Cohort of epileptic patients*

Lamminpaa et al [112] identified a cohort of Finnish patients who had received monetary reimbursement for antiepileptic drugs between 1979 and 1981; 14,487 men and 13,932 women were followed up until 1997 or until death. Thirty seven cases of liver cancer were observed compared to an expected number of 22 within the general population (Standardised Incidence Ratios, SIR: 1.71; 95% CI: 1.2 - 2.35). There was a statistically significant increased risk for liver cancer among patients receiving antiepileptic drugs. Similar findings were observed among patients aged 60 years or more (observed: 28; SIR: 1.60; 95% CI: 1.06 - 3.31). However, there were no

observed increased risks of liver cancer observed among patients that were less than 60 years of age (observed: 9; SIR: 2.12; 95% CI: 0.97 - 4.02).

The authors were unable to identify any patients that had obtained reimbursement for the drug PB. The aim of the study was to investigate whether an association existed between enzyme induction and liver cancer. The records utilised by the authors, grouped antiepileptic drugs according to their enzyme inducing properties. PB was among this group of compounds. The cases of liver cancers observed in the cohort of patients followed up were therefore not necessarily associated with PB exposure. In addition, the authors noted that initial methods of recording data into the reimbursement registers were not as thorough in excluding some anti-enzyme inducing drugs such as valproate. The authors however noted that as valproate is mostly prescribed to paediatric patients, it was unlikely that the cancers observed would have been influenced by valproate. Although a lifestyle study was not carried out to examine the direct influence of alcohol consumption among the cohort investigated, alcohol consumption was also suggested by the authors as a potential contributing factor for the observed increase risks of liver cancers, owing to the fact that it was acknowledged that 20% of newly diagnosed epileptic adults were linked to alcohol consumption [112, 115]. It was concluded that there was an increased risk of liver cancer and other cancers among epileptic patients exposed to enzyme inducing antiepileptic drugs.

It was noted that thorotrast was administered to patients in one Finnish hospital during the 1950s; however, this was not considered by the authors to have greatly influenced the observed results. Table 3.6 summarises the risk estimates from the five studies.

Table 3.6: Summary of overall results reported in the studies evaluated

Cohort studies							
First author	Year	Estimation of risk	n	Observed cases of liver cancer/ deaths from liver cancer	Expected number of cases	SMR/ SIR	95% Confidence Interval
Lamminpaa	2002	Standardised Incidence Ratio	Patients 14,487 men 13,932 women	37	22	1.71	1.2 – 2.35
McLean	1992	Observed/ Expected	4488 epileptic driving licence holders	2 ^a	1	2.0	
Shirts	1986	Standardised morbidity ratio	959 residents with seizure disorders	0	0.2 ^{*1}		
White	1979	Standardised mortality ratio	Patients 1448 men 651 women	0	0.3 [*]	0 ^b	
Nested case control study							
First author	Year	Estimation of risk	n	Observed cases of liver cancer	Number of controls	Odds Ratio	95% Confidence Interval
Olsen	1995	Odds ratio	26 out of the cohort of 8004 patients	6 ^{*2}	11	1.0	0.1 - 8.0

^{*1} Estimated values

^a Not clear whether these cases were due to liver cancer or bile tract cancer

^b Estimated frequency of the occurrence of deaths from liver cancer thought to lie between 0 and 3.7 (95% CI) and the limits for the mortality ratio were estimated to be between 0 and 12.

^{*2} Out of identified 26 primary liver cancer patients in the cohort, 17 were known to have received thorotrast and only 6 out of the remaining 9 patients actually received PB.

3.3 Discussion

One of the main objectives of this research project is to illustrate, through the evaluation of selected case study compounds, the means by which to carry out a systematic evaluation of the weight of evidence for a specific chemical using a conceptual framework approach to facilitate the development of such methodology. Using a conceptual approach proposed by the IPCS HRF, such an analysis is intended to inform the hazard identification and hazard characterisation steps of the risk assessment for a chemical or substance under investigation.

This chapter documented the systematic review and evaluation of epidemiological data available for PB. The aim was to critically examine the weight of epidemiological evidence through the application of quality assessment methods to thoroughly evaluate evidence available for the investigation of the association of PB with liver cancer. Using set criteria to evaluate the studies, qualitative score weightings were assigned for each study evaluated in order to determine the reliability and robustness of any findings reached by the authors of the individual studies. The result of this initial assessment is intended to form an integral part of the human relevance framework analysis for PB.

Narrative reviews of the weight of evidence for PB have been carried out in the past in an effort to investigate the different types of cancers induced in animals and considered to be associated with exposure to humans [55, 56, 58]. Most relevant to this report has been the paper published by Singh et al [55] which reviewed available human evidence investigating the carcinogenic effect of PB as well as other antiepileptic drugs. While this 2005 review paper discussed the findings of studies that investigated the occurrence of liver cancers among epileptic patients taking PB, the latter issue only formed a small aspect of the overall aim of the review paper which was to address the issue of general cancer risks among people with epilepsy and the association of the of these risks with antiepileptic drugs.

Eight relevant publications were identified using the search strategy developed. This was identical to the same studies identified by Singh et al [55] in their review. A more in-depth assessment of the epidemiological evidence was carried out in this

report in contrast to other reviews. Using set criteria for evaluating the studies, a qualitative assessment was carried out to assess the reliability of the available evidence and in turn the relevance and robustness of the study. The focus of the quality assessment was to evaluate how well the studies were able to address the review question. The studies were therefore qualitatively assessed on how well the authors: addressed issues of bias and confounding; defined their cohort of patients taking PB, confirm observed cases of liver cancer and what types of methods were used to assess exposure to PB.

Methodological Considerations

3.3.1 Methods of Exposure Assessment

Owing to the fact that exposure had already occurred in the subjects of study, various methods were employed by the different authors to determine the levels of PB to which they would have been exposed. Depending on the type of information available and the completeness of the records for individual patients, the authors had to make various assumptions regarding the actual drug therapy administered to each patient and also, the duration of the drug administration. Since the individual exposure levels to anticonvulsants drugs could not be adequately quantified with absolute certainty, surrogates were therefore used to estimate drug exposure. In rare cases and when available, information could be abstracted from the medical records of the actual duration of drug therapy [110, 111]. However, in most studies reliance was placed on abstracting patient admission and discharge dates from medical records or any other available information in order to estimate the duration of exposure; this was usually taken as the length of time of hospitalisation (Olsen et al., [108, 114, 116] or alternatively the number of person-years while taking relevant medication was used as a measure for cumulative exposure [112, 113]. Olsen and colleagues [109] attempted to quantify the actual cumulative doses of exposure by using computational methods. By obtaining information relating to the daily doses of PB that was prescribed to patients (100 - 300 mg) from the medical records, assumptions were then made about the drug-taking patterns of each individual patient and the cumulative doses were computed.

3.3.2 *Measures of Effects*

In all studies reviewed, the authors attempted to estimate the cancer incidence or mortality rates in the established epilepsy cohorts. The Danish [109] and one of the English cohorts [110] accomplished the latter by comparing the data obtained for the epileptic patients with data available through cancer registries or demographic databases. Methods of indirect standardisation were applied for the American [113], Finnish [112] and the second English [111] cohort of patients. For the latter, the observed numbers of incident cases or deaths were compared to the number of cases expected among the general population (standardised for age, sex and calendar period). Where possible, results of the original diagnosis were confirmed by carrying out histological evaluation of tissue samples from the relevant patients.

3.3.3 *Identification of cohort of patients treated with PB*

An overview of the available epidemiological evidence highlighted the methodological difficulties associated with trying to investigate the risks of liver cancer in epileptic patients receiving long term treatment of PB. Only a few studies were able to specifically identify cohorts of epileptic patients receiving predominantly PB as treatment and even in these circumstances some of the datasets were incomplete owing to the fact that some medical records had incomplete information and omitted vital information relating to the type of drug therapy administered and the duration of drug therapy. Cohorts established on the basis of relatively good quality data on epileptic patients administered PB included the English and Danish cohorts.

For the majority of the studies reviewed it was not possible to determine with absolute certainty whether PB was the only treatment administered for the long term treatment of epilepsy. In most cases PB was presumed by the authors to be the primary drug administered although it is more likely that PB would have been administered in combination with other antiepileptic drugs. The literature suggests that the practice of "polypharmacy" was common in Europe especially for the treatment of epilepsy whereby multiple drugs were administered even when PB was the main drug on the market [117]. When phenytoin and primidone were introduced into the market it was

common to have dosing regimens that included caffeine + phenytoin + phenytoin sodium salt + PB administered as a combined pill or as separate pills. Another combination of dosing regimen may have been the dosing of similar classes of compounds like PB + primidone [117].

The English and Danish cohorts investigated patients admitted to institutional centres for epilepsy treatment. The follow-up of these patients by the study investigators was relatively easy as the treatment centres maintained good patient medical records. The English cohort compiled data on patients admitted to the Chalfont Centre for epilepsy between 1931 and 1971 and mortality rates were examined during the period 1951 to 1977 [110, 111]. The Danish cohorts followed up patients admitted to the Filadelfia Epilepsy Centre in Denmark between 1933 and 1962. The original dataset was compiled and evaluated by Clemmesen [114]. However, three further papers were published and further analysis was carried out on the original dataset. The later publications extended the follow up period of each preceding paper and refined the dataset somewhat to take into account any patient deaths or exclusions due to loss of patient information resulting therefore in the loss of patient follow up [63, 108, 109]. The publication by Olsen et al [109] was the only Danish study included in the qualitative assessment to avoid duplication of results and also because its publication specifically identified patients within the Danish cohort that were administered predominantly PB.

3.3.4 *Methods of dealing with confounding*

One of the major difficulties in interpreting findings associated with increased risks of liver cancers among epileptic patients is the lack of thorough investigation into how varying lifestyles of epileptic patients may have impacted on the disease outcomes being evaluated. Owing to the retrospective designs of the studies evaluated, the authors could only rely on information available through public and medical records. Important confounding factors such as smoking, alcohol consumption and other lifestyle characteristics are not normally recorded in such documents; therefore the impact of such factors on the outcome of diseases observed among the epilepsy cohorts have not been thoroughly investigated or adjusted for using statistical

techniques in most of the studies reviewed. The consideration of such factors are important because disease outcomes such as cirrhosis of the liver may be an underlying cause of tumour formation in the liver and the former is most likely to be associated with alcoholic liver disease or viral hepatitis [109].

Another important confounding factor among epileptic patients treated with PB is the exposure of these patients to thorotrast (thorium-232). During the 1930s and 1940s thorotrast, a radioactive colloidal solution of thorium dioxide was used in many countries including Europe, North America and Japan during radiological procedures. During this period it is likely that many epileptic patients may have been exposed to thorotrast while undertaking procedures such as cerebral angiography. Injection of thorotrast leads to lifelong accumulation of the compound in the body, the majority of which is deposited in the liver (60%) and to lesser extents in the spleen (30%) and bone marrow (10%). Patients injected with thorotrast would have been exposed to low doses of radiation emitted from thorium-232 at the organs of accumulation. Growing concerns for the toxicity exhibited by thorotrast led to its withdrawal from the market in the 1950s. Epidemiological studies instigated as a result of the concerns raised resulted in the follow up of patients known to have received thorotrast. The results obtained from the studies showed that patients exposed to thorotrast had significantly increased risks of developing liver cancer compared to the groups of patients who had not been exposed to this compound [118-120]. The different confounding factors addressed above were considered to different extents by the different authors in the studies reviewed.

3.3.5 *Findings for the association between PB and liver cancer*

Three out of the five studies reviewed reported findings of liver cancer among their respective cohort of epileptic patients. Shirts et al [113] observed no cases of liver cancers among a cohort of 730 people suffering from epilepsy. This study received a low quality assessment score of 5 as they did not consider issues of bias, confound or limitations in the study nor did they mention any of the drugs that were administered to the patients with seizure disorders.

White et al [111] also reported no deaths as a result of primary liver cancer among 636 patients who died during the period of their study. This study received a quality score of 8. Although the authors made a poor attempt to address any issues of bias, confounding or limitations in the study, the authors were able to use patient drug records to identify patients that had be treated with PB.

Although McLean et al [110] observed two cases of liver cancers the authors concluded that these observations did not demonstrate a significant increase in cases of liver cancer. In addition the study assessed to be of low quality (score = 5) compared to the other studies reviewed. Although the authors adequately identified a cohort of patients that were treated with PB, the methods used to confirm the disease status was however considered to be of poor quality because it was not possible to determine whether the patients were suffering from liver cancer or bile tract cancer.

The remaining two studies were the largest epilepsy cohorts and both reported unequivocal findings of cases of liver cancer. Olsen et al [109] reported six cases of liver cancer matched with 11 controls (nested in a cohort of 8004 Danish epileptic patients). The study was judged to be of a high quality (score = 12) as the authors thoroughly addressed sources of bias and confounding. In addition the authors were able to identify patients that were treated with PB and were able to confirm the original diagnosis of the cases of liver cancers described. Lamminpaa et al [112] observed 37 incidents of cancer out of a total of 28,419 people reimbursed for anti-epileptic drugs during the study period. This study was assessed to be adequate and received a quality score of 8. These two studies will be discussed in more detail in the following sections.

3.3.6 *The Finnish cohort: Lamminpaa et al, 2002*

The study carried out by Lamminpaa et al (2002) based on the Finnish cohort of epileptic patients reported statistically significant increased risks of liver cancer associated with exposure to antiepileptic drugs (SIR: 1.7; CI: 1.2-2.35). This study had the largest cohort of all the studies reviewed, following up almost 30,000 epileptic patients for a minimum of 17 years and potentially up to 30 years.

Although this study obtained a quality score of 8, one of the main limitations of the study was the inability of the authors to relate the observed incidences of liver cancer with the different types of drugs consumed, owing to methods used to record data. The authors noted that antiepileptic drugs used in the 1980s included carbamazepine, phenytoin, PB and primidone. The review previously carried out by Singh et al [55] noted that the main exposure could be presumed to be PB. However, it is difficult to conclude with any certainty that the incidences of liver cancers observed were associated with exposure to PB. A review carried out on the use of antiepileptic drugs in Europe (Loiseau, 1999) confirms that the main antiepileptic drugs to have dominated the market between 1912 and 1963 were; PB, marketed in most countries from 1920, phenytoin available from the 1940s and primidone introduced in the 1950s. Carbamazepine was introduced in most European countries in 1963 and its efficacy was considered as comparable to that of both PB and phenytoin. By the 1970s carbamazepine was considered to be the drug of choice in most Scandinavian countries. The latter is an important point to consider when one bears in mind the date at which point the Lamminpaa study was initiated (1979-1981).

Although the methods of estimating exposure to PB/antiepileptic drugs were clearly stated in the papers (taken as the number of years reimbursed antiepileptic drugs), a limitation associated with this type of exposure assessment method includes, as noted by the authors, the impossibility of estimating a dose response relationship. Confounding issues were not thoroughly addressed by the authors investigating this cohort of patients. Although it was noted that thorotrast was used in Finland in the 1950s at one hospital it was not considered to have impacted on the observed incidences of liver cancers among the cohort. The authors, however, provided no justifications for this reasoning or confirmation that any of the patients within the cohort were treated at this hospital. There was also no mention of any additional methods employed by the authors to confirm the diagnosis of liver cancer observed among the cohort.

3.3.7 *The Danish cohort: Olsen et al, 1995*

The authors evaluating the association of PB with liver cancer found no increased risks (Odds Ratio: 1.0, unadjusted for other anticonvulsants therapy due to small numbers; 95% CI: 0.1-8.0) for this disease outcome among this cohort of epileptic patients. One of the limitations of the study however, was the small number of liver cancer patients matched with control subjects to be included in the study.

This study was assessed to be of a high quality and received a quality score of 12. The main strength of the study was its ability to identify the group of patients that were administered PB out of the original cohort of patients that were admitted between 1933 and 1962. Owing to the period that the patients were recruited for the study, it can be presumed that PB was predominantly the main antiepileptic drug administered to the patients. Data relating to this cohort of epileptic patients has been reviewed in the past in three separate publications, each author in turn refining the data and excluding any patients lost to follow up. Olsen et al [109] through the use of a nested case-control study design, carried out a thorough investigation of all observed cases of liver cancer identified among this cohort. The authors also applied quantitative methods to estimate the cumulative dose of PB to which the patients may have been exposed, allowing for the potential use of this analysis for dose response assessments.

The authors carried out a thorough evaluation of the potential confounders for liver cancers. Patients that were exposed to thorotrast were identified and excluded from the analysis and where tissue samples were available, histopathological evaluations were carried out for the liver cancer cases. The latter approach allowed for the confirmation of the original diagnosis of liver cancer as stated in the medical records and confirmed the status of "non-exposure to thorotrast" by confirming the absence of thorium residuals in the tissue samples. This served the purpose of also allowing for the consideration of the biological relevance of the tumours by determining whether the types of tumours observed were similar to those observed in rodents that had been administered PB.

3.4 Conclusions

This systematic review set out to evaluate the epidemiological evidence designed to investigate the association of PB with liver cancer. The search strategy that was developed identified relevant studies that sought to address this review question. Among the studies able to specifically investigate the occurrences of liver cancers among patients predominantly treated with PB, the study by Olsen et al [109] scored highly against the quality assessment criteria. It was therefore considered to be reliable and robust in its investigation of the association of PB with liver cancer. An unfortunate limitation of the study was however the small study size of patients investigated and upon which the conclusions were based.

The study by Lamminpaa et al [112] reported a statistically significant increased risk of liver cancer associated with antiepileptic drugs (SIR; CI: 1.2-2.35) however the results have to be interpreted with caution. While this study obtained a quality score of 8 as a result of the scores of “adequate” for the different pre defined criteria for the quality scoring, what this score actually reflected was the ability of the study to attempt to address the different criteria considered to play an important factor in the investigation of the association of PB and liver cancer. However the study did not go far enough to address important areas of confounding and more crucially, the study was not able to identify the patients that were actually treated with PB.

Quantitative systematic review methods such as meta-analysis could not be applied to the assessment of the epidemiological evidence owing to the heterogeneity of the studies reviewed and the lack of sufficient data for such an analytical approach. The systematic qualitative methods employed however, allowed for the application of qualitative scores as a way of facilitating the transparent justification of any conclusions from the studies reviewed; this was in keeping with the objectives of the IPCS human relevance framework.

This review has highlighted the methodological difficulties associated with trying to investigate the association between PB and liver cancer. It also identified the limitations associated with current epidemiological evidence investigating the potential cancer risks associated with antiepileptic drugs. While it may not be feasible

to establish any new cohorts of epileptic patients taking PB, where possible, it may be useful to extend the work already done in the identified cohorts of epileptic patients. The latter could include the refinement of the data collected for the Finnish cohort in order to establish if possible, the number of reimbursements that were actually made for PB, therefore allowing the determination of the true number of liver cancer cases linked to patients that were taking PB. Such an attempt would add further weight to the currently available evidence and address issues of small sample sizes.

Regardless of the limitations highlighted from the study carried out by Lamminpaa et al [112], the large cohort investigated in this study makes it difficult to disregard the positive findings of association reported by the authors. The focus for any conclusions to be drawn has to be placed on this larger study. However, it is currently difficult to draw any firm conclusions about the effects of PB from potential patients exposed for reasons already stated.

These findings highlight the need for this study to be revisited. A nested case control study on the cohort identified by Lamminpaa et al [112] would enable the identification of patients that were exposed to PB and go a long way to refine the results obtained to enable conclusions to be drawn about the association of PB with liver cancer among the epileptic patients exposed.

This systematic review of the epidemiological data for PB has enabled the thorough and transparent evaluation of the weight of epidemiological evidence available for PB. Although no conclusions have been drawn about the association of PB with liver cancer owing to the unreliable evidence evaluated; the limitations associated with the interpreted findings have been explicitly described and the rationale for assessing the individual studies have been thoroughly described. The application of the systematic approach applied here would go a long way to improve the transparency of evidence evaluated as part of the IPCS MOA/HRF.

As discussed earlier in the introductory chapter, in addition to exploring the use of systematic review techniques for as a way of improving the transparency of evidence considered as part of the IPCS HRF, another important factor to consider in the development of the IPCS HRF, is the investigation of the extent to which non

conventional data can be incorporated into the MOA/HRF analysis. The latter will be explored in the next two chapters.

Chapter 4

Toxicogenomics: Methodology for the Transcriptional Analysis of the Hepatocarcinogenic effects of PB

This chapter provides a brief background to the subject area of toxicogenomics and details the methodology developed for the evaluation of publicly available transcriptomics (microarray) datasets to investigate the hepatocarcinogenic effects of phenobarbital (PB). The aim was to develop a methodology for the investigation of the effects of PB on gene expression changes in different species. In addition, the extent to which non-commercial or commercially available pathway analysis software could be used to investigate statistical and biologically relevant pathways was evaluated and the relationship between these functional pathway changes and clinical pathology end-points investigated. More importantly, this methodology was developed to evaluate how the results output from the pathway analysis software could be used to inform mode of action and human relevance analysis. More broadly, this methodology was developed to investigate the extent to which readily published information on toxicogenomics could be utilised meaningfully in risk assessment and how this might be improved.

4.1 Brief Background to Toxicogenomics

Technological advancements in the field of genomics has allowed for increased efficiency and speed in the sequencing of DNA allowing for complete sequencing of prokaryotic and eukaryotic genomes. One of the greatest achievements in recent history was the sequencing of the entire human genome, accomplished in 2001 [121-123]. These achievements has increased scientific understanding of biological and toxicological processes resulting in the study of the function of a genome in relation to gene expression (transcriptomics), protein levels (proteomics) or metabolites (metabonomics) [124]. Genomics technologies have been used for decades to aid the

mechanistic evaluation of adverse toxic effects. Traditionally the approach to evaluating the mechanism of action relied on evaluating a few genes at a time with the aim of discovering the gene function in relation to the observed toxic effect [124, 125]. Associated technological advancements surrounding these -omics approaches have led to the field of "toxicogenomics" which can be defined as *the study of the structure and output of the entire genome as it relates and responds to adverse xenobiotic exposure* [126]. Toxicogenomics involves the application of various aspects of functional genomics i.e. mRNA (transcriptomics/gene expression profiling), protein (proteomics) and metabolite (metabolomics) analysis and its associated technologies in toxicological assessments [124, 125, 127].

The technology behind transcriptomics is the DNA microarray. The DNA microarray consists of a small glass or a membrane to which potentially thousands of known gene-specific sequences are fixed at specific points. The mRNA extracted from biological samples (treated with chemicals of interest at specific doses and time points) are treated to enable hybridisation with the complimentary DNA strand on the glass or a membrane which enables the hybridised complex to be detected [128]. The advantage of DNA microarrays is that they enable the measurement of the gene expression of tens of thousands of genes at one time taking within the context of dose and time, thereby identifying genes that are associated with the relevant disease at the time and dose of measurement [127]. Some of the limitations associated with DNA microarrays are that the measurements are semi quantitative owing to issues such as cross hybridisation and sequence specific binding anomalies. The number of samples that can be processed at any one time is also a limitation, as well as the amount of time it takes to process and analyse samples (it can take several days to generate the data required and take a considerable amount of time to analyse the data generated) [127].

Unfortunately gene expression profiling alone is insufficient to enable a thorough understanding of disease outcomes. Exposure to toxicants results in abnormalities in protein production and function. Owing to the fact that there is no direct functional correlation between the expression of individual genes and their corresponding proteins, other toxicogenomics tools such as proteomics are employed to investigate the expression of proteins that are affected by toxicant exposure [127]. Proteomics is

defined as the “*systematic analysis of expressed proteins in tissues, by isolation, separation, identification and functional characterisation of proteins in a cell, tissue or organism*” [127]. Proteins perturbed by toxicant exposure are functionally annotated and validated. Proteomics requires the use of 2-dimensional (2-D) gel electrophoresis for protein separation followed by mass spectrometry analysis of the relevant proteins. One of the main limitations of the technology used in proteomics is the low sensitivity of 2-D gel electrophoresis. The detection of low abundance proteins can be difficult owing to the presence of high abundance proteins (e.g. albumin and immunoglobulin) [127]. To mitigate against this problem, these high abundance proteins have to be removed from the protein samples using immunoaffinity techniques which allow more samples to be loaded onto the gels thereby enabling the visualisation of the lower abundant proteins[127, 129].

Notwithstanding the benefits of both transcriptomics and proteomics (especially when used in combination), neither technique provide insight into the metabolic and cellular functions of the whole organism under investigation in response to toxicant exposure. The final tool applied in toxicogenomics is metabonomics which is based on the idea that exposure to toxicants leads to changes in the relative concentrations of endogenous biochemicals [127]. Metabonomics is defined as the “*quantitative measurement of the dynamic multiparametric metabolic response of living systems to the pathophysiological stimuli or genetic modification*” [130]. Metabolites found in bodily fluids (e.g. urine, blood, cerebrospinal fluid) are affected by toxicant exposure as these metabolites are in equilibrium with surrounding cells and tissues [127]. One of the main advantages of metabonomics is that samples are more readily from human subjects to enable the analysis of the different metabolites. High resolution NMR spectroscopy is used for the detection of metabolites in biological samples. Results obtained from metabonomics analysis is intended to complement information obtained from gene expression profiling and proteomics [127, 129].

Owing to the availability of publicly available data for the selected case study compound (PB), this thesis will address only issues associated to transcriptomics. The following sections describe briefly some of the techniques and methods involved in the identification and evaluation of gene transcripts.

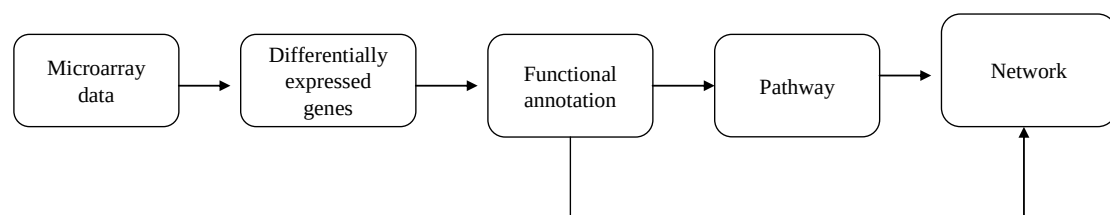
4.1.1 Microarray Study Design

The main goals of microarray studies are to identify genes that are differentially expressed under given conditions. As such, microarray study designs can be grouped into three different categories;

- **Class comparison:** these studies aim to identify differentially expressed genes between two or more groups.
- **Class prediction:** these studies aim to predict the class membership of a given sample on the basis of its gene expression profile.
- **Class discovery:** the aim of these studies is to be able to group together samples or sets of genes on the basis of shared similar gene expression profile.

Regardless of the study objectives, the main steps of microarray data analysis involve the identification of differentially expressed genes (presented in the form of lists of up/ down regulated genes or patterns of gene expression) and the evaluation of the biological relevance of the differential expression of individual or sets of genes (Figure 4.1) [128, 131]. These are discussed in more detail in the sections below.

Figure 4.1: Steps of Microarray Analysis



4.1.2 Identification of differentially expressed genes

The analysis of microarray data begins with the normalisation of the available data using different statistical methods that aim to reduce the experimental variability in

the dataset [132]. Different methods are used to compare datasets (i.e. comparison between experimental and control sample) to enable the identification of differentially expressed genes. The latter include identification of the level of fold change or the use of statistical methods to identify differentially expressed genes.

Fold Change

One of the simplest approaches for determining whether genes are differentially regulated is to compare the fold change between the experiment and control genes. A fold change of between two and three is normally considered to be indicative of a biological significant change between experiment and control. The main disadvantage of this simplistic approach to identifying differentially expressed genes lies in the methods used to decide on the threshold of significance. The arbitrarily chosen threshold level may not always be appropriate for every microarray experiment. The latter is especially true in cases where genes are affected below this two-fold threshold so they would not otherwise be selected as significantly different from the control. Under these circumstances, this can lead to reduced experimental sensitivity [132].

Use of Comparison Statistics

The use of the fold change method provides no idea of the reproducibility of the data observed. To address this, a statistical approach is used to identify genes that are observed to be perturbed compared to controls. The use of experimental replicates (biological⁶ or technical⁷) facilitates the consideration of the levels of variability in the data and therefore enables a confidence level or a *p*-value to be assigned. The choice of comparison statistics is dependent on the number of replicates and factors under consideration. The t-test is used for comparisons between two groups and ANOVA for comparisons of three or more groups [128, 131].

⁶ **Biological replicates** measure different samples on different arrays-this allows for the measurement of biological variability such as genetic variability or environmental effects.

⁷ **Technical replicates** measure the same sample on different arrays-this focuses on addressing the variations inherent in the techniques applied.

In addition to the use of comparison statistics, experimental techniques are sometimes employed to provide independent validation of results obtained from microarray experiments. Qualitative polymerase chain reaction (PCR) techniques are commonly applied for the validation of microarray experiments to test whether or not a particular gene (at mRNA or DNA level) is present or not [133]. Real Time Quantitative PCR (qPCR) is fast becoming the most accepted form of experimental validation. It requires only a small amount of biological material and the speed in which the experiments can be performed (30-45 minutes) and the user is able to carry out multiple gene expression measurements in a single reaction enabling the validation of multiple genes in a single reaction [133].

Correcting For Multiple Testing

The derivation of a p value for each individual gene often results in multiple tests being performed. In order to validate the statistical results obtained, it is therefore necessary to correct for multiple testing. Storey and Tibshirani [134] propose the calculation of the Q value to represent the "false discovery rate" (FDR). The FDR is "*the rate that truly null features are called significant*". The p value measures the level of significance in terms of the false positives rate for each individual gene, the Q provides a measure of the significance of each individual p value, thereby accounting for any multiple testing. When interpreting statistical results to identify differentially regulated genes or pathways (in the case of pathway analysis), a 5% Q value cut-off (as a cut-off criterion to identify differentially expressed genes) therefore provides a better indication of the proportion of significant genes (or pathways) that turnout to be false positives [131, 134].

4.1.3 Biological Relevance of Differentially Expressed Genes

To provide a means of analysing lists of genes within a biological context, annotation methods have been developed to facilitate biological function analysis of individual genes (or sets of genes) among researchers. A controlled vocabulary has been developed for this and is called "*ontology*". Ontology in the context of gene

annotation, can be defined as “*comprising a set of well-defined terms within well-defined relationships*” [135]. Depending on the availability of supporting literature, the annotations reflect biological knowledge about the particular genes as well as providing the basis and structure for which new genes can be annotated.

Ontologies have been developed to aid the conceptualisation of the biological functional underpinnings of gene expression. The latter have included Gene Ontology (GO) [135], GeneX (<http://ncgr.org/genex/>), eVOC [136] and KEGG orthology (KO) [137]. Gene Ontology (<http://www.geneontology.org>) is the most widely used. The GO project provides an expert curated public database that attributes genes to the various biological categories including biological processes, molecular function and cellular components.

Pathway analysis

Although lists of differentially expressed genes can be created using methods described previously, it is more relevant to consider these genes in the context of their biological significance. Gene changes do not always relate directly to the experiments carried out since genes do not operate independently of each other but rather, operate within the realms of many functional contexts in an interdependent manner [138]. By placing the emphasis on the evaluation of the relationship between different genes under specific experimental conditions, the evaluation of single genes/proteins on an individual basis would also result in the loss of information.

Pathway analysis of microarray data allows for the integration of normalised array data with their functional annotations such as gene ontology. Analysis involves placing significantly regulated genes that have been functionally annotated using gene ontology or other suitable functional terms, into pre-compiled pathways. This allows for the consideration of the physical and functional interactions between genes rather than the assessment of individual genes on the basis of functional annotation alone [138, 139]. The latter can potentially lead to the identification of more subtle changes in gene expression rather than just compiling lists of differentially expressed genes. There are various commercial and academic web-accessible tools available for

investigating pathways. Pathways are manually compiled on the basis of available literature. Considerable effort is required on the part of curators whose job it is to continually update pathways on the basis of new evidence [138].

4.2 ***Development of Methodology for Transcriptomics Analysis***

4.2.1 *Search Strategy to Identify Relevant Microarray Studies for PB*

The search for microarray data for PB focused on two microarray repositories; ArrayExpress managed by the European Bioinformatics Institute (EBI) and Gene Expression Omnibus (GEO) managed by the National Institute for Biotechnology Information (NCBI). Both repositories comply with Minimum Information about a Microarray Experiment (MIAME); a set of guidelines that outline the minimum set of information that are required to describe adequately a microarray experiment [140].

The single search term “phenobarbital” was used to search the datasets in ArrayExpress. No filters were applied to the search to limit the search to any species or type of array. This search identified 6 studies.

Datasets within the GEO database were found using the search terms: [“phenobarbital” AND “liver”]. The search produced 17 different studies.

Selection of Studies

Although the main emphasis was on obtaining studies that primarily focused on investigating PB, where available, studies that employed PB as a positive control were also selected for further investigation. Five out of the six studies found in ArrayExpress and 7 out of the 17 studies found in GEO were considered relevant for the purposes of the investigation. The 5 relevant studies obtained from ArrayExpress were found to be the same as that obtained from GEO.

In addition to the data obtained from ArrayExpress and GEO, unpublished data linked to one of the studies deposited in GEO was obtained via personal communication from the study investigators [141].

Selected Dataset

The datasets were exported from GEO. The 7 different experiments classified as GEO series are summarised in Table 4.1. Microarray data for five different time points (10hrs, 12hrs, 2, 4 & 30 days) were obtained from four separate mouse experiments, four time points (6hrs, 1, 3 & 5 days) were obtained from two separate rat experiments and only one time point from one human hepatocytes experiment were obtained from the repository. A full summary of the experiments from which the data were obtained is provided in Appendix IV (A4.1).

4.2.2 Pre-processing of Microarray data (Statistical Analysis of Microarray Data)

The statistical manipulation of the microarray data was carried out in consultation with and based on the advice from Susan Hester, PhD, a Genomics expert at the United States Environmental Protection Agency.

The raw datasets downloaded from the GEO repositories comprised the signal intensities from the respective microarray scans. The files were imported into the Gene Expression Data Analysis system Rosetta Resolver (Version 7.1) (Rosetta Inpharmatics, Kirkland, WA). The software was used to identify sequences within the treatment groups (PB) that were statistically different from the control groups for each study. Using the Rosetta Resolver error model, the microarray scans were converted to sets of normalised data called “intensity profiles” [142].

Table 4.1: Summary of experimental data exported from GEO

GEO series (Experiment)	Authors/ study contributors	Species & strain	Type of study	Time points investigated	Route of PB administration	Dose	Biological replicate samples per time point investigated
GSE6721	Rezen et al [143]	Mouse (C57BL/6)	<i>In vivo</i>	10hrs	Intraperitoneal injection.	50mg/kg in vehicle (5% DMSO in corn oil)	4 PB samples: 4 controls samples
GSE16777	Nesnow et al [144]	Mouse (CD-1)	<i>In vivo</i>	2 ⁸ , 4 & 30 days	Diet	5.9 mg/kg/day	5 PB samples: 5 controls samples
GSE14712	Ren et al [145]	Rat (Sprague-dawley)	<i>In vivo</i>	6hrs, 1 & 5 days	Gavage	100 mg/kg	5 PB samples: 5 controls samples
GSE9387	No publication from study	Rat (information on strain used not available)	<i>In vitro</i>	3 days	In vitro exposure via medium	200µM	3 PB samples: 3 controls samples
GSE10410	Goetz et al [146]	Human	<i>In vitro</i>	3 days	In vitro exposure via medium	1000µM	4 PB samples: 4 controls samples
GSE12489*	Tamasi et al [147]	Mouse (C57BL/6J)	<i>In vivo</i>	12 hours	Intraperitoneal injection.	100 mg/kg	6 PB samples: 6 controls samples
GSE12529*	Tamasi et al [147]	Mouse (C57BL/6J)	<i>In vivo</i>	12 hours	Intraperitoneal injection.	100 mg/kg	6 PB samples: 6 controls samples

⁸ The Day 2mouse dataset from experiment GSE16777 was obtained via personal communication by one of the study investigators involved in the published study by Nesnow et al., 2009. Although the dataset at Day 2 was not published, the treatment of the mouse followed the same protocol for in-life procedures, mRNA isolation and hybridization, as that of the treatment of the mice at Days 4 and 30.

*The datasets obtained for GSE12489 and GSE12529 were excluded from the studies owing to errors in the file obtained from GEO and the resulting pre-processing difficulties encountered in Rosetta Resolver (V7.1).

One-way ANOVA with Error-Weighting

Analysis of Variance (ANOVA) is used to test for difference between two or more independent groups and is based on comparison of the variation between the different groups with variation within groups. In the processing of the microarray data, ANOVA also measures the within-group variance. The use of small numbers of biological replicates in microarray studies can lead to small within-group variance resulting in a large P-value and high false positive rates, whereby genes are highlighted as differentially expressed when they are in fact not. To mitigate this problem, the Resolver software enables the user to select the error-weighting one-way ANOVA test function for the statistical analysis. The error weighting within Resolver provides a conservative estimate of the measurement errors attributed to factors such as sample preparation, labelling and dye related bias, hybridization, chip quality variation, chip scanning and image feature extraction. Carrying out error weighting as well as the ANOVA test therefore serves the purpose of improving the variance estimation [142] (Rosetta Inpharmatics, Kirkland, WA).

The one-way ANOVA was performed on the intensity data associated with the different treatment groups (PB or controls) and factor levels (number of groups under comparison = 2; PB & control). The one-way ANOVA test compared the between-group variance to the within-group variance of the measurements. The calculated within-group variance was the variation of replicated measurements within individual treatment groups. The between-group variance was measured among the mean measurements of the different treatment groups. The means of the treatment groups were identified as significantly different if the between-group variance was significantly larger than the within-group variance (Rosetta Inpharmatics, Kirkland, WA).

Multiple Test Correction (Benjamini and Hochberg FDR)

Multiple test correction was carried out on the different microarray datasets to reduce the chances of false positives (genes identified to be statistically different from controls when in reality they are not). The Benjamini and Hochberg false discovery rate (FDR) was applied to all datasets investigated to adjust the p-values derived from the multiple statistical tests and correct for the occurrence of false positives.

Differentially Expressed Genes (DEGs)

Statistically filtered sequences were identified by carrying out the one-way ANOVA with a false discovery rate (Benjamini-Hochberg test) of $p \leq 0.05$. For smaller datasets (rat, all time points and mouse 10 hrs) the applied p -value of 0.05 for FDR was observed to be too stringent (producing exceptionally small numbers of DEGs), as such a $p \leq 0.25$ was applied instead (the justifications for this approach and the associated limitations will be discussed in detail chapter 5, see section 5.6.7.).

4.2.3 Selection of Pathway Analysis Tools

A considerable amount of time was spent investigating the applicability of various freely available academic web-accessible tools to evaluate the data obtained. Two non-commercial pathway analysis tools tested included KOBAS⁹ and DAVID¹⁰. The KOBAS software required the annotation of the genes in KEGG¹¹ orthology (KO) and the first step of using KOBAS involved the annotation of the genes with KO terms. The non-commercial database KEGG, available on the public domain, was developed to combine genomic information with functional information (also known as enrichment analysis). KEGG functionally assigns genes into networks of interacting

⁹ KOBAS - KEGG Orthology-Based Annotation System (<http://kobas.cgi.pku.edu.cn>)

¹⁰ DAVID - Database for Annotation, Visualization and Integrated Discovery (<http://david.abcc.ncifcrf.gov>).

¹¹ KEGG - Kyoto Encyclopaedia of Genes and Genomes (<http://genome.jp/kegg>)

molecules, to take into account the fact that biological functions are the result of interacting genes. Three databases exist within KEGG:

- PATHWAY which represents various functions in the form of a network of interacting genes,
- GENES which provides a database to catalogue completely sequenced and partial genomes and
- LIGAND which is a database for chemical compounds in the cell enzymes and enzymatic reactions.

The KO has also been applied to functionally annotate each molecule or gene [148].

While KOBAS could be used to find the most frequent and statistically significant enriched pathways [149], one of the major shortcomings of the software and the annotation system was the unavailability of the KO terms for all the genes of interest and therefore the inability of KOBAS to annotate all the genes uploaded onto the system. This led to the loss of significant numbers of genes that would have otherwise been analysed. In addition, KOBAS could only be used for pathway analysis so a separate software was required to carry out the network analysis.

Consideration of the interactions of individual genes (using pathway analysis) is just one aspect of the overall biological process. Interactions of more than one pathway result in the formation of a regulatory network. The components of each network change according to the biological context, therefore, the pathways involved may sometimes contain only a few genes as it is entirely context dependent [150]. It was therefore essential to carry out network analysis in order to consider the interactions of individual pathways since biological pathways often involve more than one pathway.

Similar problems were encountered using the DAVID software, which annotates genes using Gene Ontology (GO). The software was unable to analyse the large lists of genes uploaded and often resulted in the loss of significant numbers of genes presumably because all the genes could not be annotated to GO. In addition, the software could not be used to carry out analysis to determine the statistical significance for the association of the genes of interest with pre-compiled pathways

(groups of genes grouped *a priori* according to their functions) as defined by GO. It was therefore difficult to determine which genes were most important and which pathways were more significant than others [151, 152].

Owing to the problems encountered using the non-commercial tools, the commercial software Ingenuity Pathway Analysis, IPA (www.ingenuity.com) was employed to carry out the analysis of the genes of interest. IPA enabled the evaluation of both the biological pathways and networks that featured genes of interest. The methodology associated with the analysis is discussed in greater detail in the following sections.

4.3 Pathway and Network Analysis of DEGs Using Ingenuity Pathway Analysis (IPA)

The DEGs obtained from Rosetta Resolver were evaluated using Ingenuity Pathway Analysis [153]. IPA was used to gain a better understanding of the biology and the significant interactions involved within groups of genes found to be differentially expressed. The biological significance of the changes observed within the DEGs identified was investigated through the use of tools within IPA such as network analysis generation, canonical pathways and toxicological function/ lists analysis.

4.3.1 Core Analysis in IPA

IPA was used to run a Core Analysis of the dataset. This analysis enabled the rapid assessment of the signalling and metabolic pathways, molecular networks and biological processes that were most significantly perturbed, within the dataset uploaded. The Analysis in IPA enabled groups of genes to be mapped onto Canonical Pathways (well characterized cell signalling and metabolic pathways that were most relevant to the uploaded dataset), IPA Tox Lists (sets of genes known to be perturbed upon compound treatment that will eventually lead to a measurable pathology) and Toxicity Functions (functions that relate to hepatotoxicity and marked clinical endpoints). The selected results outputs provided in the form of the Tox lists, Canonical Pathways and Tox functions were all different options available in IPA to

view the results. The outcome provided by the three results outputs relate to each other in the sense that each output enables the user to view and interpret the results with the desired biological context. The direct and indirect relationships between the genes were also explored using tools that enabled the functional representation of genes that were involved in relevant Toxicity functions [153].

It is important to note that regardless of the selection of the type of species dataset selected under the general settings when creating a Core Analysis (Figure 4.2), the human orthologs for each gene were provided as the default in the result outputs. This was unchangeable in IPA and it meant that genes produced as part of the results from the rat and mouse experiments had to be changed manually where necessary.

Figure 4.2: Screen shot showing the analysis page in IPA which allowed the different criteria for the analysis for each dataset

NEW

Genes and Chemicals

Functions and Diseases

Pathways and Tox Lists

Enter gene names/symbols/IDs or chemical/drug names here

SEARCH

Advanced Search

INGENUITY ANSWERS

Create Core Analysis - [analysis : DEG MOUSE 2D.xls]

Filters and General Settings for Analysis

General Settings

Data Sources Argonaute 2...

Species Mouse

Tissues & Cell Lines All

Select all

Mammal

Human

☒ Mouse

Rat

Uncategorized (e.g. chemicals)

☒ Stringent filter (filter molecules and relationships)

☐ Relaxed filter (filter molecules)

Analysis Filter Summary

Consider only molecules and/or relationships where (species = Mouse) AND (data sources = Additional interactions OR Ingenuity Expert Findings OR MicroRNA-mRNA interactions OR Protein-protein interactions)

SAVE AS DEFAULT FILTERS

Expression Value Parameters

Expression Value Type

Expression Value Cutoff

Range

Focus On

1: Fold Change

-24.9304 to 100.0

Both Up/Downregulated Identifiers

Define expression value parameters.

2030 molecules eligible for generating networks

RECALCULATE

ADVANCED SETTINGS

More Info

Dataset File Mapping for "DEG MOUSE 2D.xls"

Mapped IDs (5485)

Unmapped IDs (535)

All IDs (6020)

Network Eligible (2030)

Functions/Pathways/Lists Eligible (1845)

ADD TO MY PATHWAY

ADD TO MY LIST

CUSTOMIZE TABLE

Rows: 1 - 50

	Fold Change	ID	Notes	Symbol	Entrez Gene Name	Location	Type(s)	Drug(s)
☐	↓-1.354	1434906_at		0610005C13RIK	RIKEN cDNA 0610005C13 ge	unknown	other	
☐	↓-1.401	1428926_at		1110003O08RIK	RIKEN cDNA 1110003O08 ge	unknown	other	
☐	↓-1.440	1451250_at		1110003O08RIK	RIKEN cDNA 1110003O08 ge	unknown	other	

Source: Ingenuity Pathway Analysis™

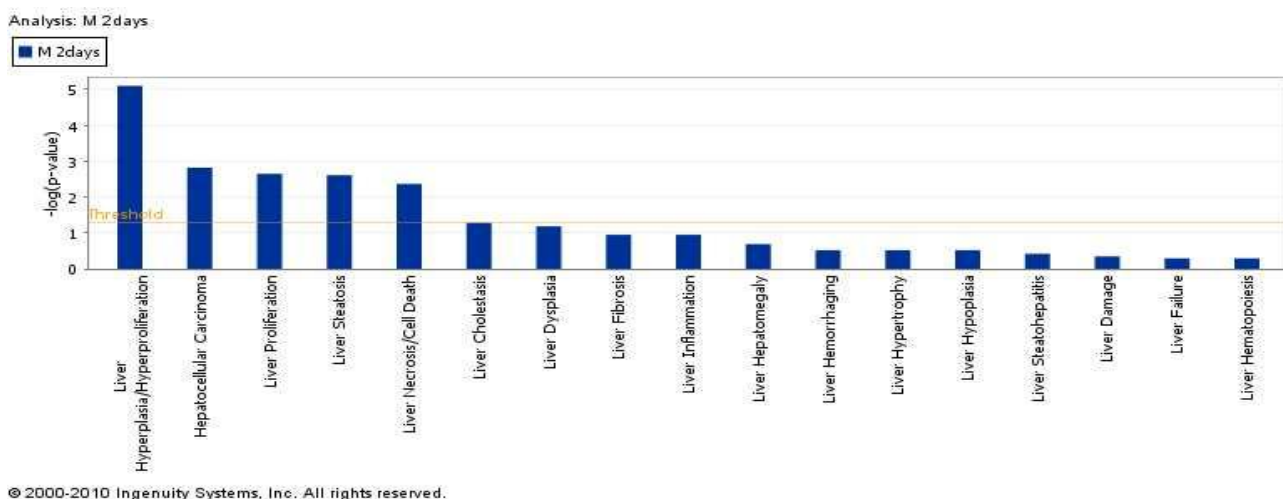
4.3.2 Levels of Significance/ P-value Calculated for Functions and Pathways in IPA

Significance values are provided in Functional Analysis for a dataset as a measure of the association between a set of Functional Analysis genes in the uploaded experiment and a given process or pathway. IPA applied the default of $p\text{-value} \leq 0.05$ which was left unaltered [153].

4.3.3 IPA Result Output: Functional Analysis of dataset

The functional analysis identified the biological functions and/or diseases that were most significant to the dataset. Genes from the dataset that met the p-value cut off and were associated with biological functions and/or disease in the IPA knowledge base were considered for the analysis. Fischer'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 [153].

Figure 4.3: An example of the results output for the functional analysis in IPA. The bar chart produced shows the different toxicological functions into which the datasets were grouped.



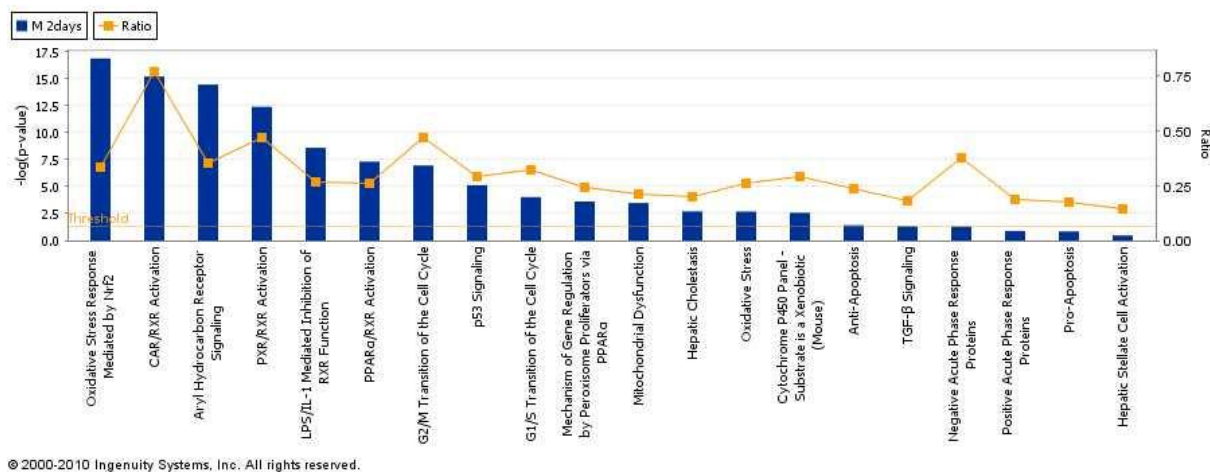
Source: Ingenuity Pathway Analysis™

(The figure shows the Toxicity Function analysis from the mouse day 2 dataset).

4.3.4 IPA Result Output: Toxicology Lists (Tox List)

The toxicity lists produced by IPA comprised of molecules known to be involved in a particular type of toxicity. The lists of genes were grouped according to critical biological processes and adaptive or reparative responses as observed upon exposure to xenobiotic chemicals. The Tox Lists enabled closer examination of the functional changes across the different time points studied for genes exposed to PB [153] .

Figure 4.4: An example of the results output for the Toxicity List in IPA. The bar chart produced shows the different types of toxicities into which the datasets were grouped.



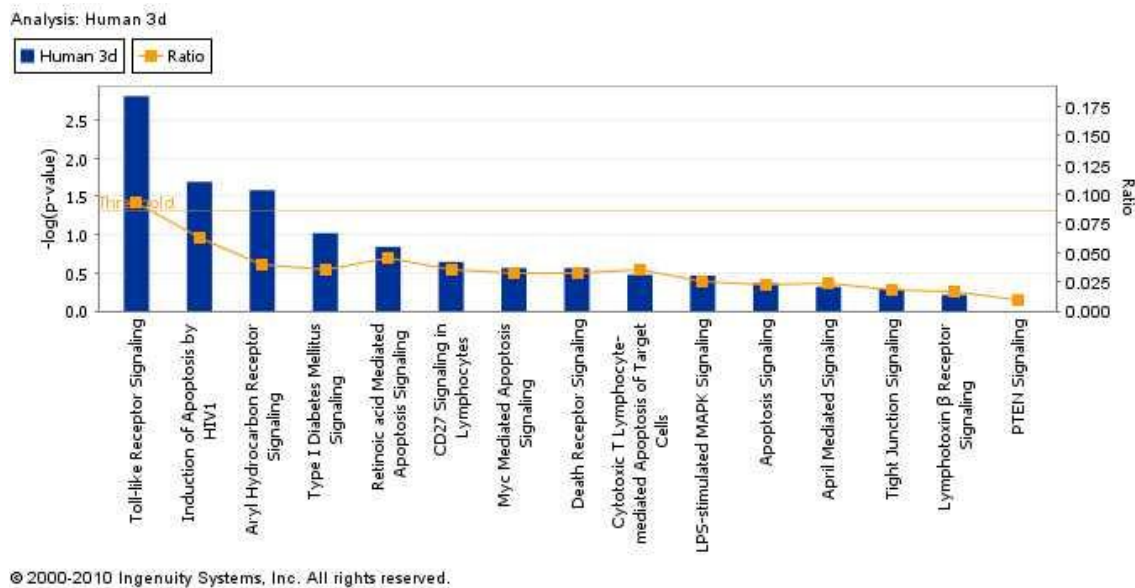
Source: Ingenuity Pathway Analysis™
(The figure shows the Tox List from the mouse day 2 dataset).

4.3.5 IPA Result Output: Canonical Pathways

Canonical pathway analysis identified the pathways from the IPA library of canonical pathways that were most significant to the dataset. Genes from the dataset that met the p-value cut off and were associated with a canonical pathway in the IPA knowledge base were considered. The significance of the association between the dataset and the canonical pathway was measured in two ways: 1) A ratio¹² of the number of genes that map to the canonical pathway was displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone [153].

¹² The ratio is the number of molecules in a given pathway that meet the cut criteria divided by the number of molecules that make up that particular pathway.

Figure 4.5: An example of the results output for the Canonical Pathways produced in IPA. The bar chart produced shows the different pathways into which the datasets were grouped.



Source: Ingenuity Pathway Analysis™

(The figure shows the apoptosis signalling canonical pathway from the human day 3 dataset).

4.3.6 Graphical representation of Networks

The displayed genes that form a network or pathway represented the molecular relationships between genes/gene products. Genes or gene products were represented as nodes, and the biological relationship between two nodes was represented as an edge (line). All edges were supported by at least one reference from the literature, from a textbook, or from canonical information stored in the IPA knowledge base. Human, mouse and rat orthologs of a gene were stored as separate objects in the IPA knowledge base, but were represented as a single node in the network. The intensity of the node colour denoted the degree of up-(red) or down-(green) regulation. Nodes were displayed

using various shapes that represented the functional class for the gene product. Edges were displayed with various labels that described the nature of the relationship between the nodes (e.g. P for phosphorylation, T for transcription) [153]. An example of such a diagram is A4.2.1 located in Appendix IV.

4.4 Biomarkers for Non-genotoxic Carcinogenicity and Reference Genes for Hepatocellular Carcinoma

A list of gene signatures for predicting non-genotoxic carcinogenicity were obtained from 2 separate investigations that attempted to identify biomarkers for non-genotoxic carcinogenicity. Fielden et al [154] identified 37 gene signatures that were independently tested against 47 chemicals. The authors reported assay sensitivity and specificity of 86% and 81%. Nie et al [155] identified 6 signature genes to predict nongenotoxic carcinogenicity and reported 88.5% prediction accuracy estimated by cross-validation. A review carried out by Pei et al [156] investigating the use of omics-based methods applied to the study of hepatocellular carcinoma identified 19 genes that had been highlighted in hepatocellular carcinoma microarray studies.

The 62 genes identified from the 3 different studies were converted to Entrez Gene identifiers using tools in PubMed and the corresponding mouse, rat or human orthologs were identified. The different gene symbols/names associated with each gene identifier were also obtained to enable efficient comparison between the different datasets. The DEGs from all time points investigated for each species were combined, duplicates were removed and then they were compared with the biomarkers and reference genes identified using the VLOOKUP tool in Microsoft Excel.

Chapter 5

Results of Transcriptional Analysis to Investigate the Hepatocarcinogenic Effects of Phenobarbital

This chapter details the results of the transcriptional analysis carried out on publicly available transcriptomics (microarray) data to investigate the hepatocarcinogenic effects of PB. The aim of this comparative analysis of microarray data was to investigate the effects of PB on gene expression changes in different species and to determine how the functional pathway changes observed, could be related to clinical pathology endpoints for the liver (e.g. proliferation, necrosis/inflammation or Cholestasis) and more importantly, how these results could be used to inform mode of action and human relevance analysis. More broadly, this was an investigation into how readily published information on toxicogenomics could be utilised meaningfully in risk assessment and how this might be improved.

5.1 *Analysis of Differentially Expressed Genes (DEGs) using Ingenuity Pathway Analysis (IPA)*

Groups of DEGs generated from Rosetta Resolver (Table 5.1) were analysed using the Core Analysis function in IPA. This allowed the interpretation of the dataset within the context of biological processes. The evaluation of the results focused on assessing the relevance of the toxicity functions associated with the datasets of interest.

Table 5.1: Statistically significant DEGs obtained from analysis carried out in Rosetta Resolver

Species	Rat				Mouse				Human
Type of study	<i>In vivo</i>		<i>In vitro</i>		<i>In vivo</i>				<i>In vitro</i>
Time Point	6hrs	1d	5d	3d	10hrs	2d	4d	30d	3d
Number of DEGs	123	321	305	25	327	3045	3771	1268	1663

5.2 Toxicology Lists (Tox List)

The lists of genes from the different time points available for the datasets from different species were mapped onto the IPA Tox List¹³ upon the application of the Core Analysis¹⁴ function in IPA. The analysis identified a series of 46 different Tox Lists that were associated with the genes obtained from one or more of the different species at different time points. The IPA Tox Lists were produced in the form of bar charts. On the basis of evidence obtained from the literature (with regards to the basis of known mechanisms of hepatotoxicity and their involvement in the postulated key events that are involved in PB induced MOA for liver tumour formation as described in chapter 2), 18 out of the 46 observed Tox Lists were selected for further investigation. The transcriptional changes across the 18 selected Tox Lists were investigated and the temporal relationships of the key gene changes examined. The transcriptional changes of the genes involved in the different Tox Lists were summarised in tables that were essentially simplified heat maps.

Table 5.2 provides a summary of the Tox Lists produced by IPA and their occurrence within the rat, mouse and human datasets. The gene changes involved in a selection of toxicities/biological processes relating to hepatotoxicity were represented in table format to enable closer examination of the temporal relationships of the gene changes across the different datasets.

A full list of the temporal comparison of the genes involved in the selected Tox Lists, are provided in Appendix V (A5.1.1 to A5.1.20).

¹³ See Chapter 4, section 4.3.4 for a description of the IPA Tox Lists

¹⁴ See Chapter 4, section 4.3.1 for a description of the IPA Core Analysis function

Table 5.2: Table showing Ingenuity Pathway Analysis (IPA) Toxicity Lists produced from all datasets

Ingenuity Toxicity Lists	Time points studied								
	Rat				Mouse				Human
	6hrs	1d	3d	5d	10hrs	2d	4d	30d	3d
Oxidative Stress Response Mediated by Nrf2	X*	X*		X*	X*	X*	X*	X*	X*
CAR/RXR Activation	X*	X*	X*	X*	X*	X*	X*	X*	X*
Aryl Hydrocarbon Receptor Signalling	X	X*		X*	X*	X*	X*	X*	X*
PXR/RXR Activation	X*	X*	X	X*	X*	X*	X*	X*	X*
Xenobiotic Metabolism	X*	X*	X	X*	X*	X*	X*	X*	X*
Fatty Acid Metabolism	X*	X*	X*	X*	X*	X*	X*	X*	X*
LPS/IL-1 Mediated Inhibition of RXR Function	X*	X*		X*	X*	X*	X*	X*	X*
PPAR α /RXR Activation		X		X	X*	X*	X*	X*	X*
G2/M Transition of the Cell Cycle						X*	X	X*	
p53 Signalling						X*	X	X	
FXR/RXR Activation	X	X*	X	X*	X*	X*	X	X*	X*
G1/S Transition of the Cell Cycle						X*		X	
Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR α		X*		X	X*	X*	X	X*	X
Hypoxia-Inducible Factor Signalling		X*		X	X	X	X	X	
TR/RXR Activation		X*	X	X	X	X*		X	X*
Mitochondrial Dysfunction	X	X*		X	X*	X*	X	X	
Cytochrome P450 Panel - Substrate is a Xenobiotic (Human)	X	X*		X	X*	X*	X	X	X*
Hepatic Cholestasis		X*		X	X*	X*	X	X*	X*
Oxidative Stress		X*		X*	X*	X*	X*	X*	X*
Cytochrome P450 Panel - Substrate is a Xenobiotic (Mouse)	X	X*		X*	X*	X*	X*	X	X*
RAR Activation	X	X*				X*	X	X	
NF κ B Signalling Pathway						X*		X	X
Cytochrome P450 Panel - Substrate is a Xenobiotic (Rat)	X*	X*		X*	X	X*	X*	X*	X
Cytochrome P450 Panel - Substrate is a Fatty Acid (Mouse)				X*	X	X*		X	X
Cholesterol Biosynthesis						X*	X	X	X
Anti-Apoptosis						X*	X	X	
TGF- β Signalling		X		X	X	X*	X	X	

Ingenuity Toxicity Lists (continued)	Time points studied								
	Rat				Mouse				Human
	6hrs	1d	3d	5d	10hrs	2d	4d	30d	3d
Cytochrome P450 Panel - Substrate is a Fatty Acid (Human)				X*	X	X*		X	X
Negative Acute Phase Response Proteins		X		X*	X	X*	X	X	X*
Cytochrome P450 Panel - Substrate is a Fatty Acid (Rat)				X*	X	X*		X	X
Cytochrome P450 Panel - Substrate is an Eicosanoid (Human)					X*	X			X
Cytochrome P450 Panel - Substrate is an Eicosanoid (Rat)					X*	X			X
Positive Acute Phase Response Proteins		X*	X*	X		X		X*	X
Cytochrome P450 Panel - Substrate is an Eicosanoid (Mouse)				X	X	X			X
Pro-Apoptosis						X	X	X	X
Hormone Receptor Regulated Cholesterol Metabolism						X	X	X	X*
LXR/RXR Activation		X*	X*	X	X	X		X	X*
Hepatic Stellate Cell Activation				X		X	X	X	X*
VDR/RXR Activation	X	X	X		X	X		X	X
Cytochrome P450 Panel - Substrate is a Vitamin (Human)						X		X	
Cytochrome P450 Panel - Substrate is a Vitamin (Mouse)						X		X	
Cytochrome P450 Panel - Substrate is a Vitamin (Rat)						X		X	
Cytochrome P450 Panel - Substrate is a Sterol (Human)		X*					X	X	X
Cytochrome P450 Panel - Substrate is a Sterol (Mouse)		X*					X	X	X
Cytochrome P450 Panel - Substrate is a Sterol (Rat)		X*					X	X	X
Hepatic Fibrosis	X	X	X	X*	X		X*	X	X

NB: The crosses at the different time points indicate that the genes within that particular dataset are involved in the indicated toxicity.

X* – Indicate the statistically significant (p-value ≤ 0.05) association of the genes within the species at the time point and the toxicity indicated.

5.2.1.1 *CAR/ RXR Activation (Appendix A5.1.1)*

The constitutive androstane receptor (CAR) is part of a steroid family of nuclear receptors. Following activation by compounds like PB, CAR binds to the retinoid X receptor (RXR) forming a heterodimer, which binds to upstream enhancers of target genes, including CYP2B genes and others that are involved in drug metabolism [71]. The “CAR/ RXR activation” list consists of genes associated with CAR/ RXR activation [153].

Although no common genes were found across the different datasets, the majority of the genes involved in the CAR/ RXR activation Tox List across all species were up-regulated and involved in phase I and II drug metabolism. The latter included the cytochrome P450 enzymes (CYP2B, CYP2C), aldehyde dehydrogenase (ALDH1A1), sulfotransferases, glutathione S- transferases (GST). Changes in CAR (NR1I3) itself were only observed at Day 2 carried out in the mouse where it was down-regulated. Retinoid X receptor alpha (RXRa) was observed to be down-regulated in the mouse at two days but upregulated at Day 30. The latter was also down-regulated in the human dataset at Day 3.

5.2.1.2 *PXR/ RXR Activation (Appendix A5.1.2)*

The pregnane X receptor (PXR) is a nuclear receptor activated by compounds such as PB. It forms a heterodimer with RXR, which binds to enhancer regions of target genes such as some cytochrome P450 enzymes involved in drug metabolism [71].

Phase I and II drug metabolising enzymes were consistently up-regulated across all species and time points. The nuclear receptor pregnane X receptor (PXR, NR1I2) was also up-regulated but only at day 4 in the mouse.

Genes found to be down-regulated at one or more days were those known to play a role in hepatic energy metabolism. The latter were only observed in the mouse and included the

forkhead transcription factor (FOXO1), glucose-6-phosphatase (G6PC) and 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (HMGCS2). In addition, hepatocyte nuclear factor 4 α , insulin-like growth factor binding protein 1, peroxisome proliferator-activated receptor γ co-activator 1 α , peroxisome proliferator-activated receptor α (HNF4A, IGFBP1, PPARGC1A, PPARG) and protein kinase, cAMP-dependent, regulatory, type I, α (PRKAR1A) were down-regulated at one or more days. RXRa was also down-regulated in both the mouse and human datasets at days 2 and 3 respectively.

5.2.1.3 *PPAR α / RXR Activation (Appendix A5.1.3)*

The perioxosome proliferator-activated receptor alpha (PPAR α) is a ligand activated transcription factor that belongs to the family of nuclear receptor and forms a heterodimer with RXR [157].

The majority of the gene changes observed were from the dataset collected from the mouse. Genes found to be up-regulated within this list of genes included the cytochrome P450 family of enzymes (CYP2C) involved in drug metabolism, synthesis of cholesterol steroids and other lipids. Other genes found within this list of genes fell predominantly within the roles of cellular growth, proliferation, fatty acid metabolism and apoptosis.

RXR was observed to be down-regulated in both the mouse (days 2 & 30) and human (day 3) dataset.

5.2.1.4 *Mechanism of gene regulation by Peroxisome Proliferators via PPAR α* (Appendix A5.1.4)

One response to exposure to foreign compounds is the occurrence of peroxisomal proliferation. Peroxisomes are microbodies that are abundant in hepatocytes and have been observed to increase after exposure to certain compounds [158]. The induction of peroxisome proliferation is mediated by the ligand dependent nuclear receptor, PPAR α .

Changes in genes involved in this Tox List occurred predominantly in the mouse between days 2 and 30. There were 31 different observed gene changes in the mouse compared to 10 and two from the rat and human dataset, respectively. Although the results of the two genes in the human dataset (RXR, IKBKG) were similar to the results observed in the mouse, they were not specific to PPAR as these genes were observed to occur across many of the other Tox Lists evaluated. Three out of the 10 genes in the rat dataset showed contrasting results to the mouse. The transcription regulator CITED2 was down-regulated in the rat at day 1 but up-regulated in the mouse at day 4. The heat shock protein, HSP90AA1 (known as HSP86 in the rat) was down-regulated in the rat at day 1 and observed to be up-regulated in the mouse at 10 hrs and 2 days. The RAF1 gene was up-regulated in the rat at day 5 but down-regulated in the mouse at 10hrs and at days 2 and 30.

With the exception of one gene (GRB2), which was up-regulated at day 2 and down-regulated at day 4, all other genes maintained a consistent direction of regulation at either 1 or more days within the mouse dataset.

Most of the genes identified in this list are involved in lipid metabolism, cell proliferation, apoptosis and oxidative reduction.

5.2.1.5 *Oxidative Stress & Oxidative Stress Response Mediated by Nrf2* (Appendix A5.1.5 & A5.1.6)

In the presence of foreign compounds like PB, reactive oxygen species may be produced. The oxidation and reduction cycle that ensues, results in the donation of an electron to oxygen to produce superoxide. Oxidative stress results from an imbalance between the production of reactive oxygen species produced and the detoxification of reactive intermediates [87].

IPA identified 133 genes within the Tox List of genes involved in Oxidative Stress mediated by Nrf2. This comprised of 86 genes from the mouse, 29 from the rat and 18 from the human. In addition, IPA grouped another 25 genes within a separate Tox List, Oxidative Stress which comprised of 18 genes from the mouse, 11 from the rat and 5 from the human dataset.

Metabolising enzymes with key roles in the detoxification of xenobiotics to reduce cellular stress were observed to be up regulated. The latter included glutathione transferases (GSTs), dehydrogenases (NQO), reductases (AKR, TXN) and peroxidases (PRDX). Glutathione S-transferase theta-2 (GSTT2) was down-regulated at 6hrs in the rat but up-regulated at several time points in the mouse. For genes common to both the mouse and the human, the direction of gene regulation was found to be the same.

The key genes identified from the general literature to be involved in the regulation of this pathway included NQO1, KEAP1, MAFG and NFE2L2 (Nrf2). KEAP1, involved in the regulation of Nrf2 (Nguyen et al. [159] & Pi et al. [207]), was observed to be up-regulated from days 2, 4, and 30 in the mouse data only. The gene encoding Nrf2 (NFE2L2) was up-regulated in the mouse at Day 2 and also in the human dataset. NADPH: quinone oxidoreductase 1 (NQO1) which possesses antioxidant response elements (ARE) within the promoter region of its sequence, was observed to be up-regulated in the rat (Days 1 and 5) and mouse at Day 2 [159]. The gene encoding the

small MAF protein (MAFG) was only observed to be up regulated in the human data. The latter is known to form a heterodimer with NrF2 to amplify its signalling [160].

5.2.1.6 *Aryl Hydrocarbon Receptor Signalling (Appendix A5.1.7)*

The aryl hydrocarbon receptor (AhR) is involved in the mediation of numerous biological processes that result from exposure to foreign compounds [161].

In total 66 genes were grouped into the Aryl Hydrocarbon Receptor Signalling Tox List, of which 62 genes were from the mouse dataset, 16 from the rat and 11 from the human dataset. Most of the genes across all datasets were up-regulated and included phase I metabolizing enzymes (CYP, ALDH), phase II metabolizing enzymes (GSTs, NADPH-quinone oxidoreductases), different other nuclear receptor co-activators (NCO) and proteins and growth factors involved in cell cycle progression (CCN, CDK) and apoptosis (BAX).

The aryl hydrocarbon nuclear translocator (ARNT) known to dimerize with AHR was only present and observed in the mouse list, where it was down-regulated at days 2 and 4. AHR observed only in the mouse dataset and it was up regulated at all time points investigated.

5.2.1.7 *P53 signalling (Appendix A5.1.8)*

P53 is a tumour suppressor protein involved in the regulation of cellular stress such as DNA damage, UV radiation and hypoxia [162]

The P53 Tox List was only identified in the mouse dataset at Days 2, 4 and 30 and comprised of 28 genes and proteins. IPA did not produce this Tox List from the datasets uploaded for both rat and the human datasets.

Most of the genes and proteins in this list were up-regulated especially at Day 2. The majority of the genes within this list are those involved in cell cycle progression (KAT2B, MDM2, CCND1, CDK4, PCNA, CCNG1, PLAGL1 and PTEN) and almost all are involved in apoptosis and cell proliferation and growth.

5.2.1.8 *G2/M Transition of Cell Cycle (Appendix A5.1.9)*

Gap 2 (G2) is one of four phases of the cell cycle of a proliferating cell. The four phases include: Gap 1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis (M). During G2, cells grow and prepare for division [163].

The G2/M transition of the cell cycle Tox List was identified from the mouse dataset only. The results produced from the analysis in IPA did not identify this Tox List from the rat or human datasets. 16 genes and proteins from Days 2, 4 and 30 were identified to be part of this Tox List and the regulation of the G2/M transition of the cell cycle. Most genes were up-regulated particularly at Day 2.

Most of the genes in this list were identified in IPA to play a role in apoptosis and cell proliferation. In addition genes have more specific roles in G2 and mitosis (CDC, CCNB, GADD45A, PLK1, WEE1, YWHAE). The genes within the list are also involved in segregation of cells (TOP2A), DNA damage response (CHEK1) and cell cycle progression (MDM2, KAT2B, CDC, GADD45A, PLK1, YWHAE) [153].

5.2.1.9 *G1/S Transition of the Cell Cycle (Appendix A5.1.10)*

During the G1 phase of the cell-cycle when cells are primarily involved in growth, the cells usually proceed to S phase, proliferate or exist in a quiescent state (G0) [163].

This Tox List was only observed in the mouse dataset and comprised of 15 genes, of which the majority were up-regulated at Day 2 and some at Day 30. . IPA did not produce this Tox List from the datasets uploaded for both rat and the human datasets.

A number of genes involved in S phase of cell growth (E2F, CCND) were up-regulated at Days 2 and 30. The latter are also involved in the G1/S phase transition, cell cycle progression and apoptosis.

5.2.2 *Mitochondrial Dysfunction (Appendix A5.1.11)*

The mitochondrion hosts many redox carriers that are able to transfer single electrons to oxygen leading to the formation of ROS. As well as being primary consumers of oxygen, the mitochondria also play host to an antioxidant defense system capable of detoxifying ROS. This defense system protects against oxidative damage to cellular components [164].

This Tox List was only identified in the mouse dataset which comprised of 33 genes and proteins. Phase I (COX, CYB5R3, GPD2, GPX4, SDH, PDHA1) and II (GSR) metabolizing enzymes were found to be mostly up-regulated at Day 2 or later, although some members of the cytochrome oxidase, oxidoreductase (COX) were also down-regulated at a combination of one or more days. Some members of the family of NADH dehydrogenases were also down-regulated at one or more days.

5.2.2.1 *Pro-Apoptosis (Appendix A5.1.12)*

Genes identified by this Tox List included those involved in pro-apoptosis signalling and regulation. External factors such as exposure to a foreign compound can trigger a cell to program its death by the process of apoptosis.

Thirteen genes from the mouse dataset were observed to be part of the pro-apoptosis Tox List compared to one gene observed at Day 5 in the rat dataset and one gene observed in the human dataset. Most genes were down-regulated at more than one time point and all had a role to play in apoptosis and cell death. Apoptosis (killer genes) specific genes (BOK and BAD) were down-regulated at Day 2 and 4 respectively within the mouse dataset. The gene CASP3 up-regulated at Day 2 in the mouse is part of the caspase family that plays a central role in the execution phase of apoptosis.

5.2.2.2 *Anti-Apoptosis (Appendix A5.1.13)*

IPA identified 8 genes as part of the Anti-Apoptosis Tox List. All the genes in this list were associated with the inhibition of apoptosis signalling and regulation. Only three genes (BAG3, BIRC5, TMX1) were up-regulated at Day 2 or later. The gene changes were only observed in the mouse dataset. The up-regulated genes are known to inhibit apoptotic function.

5.2.2.3 *Hepatic Cholestasis (Appendix A5.1.14)*

Bile acids and their salts are produced in the liver and have an essential role in the digestion of fatty acids. Hepatic cholestasis occurs when bile produced in the liver is unable to flow to the small intestine. Hepatic cholestasis is clinically characterized by increased plasma concentration levels of cytotoxic biliary constituents such as bilirubin,

reduced absorption of fats and damage to the liver [165]. Exposure to foreign compounds may result in the development of hepatic cholestasis.

Fifty genes were identified to be part of the hepatic cholestasis Tox List of which 34 were from the mouse dataset, nine from the human and seven from the rat dataset. The majority of genes within this list were from the family of transporters, kinases and ligand dependent nuclear receptors. The ABC transporter proteins (ABCC2, ABCC3, ABCB11) associated with transport of phospholipids from liver hepatocytes into bile were up-regulated at one or more days and were found to be common between the rat, mouse and human datasets. Members of the solute carrier family (SLC), which are co-transporters of sodium/bile and aid in the circulation of bile acids, were found to be up-regulated predominantly in the mouse at day 2, although SLC10A1 was found to be common between the mouse and the rat.

5.2.2.4 *LPS/IL-1 Mediated Inhibition of RXR Function (Appendix A5.1.15)*

Eighty-nine genes/proteins were identified to be part of the LPS/IL-1 mediated inhibition of RXR function. The latter comprised of 31 genes from the rat dataset, 64 from the mouse and 26 from the human dataset.

The genes involved in this Tox List were mostly up-regulated and included metabolizing enzymes (ALD, CYP, GST, FMO, SULT). The gene encoding the enzyme CYP2B6 (CYP2B2/ CYP2B10 in the rat and human respectively) was observed to be up regulated across all species and time points investigated. Some members of the aldehyde dehydrogenase family (ALDH6A1, ALDH9A1) were down-regulated in the mouse at Day 2. Members of the ABC transporting proteins involved in the transportation of molecules within the intra- and extra-cellular membranes (and thought to be involved in biliary transport and members of the solute carrier family (SLC) known to be involved in ion transport and fatty acid transportation) were found to be common between the different species and were mostly up-regulated at the different time points. ABCA1 was

down-regulated at days 4 and 30 in the mouse and also at day 3 in the human dataset. In addition, proteins involved specifically in lipid transport- apolipoprotein (APOC4) and the phospholipids transfer protein (PLTP) were down-regulated at Days 2 and 30 respectively in the mouse.

Members of the acyl CoA synthase long chain family (ACSL) that play a key role in lipid biosynthesis and fatty acid degradation, were all down-regulated from Days 2 to 30 in the mouse and at Day 5 in the rat (ACSL1).

There were observed differences between the expression of genes within the Tox List between the three species. Members of the glutathione transferase family (GSTT2, GSTM1) were observed to be down-regulated in the rat at 6hrs and Day 5 respectively in comparison to the mouse where the genes were up-regulated. Differences were also observed between the rat and the human dataset for genes belonging to the sulfotransferase family. SULT1A1 was down-regulated at Days 2 and 5 in the rat compared to being up-regulated in the human dataset at Day 3. SULT2A1 was down-regulated in the rat at Day 5 while SULT1A2 was up-regulated at Day 3 in the human. SULT1A3 was however down-regulated in the human. The enzyme catalase (CAT) was observed to be up-regulated in the rat at Day 1 but down-regulated at Day 30 in the mouse.

5.2.2.5 *Negative Acute Phase Response Proteins (Appendix A5.1.16)*

Six genes were identified within this Tox List, which comprised of three genes from the rat dataset, 4 genes from the mouse and 2 genes from the human dataset. The genes involved in negative acute phase response were observed to be mostly down-regulated across all 3 species.

The plasma proteins albumin (ALB) and apolipoproteins (APOA1) were down-regulated in the mouse and human datasets, while APOA2 was observed to be up-regulated at 5

days in the rat. The gene AHSG now known as fetuin A was also down-regulated in the mouse and human dataset at 2 and 3 days respectively. The latter exhibits pro-inflammatory effects by playing a key role in the regulation of cytokine expression, most notably interleukin 6 (IL6) which is involved in the inflammation and maturation of B cells [166]. The inter-alpha trypsin inhibitors (ITI) a family of plasma serine protease inhibitors were also down-regulated in the mouse and rat dataset.

5.2.2.6 *Positive Acute Phase Response Proteins (Appendix A5.1.17)*

IPA identified 14 genes to be part of the positive acute phase response Tox List. This comprised of five genes from the rat, 9 from the mouse and 2 from the human dataset. The positive acute response proteins identified were mostly up-regulated across the three species.

Key genes encoding acute phase response proteins observed to be up-regulated in the rat and mouse included the C-reactive protein (CRP) which is involved in a host of defence-related functions, and orosomucoid proteins (ORM1, ORM2) that are known to increase due to inflammation. Most of the proteins encoded by the genes involved in this Tox List were plasma proteins. Genes that were down-regulated included the metalloprotein ceruloplasmin (CP) involved in iron transport, alpha 2-macroglobulin (A2M) a protease inhibitor and cytokine transporter and the plasma proteins serpin peptidase inhibitors SERPING1 and SERPINA3 involved in the regulation of the complement cascade and protease targeting respectively [153].

5.2.2.7 *TGF- β -Signalling (Appendix A5.1.18)*

The transforming growth factor- β (TGF- β) signalling Tox List comprised of 18 genes, three of which were observed in the rat and 17 from the mouse dataset. The TGF- β involved in the regulation of growth and the proliferation of cells was expressed only in the mouse dataset where it was observed to be up-regulated at Days 2, 4 and 30. Genes encoding key proteins known to bind and activate TGF- β were also present in this Tox List and they included the bone morphogenic proteins (BMP2, BMPR2), observed to be down-regulated at Day 2 in the mouse and the SMAD family of transcriptional regulators. SMAD1 was up-regulated at day 2 and down-regulated at Day 4 in the mouse while SMAD4 was down-regulated only at Day 4.

Members of the mitogen-activated protein kinases (MAP2K1, MAP2K2, MAPK8) were also up-regulated in the mouse in addition to members of the Ras family of genes (HRAS, RRAS, RRAS2). HRAS and RRAS were both up-regulated at Day 2 in the mouse while RRAS2 was down-regulated at Days 2, 4 and 30, also in the mouse. Ras genes encode proteins that are involved in cellular processes that involve cell growth, differentiation and survival. They are also involved in the regulation of MAP kinases [153].

5.2.2.8 *Hepatic Fibrosis (Appendix A5.1.19)*

Hepatic fibrosis is a chronic liver disease associated with accumulation of extracellular (ECM) matrix proteins.

IPA identified 18 genes as part of the Hepatic Fibrosis Tox List. This comprised of 14 genes from the mouse, five from the rat and four from the human dataset. Most of the genes in this group were down-regulated at Day 2 or more and involved in cell migration, growth and proliferation.

The collagen-type proteins (COL), which are part of the extracellular matrix of proteins were up-regulated at Day 2 in the mouse. COL3A1 was, however, down-regulated in the rat at 6hrs in contrast to the mouse in which it was up-regulated at Day 2.

5.2.2.9 *Hepatic Stellate Cells Activation*

IPA identified 8 different genes encoding proteins associated with the process of hepatic fibrosis and hepatic stellate cell activation within this Tox List. The latter was made up of one gene from the rat, 6 from the mouse and 3 from the human dataset.

Genes up-regulated included the growth factors PDGFA and TGFA at Day 2 and 30 respectively in the mouse dataset. The gene encoding Cluster of differentiation 14 (CD14) able to detect lipopolysaccharide (LPS) and bind with apoptotic cells and LPS (in the presence of the LPS binding protein), was up-regulated in the human dataset. Transforming growth factor (TGF)-beta genes (TGFB2, TGFB3) were both present within the mouse data. TGFB2 a member of the kinase family and TGFB receptor subfamily were up-regulated at Days 2, 4 and 30 in the mouse. TGFB3, a multifunctional cytokine involved in extracellular matrix production was down-regulated at Day 2 in the mouse.

The insulin-like growth factor (IGF1) was down-regulated in the mouse and human dataset at all days investigated. The gene encoding CYP2E1 was down-regulated in the rat at Day 5 and also in the human at Day 3 [153].

5.3 Toxicological Function (Tox Function)

IPA Tox Function analysis enabled genes to be grouped according to their known associations with clinical pathology endpoints. Applying a similar approach as that carried for the selection of the Tox Lists, the Tox Functions results were selected on the basis of the association with postulated PB key events for liver tumour formation.

Eight functional categories associated with hepatotoxicity clinical pathology endpoints were selected for further analysis. This included liver proliferation, hyperplasia/hyperproliferation, hypertrophy, liver necrosis/cell death, liver damage, liver steatosis, liver cholestasis and hepatocellular carcinoma. The toxicity functions were selected for further analysis on the basis of a) the number of genes involved from the experimental datasets and b) the assigned statistical significance that measured the likelihood of association between the genes within the dataset and a particular clinical pathology endpoint as denoted by the functional toxicity. Table 5.3 summarises the toxicity functional analysis findings and the corresponding scores and statistical significance rankings for each function category from the individual datasets.

The Path Designer tool function in IPA was used to generate functional networks for the different datasets to investigate the interactions and relationships of the molecules involved in the different toxicity functions. The results of the functional analysis carried out for each time-point were combined for each particular toxicity function. The functional network was built using the published dataset rather than data available within the IPA Knowledge base. The functional networks for the toxicity functions showed the interactions and relationships (direct or indirect) of molecules up- and downstream of the key genes. The network interactions were built to expand the key genes by 10 more molecules using data only available from the uploaded dataset. The generated networks for the different toxicity functions within the different species datasets are provided in Appendix V (A5.2 - A5.2.11).

Table 5.3: Toxicity Functions Related to Hepatotoxicity across all species

Category	Rat 1 day		Rat 5 days	
	Occurrence	No. of genes	Occurrence	No. of genes
Hepatocellular Carcinoma	X*	4		
Liver Cholestasis	X*	9	X*	2
Liver Cirrhosis	X*	3		
Liver Hepatomegaly	X*	1		
Liver Hyperbilirubinemia			X*	2
Liver Steatohepatitis	X	1		
Liver Steatosis			X*	1

Category	Human 3 days	
	Occurrence	No. of genes
Liver Cholestasis	X*	12
Liver Hyperbilirubinemia	X*	2
Liver Cirrhosis	X*	4
Liver Fibrosis	X*	4
Liver Proliferation	X	1
Liver Steatosis	X	1
Liver Necrosis/Cell Death	X	1
Liver Steatohepatitis	X	1
Liver Hepatitis	X	1

NB: X* –indicates statistically significant association ($p\text{-value} \leq 0.05$) of the genes involved in the Tox Function indicated and genes known to be associated with that Tox Function in the IPA Knowledge base.

Table 5.4: Summary of Tox Functions According to the Different Hepatotoxicity Categories

Category	Mouse 10 hrs		Mouse 2 days		Mouse 4 days		Mouse 30 days	
	Occurrence	No. of genes	Occurrence	No. of genes	Occurrence	No. of genes	Occurrence	No. of genes
Hepatocellular Carcinoma			X*	9	X*	6	X	3
Liver Cholestasis	X*	2	X*	3	X	1	X*	2
Liver Cirrhosis								
Liver Congestion					X	1		
Liver Damage	X	1	X	10	X	4	X*	4
Liver Degeneration					X	2		
Liver Dysplasia			X	2				
Liver Hematopoiesis			X	1			X	1
Liver Hepatomegaly			X	3	X	2	X	2
Liver Hepatitis			X	1			X*	4
Liver Hemorrhaging							X*	2
Liver Hyperplasia/Hyperproliferation	X*	2	X*	11	X*	6	X*	7
Liver Hypertrophy			X	1	X	1	X	1
Liver Hypoplasia			X	1	X	1	X	1
Liver Failure			X	1	X	1	X	1
Liver Fibrosis	X*	1	X	3	X	3	X*	3
Liver Hyperbilirubinemia								
Liver Inflammation			X	4	X	2	X*	5
Liver Proliferation	X	2	X*	15	X	6	X*	8
Liver Regeneration	X	1			X	2	X	3
Liver Necrosis/Cell Death	X*	4	X*	23	X*	9	X*	14
Liver Steatosis	X	1	X*	11	X	5	X*	12
Liver Steatohepatitis			X	2	X	2	X*	2

NB: X* –indicates statistically significant association (p-value ≤ 0.05) of the genes involved in the Tox Function indicated and genes known to be associated with that Tox Function in the IPA Knowledge base.

5.3.1 *Liver Proliferation*

Nineteen genes combined from the mouse datasets (10hrs & 30 days) were associated with liver proliferation. These genes were used as the focus molecules from which the functional network for liver proliferation was built. A comparison of the network with the IPA knowledge base on relevant biological functions (cellular growth and proliferation) confirmed that all 19 genes were statistically associated (P value = $2.2\text{E-}32$) with the proliferation of liver cells when compared with genes within the IPA knowledge base. Most of the genes up- or down-stream from the key molecules were also identified to be involved the proliferation of the cells (P value = $1.61\text{E-}22$).

Only one molecule from the human dataset was identified by IPA to be associated with liver proliferation (IGF1). The Insulin-like growth factor is involved in the mediation of cell growth and development. The surrounding genes up- or down-stream of IGF1 between the mouse and the human dataset were very different. In addition, only two of the genes (CAV1 & VTN) that interacted directly with IGF1 were identified to play roles in the proliferation of particular types of cells.

A search carried out in the IPA Knowledge base found 75 genes associated with liver proliferation. These genes were compared to those obtained from the Tox Function results for the mouse and human dataset (A5.3 and A5.3.1 in Appendix V). Nineteen genes were observed to be from the mouse and only one gene from the human dataset were found to be part of the IPA liver proliferation associated genes.

5.3.2 *Liver Hyperplasia/ Hyperproliferation and Liver Hypertrophy*

IPA also grouped 13 genes within a separate Tox function category of hyperplasia/ hyperproliferation from the mouse datasets. This Tox function was identified in all mouse time points investigated. All molecules were combined to create the functional network (A5.2.3 in Appendix V). The key focus genes that included the ligand dependent nuclear receptors (AHR, PPARA), transcription regulators (MYC) and kinases (MAPK8, IKBKG, TGFBR2) were categorised in IPA Tox Function to have roles in proliferation and hyperplasia. The genes up- or down-stream of the key molecules were also shown to be involved in cell growth and proliferation.

Genes associated with the liver hypertrophy Tox function were only observed within the mouse dataset at day 4. The gene encoding the enzyme CYP1A2 was categorised within IPA to play a role in liver hypertrophy. CYP1A2 was shown to interact directly with key ligand dependent nuclear receptors such as AhR and indirectly with NR1I2. It was also shown to interact directly with transcription regulators NFIA, PPARGC1A and indirectly with HNF4A.

Only six genes associated with liver hyperplasia/ hyperproliferation were found within the IPA Knowledge base. Comparison of these genes with the mouse datasets (A5.2.3 in Appendix V) showed two genes in common with those identified by the IPA Knowledge base. Similarly, only three genes specifically associated with liver hypertrophy were identified within the IPA Knowledge base, of which only one was actually found in the mouse dataset.

5.3.3 *Liver Necrosis/ Cell Death*

Changes in genes associated with liver necrosis/cell death for the mouse datasets, were observed to be statistically significant at all four time points. 33 key genes common to all datasets were identified from the toxicity function results to create the functional network showing the immediate interacting genes. Overlay of the network with the IPA functions and disease database identified only the key genes associated with death of liver cells (P value = $7E-38$) and necrosis (P value = $1.24E-9$).

A search for genes commonly associated with cell death identified 65 genes within the IPA Knowledge base. 35 of these genes were found to occur within the mouse dataset and were observed to be mostly up-regulated.

5.3.4 *Liver Cholestasis*

Genes thought to be involved in liver cholestasis were observed across all three species investigated. The gene encoding the transporter protein ABCC2 was the only common gene across the three functional networks. The genes encoding transporter proteins from the ABC and solute carrier family were mostly perturbed across the three datasets.

A search carried out in the IPA Knowledge base identified 83 genes associated with liver cholestasis. Only four, 10 and 12 of the latter genes were found within the mouse, rat and human dataset respectively.

5.3.5 *Liver steatosis*

The functional network for liver steatosis comprised of 19 key genes compiled from the four time points investigated in the mouse. Overlay of the functions and disease database in IPA identified the key genes to be associated with hepatic steatosis (P value = $2.68E^{-34}$).

Fifty-six genes associated with hepatic steatosis were found within the IPA knowledgebase. Comparison of the latter with the mouse dataset showed that 18 of these genes were perturbed within the mouse datasets. Most of the genes from the mouse dataset were shown to be down-regulated.

5.3.6 *Hepatocellular Carcinoma (HCC)*

Genes associated with hepatocellular carcinoma were only identified in the results obtained from rat (Day 1) and mouse datasets (all time points investigated). Comparison of the functional network to the functions and disease database in IPA showed the key genes were statistically associated with molecules known to be involved in hepatocellular carcinoma (Mouse: P value = $4.8E^{-16}$; Rat: P value = $1.49E^{-7}$).

A search within the IPA Knowledgebase identified 107 genes associated with hepatocellular carcinoma. Comparison of the latter with the dataset showed that only five and 12 from the rat and mouse were common with the IPA genes.

5.4 *Comparison of All Datasets with Published Multi-Gene Signatures for the Identification of Non-Genotoxic Hepatocarcinogens and Reference Genes for Hepatocellular Carcinoma as Identified by the Literature*

The DEGs from all time points investigated across the three species were combined and any duplicates observed were removed. The common genes observed were compared to the 19 genes identified by Pei et al [156] to be associated with HCC. The comparison showed that there were no common genes between the different DEGs obtained from either the mouse or the rat. The gene, paternally expressed 10 (PEG10) was, however, identified from the human dataset (Table 5.5).

There was no commonality between the multi-gene signatures identified by Fielden et al [154] and Nei et al [155] for the identification of non genotoxic hepatocarcinogens. In addition, the comparison of these multi-gene signatures with the grouped DEGs obtained from the datasets obtained for the three species investigated here showed that there was no commonality between the different lists of genes (Table 5.5).

Table 5.5: Summary of results from the comparison of all datasets with literature derived biomarkers for non-genotoxic chemicals

Source	Multi-gene signatures for the identification of Non-genotoxic hepatocarcinogens	Mouse (3466 DEGs)	Human (831 DEGs)	Rat (285 DEGs)
Nie et al. [155]	6	0	0	0
Fielden et al. [154]	37	0	0	0

Table 5.6: Summary of results from the comparison of all datasets with genes identified in the literature to be associated with hepatocellular carcinoma

Source	Genes Associated with HCC	Mouse (3466 DEGs)	Human (831 DEGs)	Rat (285 DEGs)
Pei et al. [156]	19	0	1	0

5.5 *Canonical Pathways*

A similar approach applied for the selection of results obtained from the Tox Lists and Tox Functions, was employed for the selection of the canonical pathways. The focus was placed on observed signalling pathways and those considered to have a potential involvement in the MOA of PB were selected for further analysis. Selected signalling pathways included cellular growth, proliferation, apoptosis and cellular stress. For illustrative purposes, only one experiment was selected to represent each species (rat Day1, mouse Day 2 and human Day 3) for the evaluation of the MOA related pathways. The MOA related signalling pathways from the three different experiments are provided in Appendix V (A5.4 to A5.4.18).

5.5.1 *Cell Growth, Proliferation and Development*

The exposure of PB to all three species affected numerous signalling pathways involved in cell growth, proliferation and development. Although there was strong similarity between the pathways affected across the three species in relation to cell growth, proliferation and development, the percentages of genes involved in these different pathways were significantly higher in the experiment carried out in the mouse compared to the rat and human.

5.5.2 *Apoptosis*

The genes involved in the signalling pathways involved in apoptosis were mostly affected in the mouse and human compared to the rat. The percentage of genes in the mouse experiment was again shown to be higher when compared to the level of genes actually perturbed in similar pathways in the rat and human experiments. The AhR signalling pathway was however observed across all three species to be greatly affected by PB.

5.5.3 *Cellular Stress and Injury*

The three species showed that similar pathways involved in cellular stress and injury were affected. Genes in the mouse were observed to be mostly affected by exposure to PB. The NRF2-mediated oxidative stress response pathway was observed to be significantly affected across all three datasets. This indicated that exposure to PB resulted in the induction of genes involved in the regulation of oxidative stress.

Where available, the signalling pathways associated with the Tox Lists and Tox functions discussed previously, were examined to provide a better understanding of the levels of genes that were affecting the pathways of interest. The relevant pathways¹⁵ for the different time points across the different species datasets are displayed below as stacked bar charts in Figures 5.1.1 to 5.1.9. The figures also illustrate the overlap between the signalling pathways relevant for the MOA and the observed toxicities as depicted by the Tox List and Tox functions.

The selected signalling pathways for the IPA Tox Lists included: NRF2-mediated Oxidative Stress Response, AhR Signalling, PXR/RXR Activation, LPS/IL-1 Mediated Inhibition of RXR Function, PPARA/RXR Activation, Cell Cycle: G2/M DNA Damage Checkpoint Regulation, Cell Cycle: G1/S Checkpoint Regulation, Acute Phase Response Signalling, PPAR Signalling, p53 Signalling, Apoptosis Signalling, Mitochondrial Dysfunction, NF-KB Signalling TGF- β^2 Signalling, Growth Hormone Signalling, Hepatic Cholestasis and Hepatic Fibrosis / Hepatic Stellate Cell Activation. The IPA Tox List for CAR/RXR was not included as a signalling pathway.

¹⁵ The pathways represented by stacked bar charts show the number of up-regulated (red), down regulated (green), and unchanged molecules (grey) in each Canonical Pathway. The selected canonical pathways involved in the analysis are shown on the x-axis. The y-axis on the left shows the percentage of genes within the dataset that are involved in the relevant pathway, while the right y-axis shows the level of significance (P-value = 0.05). The total number of genes that make up the individual IPA pathway is displayed above each bar chart.

5.5.4 *Tox Function: Rat*

The pathways most affected by PB treatment in the rat dataset were PXR/RXR Activation, NRF2-mediated Oxidative Stress Response, LPS/IL-1 Mediated Inhibition of RXR Function and AhR Signalling. At 6 hours, only the PXR/ RXR activation pathway was slightly affected. At Day 1 an increase in the involvement of the genes in all 4 pathways were observed and this increased further at day 5. The most affected pathway at Day 5 was the PXR/RXR activation (15%) followed by LPS/IL-1 Mediated Inhibition of RXR Function (12%), NRF2-mediated Oxidative Stress Response (10%), and AhR Signalling (8%). At Day 1, 6% of the genes involved in the Acute Phase Response Signalling pathway were shown to be predominately down-regulated and this was reduced to 3% at Day 5.

5.5.5 *Tox Function: Mouse*

Almost all the pathways selected were affected in the mouse compared to the rat. Similar to observations in the rat, the PXR/RXR Activation pathway was observed to be most affected at the earliest time point of 10hrs. In general, the highest percentages of genes involved in the selected pathways were observed at Day 2 with levels decreasing by days 4 and 30. At day 2, the Cell Cycle: G2/M DNA Damage Checkpoint Regulation was most affected by PB with about 44% of the genes in that pathway perturbed. More than 30% of the genes within the NRF2-mediated Oxidative Stress Response, Aryl Hydrocarbon Receptor Signalling, and LPS/IL-1 Mediated Inhibition of RXR Function were affected. In general, the percentage of genes perturbed by the latter 4 pathways decreased significantly by Day 30. Interestingly, however, although the genes involved in the PXR/RXR activation pathway decreased from 34% at Day 2 to 14% at Day 4, the percentage of genes involved in this pathway increased again by Day 30 to 23%.

5.5.6 Tox Function: Human

Only a small fraction of the selected pathways were significantly affected by PB within the human dataset. Similar to observations in the rat and mouse, the PXR/RXR Activation pathway was the most affected at the time point investigated showing that at least 15% of the genes involved in that pathway were perturbed. This was followed by the LPS/IL-1 Mediated Inhibition of RXR Function and NRF2-mediated Oxidative Stress Response pathways which showed 12 and 7% of the genes in their respective pathways were affected by PB treatment.

Figure 5.1.1: Selected signalling pathways for Rat 6 hours

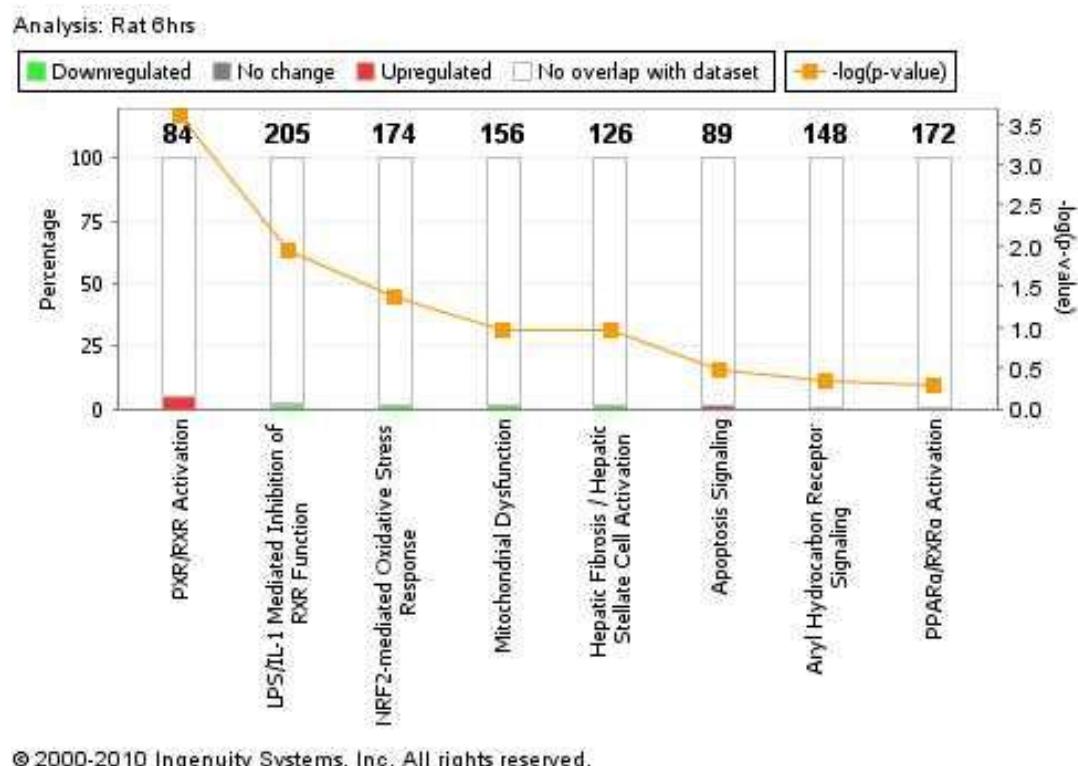


Figure 5.1.2: Selected signalling pathways for Rat Day 1

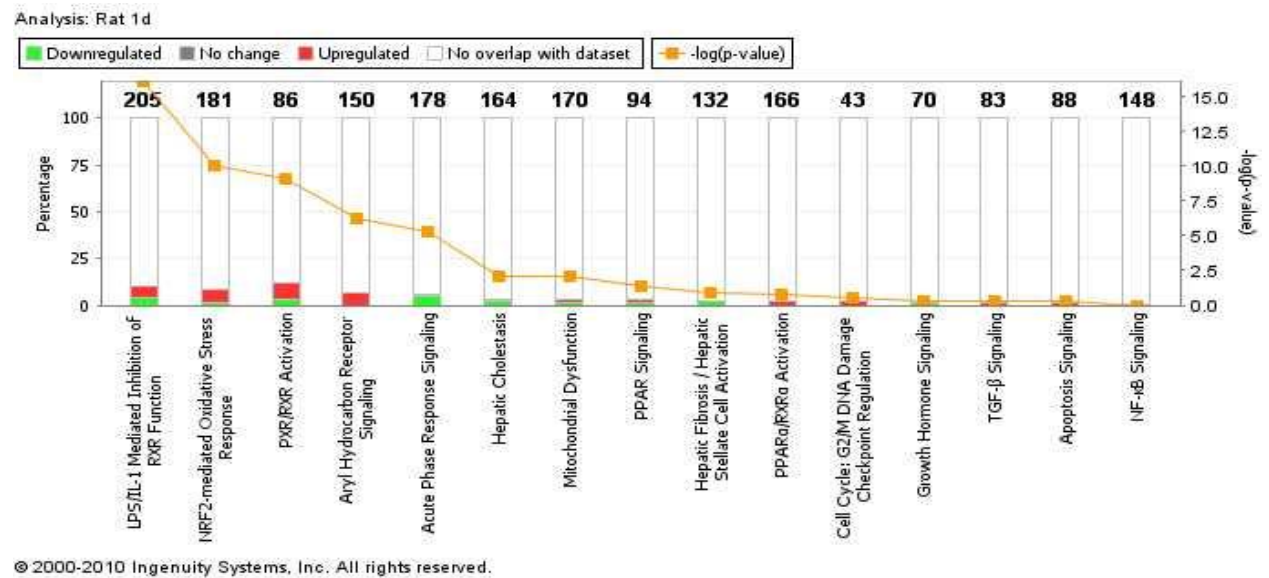


Figure 5.1.3: Selected signalling pathways for Rat Day 3

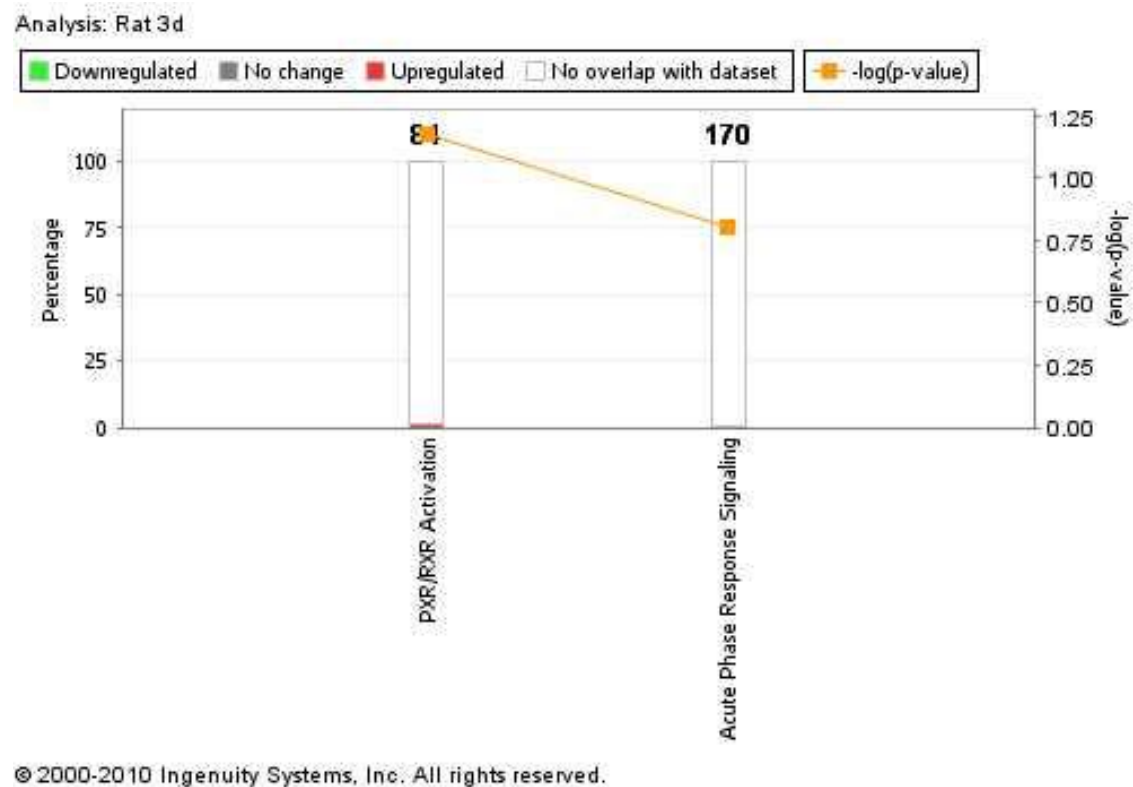


Figure 5.1.4: Selected signalling pathways for Rat Day 5

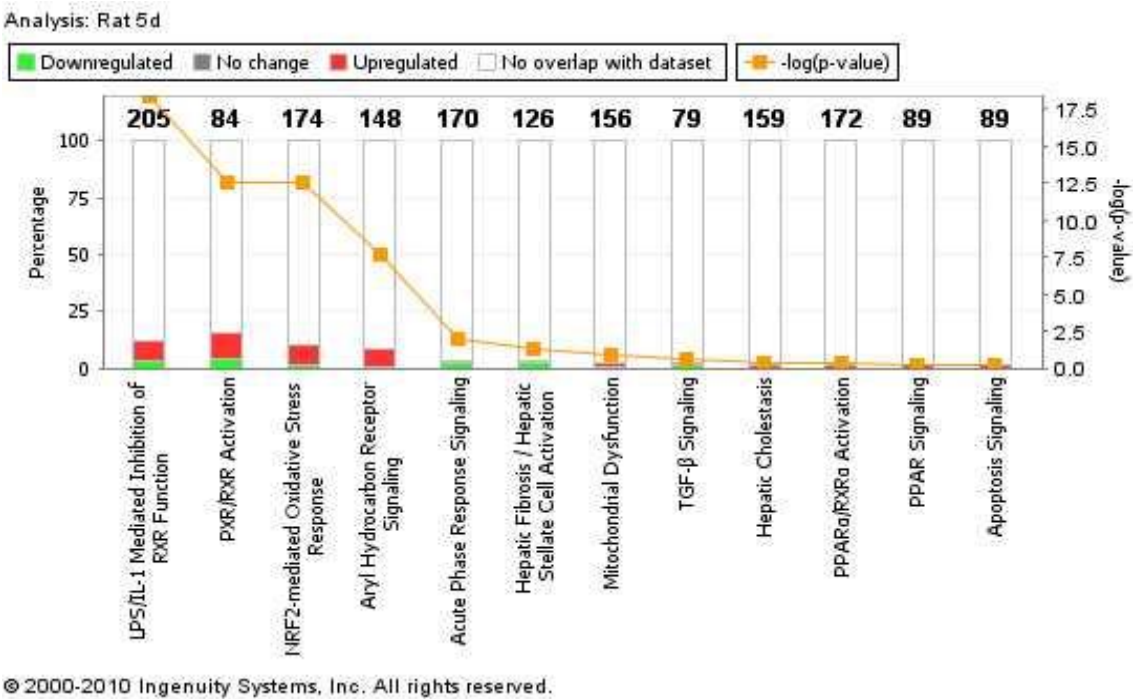


Figure 5.1.5: Selected signalling pathways for mouse 10 hours

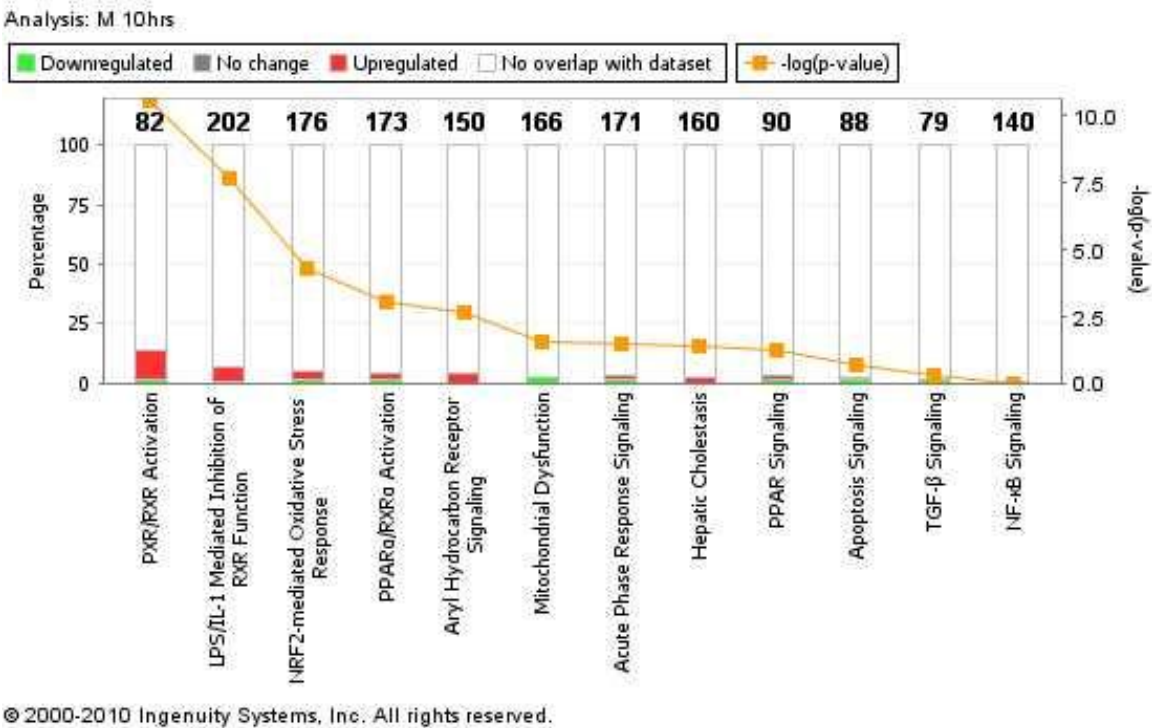


Figure 5.1.6: Selected signalling pathways for mouse Day 2

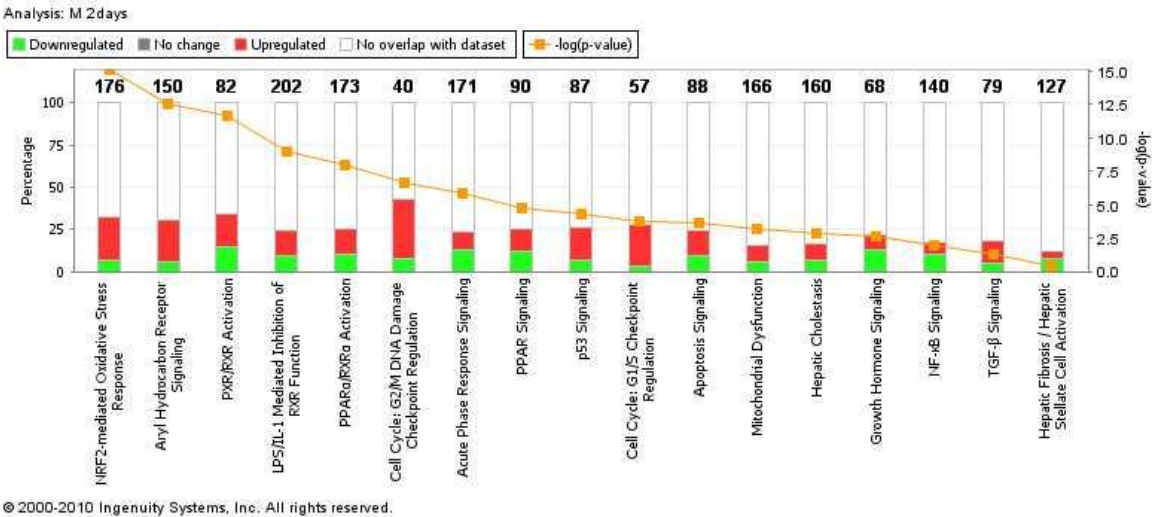


Figure 5.1.7: Selected signalling pathways for mouse Day 4

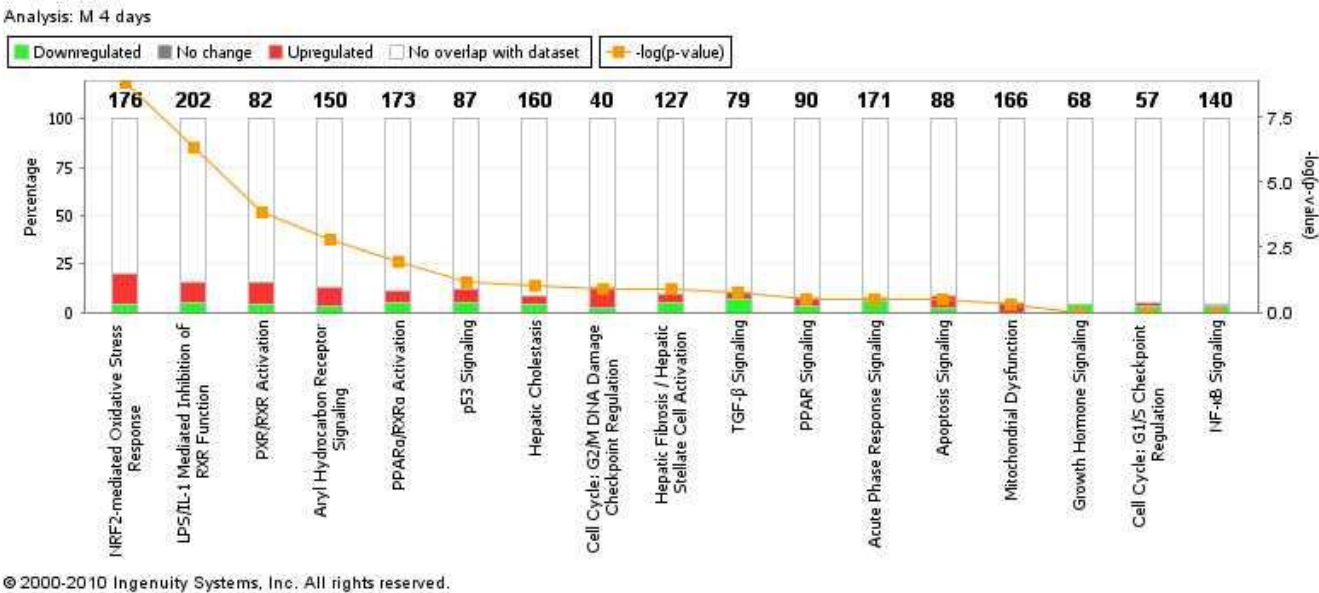


Figure 5.1.8: Selected signalling pathways for mouse Day 30

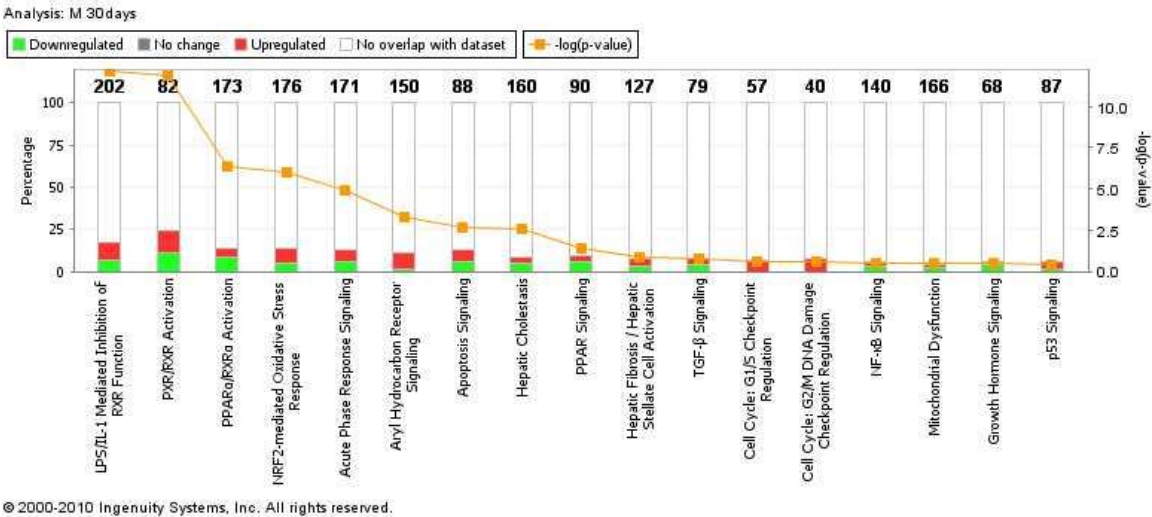
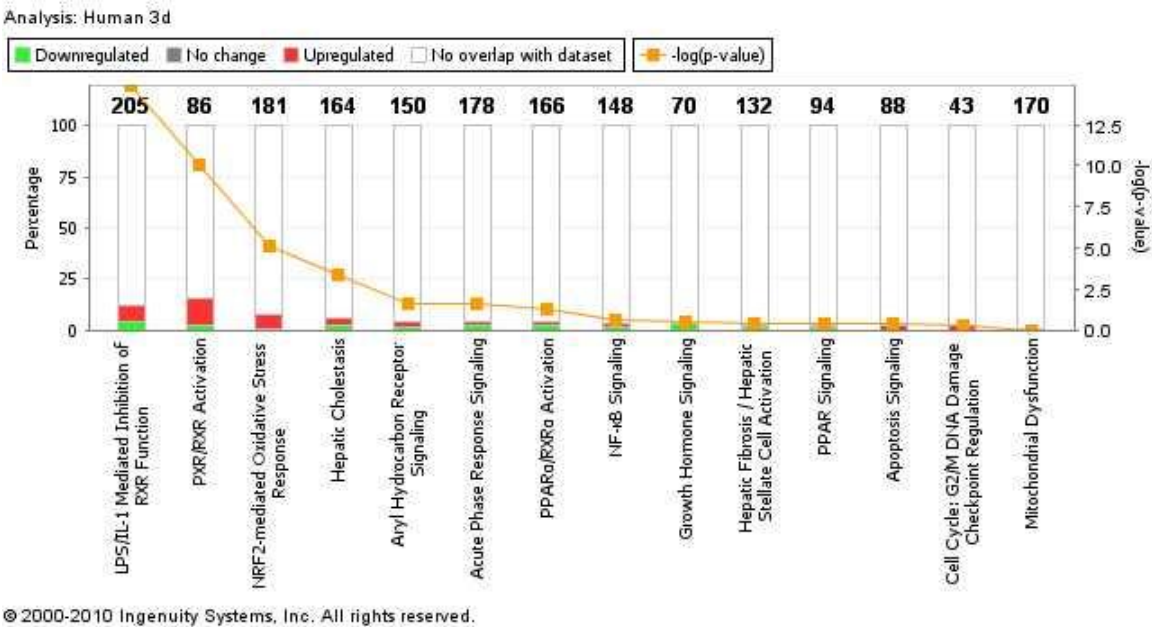


Figure 5.1.9: Selected signalling pathways for human Day 3



5.6 Discussion

The use of toxicogenomics in different biological disciplines has increased significantly in the past decade [167]. Numerous studies have been published to describe the use of toxicogenomics to predict toxicological outcomes such as carcinogenicity. In addition to the use of transcription data in a predictive capacity, numerous studies have also been published that have attempted to predict the mode of action of a chemical, solely on the basis of observed gene expression changes. A recent review by Waters et al [168] identified 11 studies that used toxicogenomics methods in their characterisation of modes of action for different chemicals, using transcription profiling and supervised clustering methods. In the latter review, the authors identified papers that had published signature genes/ biomarkers that appeared to discriminate between direct vs. indirect carcinogens and non-genotoxic and non-carcinogens.

To date, no toxicogenomics studies have been published that thoroughly examine the nature of a MOA through the examination of potential molecular pathway changes that may be indicative of toxicological end points/ pathologies that are key to a MOA [169]. Indeed, the extension of the IPCS framework to include additional types of information, such as that from toxicogenomics, has been highlighted as a key potential development.

The availability of microarray data in public repositories has made it easier to obtain such data and for scientists to carry out toxicogenomics analysis. Problems relating to technological issues and quality control of data produced from microarray experiments are also being overcome through the development of internationally recognized standards for microarray experiments [167]. The latter includes the Minimum Information About a Microarray Experiment (MIAME) [140], External RNA Controls Consortium (ERCC) [170] and the Microarray Quality Control Project (MAQC) [149]. The majority of journals have made it compulsory for published experiments using DNA microarrays (and submissions of microarray data to be made publicly available in repositories) to meet MIAME standards [167].

A comparative assessment of MIAME compliant microarray data obtained from public repositories was carried out as part of this project to explore the use of toxicogenomics data in MOA analysis. Different analytical tools were used to analyse the transcriptional responses of liver tissues obtained for the mouse and rat in-vivo and for human hepatocytes treated with PB at dose levels (rat and mouse) that induce tumourigenic response in rodents.

The functions, pathways and networks associated with the identified differentially expressed genes (DEGs), were investigated through the use of IPA Tox Lists, IPA Tox Functions, IPA Canonical Pathways and IPA Functional Networks.

The diverse effect of exposure to PB was illustrated by the Toxicity List and Canonical Pathway analysis in IPA, which showed that the differentially regulated genes were grouped and associated with a wide range of Tox Lists and signalling pathways across all the experiments from the different species and time points investigated.

Phenobarbital has been shown in experimental studies to induce hepatic drug metabolism as well as a range of pleiotropic effects on liver function that include cell proliferation, cell growth and communication, tumour promotion, glucose metabolism, inhibition of apoptosis, hypertrophy and development of hepatic foci (although this list is not exhaustive). Experimental studies carried out in rodents have shown that PB exerts many of its diverse effects on liver function through its activation of the nuclear receptor CAR [56, 59, 71].

5.6.1.1 *Evidence for CAR Activation and CAR-Related Effects on Liver Function*

The activation of CAR has been postulated to be an essential key step for PB-induced liver tumour formation [56]. The genes that encode the various enzymes and transporter proteins regulated by CAR have been shown to be involved in the excretion of steroid and thyroid hormones, bilirubin and bile acids. In addition to metabolism key toxicological responses to the observed tumourigenic effects of PB

(i.e. increased cell proliferation, hypertrophy, development of altered hepatic foci (leading to advanced liver tumours) have all been shown to be dependent on CAR activation [64, 72]. It has been suggested that the nuclear receptors CAR and PXR work together in a manner referred to as “crosstalk” to interact with hormone responsive transcription factors that work to repress the transcription of genes involved in glucose homeostasis, lipid metabolism and inflammatory response [67, 171]. Indeed the two nuclear receptors have been observed to share the same target genes such as phase I metabolising enzymes (CYP2B, CYP3A CYP2C) and phase II metabolising enzymes (GSTs, UGTs and SULTs) [68, 172].

The CAR/RXR Activation Tox List was observed across all time points and species investigated. The direction of transcriptional regulation was observed to be the same across all the datasets investigated and as expected, genes affected were mostly those encoding metabolising enzymes (CYP2B & CYP2C) and transporter proteins. These genes were all up-regulated across all time points and species investigated. The genes encoding CYP2B10 (orthologous to human gene CYP2B6 and rat gene CYP2B2) and CYP3A1 (orthologous to human gene CYP3A4 and mouse gene CYP3A4) have been identified as the target genes for CAR and PXR respectively [68]. With the exception of Day 3 in the rat, the gene encoding the human gene CYP2B6 was observed to be up-regulated across all other time points across the 3 different species. PB induced CAR induction of CYP2B6 has been demonstrated in numerous experiments [67, 173]. Honkakoski et al [68] investigated the time course for the induction of CYP2B10 in mice after exposure to PB and observed mRNA levels of was slightly induced after 1hr and was markedly raised after 24hrs. The authors demonstrated that the increase in levels of CYP2B10 occurred after the binding of mCAR1 with RXR following PB treatment.

In an effort to elucidate the structural and functional relationship between CAR and PXR, Moore et al [174] tested the effects of PB on mouse and human CAR (hCAR/mCAR) and PXR (hPXR/mPXR) ligands. The authors observed that the hPXR ligand was induced to a much greater extent (9-fold change) compared to the mPXR (no change in expression compared the controls). It is therefore likely that the up-regulation of CYP2B6 observed in the human dataset may reflect the effect of PXR rather than CAR on this target gene. Hariparsad et al, [175] employed PB as a positive

control in their investigation of the induction of CYP3A4 in human primary hepatocytes. The authors observed a marked increase in the mRNA and protein levels (1.9 ± 0.3 -fold increase) of CYP3A4 after treatment with PB. This increase in CYP3A4 activity level corresponded with an observed 30.9-fold increase in the expression level of hPXR following treatment with PB.

Within the PXR/RXR Tox List, the gene encoding CYP3A4 was also observed to be up-regulated in all three species (rat Days 1 and 5; mouse Day 2 and human Day 3). Although all three species showed statistically significant transcriptional changes within the set of genes identified by IPA to be associated with this type of toxicity (A link to the full list of genes involved in all Tox Lists investigated is provided in Appendix V), the gene encoding CAR (NR1I3) was down-regulated and only observed in the mouse at 2 days, while RXRA was observed in the mouse at Day 2 (down-regulated) and at Day 30 (up-regulated) and down-regulated in the human dataset (Day 3). The observations for CAR here were similar to evidence obtained from *in-vivo* experiments which showed that CAR was down-regulated in both mouse and rat hepatocytes [176-178]. Moore et al [174] also observed mCAR and hCAR to be induced at very low levels (0.4 and 0.5 respectively) by PB.

5.6.1.2 *Evidence for Crosstalk between CAR and PXR: Glucose & Lipid Metabolism*

IPA identified a larger set of genes to be associated with PXR/RXR Activation compared with the number of genes identified to be associated with CAR/RXR Activation. All three species showed significant levels of genes involved in PXR/RXR Activation toxicity across the different time points. In addition to genes encoding drug metabolising enzymes and transporter proteins, genes known to encode proteins that play significant roles in hepatic energy metabolism were also observed to be perturbed by PB treatment. The latter was only observed within the mouse dataset at the different time points investigated. The gene encoding PXR, NR1I2 was only observed to be up-regulated in the mouse at 4 days, this result is consistent with

evidence from experimental studies in the mouse [178] which shows that PXR is up-regulated in response to PB exposure.

The gene encoding CAR was down-regulated at day 2 in contrast to PXR which was up-regulated at day 4. The genes identified within the PXR/RXR activation toxicological list were predominantly those involved in hepatic energy metabolism.

Key genes known to be involved in hepatic energy metabolism that were perturbed by PB treatment within this Tox List included FOXO1, G6PC, HNF4A and PRKARIA. Experimental studies show that the pancreatic hormones insulin and glucagon, control the amount of energy available in the liver through the regulation of glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase (PCK1/PEPCK1) which control the rate limiting steps in glucose and lipid metabolism [67, 179, 180]. The down-regulation of the gene G6PC (mouse day 30) is consistent with observations from studies carried out in using mouse hepatocytes [181, 182].

Insulin decreases the production of glucose by repressing the transcription of G6PC and PEPCK1 genes. This brings into play the forkhead transcription factor (FOXO1) which is able to activate insulin response sequence (IRS) bearing genes such as G6PC and PCK1, in the absence of insulin [67, 183, 184].

Both CAR and PXR have since been shown to bind directly to FOXO1 thereby preventing its interaction with G6PC. It has been suggested that this crosstalk with the same gene is the underlying mechanism for the repression of the G6Pase in response to PB and the resulting repression of gluconeogenesis, an observed effect following exposure to PB [67]. A study carried out by Kodama et al [185] demonstrated that both CAR and PXR repressed FOXO1-IRS activity in cotransfected HepG2 cells after exposure to PB for 48 hours.

In addition to the cross-talk between PXR and CAR to interact with the insulin responsive transcription factor FOXO1 to prevent gluconeogenesis, PXR has also been shown to interact with cAMP response element binding protein (CREB). Since G6PC contains CRE, interaction with PXR leads to the repression of glucagon [186].

Other key genes observed in this Tox List observed to be down-regulated at Days 4 and 30 in the mouse were the nuclear factor hepatocyte nuclear factor 4, α (HNF4A) and the peroxisome proliferator-activated receptor γ co-activator 1 alpha (PRKAR1A) which were also down regulated in the mouse at Days 2, 4 and 30. Studies show that PRKAR1A which is activated by glucagon, binds to and co-activates HNF4 α mediated transcription. Activation of CAR and/or PXR has been shown to lead to the dissociation of PRKAR1A from HNF4 α resulting in the repression of G6PC and PCK1 (PCK1 was observed to be down regulated at Days 2, 4 and 30 in the mouse within the CAR/RXR Tox List). Ueda et al [67] investigated the effects of PB using CAR knockout mice and observed that exposure of wild type mice after 12 hours of exposure to PB resulted in the repression of the gene PEPCK1 (among others) genes involved in glucose synthesis. Kodama et al [185] also observed a decrease of PEPCK1 mRNA in CAR wild type mice after chronic treatment with PB (32 weeks). Both CAR and PXR therefore repress glucose production by directly binding to the transcription factors that activate genes involved in gluconeogenesis [67, 187].

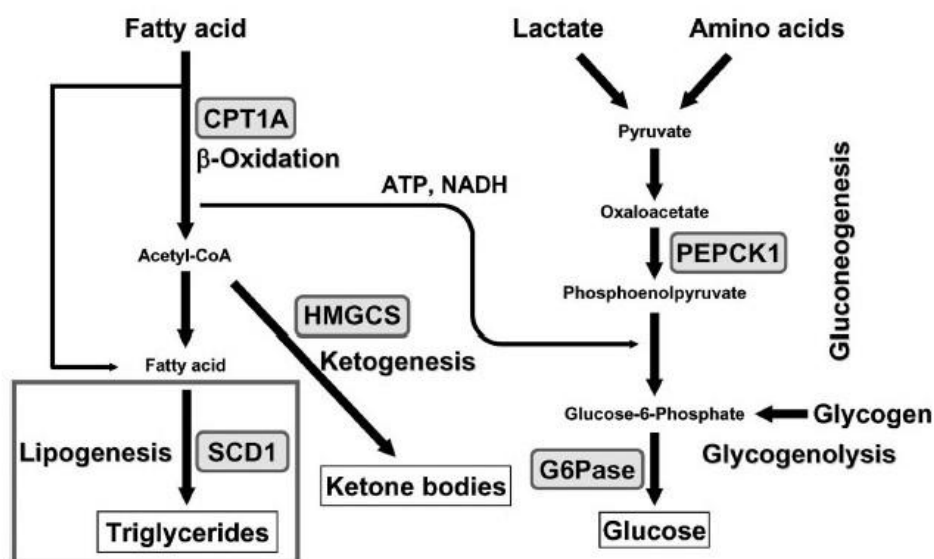
It is important to note that insulin has been shown to reduce PB induction of CYP2B in rat hepatocytes [188]. The levels of CYP2B, CYP3A and CYP4A have also been shown to increase in diabetic rats and mice and return to normal levels after treatment with insulin [189]. It has been suggested that the repression of gluconeogenesis may actually serve the purpose of increasing the capability of hepatic cells to metabolise foreign compounds. Repression of G6PC leads to increased levels of hepatic glucose-6-phosphate, an essential component of the pentose phosphate pathway. Glucose-6-phosphate is converted to ribose-5-phosphate by glucose-6-phosphate dehydrogenase, generating NADPH in the process. It is therefore thought drug-induced CAR and PXR activation (as in the case of PB treatment resulting in repressed gluconeogenesis) may help hepatic cells maintain high enough levels of NADPH required for drug metabolism [67, 180].

Oxidation of fatty acid and ketogenesis occurs in response to low blood glucose to provide hepatic tissues with extra ketone bodies in the liver. The key enzymes involved in this process of β -oxidation and ketogenesis are carnitine palmitoyltransferase 1A (CPT1A) and mitochondrial 3-hydroxy-3-methyl-glutarate-CoA synthase (HMGCS2). Stearoyl-CoA desaturase 1 (SCD1) is the key enzyme

involved in the synthesis of unsaturated fatty acid and is regulated by glucose and fructose. The transcription factor FOXA2 activates both CPT1 and HMGCS2, however, in the presence of insulin both genes are repressed through the inactivation of FOXA2. Insulin has also been shown to increase the transcription of SCD1 through the activation of the sterol regulatory element-binding protein (SREBP). Studies carried out in mice have shown that the mRNA levels of CPT1A and HMGCS2 are down-regulated in wild-type mice but not in PXR-null mice. The opposite effect was observed for SCD1 which was shown to be up-regulated in wild-type mice but not in PXR-null mice. The drug-activated PXR was shown to bind directly to FOXA2 thereby preventing its binding with CPT1A and HMGCS2. PXR activation was also shown to increase the transcription of SCD1. It is therefore thought that although mechanisms may differ, drug-activated PXR mimics the behaviour of insulin by repressing hepatic metabolism through the reduction of β -oxidation and ketogenesis and results in an increase of triglycerides. The pathway for the energy metabolism is depicted in Figure 5.2 [180].

The key genes involved in lipid metabolism discussed above were identified within the PXR/RXR Tox List. CPT1A was down-regulated in the mouse at Day 2 while HMGCS2 was also down-regulated in the mouse at Days 2 and 30. SCD1 was however observed to be down-regulated at Day 1 in the rat and also at Day 30 in the mouse and at Day 3 in human hepatocytes. This result was inconsistent with the experimental results described above.

Figure 5.2: Energy Metabolism in the Fasting Liver



Source: Konno et al., 2008

The key genes discussed above were also identified within other Tox Lists in IPA. CPT1A was also grouped within genes involved in the LPS/IL-1 Mediated Inhibition of RXR Function, Hepatic Cholestasis and Mitochondrial Dysfunction. SREPF1 which is involved in the activation of SCD1 was observed to be down regulated at Day 1 in the rat within the LPS/IL-1 Mediated Inhibition of RXR Function and Hepatic Cholestasis Tox Lists. The down-regulation of the latter gene by PB may explain why SCD1 was also observed to be down regulated in the rat and mouse.

5.6.1.3 Inhibition of RXR

Significant numbers of genes were observed to be involved in the LPS/ IL-1 Mediated Inhibition of RXR Function Tox List and Canonical pathway across the three species. Lipopolysaccharide (LPS) found on the outer membrane of gram-negative bacteria is known to bind to the CD14/TRL4/MD2 complex which promotes the secretion of pro-inflammatory cytokines [190]. Inflammation down-regulates the expression of hepatic genes involved in different physiological processes collectively known as negative acute phase response. Beigneux et al [191] demonstrated that mice injected

with LPS led to an 80% decrease in the level of mRNA for CYP2B10 after 4 hours, the effect was sustained for at least 16hrs and resulted in a 95% decrease in mRNA level compared to controls. Similarly the mRNA levels for CYP3A was markedly reduced by 99% (compared to controls) by 16hrs following treatment with LPS.

Many of the genes involved in acute phase response are regulated by RXR. In response to the pro-inflammatory cytokines RXR undergoes a JNK-mediated CRM-1 dependent nuclear export that leads to the reduction in the levels of nuclear RXR. When this occurs, the knock on effect is the reduction of the expression of hepatic transport proteins (e.g. ABC), metabolising proteins (e.g. CYP, GST) and biosynthesis (CYP7A1), leading to impaired metabolism, transport/ and or biosynthesis of lipid, cholesterol, bile acid and xenobiotics [192]. Beigneux et al [191] observed a 90% and 76% decrease in mRNA levels for both CAR and PXR respectively at 16 hours following treatment with LPS. The authors observed that this reduction also coincided with a 76% and 81% decrease in the mRNA level of RXRa after 4 and 16 hours respectively following LPS treatment.

Evidence for LPS/IL-1-mediated inhibition of RXR, included the observed up regulation of the CD14 gene in the human experiment. RXRA was down-regulated in the mouse at Day 2 and at Day 3 in the human experiment in addition to CYP7A1 observed to be down-regulated in the rat at Day 1. Finally, members of the acyl CoA synthase long chain family (ACSL) that play a key role in lipid biosynthesis and fatty acid degradation, were all down-regulated from Days 2 to 30 in the mouse and at Day 5 in the rat (ACSL1).

A significant number of genes were categorised by IPA to be associated with the LPS/IL-1 mediated inhibition of RXR function pathway, to suggest the inhibition of RXR. The observed up regulation of metabolising enzymes (particularly the target genes for CAR and PXR; CYP2B and CYP3A) as observed in other pathways, suggests that this pathway is not relevant and does not illustrate a real effect from PB exposure among the datasets examined. In addition, AHSG (grouped with genes involved in the Negative Acute Phase Response Proteins Tox List) which exhibits pro-inflammatory effects by playing a key role in the regulation of cytokine

expression was also down-regulated in the mouse and human dataset at Days 2 and 3 respectively.

The evidence from the latter suggests that although a significant number of genes were shown to be involved in the LPS/IL-1 mediated inhibition of RXR function, the inhibition of RXR via this pathway clearly did not affect the ability of RXR to bind with either CAR or PXR. The up-regulation of target genes for PXR and CAR was an indication that these nuclear receptors were able to bind with RXR to induce the genes involved in the various physiological processes. The observations from the genes categorised in this pathway illustrates the importance of interpreting the biological significance of the effects observed at both the individual gene level as well as at the pathway level. It also highlights the importance of not restricting the focus of the biological interpretation to only “top pathways/Tox Lists” as indicated by the level of assigned statistical significance.

5.6.1.4 *Evidence for Cell Proliferation and Growth*

Numerous signalling pathways associated with cellular growth and proliferation were affected by exposure to PB across the three species (see Appendix 4.1D). The genes involved in these pathways were also grouped within different Tox Lists that included PPAR α /RXR Activation, G2/M Transition of the Cell Cycle, G1/S Transition of the Cell Cycle, Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR α , TGF- β Signalling, Aryl Hydrocarbon Receptor (AhR) Signalling and p53 Signalling.

Key genes such the retinoblastoma-like 1, (RBL1/p107) and retinoblastoma-like 2 (RBL2/ p130), D-type cyclins (CCND1, CCND3), cyclin-dependent kinase 4 (CDK4) and E2F transcription factors (E2F1, E2F2, E2F3, E2F4, E2F6), were observed within the G1/S Transition of the Cell Cycle Tox List. The latter genes have been associated with playing key roles in the regulation of cell proliferation.

The D-type cyclins are known to interact with the kinases, CDK4 and CDK6 leading to the initiation of phosphorylation of the Rb family members p107 and p130 thereby preventing their interaction with the E2F transcription factors. The phosphorylation of

p107 and p130 leads to the accumulation of the E2F transcription factors, particularly E2F1, E2F2 and E2F3 which are involved in the transcription of many genes involved in DNA replication and cell cycle progression. An additional consequence of the phosphorylation of p130 is the disruption of the formation of complexes with E2F3b, E2F4 and E2F5 found in quiescent cells which act as transcriptional repressors of S phase genes [193, 194].

It is postulated that the deregulation of these key genes and others that encode proteins involved in this RB/E2F pathway which would result in the loss of the tumour suppressor protein Rb or over expression of D-type cyclins may be an important key step in the development of tumours [194, 195]. With the exception of RBL2 (p130), the key genes discussed above were all up-regulated in the mouse experiment at Day 2 within the G1/S Transition of the Cell Cycle Tox List. E2F2 and E2F3 were also observed in the mouse experiment at 30 days. The results observed here are also corroborated by observations in experimental studies. A two week *in-vivo* study by Kinoshita et al [92] showed that rats administered with 0.05% PB led to the induction of cyclin D1 (CD1/ CCND1) and p21^{WAF1/Cip1} mRNA expression. The authors observed the highest induction of CD1 mRNA at day 4 of the experiment; this preceded the highest elevation of proliferating cell nuclear antigen (PCNA) indices observed on day 6 of the experiment. The induction of CD1 was followed by the induction of p21^{WAF1/Cip1} mRNA expression at day 6 during the significant increase of PCNA and prior to the observed elevation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (a known marker for oxidative DNA Damage) and apoptosis.

5.6.1.5 *Evidence for the Inhibition of Apoptosis*

Within the Canonical Pathways produced for the different datasets, numerous signalling pathways were identified for apoptosis. Among the signalling pathways identified, the AhR signalling pathway was observed to be common across the dataset obtained from the 3 different species, and was observed to be particularly perturbed in the mouse Day 2 dataset.

The Anti-Apoptosis Tox List identified only eight genes that were associated with anti-apoptotic function. Only three of these genes BAG33, BIRC5, TMX1, known to play a role in the inhibition of apoptosis were observed to be up regulated in the mouse.

As the results observed from the Canonical Pathways implicated the role of the AhR Signalling in apoptosis, some of the key genes observed to be up-regulated and identified to play a role in the inhibition of apoptosis within Tox List and Canonical Pathways included: CCND1, CDK4, CHEK1, MYC and MDM2 (as observed in the mouse data). In addition TGFBR2 was observed to be up-regulated in the mouse experiments carried out at Days 2, 4 and 30 while SMAD4 was down regulated at mouse Day 4 within the TGF- β signalling Tox List.

The ligand-activated transcription factor aryl hydrocarbon receptor (AhR) has been shown to be involved in the mediation of a wide range of toxicological responses through the activation of multiple signalling pathways upon exposure to xenobiotic chemicals. Evidence for AhR -mediated inhibition of apoptosis and cell-cycle progression has been obtained from studies carried out using 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (reviewed in Marlowe & Puga [161]). The effect of AhR on different signalling pathways results from its induction on genes that include MYC, MYB and members of the FOS and JUN family. Tumour promotion was not observed in AHR-null rats treated of with TCDD, suggesting that AhR was required for this effect [161, 196]. In addition, TCDD was shown to modulate MDM2 protein levels which have been shown to negatively regulate p53 by promoting its degradation. It has been suggested that AhR may crosstalk with the p53 signalling pathway initiated by the activation of MDM2 by tumour promoters like TCDD, resulting in a reduced p53 response to deal with DNA damaging agents through either arrest of the cell cycle or apoptosis [194, 196] MDM2 was observed to be up-regulated at Day 2 in the mouse and was grouped within genes associated with both p53 signalling and AhR Signalling pathways and Tox Lists.

Evidence for the role of AhR-mediated inhibition of apoptosis as a result of PB exposure is however not corroborated by the experimental evidence. In fact, PB has been shown to have a low binding affinity for the AhR although it has been shown to

induce expression of CYP1A2 (an AhR target gene) in mouse liver [197]. The latter effects have been observed to occur in both wild type and AhR knockout mice, which suggests that PB induces CYP1A2 independently of the AhR [197]. In addition, Nelson et al [162] observed a 2-fold increase in the mRNA levels of MDM2 in rats after single or repeated treatment (5 days) with PB this was in contrast to observations in the mouse where PB did not induce MDM2 mRNA in the liver. The authors did not observe any changes in other p53-dependent genes and concluded that the observed increase in hepatic protein levels of MDM2 demonstrated the nuclear localisation of MDM2 and confirmed its interaction with P53 resulting in its degradation. Indeed, the authors observed a 35% decrease in p53 protein levels after 5 days of PB treatment. The mechanism by which PB induces MDM2 is yet to be determined [162].

Induction of the transcription regulator MYC plays a key role in cell proliferation control through the direct transcriptional activation of G1 cyclin D1 and D2 as well as the cyclin D partner CDK4. Over-expression of MYC has been shown to induce E2F binding of DNA. This activity is thought to be the result of the MYC mediated induction of the cyclin D/CDK4 which results in the phosphorylation of and subsequent inactivation of members of the Rb family leading to the accumulation of E2F [194, 198].

Another gene that plays a key role in the regulation of cell proliferation is the transforming growth factor- β (TGF- β) which is a potent cell growth inhibitor and inducer of apoptosis. TGF- β is initiated by the binding of the ligand to a heterodimeric complex of two transmembrane receptors namely TGF- β receptor type I (TGFR1) and TGFR2 both of which possess serine/threonine kinase activity. In the presence of a ligand, the two transmembrane receptors interact with each other resulting in the phosphorylation of TGFR1 by TGFR2. This event triggers the phosphorylation of the SMAD3 which forms a heterodimeric complex with SMAD4 in the cytoplasm and later translocates to the nucleus where it represses c-Myc [199].

The observed up-regulation of TGF- β and MYC were consistent with *in-vivo* studies in the mouse. The co-expression of c-Myc and TGF- β frequently expressed in human tumours including hepatocellular carcinoma [200] has been studied extensively. Studies carried out to investigate the synergistic effects of c-myc and TGF- α using c-

myc/ TGF- α double transgenic mice showed that co-expression of c-myc and TGF- α resulted in increased cell proliferation and a reduction of apoptosis in hepatic pre-neoplastic cells postulated to be the result of the disrupted Rb/E2F pathway [201-203]. Sanders & Thorgeirsson [201] showed that administration of PB in mice resulted in a decrease in TGF- β 1 accompanied by a decrease in apoptosis. Dramatic increases in apoptotic activity were observed upon the removal of PB from the diet. These results, were supported by observations in studies carried out by Santoni-Rugiu et al [203], showed that the induction of c-Myc (presumably as a result PB treatment) disrupts cell cycle progression in the liver leading to the TGF- β acting as survival factor in hepatocytes. The reduced apoptotic activity therefore appears to be due to the increased levels of c-Myc and E2F and the induction of TGF- β 1. It has been suggested that under these conditions, PB and TGF- β act via a common pathway to promote cell survival by inhibiting apoptosis leading to accelerated cell growth.

5.6.1.6 *Evidence for Cellular Stress (Oxidative Stress)*

The Tox List and Canonical pathway for Oxidative Stress Response Mediated by Nrf2 was observed across all species to be significantly affected by PB treatment, the highest percentage of perturbed genes were however observed from the mouse dataset at day 2.

In response to PB treatment, various cytoprotective enzymes involved in mechanisms of detoxification to reduce oxidative stress were perturbed and observed to be predominantly up-regulated across all datasets investigated. Antioxidant response has been shown to require the involvement of four key components that consist of NF-E2 related factor 2 (NFE2L2/ Nrf2), Kelch-like ECH-associated protein 1 (KEAP1) small musculoaponeurotic fibrosarcoma (Maf) proteins (MAF & MAFG) and a cis-acting enhancer known as the antioxidant response element (ARE) which is found in the promoters of the major detoxifying enzymes; glutathione S transferase A2 (GSTA2) and NADPH: quinone oxidoreductase 1 (NQO1) [159, 160, 204]. All four components were affected by PB treatment and the four genes NQO1, KEAP1, NFE2L2, and MAFG were observed within the Tox List obtained from analysis carried out in the mouse and human datasets.

The transcription factor, NrF2 is an unstable protein with a half-life of approximately 15 minutes [159]. It is located in the nucleus and is able to bind and activate the AREs to enable expression of the metabolising enzymes. NrF2 is constitutively expressed in the cell and is able to translocate directly to the nucleus. Under homeostasis, it is stabilized and targeted for proteasomal degradation through its interaction with KEAP1. KEAP1 thus represses NrF2 in a redox-dependent manner under homeostatic conditions [205].

Under conditions of oxidative stress, the interaction between NrF2 and KEAP1 is antagonized, leading to the accumulation of NrF2. Although the mechanism of the disrupted NrF2-KEAP1 interaction is not fully understood, it is thought that the dissociation is likely to be due to mechanisms that either prevent or reduce access of KEAP1 to NrF2, by oxidation of key –SH groups on KEAP1 [159]. The accumulation of NrF2 in the nucleus is enabled through its stabilisation by the interaction with members of the small MAF family of transcription factors [204]. Li et al [160] carried out an in-vitro study using human cells and showed that NrF2 formed a heterodimer with MAFG. The MAFG gene has been shown to possess an ARE enhancer in its promoter region [206]. The stabilization of NrF2 through the formation of the NrF2-MAFG heterodimer therefore amplifies NrF2 signalling [160] to enable the increased expression of ARE-driven genes such as NQO1.

The gene encoding NrF2 (NFE2L2) was observed to be up-regulated in the mouse at Day 2 and also in the human experiment at Day 3. KEAP1 expression was only up-regulated in the mouse dataset at Days 2, 4 and 30. Up-regulation of the ARE-driven gene NQO1 was observed in the rat experiments at Days 1 and 5 and also in the mouse experiment at Day 2. Increased expression of the MAFG gene was only observed in the human experiment, at Day 3.

The observed up-regulation of KEAP1 in the mouse experiments was consistent with experimental observations of the transcriptional expression of KEAP1 in response to oxidative stress. A study carried out by Pi et al [207] to examine the effect of arsenic (a potent inducer of oxidative stress) showed marked increases of KEAP1 at both the transcriptional and protein levels after treatment of human cells with arsenic. The

increase in KEAP1 expression levels also occurred within the same time-frame as the observed maximum Nrf2 production/nuclear accumulation and preceded the Nrf2 activation of NQO1.

Although both Nrf2 and MafG were observed to be up-regulated in the human experiment the ARE-driven gene NQO1 was not observed to be differentially expressed. This was in contrast to the mouse experiment, which showed that NQO1, KEAP1 and Nrf2 were all up-regulated. The latter suggests that the observed transcriptional changes were in response to dealing with cells under oxidative stress. The fact that NQO1 was not increased in the human experiment raises the question of whether the Nrf2-ARE signalling pathway was indeed relevant in the human experiment. It is not possible to tell in the case of the human hepatocytes whether the observed changes are secondary to the toxic effect of PB exposure.

A two-week study in rats carried out by Kinoshita et al [92] showed that PB administered at a high dose induced oxidative stress in the rat hepatocytes. The authors investigated the relationship and mechanisms involved between the induction of oxidative stress, cytochrome P-450 and hydroxyl radicals and the formation of 8-OHdG. Kinoshita et al [92] measured the formation of oxidative stress using electron spin resonance (ESR) which showed statistically significant formation of OH[•] by Day 4 of PB exposure and reached a maximum by Day 8 of exposure ($82.1 \pm 6.0\%$ $p < 0.001$). A time-dependent significant elevation of total P-450 content and protein levels of CYP2B1/2 and CYP3A2 were also observed. The activity levels of the enzymes were also observed to increase rapidly until Day 8 at which point the levels remained constant, similar to the levels of OH[•]. The authors measured the effect of the observed oxidative stress on DNA damage by measuring the levels of 8-OHdG by immunohistochemistry. Statistically significant increases in 8-OHdG-specific immunoreactivity were detected from Day 4 following treatment with PB (this effect was observed to be reversible). The authors noted that the observed accumulation of 8-OHdG in the nucleus and elevated levels of CYP2B1/2 and CYP3A2 in the cytoplasm were evidence that the observed increase in DNA damage post PB exposure in rat was a direct result of the production of oxidative stress. The authors suggested that this observed effect was related to the increase in protein and activity levels of the 2 P-450 isoenzymes, although this was not demonstrated unequivocally

by the authors. It should be noted however that neither KEAP1 or NRF2 changes were observed in the rat dataset examined here.

5.6.2 *Evidence for Hepatocellular Carcinoma (HCC) – Comparison with Reference Gene Lists*

The IPA Toxicity function, which groups the DEGs according to clinical pathology endpoints, enabled the overview of the dataset within the context of the toxicological endpoints. The Tox functions that could be associated with known key events in PB induced hepatocarcinogenesis included: liver proliferation, hyperplasia/hypertrophy, necrosis/cell death and hepatocellular carcinoma. The genes identified to be part of the selected Tox functions were compared with genes obtained from the IPA Knowledge Base for their association with the relevant toxicological end-points. The search within the IPA Knowledge Base identified large reference gene lists for liver proliferation (75 genes), cell death (65 genes) and HCC (107 genes). This was in contrast to the six reference genes obtained for hyperplasia/ hyperproliferation and hypertrophy (3 genes). The low numbers of genes obtained for the latter was most likely due to the focus on identifying liver only related genes.

Cross comparison of the different datasets with the IPA reference genes showed that for most of the end points selected, particularly for liver proliferation, cell death and HCC, only the data from the mouse datasets (genes combined from all end points investigated in the mouse) showed some correlation with the IPA reference genes. For liver proliferation 25% of the IPA genes were observed within the mouse dataset.

With regards to the IPA genes identified for liver proliferation, cell death and HCC, the most meaningful correlations were obtained by comparison of the reference gene lists with the mouse data, compared to 0.01% in the human dataset and 0% in the rat. 38% of the IPA genes identified for cell death were observed in the mouse compared to no genes in the rat and human dataset. Finally, for HCC, 11% and 0.04% of the IPA genes were identified in the mouse and rat datasets respectively.

The above exercise was intended to identify IPA derived reference genes that were grouped according to biological functions that could be associated with key events that are related to the MOA for PB induced hepatocarcinogenesis. It was hoped that a comparison of the Tox function results (obtained from the Core Analysis of the different datasets) with the groups of genes categorised in IPA to be associated with the relevant key events would provide a better indication of the extent to which the datasets under investigation identified the same genes as those identified by the IPA Knowledge Base.

The fact that meaningful comparisons could only be made from the mouse dataset highlighted some of the limitations (e.g. reliability of the datasets) associated with the rat and human dataset investigated. It also highlighted some of the problems associated with the categorisation of genes within biological functions. As illustrated by the example for the genes identified from the IPA Knowledge Base for liver hypertrophy (which identified only 3 genes), it is not always possible to identify genes that are associated with common pathways for the key events. In addition the identification of CYP1A2 as being associated with liver hypertrophy was somewhat odd as this gene isn't necessarily associated with liver hypertrophy and its induction may be more of a reflection of a more general response to the exposure to a foreign compound. The fact that liver hypertrophy which has been demonstrated in biochemical studies to be a real key event for PB induced hepatocarcinogenesis but was not represented among the pathway changes observed in IPA indicated that while many of the genes that are associated with liver hypertrophy were probably present within the dataset investigated, they may have been categorised under different biological functions and so they were not identified as a significant pathway.

To enable a more objective comparison of these datasets, the DEGs obtained from the 3 species were compared to genes that had been identified to be associated with HCC and biomarkers that had been identified to show predictive capability for non-genotoxic MOAs. The comparison of all the datasets from the 3 species with the genes obtained from the 3 studies [154-156] showed that 1 of the HCC associated genes was identified from the human dataset but overall, none of the reference genes or biomarkers were observed across the different species. This result was somewhat surprising considering PB is a classic non-genotoxic chemical and PB was also

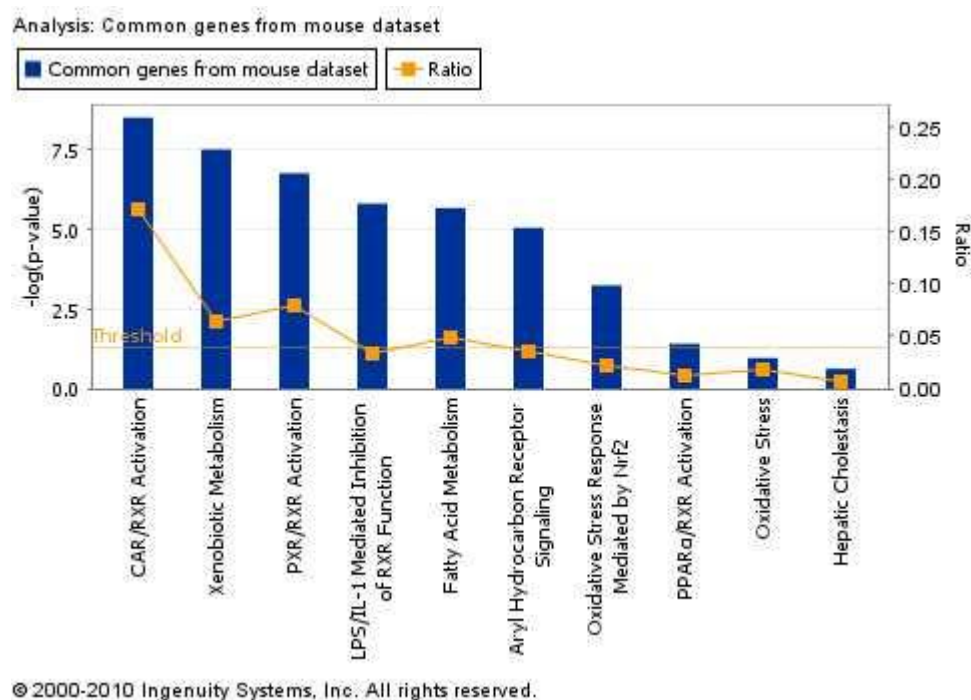
selected as a reference compound by Fielden et al [154] to verify the MOAs of their test compounds using clustering methods.

By their own admission, Fielden et al [154] acknowledge that the accuracy of prediction of their identified gene signatures for nongenotoxic hepatocarcinogens are only as good as the accuracy of the results being modelled. The latter is especially true of the different datasets investigated here particularly for the rat datasets for which significantly lower numbers of DEGs were obtained when compared with those obtained from the mouse and human datasets. The experimental variability between the different datasets is also likely to have had a significant impact on the results obtained from the different data sources. As such IPA was used to run a comparison analysis between the DEGs obtained from the data at the different time points investigated for each species. No genes were found to be common between the rat, mouse and human datasets or between the rat and mouse datasets. The gene, UDP glucuronosyltransferase 2 family (UGT2B4) was the only molecule found to be common in the combined rat datasets. In contrast, 35 genes were found to be common among the combined mouse datasets. A full list of the genes and the results of a functional analysis is provided in Appendix V.

A comparison of all the datasets investigated in the mouse identified 35 common genes from the four time-points investigated. A Core Analysis run on the identified 35 genes enabled the categorisation of the genes according to the different Tox Lists, Tox Functions and Canonical pathways as shown in Figures 5.3 to 5.5. As expected, the results showed that the genes are associated with a wide range of pathways and toxicities as already discussed above.

While the pathways and Tox Lists identified are characteristic of compounds like PB, it would be premature at this stage to refer to these 35 genes as biomarkers for PB induced toxicity. However, the fact that the analysis of these genes show that they are involved in a cross section of toxicities that are indicative of a wider range of functions associated with for example CAR/PXR activation, as observed from the analysis of the full datasets is reassuring evidence for the methodology applied in this investigation.

Figure 5.3: Tox List Analysis of genes common between all mouse datasets.



NB:

In IPA the significance of the association between the dataset and the different pathways were measured in 2 ways:

- 1) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the pathway is explained by chance alone (left Y-axis).
- 2) A ratio¹⁶ of the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the pathway (right Y-axis) [153].

¹⁶ The ratio is the number of molecules in a given pathway that meet the cut criteria divided by the number of molecules that make up that particular pathway.

Figure 5.4: Canonical Pathway Analysis of genes common between all mouse datasets.

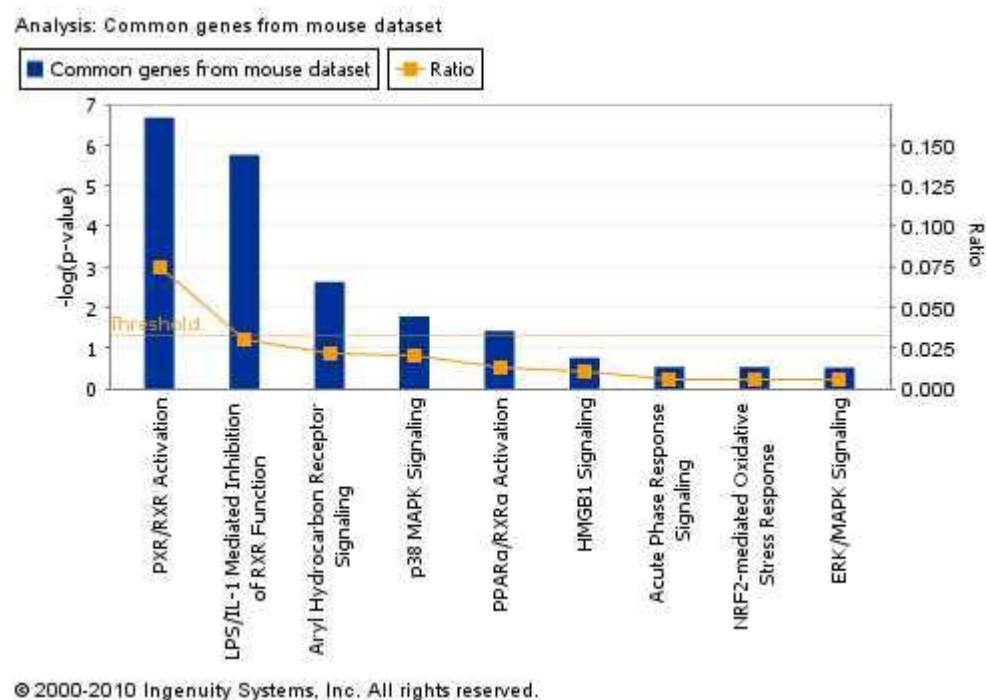
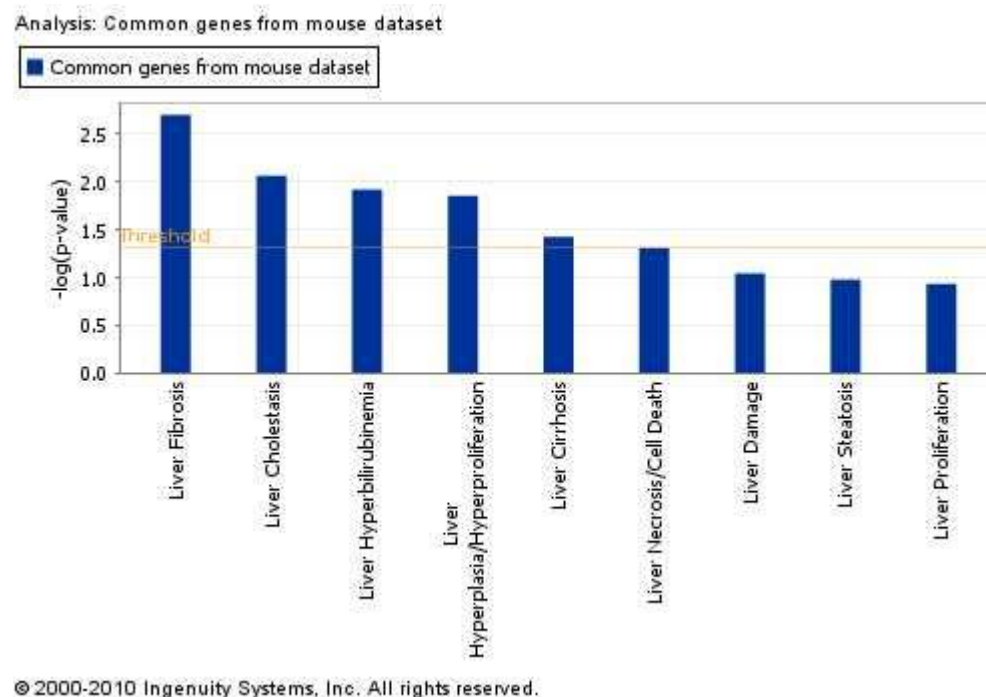


Figure 5.5: Tox Function Analysis of genes common between all mouse datasets.
Tox function

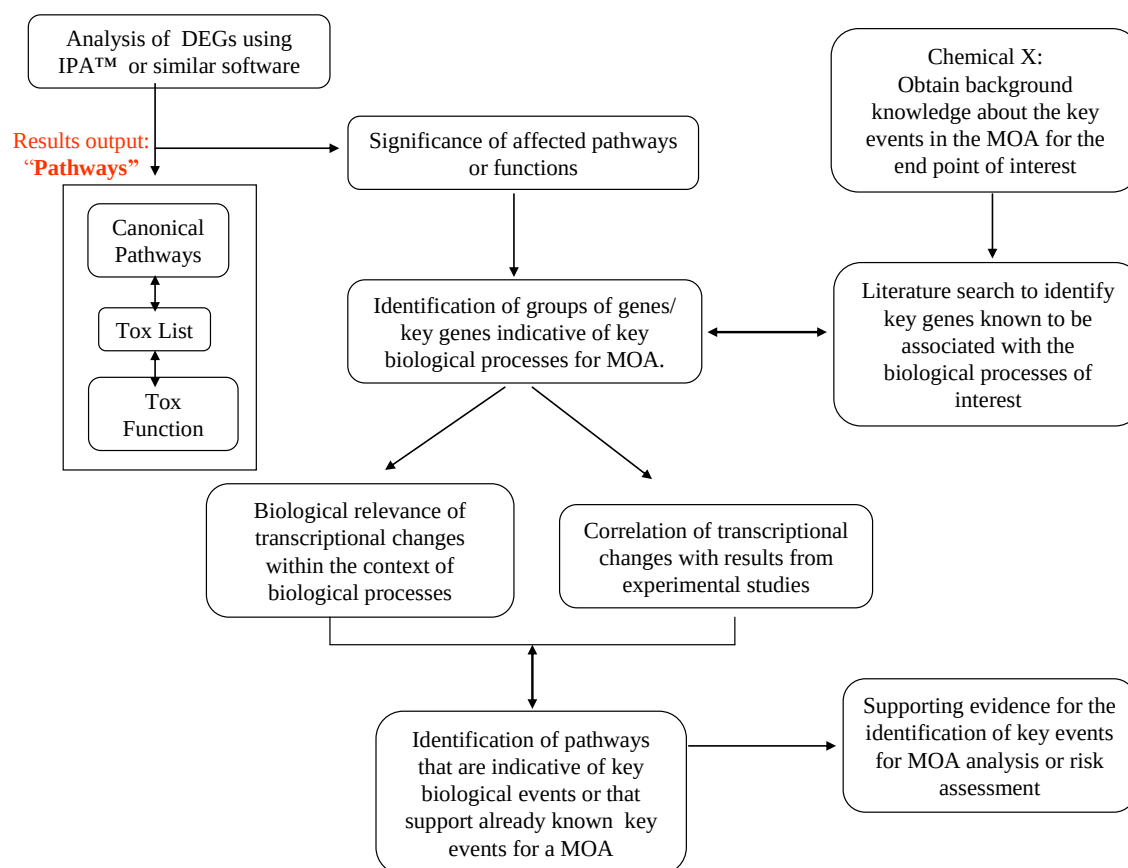


5.6.3 *Discussion of Applied Methodology: Application of evidence from transcriptomics analysis to MOA analysis – learning points and recommendations*

One of the main challenges of this transcriptomics analysis was on the presentation of the result output produced by IPA and the presentation of the information within the context of MOA analysis.

The approach taken for the analysis of the results obtained from IPA is summarised in Figure 5.6 below. A key learning point from this investigation was that information obtained from the different result outputs produced by IPA could not be viewed or interpreted in isolation. The Core Analysis carried out in IPA enabled the assessment of the signalling, metabolic pathways, molecular networks and biological processes that were most significantly perturbed in the PB datasets (based on the DEGs uploaded to IPA) investigated. The evaluation of the results focused on the analysis of the canonical pathways, Tox Lists and Tox functions. IPA suggest that the results produced are intended to facilitate a better understanding of the relative impact of the gene changes within the context of well characterised pathways (descriptions of the different result outputs can be found in chapter 4, section 4.3). The collective evidence from the three result outputs will be referred to as simply as “pathways” for the ease of discussion

Figure 5.6: Strategy for Pathway and Network Analysis of Transcriptomics Data for MOA Analysis.



5.6.4 Identification of Toxicologically Informative Pathways

Regardless of the assigned statistical significance, pathways were selected for further analysis on the basis of plausible mechanisms and MOAs for hepatotoxicity. An important factor for the consideration of the biological significance of a pathway of interest is the extent to which the genes identified to make up a pathway are perturbed by the compound under investigation.

The CAR/RXR Activation pathway for example was heavily perturbed by PB treatment as illustrated by almost 80% of the genes being perturbed (mouse Day 2 experiment). This observed effect as a result of PB treatment was an indication that

the effect on this pathway was extremely unlikely due to chance alone. Although the consideration of data from other species datasets investigated showed that the same pathway was affected to a lesser extent, the fact that similar target genes (e.g. CYP2B6, RXR and other metabolising enzymes) were observed to be affected across all species indicated that the involvement of less genes from the rat and human datasets on this pathway may be due to the lower number of DEGs available for analysis more than the reduced impact on this pathway.

The effect of a compound on a pathway, as indicated by the number of affected genes within that pathway, is a good indication that the pathway is affected. However that is not to say that this pathway would play a critical biological role in the toxicity but it does provide a good indication of pathways that should not be ignored and that should be considered for further investigation. In the face of overwhelming evidence such observations, particularly from unsupervised analysis, can be used to develop hypotheses about a compound's MOA.

5.6.5 *Identification of Key Genes within a Pathway that are Indicative of Key Biological Processes for a MOA*

Owing to the sheer volume of genes involved in some of the pathways investigated across all the datasets examined, it was impossible to consider all the genes individually. Instead, the focus was placed on some of the key genes within the pathways that were known to co-ordinate or be involved in key biological processes.

For the purposes of this risk assessment led exercise, the identification of key genes involved focusing on signalling pathways within the identified Canonical Pathways and Tox Lists that were observed as being associated with the biological processes of interest and key events postulated to be part of PB's possible MOA e.g. CAR activation, apoptosis, cell proliferation, hypertrophy or oxidative stress. For example, PXR/RXR Activation was observed as one of the Canonical Pathways and separately as a Tox Lists. The genes involved in both these pathways were observed to be highly perturbed across the different time points investigated across the three species. This observation was considered to be significant as there has been considerable documentation on the effects of PB on nuclear receptors, most notably CAR and PXR.

As both CAR and PXR were identified within the Tox Lists, the search within the literature focused on the identification of key genes regulated by these two nuclear receptors upon exposure to PB. The search enabled the identification of mechanistic studies that provided evidence for the cross-talk between PXR and CAR and the induction of key genes involved in the regulation of hepatic metabolism. The key genes identified by the literature were then examined to determine whether the same genes were observed in the different time points investigated within the studied datasets and whether the perturbed changes observed were consistent with the evidence suggested by the literature.

5.6.6 *Biological Relevance of Transcriptional Changes Observed for Key Genes; Consideration of Experimental Evidence*

Once the key genes had been identified, an important factor for consideration was the biological relevance of the observed transcriptional changes. In general it is important to consider studies that have investigated the genes of interest using the compound of interest or a compound with a similar MOA. Where available it is helpful to correlate transcript levels of genes of interest and also consider the protein levels for the corresponding genes to establish weight of evidence. While it may not always be possible to find results for the compound of interest, a search for results on the basis of similar pathways, endpoints (or effects from other published studies from compounds with similar MOAs) can sometimes prove useful. In addition, it is often useful to obtain evidence on mechanistic studies that have sought to investigate the toxicological effect in relation to the key event for the compound under investigation. This was illustrated with results compiled for oxidative stress in which the transcript levels for the gene KEAP1 were obtained from studies carried out using arsenic. Although no evidence for the induction of KEAP1 by PB was found in the literature, it was possible however to obtain mechanistic studies investigating the effect of PB on oxidative stress. Mechanistic studies carried out by Kinoshita et al [92] showed that high levels of PB in the rat induced DNA oxidative base modifications in rat hepatocytes via the generation of oxidative stress.

The pathway for Oxidative Stress Response Mediated by NrF2 (as observed from the Canonical Pathways and Tox Lists) was significantly perturbed in the human experiment, similar to observations in the mouse. Although the key genes identified from the literature were common between the two species, the expression of NQO1 did not change in the human experiment. Therefore, regardless of whether the other key genes were observed in the human, the fact that one of the target genes for this pathway did not show a change in expression, led to the biological relevance of this observed pathway being questioned in the human experiment. A search within the literature however identified an experiment carried out by Lambert et al [208] which showed that NQO1 is up-regulated in human hepatocytes following exposure to PB. This study raised issues of consistency among different toxicogenomics studies; it is clear that different study approaches will inevitably affect the repeatability of results and potential reliability of the output from one study. It also raises the issue of the extent to which any one dataset can be used to form conclusions about potentially significant pathways (as informed by the presence of perturbed key target genes).

Similarly, results obtained from the pathway changes observed in the three species pointed to a potential role for the AhR in the regulation of apoptosis related events. Although evidence for the role of AhR for the mediation of the observed effects of inhibition of apoptosis was obtained from mechanistic studies carried out in compounds such as TCDD; mechanistic evidence in studies carried out with PB showed that it has a low binding affinity for the AhR. In this situation, although the results obtained in IPA pointed to a potential role of AhR in the mediation of this key event, mechanistic evidence obtained from the literature showed that while PB induced the same key genes as those mediated by hAHR, the inhibition of apoptosis by PB was achieved via a different mechanism, although the manner in which it does this is still unknown [162].

While it is important to pay close attention to pathways that are shown to be highly perturbed by the compound under investigation, inferred pathways should be verified with evidence obtained from mechanistic studies of the chemical of interest. Examination of the presence of key genes that are involved in the regulation of the key biological processes is also essential. Where possible, it is also useful to consider the correlation of the transcriptional changes observed with results from experimental

studies to ensure that the observed transcriptomics results are as would be expected in experimental studies and not the result of false positives or false negatives from the microarray techniques applied.

5.6.7 *Study Limitations*

As the discussions of the results show, most many of the results interpreted as evidence for the key event were findings from the mouse datasets. The lack of adequate rat datasets rendered it impossible to carry out a thorough species comparison analysis.

The availability of PB data for analysis was a major limitation in this investigation, particularly for studies carried out in rat and human hepatocytes. As this investigation illustrates, the application of toxicogenomics evidence to aspects of chemical risk assessment like MOA analysis, can be very much dependent on the availability of public microarray data such as those stored in public microarray repositories. The availability of data at more varied time points for the different species would have enabled a more in-depth analysis of the temporal relationship to examine the effect of PB on the observed gene changes.

There were also shortcomings in relation to the statistical manipulation of the microarray data. For smaller datasets like those obtained from the rat, the statistical filter applied to identify differentially expressed genes (DEGs) had to be relaxed quite substantially, in some cases a p-value cut off of 0.25 was applied in order to obtain enough genes to proceed to the next stage of analysing the DEGs in IPA. While the lack of uniformity in statistical analysis increased the likelihood of obtaining false positives in the genes identified to have been affected by PB treatment, the practice is justified given that the investigation sought to conduct the analysis as an illustration of the use of toxicogenomics data in risk assessment. The use of such a high p-value cut off in this instance therefore served the purpose to enable the availability of enough data for the assessment in IPA. While this was a major limitation with some of the datasets, this relaxed statistical approach for some of the datasets enabled the evaluation of some genes rather than none at all. It is however acknowledged that this

increases the uncertainties in the results obtained. One would imagine, however, that this is likely to be the same approach that a risk assessor with limited toxicogenomics data would apply, as this would probably be the case for many chemicals.

The data was insufficient to carry out a thorough analysis of the similarities or differences between *in-vivo* and *in-vitro* studies as well as an in-depth assessment of the temporal relationships for the individual species. However, the similarities observed between the mouse and rat dataset was an indication of the possibility to interpret findings for MOA using a combined data approach.

5.6.8 *Problems encountered using IPA*

The use of IPA in this transcriptomics analysis was essential for the consideration of the biological relevance of the gene changes observed as a result of PB treatment. Notwithstanding the benefits of applying this tool for the analysis, many challenges were encountered with using the software.

One of the minor difficulties encountered using IPA was the inability to change the gene symbol output from the default of human, regardless of the dataset specified in the analysis settings. This meant that for the mouse and rat dataset analysed, for which the settings for mouse and rat were selected respectively, IPA instead provided the human equivalent gene symbol for that gene. In the case of the lists of genes obtained from the Tox List analysis, care had to be taken to ensure the correct names for the genes from the different species were obtained and interpreted correctly.

This investigation illustrated that while various microarray datasets can be evaluated for risk assessment purposes such as MOA evaluation, the experimental variability inherent (such as those obtained from different sources in this investigation) in the experimental derivation of the datasets means that some datasets will provide more informative results than others. However all datasets need to be considered to help paint a better picture of the biological functions involved in the evaluation of the compound of interest.

The genes within the IPA Knowledge base are curated manually by scientists. The categorisation of the genes into their biological functions and, therefore, the relevant pathways and networks that they are involved in, relies on the consistency of IPA database with the most up-to-date evidence from the literature.

While the assignment of groups of genes to various Tox Lists and Canonical Pathways enabled the consideration of the observed gene changes within the context of the key events for PB-induced liver tumour formation, the suggested results were interpreted with caution, particularly when the results were cross-referenced against mechanistic evidence available for PB. A noteworthy example of the latter case was the implication of the AhR signalling pathway in apoptosis on the basis of the significantly perturbed pathways observed from the results obtained in IPA (discussed above).

In addition, the categorisation of some genes as being involved with particular toxicological endpoints (as suggested by the Pathways) had the potential to allow the user to wrongly interpret some of the findings as being biologically relevant. This was illustrated by the large number of genes that encode transporter proteins that were grouped within the pathways for hepatic cholestasis. The changes observed for the genes that encode the transporter proteins may have been the result of other effects of PB that are not necessarily associated with hepatic cholestasis. Another example of this was the identification of CYP1A2 from the mouse dataset as being involved in liver hypertrophy. A search within the IPA Knowledge Base for genes associated with liver hypertrophy identified only 3 genes (that included CYP1A2) for this toxicological effect. It was notable that IPA did group many genes into the Liver Hyperplasia/ Hyperproliferation and Liver Hypertrophy Tox List. This was somewhat surprising as this constitutes a main key event for PB induced hepatocarcinogenesis. Without prior knowledge about the key events for PB MOA, the results obtained would now have led to the identification of this pathway as being important for further investigation.

When considering evidence for cell proliferation for PB, it was interesting to note that the RB-E2F pathways cited across numerous publications as being an important pathway for cell proliferation was not categorised within IPA as either a signalling

pathway or as a Tox List. While key genes such as E2F1, RBL1, RBL2, MDM2, MYC and the D-type cyclins were grouped into Tox Lists such as G1 and G2 Transition, P53 signalling and AhR signalling and various other signalling Canonical pathways, the interpretation for the involvement of these genes within key pathways involved in the regulation of cell proliferation was rendered a lot more complicated than it had to be. While it is not being suggested here that the results from IPA were incorrect, it was merely an observation that the categorisation of the pathways/Tox Lists were perhaps not as up-to-date as they ought to have been. For example, in addition to the signalling pathways grouped under the category of “Cellular Growth, Proliferation and Development” within the signalling Canonical pathways, a pathway relating to genes involved in the RB-E2F pathway highlighted in the literature could also be considered. The interpretation for key events within the context of risk assessment would be made a lot easier if the pathways and Tox Lists are grouped in such a way as to take into account some of the main key events that commonly occur in carcinogenesis.

Attention must also be drawn to the changing nomenclature for genes, which was a considerable source of confusion during the analysis. In an attempt to compare the datasets from this investigation with biomarkers identified for non-genotoxicity and hepatocellular carcinoma, each gene identifier provided for the biomarker first had to be converted to the correct gene symbol to enable comparison. Secondly, the different synonyms for each gene symbol pertaining to the species of interest also had to be obtained to ensure that no relevant genes were missed out. In the era of developing biomarkers for endpoints of interest, it seems logical that the issue of gene nomenclature should also be addressed. The availability of so many synonyms for a single gene is a cause for confusion in this field. Until there is more harmonisation in this area and the issue of nomenclature is resolved, it is important that risk assessors consider all the different synonyms for their gene of interest.

As a Pathway Analysis software, IPA is an extremely powerful and essential tool for the evaluation of genes that have been obtained using high throughput technology. It would be otherwise impossible to consider biological and functional relevance of thousands of genes and the use of such a tool enables the genes to be grouped together within their functional contexts. That said care has to be taken in the interpretation of

the results suggested by the analysis output. It is important that the results obtained are not interpreted in isolation from other sources of evidence obtained in the literature.

5.6.9 *Summary of Toxicogenomics Evidence for PB Key Events*

As outlined in Chapter 2, the key events postulated for the MOA for PB liver tumour formation include: activation of CAR, liver hypertrophy, development of altered hepatic foci, cell proliferation and inhibition of apoptosis. Other associated toxicological events include oxidative stress and inhibition of gap junction intercellular communication. The analysis of publicly available transcriptomics data for PB provided evidence for CAR activation, cell proliferation, inhibition of apoptosis and oxidative stress. The evidence for the key events directly inferred from the results outputs from the Tox Lists, Tox Functions and Canonical Pathways are briefly summarised below.

CAR Activation

The induction of the gene encoding the CYP2B enzyme, one of the characteristic effects elicited by the nuclear receptor CAR in response to exposure to PB was observed across the data examined from all species investigated.

Although the gene encoding CAR (NR1I3) was observed to be down regulated in the mouse, the up-regulation of key genes such as PXR, CPT1, G6PC, FOXO1, HMGCS2, HNF4A and PRKAR1A provided evidence for the cross-talk between CAR and PXR for the regulation of hepatic metabolism as suggested by evidence reviewed from the literature. Evidence interpreted for this cross-talk between the two nuclear receptors was only observed in the dataset obtained from the mouse experiments. The observed gene changes were consistent with evidence obtained from mechanistic studies carried out in the mouse.

Cell Proliferation and Growth

The evidence for cell proliferation and growth was obtained from the results observed from gene changes across different perturbed pathways that included: PPAR α /RXR Activation, G2/M Transition of the Cell Cycle, G1/S Transition of the Cell Cycle, Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR α , TGF- β Signalling, AhR Receptor Signalling and p53 Signalling.

The key genes identified from the literature to be associated with playing a key role in the regulation of cell proliferation included D-type cyclins (CCND1), E2F transcription factors (E2F1, E2F2, E2F3), cyclin-dependent kinase (CDK4) and retinoblastoma like genes (RBL1, RBL2). The latter perturbed genes identified in the literature to be associated with the regulation of cell proliferation were observed to be up-regulated in the mouse dataset only.

Inhibition of Apoptosis

Evidence for the PB-induced inhibition of apoptosis was obtained predominantly from the perturbed genes grouped within the AhR signalling pathway. Although the latter pathway was identified across the datasets obtained from the three species, which was suggestive of a role for AhR in the mediation of the observed effects of apoptosis, evidence obtained from the literature suggested that PB has a low binding affinity for AhR and therefore the gene changes observed occurred independently of the AhR.

Key genes identified by the literature to play key roles in the regulation of cell growth and the inhibition of apoptosis included: CCND1, CDK4, MYC, MDM2 and TGFBR2. With the exception of TGFBR2, all the genes were grouped within the AhR signalling pathway. Therefore, although the literature provided evidence that allowed the exclusion of role of the AhR ligand as suggested by the results obtained by IPA, the relevant key genes were observed within this pathway of genes. The five key genes whose role provided evidence for the PB-induced inhibition of apoptosis were all observed to be up-regulated in the mouse dataset only.

Oxidative Stress

Evidence for oxidative stress was obtained from the gene changes observed from the perturbed pathway for Oxidative Stress Mediated by NrF2. Four key genes identified from the literature as being involved in oxidative stress included NrF2, KEAP1, MAF(G) and NQO1.

A search in the literature identified four genes considered to be essential component for antioxidant response. All four genes were present in the mouse dataset (NrF2, MAF, NQO1 and KEAP1). While only three of the genes (NrF2, MAFG and KEAP1) were observed within the human dataset. The key genes within the mouse and human datasets were observed to be up-regulated. In the absence of the antioxidant response element responsive gene (NQO1) in the human dataset, it was not possible to conclude with certainty that the gene changes within this dataset were caused by oxidative stress or as a result of the more general toxicity effects exerted by the exposure of the human hepatocytes to PB.

The expression of the gene changes observed within the mouse and human datasets were consistent with observations in experimental studies.

Liver Hyperplasia/ Hypertrophy

Only a small number of genes were observed to be associated with the Hyperplasia/Hyperproliferation and Liver Hypertrophy pathways. The results were interpreted as being insignificant and insufficient as evidence for this key event. Based on the categorisation of the genes in IPA, the gene encoding the CYP1A2 enzyme was identified in the mouse Day 4 dataset to be associated with liver hypertrophy. Based on the known function of this gene as a drug metabolising enzyme, this result was disregarded as it was more likely that the induction of this gene was more the result of the inducing effects of PB exposure rather than as a true indication of the effects of hypertrophy.

Observed Species Differences (mouse *in vivo* dataset vs. Human *in vitro* dataset)

The gene changes observed across the datasets obtained from all three species showed that many biological pathways were perturbed as a result of exposure to PB. Of particular interest were the observed differences observed between the mouse *in vivo* dataset and the human *in vitro* dataset.

Whilst it was possible to identify biological pathways of interest that were indicative of some of the key events (and associated toxicological events) for the MOA for PB liver tumour formation, a thorough examination of the genes involved in the perturbed pathways, indicated that the key genes identified by the literature to play key roles in the key events of interest, were predominantly observed in the mouse (*in vivo*) dataset only. The only exception was for the evidence obtained for PB-induced oxidative stress, whereby some evidence for this associated toxicological event was also observed in the human (*in vitro*) dataset.

PB exposure was observed to result in the up-regulation of many of the genes encoding some of the phase I and II drug metabolising enzymes (predominantly CYP family, GST family) as well as the genes encoding transporters proteins (predominantly ABC and SLC family) in both the mouse and human datasets. In contrast, changes in the key ligand dependent nuclear receptors (CAR and PXR) were only observed in the mouse and not in the human dataset. RXR was, however, observed in both the mouse and human dataset. Similarly, most of the key transcription regulators (JUN, E2F1, MYC, MDM2 and SMAD) were only observed to be up-regulated in the mouse dataset and were not differentially expressed in the human dataset. A change in only NrF2 was observed to occur in both the human and the mouse dataset.

Similarly, many of the pathways selected for investigation were observed in both the mouse and human datasets. To determine the biological relevance of the observed perturbed pathways, where possible the key genes observed were cross referenced with information on the mRNA levels for the relevant genes from mechanistic or biochemical studies obtained from the public literature. In most cases examined, the mRNA levels for most of the key genes were found but the gene changes were

observed to occur predominantly in the mouse. This was particularly true for evidence obtained for the evidence obtained for CAR/ RXR activation, evidence for the cross talk with PXR, cell proliferation and the inhibition of apoptosis.

A lack of change in the expression of the key genes in pathways thought to be involved in the key events for the MOA for PB in the human dataset could be an indication that these effects in humans do not occur. However, owing to the problems encountered in interpreting the results obtained from this transcriptomics analysis, such a conclusion would be premature without further analysis of a much larger human dataset. Indeed, a recent gene expression profiling study carried out by Lambert et al [208] showed that primary human hepatocytes exposed to various concentrations of PB, led to the induction of genes such as MYC, CAR, PXR (albeit at low levels of induction at some doses after quantification using reverse transcription polymerase chain reaction (RT-PCR) and many other drug metabolising enzymes similar to results observed in the data investigated here. The observed induction of some of the genes as a result of PB exposure of the human primary hepatocytes contradicts some of the results obtained in the human dataset analysed here. It is therefore essential that a wider human *in vitro* dataset be evaluated using a comparative approach similar to that applied here, before any conclusions can be drawn about the differences between the *in vitro* and *in vivo* datasets in relation to the potential effects of PB on the key events for the MOA for liver tumour formation.

5.7 Conclusions

One of the main challenges of this transcriptomics analysis was on the presentation of the result output produced by IPA and the presentation of the information within the context of MOA analysis.

Based on the evaluation of the different pathways, Tox Lists and Tox functions, evidence for some of the key events for PB-induced hepatocarcinogenesis including CAR/PXR activation, cell proliferation, inhibition of apoptosis and associated the effects of oxidative stress were obtained from the combined datasets obtained from the mouse.

A sensible approach to deal with the large lists of genes obtained and grouped according to functionality, was to focus on key genes or groups of genes as informed by the available literature. This approach enabled the focus to be placed on particular sets or groups of genes that were indicative of some of the key events that have been postulated to be part of the PB MOA for liver tumour formation.

Similar to observing groups of genes within a network of genes, one of the benefits of examining the results of the Tox Lists in the form of simplified heat maps was that it facilitated an easier examination of the temporal relationships among the observed gene changes involved across the different pathways. This was exemplified by the interpretation of the results collated as evidence for proliferation and inhibition of apoptosis, which showed the involvement of key genes across different Tox Lists and pathways.

Overall, the results obtained from the analysis were difficult to interpret. The analysis in IPA produced an overwhelming amount of results and in order to make sense of the results output created from the analysis, focus had to be placed on selected key genes (on the basis of information obtained from literature) within pathways considered to be potentially relevant for the MOA for PB. It would be a cumbersome task to consider all the many functions of a single gene and such an approach would be counterproductive, particularly from a risk assessment point of view. However, the approach used to select genes to interpret findings for a biological effect risks being criticised over its subjective nature owing to the fact that the assessor chooses what

the focus for the interpretation will be. Regardless of the potential subjectivity of the approach applied here, it is the only practical solution to evaluating transcriptomics data derived from using high throughput technologies when conducting a risk assessment.

It was hoped that the examination of the individual gene changes associated with the different Tox Lists would provide insight into the temporal relationship of potentially significant genes and enable the identification of differences between the different species at the time-points investigated. Owing to the limitations in the datasets available however, no meaningful significant temporal or species differences could be found. Regardless of the shortcomings of the available dataset, the majority of the Tox Lists reviewed showed that most of the genes that were present at the different time points had similar directions of transcription.

The comparison of the datasets with biomarkers for non-genotoxicity obtained from the literature showed that there were little or no similarities between the different datasets and the identified biomarkers from the three different sources. Comparison of the DEGs with functionally related genes identified from the IPA Knowledge base showed that where it was possible to categorise the genes into the biological processes of interest, many of the genes from the mouse dataset were observed to have been affected by PB treatment.

Although 35 genes (from the combined mouse dataset only) were found to be common from the studies carried out at the different time points investigated, these have deliberately not been referred to as biomarkers in this investigation. The genes identified were representative of a wide range of biological processes affected by PB and as such illustrated some of the characteristic gene changes that represented the overall results obtained from the analysis with the full datasets.

The analysis carried out here has demonstrated that the molecular basis of some of the key events in the MOA for PB can be determined from published toxicogenomics data. However, this was not achieved using an unsupervised approach and a meaningful interpretation of the results within the context of the key events for MOA analysis was only achieved on the basis of the information obtained from the literature and through verification of the observed gene changes with evidence from

mechanistic studies. Indeed, interpretation of some of the observed pathway changes or individual genes on the basis of the results produced by IPA could have led to the misinterpretation of the significance of particular genes in the regulation of pathways that were indicative of potential key events for PB's MOA.

While it is clear from the results discussed here, that toxicogenomics can enable the MOA to be described on the basis of some of the key events (through the identification of key genes that are indicative of the involvement of a particular biological pathway), it would be premature to conclude that the potential effects for a chemical like PB can be deduced using MOA consideration, without the need to conduct long term studies, as suggested by Waters et al [168]. The experience of this transcriptomics analysis indicates that many more hurdles need to be overcome to improve the confidence placed in the results obtained from toxicogenomics data. More importantly, more publicly available datasets for case study compounds like PB need to be evaluated to improve the weight of evidence for the interpreted findings in order to reduce the level of uncertainties associated with the interpreted results.

The analysis described in this chapter has illustrated that to some extent it has been possible to explain key events in terms of Toxicity pathways provided in IPA. However it was not possible to predict key events and MOA on the basis of the pathways observed. In the future it would be helpful to examine the toxicogenomics data using specific MOA assessment criteria. This would facilitate the identification of data gaps and enable a more thorough assessment of the weight of the toxicogenomics evidence for a particular key event. In addition such an analysis would not only focus on the evaluation of the time-response but also on the dose-response, consistency and plausibility of observed gene expression changes.

Chapter 6

General Discussion and Conclusions

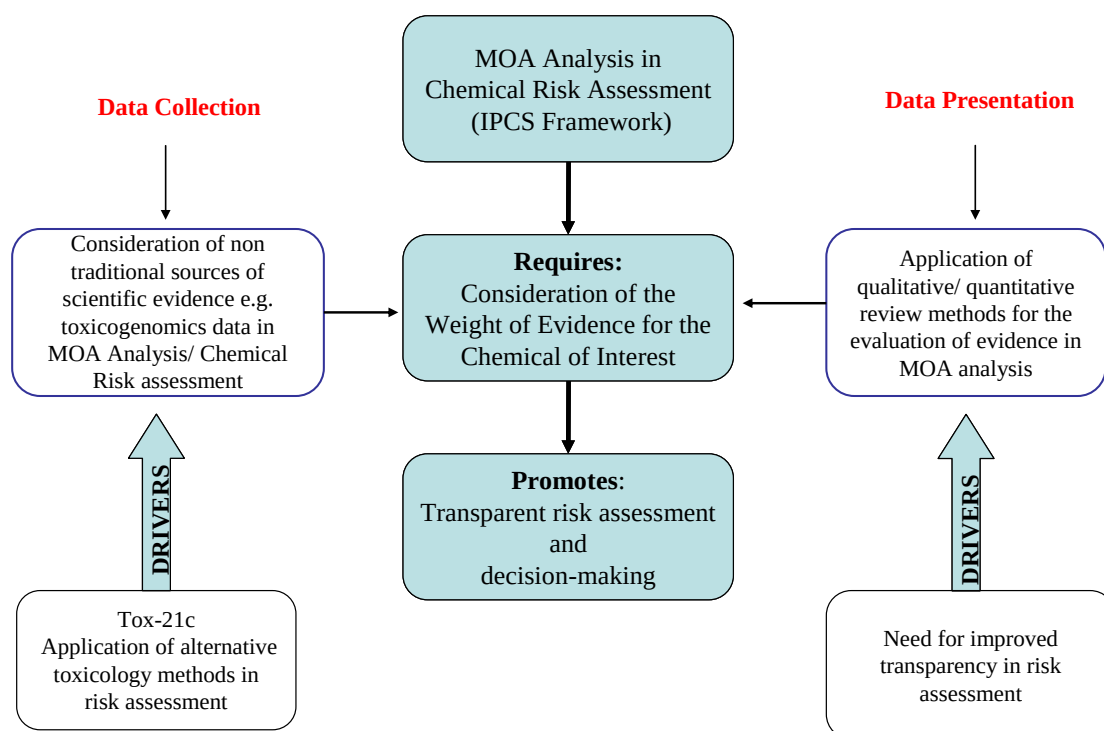
The chemical risk assessment process requires the thorough consideration and evaluation of a variety of scientific evidence deemed appropriate to evaluate the hazard from a chemical of concern as part of the effort to mediate against any potential risks to human health and the environment [54].

In the past decade the use of the MOA approach in risk assessment has been promoted widely and adopted by many regulatory and advisory bodies as a means to harmonise approaches to chemical risk assessment and to improve transparency. In this same period, the simultaneous growth of genomics data generated within the public domain has provided the impetus to investigate the extent to which such data can be considered as part of the WOE in a MOA analysis and more generally in chemical risk assessment. Alongside this, the increased need for transparency in risk assessment means that more effort needs to be put into the application of techniques that facilitate transparent evaluation of the WOE as part of MOA/HRF and risk assessment in general.

This thesis aimed to address two important aspects of the WOE analysis process pertinent to the application of the IPCS HRF and more generally risk assessment. An attempt to conceptualise these two aspects is illustrated in Figure 6.1 and includes:

- The types of data compiled and considered as evidence as part of the WOE analysis for MOA or human relevance analysis.
- The presentation of evidence to improve the transparency of the decision making process for MOA or human relevance analysis.

Figure 6.1: Conceptual illustration of fundamental aspects of a WOE-based approach applied for MOA analysis in risk assessment and current drivers for the further development of the IPCS HRF as a risk assessment tool.



6.1 Techniques to Improve the Transparency of the use of a WOE-Based Approach in MOA and Human Relevance Analysis: Systematic Review

Conclusions on the human relevance of the MOA for PB, and therefore for other compounds sharing the same MOA, rely heavily upon epidemiological studies on its carcinogenic potential in patient populations. PB was therefore used as an illustrative example, in which systematic review techniques were applied for the evaluation of available epidemiological evidence for a potential association between PB and liver cancer. The methodology and results of the review are detailed in chapter 3.

The systematic review was undertaken broadly applying the guidelines provide by the NHS Centre for Reviews and Dissemination [43] and recommendations for systematic reviews provided by Peters et al [101] and Weed et al [103]. A key feature of this review was the initial statement of the intent and purpose of the review. The methodology developed, detailed the efforts to develop a search strategy to identify all relevant epidemiology papers from different electronic databases. Inclusion and exclusion criteria for the selection of epidemiological papers considered to be relevant for the review were also elaborated and clearly stated.

A key component of the systematic review was the development of a quality assessment scoring system (also described within the methodology) that enabled the transparent assessment of the quality of the selected studies. The studies were assessed on the basis of how well the authors addressed and dealt with issues of bias, confounding and different aspects that related to the exposure, classification and assessment of epileptic patients exposed to PB. The criteria used to assess the quality of the different studies and the basis for the assigned scores was stated explicitly. The latter ensured a transparent critique of the different studies and made transparent, the basis for the conclusions and interpretations of the results obtained from the studies.

One of the main advantages of this approach to the evaluation of evidence for the HRF was the identification of areas of uncertainty for the assessment of the evidence. Most notably, the review highlighted some of the methodological difficulties encountered with trying to investigate the association between PB and liver cancer.

It was not possible to reach a conclusion about the association between PB and the occurrence of liver cancer on the basis of the epidemiological evidence reviewed in Chapter three. Although the English and Danish cohort studies [109, 110] were assessed to be of a high quality as they were the only studies to have adequately identified a group of patients that were treated unequivocally with PB predominantly, the findings reported by the authors were based on a very small cohorts of patients. In contrast the larger Finnish cohort (almost 30,000 patients) described by Lamminpaa et al [112] found a positive association but were unable to identify patients treated predominantly with PB. The results reported by Lamminpaa et al [112] were assessed to be unreliable on the basis of the limitations inherent in their study design. While the focus of any conclusions about the association of PB and liver cancer should be placed on this larger study, the uncertainty associated with the Finnish cohort study suggest that the results reported should be interpreted with caution. A refinement of the Finnish study (such as setting up a nested case control study on the cohort already identified) would go a long way to improving the reliability of the study and would add to the WOE and enable more definitive conclusions to be drawn about the potential association between PB and liver cancer.

The routine application of systematic review techniques such as that described above and in Chapter 3 would go a long way to improving the transparency of decisions reached using a WOE-based approach to the IPCS MOA/HRF and other components of the risk assessment paradigm. One of the cited advantages of applying the IPCS HRF is that it improves the transparency of aspects of the hazard characterisation stage of risk assessment [9]. It achieves this objective by facilitating the consideration of the WOE within the context of causal criteria¹⁷ as articulated by Hill [29]. What remains to be seen is how the WOE compiled for the different aspects of the individual criteria for causality can be made more transparent, particularly if they are used as the basis for key decisions about a chemical of interest.

It is worth considering how feasible it is to conduct an exhaustive systematic review for every process that applies a WOE-based approach to the narrative review as part

¹⁷ Hill emphasised that the issues he proposed useful for consideration of causality of an association identified in an epidemiological study should not be considered as criteria. However, the term 'Hill criteria' is now widely used, and has been used in this thesis for convenience and consistency with current literature.

of the risk assessment. Clearly the process of compiling and evaluating the WOE for MOA analysis can be quite labour intensive let alone the evaluation of WOE for every aspect of the risk assessment for a single chemical under evaluation. If it is envisaged that it may not be possible to apply a thorough and detailed systematic review approach to every aspect of the Framework then perhaps more guidance is needed to identify the key areas of the HRF to which a more thorough application of systematic review techniques for the evaluation of the WOE would be most beneficial.

This issue was recently addressed in a guidance document produced by EFSA on the application of systematic review methodology in food and feed safety risk assessment [42]. The EFSA guidance document states that while it may be suitable to carry out a systematic review to address a research question, it may not always be worthwhile or practically feasible. Some of the factors stated as having an impact on the feasibility of carrying out a systematic review included the consideration of the quality and quantity of the evidence to be reviewed, the availability of resources and the potential costs of applying such techniques (taking into consideration the time, budget and the expertise that would be required). To decide on whether or not to engage in a systematic review process, the guidance document recommends the use of a scoping technique to initially survey the extent of the literature. This process of assessing the extent of literature to be evaluated would enable the estimation of the amount of resources that may be required to carry out a systematic review process.

There is however the contrasting view that there is no excuse for not carrying out a systematic review using the many guidelines that are now available (e.g. Guzelian et al. [209]). Even when the evidence for a particular review question is overwhelming, it may still be possible to apply systematic review methods. The transparency of the risk assessment process is fundamental to credible risk assessment [40] and this can be facilitated by techniques such as systematic reviews applied in the evaluation of the WOE. In situations where there is overwhelming evidence for a review question, the emphasis should be placed on the thorough consideration of the criteria for the eligibility of studies for inclusion in the review. Therefore if the scoping process identifies a significant volume of evidence for a review question, the criteria for inclusion could be restricted to include only e.g. key studies, pooled studies, narrative reviews or meta-analyses etc [210]. It is the explicit description of the process behind

the evaluation of the evidence and the stated rationale for the selection of studies used as the basis for risk assessment conclusions that render the risk assessment process more transparent and the decisions credible.

An additional key issue is the consideration of the quality of toxicological studies which form the bulk of the WOE assessed as part of MOA analysis. From a UK perspective, the current approach to evaluating such data and the formulation of decisions relies on a combination of the WOE considered and application of expert judgment to draw conclusions about whether the substance under evaluation is considered to have adverse affects on human health [43]. Klimisch et al [211] proposed guidelines for a systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. The authors proposed reliability categories which varied depending on the level to which the study conformed to good laboratory practices (GLP) or other defined criteria. While such factors are already taken into account by experts who make their judgement about particular toxicological studies (quality assurance and GLP are commented on explicitly for all studies cited in JMPR monographs), this is often implicit, and the adoption of a more formal approach would render the process far more transparent and credible, a desired outcome articulated by an ILSI/HESI expert committee [40].

6.2 Consideration of Toxicogenomics data as part of the WOE for MOA analysis

The PB case-study

The results and data from relevant toxicogenomics studies are being made increasingly available for potential inclusion in risk assessment. There is therefore a need to develop a structured framework for the evaluation of such data in WOE considerations. PB was used as a case study to explore some of these issues. The methodology developed for the analysis of the transcriptomics data was described in Chapter 4 and the results of the analysis and a discussion of the results obtained were detailed in Chapter 5.

Toxicogenomics data on PB were obtained from 2 public microarray repositories (ArrayExpress and Gene Expression Omnibus). The toxicogenomics datasets obtained for PB were from both studies that had focused on investigating PB as the primary compound and those that had generated data on PB through its use as a positive control. Although it would have been more useful to consider only datasets in which PB was employed as the primary compound for investigation, the datasets compiled from the use of PB as a positive control had to be included owing to the limited number of datasets available for the analysis.

The pre processing of the data obtained and the statistical strategy were devised in consultation with a genomics expert from the USEPA. Differentially expressed genes (DEGs) were identified from data collected from 9 investigated time points in the mouse (4 time points studied *in vivo*), rat (3 time points studied *in vivo* and 1 time point studied *in vitro*) and human hepatocytes (1 time point studied *in vitro*). The aim was to carry out a comparative analysis of publicly available toxicogenomics data for PB to determine the extent to which such information could be meaningfully interpreted as part of the MOA analysis to form part of the weight of evidence for a chemical of interest. A further objective was to explore the value added by such studies to such an analysis.

To facilitate the biological interpretation of the observed gene changes, the commercial pathway analysis software Ingenuity Pathway Analysis™ (IPA) was employed for analysis of the data. Analysis of the data in IPA produced results in the form of pathways (IPA Tox Lists and Canonical Pathways) and functional networks which were interpreted within the context of the known key events for the MOA for PB induced liver tumour formation as described in Chapter 2 of this thesis. Novel insights into the MOA for PB from these data were also sought.

Based on the observed results from the Tox Lists and Canonical Pathways, evidence for PB induced CAR activation, cell proliferation, inhibition of apoptosis, and hepatocellular carcinoma was obtained, on the basis of the perturbed gene changes within the selected pathways. In addition evidence was described for associated toxicological events like oxidative stress. The results were also suggestive of

pathways that were unlikely to be involved in the MOA for PB hepatocarcinogenesis (e.g. AhR and PPAR).

Owing to the sheer volume of the results obtained, a strategic approach had to be adopted to enable a manageable but efficient interpretation of the results within the desired MOA context. The focus was therefore placed on describing the changes observed for the principle genes identified from the literature to play critical roles in the regulation of genes within the pathways identified to be essential for the key events of interest. Where possible, experimental evidence for the observed gene changes was obtained from the literature and discussed; this was done as part of an effort to determine whether the gene changes observed from the constructed microarray experiments correlated well with experimental observations. In most cases, the key genes selected for further investigation and discussion from the microarray experiments were consistent with the experimental evidence for PB. The only exception (among the genes selected) to this was for the gene SCD1 which is involved in the regulation of lipid metabolism. This gene was observed to be down regulated in the rat and mouse dataset investigated even though the gene has been demonstrated to be up regulated in studies carried out using PXR knock-out mice [180]. The gene encoding AhR was however observed to be up regulated in all the mouse time points investigated even though mechanistic studies have shown that PB has a very low binding affinity for this transcription factor. In addition there is little evidence for the involvement of the AhR signalling pathway in PB MOA yet this pathway was observed to be significantly perturbed in the results produced in IPA. Such issues of reliability of the results obtained will be discussed further in the following sections.

A clear limitation of this comparative analysis of publicly available toxicogenomics data was the amount of evidence available for PB. A thorough species comparison could not be carried out as the data was limited for the rat and human. Although there was some observed commonality between the perturbed genes across the different pathways selected, the majority of the pathways compiled as evidence for the key events were based on the data obtained from the mouse datasets particularly the mouse data generated from the study at 2 days. The evidence for the key events directly inferred from the results output from the Tox Lists, Tox Functions and Canonical Pathways provided some evidence for PB induced CAR activation, cell

proliferation, inhibition of apoptosis and oxidative stress. With the exception of evidence considered for oxidative stress (in which key genes were observed to be differentially expressed in both the mouse and human dataset) which showed consistency between the two datasets, it was not possible to evaluate the consistency of the results observed across the datasets obtained for the three species. This was because the majority of the evidence obtained for the key events were on the basis of results obtained from the mouse dataset.

6.2.1 *Generalisation from the Case-study*

The comparative analysis of the transcriptomics data for PB identified a number of methodological issues associated with trying to interpret the biological relevance of the results obtained within the context of the key events for the MOA for PB induced liver tumour formation. These issues fell into two main categories that included:

1. The inability to apply an unsupervised (i.e. without the need to be led by information in the literature) approach to the identification of key events on the basis of the observed perturbed genes or pathways alone.
2. The inability to have confidence in some of the results obtained without cross referencing mechanistic data available from the published literature (e.g. interpretation of the relevance of AHR in the regulation of apoptosis for PB induced effects).

The Inability to Apply an Unsupervised Approach to the Identification of Key Events on the Basis of the Observed Perturbed Genes or Pathways Alone

One of the advantages cited for the application of *omics* technology to MOA analysis is the lack of bias towards a particular MOA [212]. While this may be true, one of the main limitations observed from the comparative analysis carried out in this thesis, was that it was very difficult to identify where the focus of the results interpretation should be placed in order to determine which molecular pathways were most relevant for the

PB MOA. Therefore the experience of carrying out the transcriptomics analysis was that one needed to have prior knowledge of the possible MOA for PB induced hepatocarcinogenesis in order to decide how best to interpret the results and to decide on which molecular pathways were most likely to be relevant. While it is appreciated that an unbiased approach to determining a MOA is preferred, in the case of toxicogenomics data, a balance has to be struck between striving for an objective approach to determining the key events on the basis of the vast amount of data generated and being able to obtain definitive answers to inform the risk assessment process.

The experience encountered from the comparative analysis carried out in this thesis has been that one needs a sense of direction when trying to interpret the vast amount of data that have been generated and some prior knowledge about a chemical of interest certainly aids the process of interpreting the data and getting a better sense of what information is relevant and to provide direction for the interpretation of the results. Therefore it is essential that mechanistic data about the key events in the MOA for either the chemical of interest or a chemical known to operate via a similar MOA are assimilated prior to the toxicogenomics analysis in order to have a better understanding of the observed pathway changes in order to fully appreciate the biological relevance of observed perturbed pathways. This has obvious implications for the nature of toxicity testing required, at least in the short to medium-term studies.

General background knowledge about the key events involved in the MOA for PB induced hepatocarcinogenesis was found to be essential (provided in Chapter 2) for the interpretation of the toxicogenomics results. Review of the evidence for the PB key events and associated toxicological events enabled a more targeted evaluation of the toxicogenomics results produced in IPA. In addition it was helpful to have some knowledge about general mechanisms of hepatotoxicity in order to consider the involvement of different MOAs. This enabled the selection of specific pathways for further investigation rather than placing the focus on only the significantly perturbed pathways identified in IPA. This was illustrated by the selection of 18 Tox Lists (pathways) out of the 46 produced for hepatotoxicity in IPA as the focus for the analysis (the selected Tox Lists included significantly changed pathways and non-significant pathways).

Evidence gathered from biochemical and mechanistic studies were also essential to help interpret the significance and relevance of the gene changes observed. These included mechanistic data obtained from genetically modified animals (e.g. CAR and PXR knockout mice) and mechanistic studies on specific target genes known to be involved in the regulation of specific pathways. Mechanistic studies demonstrating the effect of PB on transcript and protein levels were also reviewed to help determine the biological relevance of some of the gene changes observed. An important consideration for microarray studies is that not all gene changes observed will prove to be biologically relevant.

The comparative analysis demonstrated that a range of supportive evidence is needed to facilitate the interpretation of toxicogenomics results. It is clear that the type and amount of supportive data needed for the interpretation of toxicogenomics results would be completely dependent on the intended application of the results. As illustrated in the attempt to apply the toxicogenomics data to key events for PB MOA, it was necessary to consider a whole range of evidence.

In cases where toxicogenomics data is available to enable a more targeted consideration of different factors that could affect the response observed (e.g. dose, time, species), it is clear that more supportive mechanistic data would be required. The attempt to carry out a thorough species and temporal comparison of the PB dataset was limited by the lack of available DEGs to facilitate such analysis particularly within the rat and human datasets.

In cases where there is insufficient mechanistic data to address particular aspects of the risk assessment, toxicogenomics data could prove potentially useful in helping to bridge some of the data gap about responses at particular time points or responses at specific doses or in particular species. The experience encountered here however suggests that this particular application can only be realised in cases where there is already sufficient mechanistic information available for the compound under investigation and the toxicogenomics results would be used for addressing very specific and targeted aspects of the missing knowledge. Such an approach would also require the consideration of the appropriate study design.

An outcome of this thesis was the development of a logical framework for the evaluation of toxicogenomics data within the context of MOA analysis. As illustrated in Figure 5.6 (shown in chapter 5), it is necessary to apply a constant iterative process to the evaluation of both the mechanistic data (obtained from available literature) and pathways identified as relevant from the toxicogenomics results. Interpretation of the biological relevance of the observed gene changes (as evidence for a key event) requires the verification of the observed expression changes with the mechanistic evidence that has examined the relevant end point and also the transcript changes (including protein changes) for the critical genes exposed to the relevant compound. While attempts have been made to do this in the analysis carried out in this thesis, the consideration of a more comprehensive dataset across the three species would reduce the uncertainties in the toxicogenomics evidence described here for PB. Where the mechanistic data have been obtained from *in vivo* studies, these can help confirm the consistency and predictability of toxicogenomics data developed using *in vitro* systems.

Inability to Have Confidence in Results Obtained Without Cross Reference with Mechanistic Data

The method by which individual genes are categorised in IPA into the biological functions and subsequently into biological pathways can result in a single gene being categorised under multiple functions. This can therefore make it very difficult to determine *a priori* which particular function and therefore which key event for the MOA is most relevant for the chemical of interest.

An evaluation of the different outputs (Tox Lists, Canonical Pathways and Tox functions) was required to collate the evidence for the different key events for PB MOA. Crucially the pathways selected (on the basis of known mechanisms of hepatotoxicity) and considered to be relevant for the key events of interest were not necessarily identified by IPA to be the most statistically significant or dominating pathways. While it is known from experimental studies that PB induces a range of definitive biological effects in different species that can be identified as the key events that lead to liver tumour formation, it was difficult to obtain the same clear indications

from the microarray experiments carried out at the different time points across the different species.

A targeted approach to interpreting the vast amounts of results obtained would not have been possible without prior knowledge about the key events of PB. Indeed the interpretations about the potential key events of PB on the basis of the results observed in IPA alone could have led to systematic misinterpretations of some of the results. An example of this was the identification of the LPS/ IL-1 Mediated Inhibition of RXR Function as a significantly perturbed Tox List and Canonical Pathway across all datasets from all species investigated. Although the evidence provided by the pathways were suggestive of the inhibition of RXR function as a potential effect of PB exposure, the fact that the target genes (CYP2B6 and CYP3A4) for the binding partners of RXR (CAR and PXR respectively) were up regulated suggested that this was clearly not a real effect of PB. While evidence from the literature was reviewed that confirmed the role of RXR inhibition of CAR and PXR in the presence of LPS, evidence from the same dataset examined confirmed that this was clearly not a real PB induced effect.

Similarly the pathways for Hyperplasia/ Hyperproliferation and Liver Hypertrophy were not observed to be significantly perturbed. CYP1A2 was categorised to be associated within liver hypertrophy on the basis of evidence obtained from the mouse data. This was interpreted to be more as a reflection of the inducing effects of PB rather than a true indication of the key role of this gene in liver hypertrophy. This example suggested that many of the genes involved in both liver hyperplasia and hypertrophy were probably categorised and grouped within other pathways and so this pathway (confirmed to be key event on the basis of evidence from mechanistic studies) was not identified as significant. Without prior knowledge about the key events for PB, this pathway would not have been selected for further investigation and would have been disregarded as insignificant on the basis that the pathway was not significantly perturbed.

Both these examples illustrated the importance of not placing the focus on only “top/ significant” pathways as the basis for any conclusions about the suggested effects of a chemical under consideration. It also highlighted one of the limitations of Pathway

Analysis programs like IPA, in that the results of significantly perturbed pathways do not always point to real effects and so have to be interpreted with caution and the biological relevance has to be thoroughly considered. It is effectively the case that a program like IPA throws up lots of results and the user of the program has to decide on the basis of scientific knowledge and expertise which pathways are actually, or likely to be, biologically relevant to the compound under consideration.

It has been suggested that molecular pathways can be verified *in vitro* to identify and verify pathways considered to be associated with a proposed MOA in addition to developing assays that correspond to these toxicity pathways for use in modelling [212].

The experience encountered from this research whereby publicly available toxicogenomics data was analysed to see how the results correlated with experimental observations, makes it very difficult to imagine how an untargeted approach could be applied to identifying the key events on the basis of the toxicogenomics data alone, without the unnecessary commitment of large amounts of resources. The experience encountered here suggests that the identification of pathways or functions as a key event on the basis of the functional classifications identified by pathway analysis software may not be achievable from standard microarray studies alone. Attempts to try to do this would not only be a cumbersome process, but could potentially lead to the misinterpretation of the data where adaptive toxicological responses could be wrongly interpreted as being key events in the carcinogenic process.

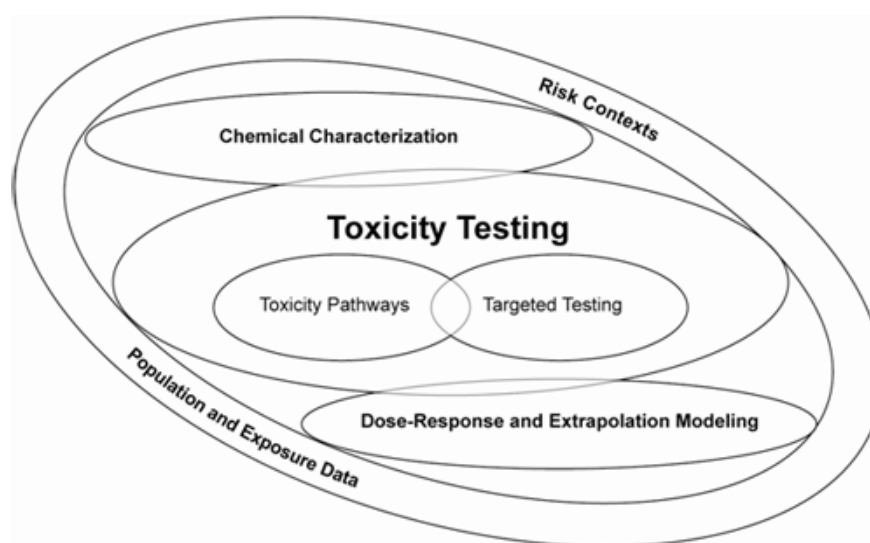
6.2.2 Challenges for the Application of Toxicogenomics Data in Regulatory Risk Assessment

The Health and Environmental Sciences Institute (HESI) Committee on the Application of Genomics to Mechanism-based Risk Assessment has highlighted that a measure of the successful impact of toxicogenomics data in risk assessment would be the inclusion of such data in regulatory submission and subsequent consideration in risk assessment applications. The vast amount of toxicogenomics data being generated has put increasing pressure for such data to be incorporated into toxicological risk assessment [213]. The goal for the intended move in this direction

was formally addressed in the report by the US National Research Council which puts forward their vision for the future of toxicity testing in the 21st century (Tox-21c). Tox-21c has formalised the intentions of what is expected from genomics technologies and puts forward a vision of how these expectations might be realised.

The landmark report by the NRC: *Toxicity Testing in the 21st Century: A vision and Strategy* (Tox-21c) was commissioned by the US EPA (and the collaborating agencies) and their co-ordination of this work includes their ToxCast program [214] which has been developed to improve their capabilities to predict toxicity based on bioactivity profiling. The Tox-21c report sets out the vision (Figure 6.1) for the future of toxicity-testing system that would rely more on the improved understanding on “toxicity pathways” to take into account the increased understanding of biological processes to chemical stressors and also to enable the incorporation of evidence obtained using high throughput technologies (e.g. genomics) and evidence from other emerging fields such as systems biology and bioinformatics. It is envisioned that such technologies would be used in a focused way rather than for broad-based genome wide studies [215, 216].

Figure 6.1: The vision for the future of toxicity testing proposed by the National Research Council [216]



The identification and use of the toxicity pathways as the basis for new approaches to toxicity testing and dose response modelling was stated as one of the long term aims of the proposed vision [216]. In addition, the vision highlighted the need to use high-throughput assays to evaluate relevant perturbations in key toxicity pathways that eventually result in disease. The latter links in closely with the requirements for MOA analysis (description of the key events for a MOA) as it highlights the need to focus on the evaluation of the key pathway changes that are obligatory for an observed effect.

While regulatory agencies like the US EPA have been keen to encourage the submission and use of toxicogenomics data and have even gone as far as to incorporate guidelines for the use of toxicogenomics data in the form of policy [52], they remain cautious, highlighting crucially that such data cannot be used in isolation as the basis for a decision [51, 52].

Similarly in the UK, the potential for the future use of toxicogenomics data has been acknowledged but caution has been expressed over its use in regulatory risk assessment. A symposium was organised by the expert advisory Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) and its sister Committees on Carcinogenicity and Mutagenicity (COC and COM) to discuss the current state of science for the use of genomics and proteomics in toxicology. The outcome of the symposium resulted in the production of a joint statement from these committees that concluded that research and validation in these technologies were required before such data could be considered for routine use in regulatory risk assessment [213, 217].

Many challenges remain to be addressed for the validation of toxicogenomics based test systems [218] before data generated using these technologies can be more widely accepted and trusted by regulatory bodies responsible for risk assessment. Indeed the vision for Tox-21c has identified validation as one of its key areas for research and development. The research and development needs highlighted in Tox-21c are split into 4 phases that include:

1. phase I: the elucidation of toxicity pathways; development of data storage, access and management systems; development of standard protocols for research methods and reporting.
2. phase II: development and validation of toxicity pathway assays and identification of markers of exposure, effect and susceptibility.
3. Phase III: evaluation of assays by comparison with traditional toxicity tests and on chemical with large datasets.
4. Phase IV: the use of validated assays in place of traditional toxicity tests [216].

6.2.3 *Practical Considerations for Use of Toxicogenomics Data in Risk Assessment*

The vision of Tox-21 to identify key toxicity pathways links closely with the requirements for MOA analysis. This suggests that a more targeted approach would be necessary for toxicogenomics study designs in order that this vision be fully realised. In this respect it is very different to the suggested use of programs like IPA which focuses on the user identification of significantly perturbed genes/ pathways that may or may not be suggestive of a toxicological effect.

The move towards the increased use of toxicogenomics data in risk assessment would rely heavily on the increased use of *in vitro* tests. Under the proposal for Tox-21c this would involve the use of a wide range of *in vitro* tests to identify biological perturbations using cellular and molecular systems based on human biology. It is the aim of the Tox-21c vision that these tests would eventually replace whole animal testing and provide improved mechanistically predictive tools for human health risk assessments [216, 219]. It is important to consider the extent to which data obtained from *in vitro* studies can be informative and reliable for MOA analysis. As illustrated with the PB case study, the data obtained from the study in human hepatocytes showed that some key genes (NQO1, MYC, PXR, CAR) identified as critical for certain pathways (e.g. oxidative stress pathway, cell proliferation and inhibition of apoptosis) were not observed to be differentially expressed within the dataset examined but were reported to be up regulated in another study identified within the literature.

The Tox-21 vision shares similarities with the vision promoted in Europe for alternative testing methods, based on the concept of the 3Rs (reduction, refinement and replacement) to strive for greater animal welfare and minimise and eventually phase out animal use [220]. The experience of striving for the 3Rs in Europe suggests that while there may have been a 60% overall reduction (spanning over 3 decades) in the use of animal usage in experimentation, this reduction has been attributed to the increased use of genetically modified mice [221] rather than the introduction of formally validated 3Rs alternatives [220]. The continued reliance on the use of whole animal models for toxicity testing in human risk assessment are attributed to the many challenges cited for the shortcomings of the use of *in vitro* testing. The latter include:

- the inability of cell-based assays to pick up some metabolic effects, for example studies carried out in hepatocytes may miss out toxicities that only occur in the whole liver.
- Adequate testing of metabolites: cell-based assays may not always provide a true reflection of the actual toxicity as the toxicity from some compounds is caused by a metabolite of the parent compound (this is also an issue *in vivo*) [222].
- Adequacy of cell models, often based on tumour-derived immortal cell lines.

It is interesting to note that most animal tests carried out for human risk assessment have not changed significantly in the last 40 to 80 years [220]. While rats and mice may not be the most predictive models for human, they are relatively inexpensive to use in terms of breeding and maintenance [220].

The vision for Tox-21c promotes a paradigm shift towards the increased use of human primary cells. The costs of generating toxicogenomics data obtained from *in vitro* or short term *in vivo* techniques are considered to be relatively low when compared to long term testing animal testing [223], however the costs of running numerous microarray experiments and analysing the data produced can render them quite expensive. This therefore hinders their use in more detailed microarray studies to consider dose and time course relationships. It also means that current microarray studies can only provide limited information and a snapshot of information [224].

A more important consideration is the costs for the validation of both methodologies and identified genomic biomarkers to enable increased acceptance of toxicogenomics data by regulatory bodies. Although advocates for the use of high throughput technologies highlight their potential to identify biomarkers for use in human risk assessment, there is currently little motivation from private companies to validate safety biomarkers partly because they do not know how they will be used by regulatory bodies but mostly because the high costs of validating identified genomic signatures [224].

One of the advantages of applying a WOE-based approach to the IPCS HRF is that it facilitates the identification of data gaps to potentially inform toxicological study design and the identification of biomarkers. The routine application of toxicogenomics data to inform more targeted testing could prove potentially expensive not least because of the inability of toxicogenomics studies to specifically answer key questions.

6.2.4 General Considerations and Identified Areas for Future Work

Expectations are high for the use of data generated from *omics* technology for addition to the WOE for MOA analysis and risk assessment in general. The comparative analysis of publicly available toxicogenomics data for PB has identified many challenging areas that remain to be resolved and it is clear that we are still very much in the early part of phase I of the vision for Tox-21c and may be here for a while yet. The experience encountered here suggests that current microarray study designs (such as the studies obtained from the public microarray repositories) may not be fit for the purposes envisioned by Tox-21. Indeed the Tox-21 report suggests that a major investment in mechanistic and method development will be required to accomplish their objectives. However if the expectations of *omics* technologies are to be realised, then more case studies are needed to help in method development on the applicability of such data particularly when using publicly available data.

If toxicogenomics data (or other forms of *omics* data) are going to be routinely generated and deposited in public databases for the intended application in risk

assessment analysis, effort needs to be put into the development of more thorough methodology on how such results should be interpreted. This is especially true when trying to determine what the focus of such interpretations should be, particularly when considering several endpoints at one time, as in the case of MOA analysis which often requires the identification of several key events. As illustrated by the comparative analysis, exposure to PB leads to the perturbation of many pathways (as would be the case for other compounds) and the challenge here was on the identification of the pathways that were most likely to inform the toxicological effect of interest.

6.2.4.1 **Future work**

One of the advantages of applying a WOE-based approach to the IPCS HRF is that it facilitates the ability to read across information to other compounds that are thought to act via the same MOA. The potential to read across toxicogenomics information about a particular compound should also be explored and is potentially very powerful.

For prototypical compounds like PB, it would be beneficial to explore the extent to which perturbed gene(s) or pathways can be read across to other compounds known or postulated to act via the same MOA. Again using publicly available data, it would be particularly useful to investigate the potential for species extrapolation for compounds where there is available toxicogenomics data but insufficient mechanistic data.

Finally, it would be beneficial to develop guidance on the extent to which toxicogenomics pathways can be verified. The verification of pathways of interest could be undertaken via independent confirmation or through the systematic evaluation of mechanistic evidence for the involvement of the pathways in toxicological effects of interest. The latter approach could be undertaken by applying the Hill criteria as appropriate (e.g. dose, time, plausibility etc.) and this approach would relate directly with the strategic framework proposed in this thesis for the analysis of toxicogenomics data for MOA analysis.

6.3 Final Conclusions

Weight of evidence analysis is an essential component of the application of the IPCS Human Relevance Framework in cancer and non-cancer hazard identification. Application of a framework approach as an analytical tool in cancer hazard identification encourages the use of harmonised approach to risk assessment and aims to improve the transparency of the risk assessment process. While the framework currently provides the means to facilitate the systematic evaluation and presentation of available evidence, the use of more formal systematic review techniques to evaluate the weight of evidence as illustrated in this thesis through the evaluation of epidemiological evidence for PB, would improve the capability of the framework to provide more transparent conclusions about an animal MOA and the human relevance (both qualitative and quantitative) of the MOA.

As demonstrated by evaluation of the epidemiological evidence for PB, the use of a systematic approach to evaluating evidence for risk assessment, particularly when it includes a quality assessment of the evidence under consideration, enables a more transparent identification of areas of uncertainty associated with the conclusions made and provides justifications for the basis of any resulting decisions.

The illustration of the potential application of toxicogenomics data as part of the weight of evidence in MOA analysis has demonstrated that while it is important to include and thoroughly consider all types of available evidence about the potential hazards of a chemical of interest, interpretation of toxicogenomics data within the context of MOA analysis, through the use of pathway analysis software is difficult. A suggested framework for the evaluation of toxicogenomics data has been proposed to facilitate a logical evaluation of toxicogenomics data within the context of MOA analysis as proposed by IPCS.

The implication of gene(s) or pathway changes as potential indicators of key events also needs to be interpreted with caution after thorough consideration of how these changes reflect changes observed or expected to occur in experimental studies. While the potential to read across the toxicogenomics evidence obtained for PB to other

compounds postulated to act via the same MOA needs to be explored, such an approach would be useful for providing supportive evidence for the results obtained here but would be dependent on the availability of public data. The experience from this comparative analysis however suggests that at the present time, the observed perturbed pathways are not robust enough to be used as stand alone evidence in MOA analysis or in general risk assessment. In addition the evidence obtained from the analysis of the perturbed pathways did not add weight to the evidence currently available for the MOA of PB induced hepatocarcinogenesis. Even though many of the “right” pathways were sometimes observed to have changed, “wrong” pathways were also observed to be perturbed

There is a clear need to improve the transparency of the communication of the interpreted findings of the results obtained from the analysis of toxicogenomics data (and more generally *omics* data) and users of pathway analysis software need to make explicit the basis for which they have interpreted their results as evidence for a key event. Where such data is used as supportive evidence as part of the weight of evidence in MOA analysis, it should be made clear how consistent such data is with what is already known from experimental studies. Guidance from IPCS in this area would be particularly useful. More importantly, genomics investigators need to be more transparent and explicit about the methodology that they have applied to investigating the biological relevance of their findings as illustrated in the analysis carried out in this thesis.

While there is a current drive to promote the use of *omics* data in the risk assessment process, a global commitment is needed from scientists to develop best practices for the interpretation of the data being generated. Such a move would go a long way to address the current disconnect between the expectations of what *omics* technologies will deliver and the practicalities from a risk assessment perspective of how easily it is to interpret the results being generated to gain a full appreciation of the pledges that have been made for these technologies.

Summary of the Outcome of the toxicogenomics comparative analysis:

- Currently, prior mechanistic knowledge about a compound under investigation is essential to enable efficient interpretation of the biological relevance of perturbed pathways identified using Pathway Analysis software.
- An untargeted approach to evaluating toxicogenomics evidence within the context of MOA analysis can lead to misinterpretation of findings for the association of significant genes/ pathways with potential key events.
- To realise the true potential for the application of toxicogenomics data in risk assessment, future toxicogenomics study designs will have to be a lot more targeted (i.e. in their considerations of dose-response, time-response and species analysis) before they can be applied to help fill in the current gaps in mechanistic knowledge.
- Current analysis based on “standard” microarray studies are unlikely to be fit for the purposes envisioned by Tox-21 to effectively assess key pathways that would be indicative of key events in the MOA for a compound of interest. Such studies are essentially a means to an end, so that the information generated is currently only of limited value in MOA analysis.

REFERENCES

1. Weed, L. Douglas, *Weight of Evidence: A Review of Concept and Methods*. Risk Analysis, 2005. **25**(6): p. 1545-1557.
2. Linkov, Igor, Loney, Drew, Cormier, Susan, Satterstrom, F. Kyle, and Bridges, Todd, *Weight-of-evidence evaluation in environmental assessment: Review of qualitative and quantitative approaches*. Science of The Total Environment, 2009. **407**(19): p. 5199-5205.
3. FAO/WHO, *Understanding the Codex Alimentarius Commission* 3rd ed. Codex Alimentarius, ed. World Health Organization/ Food and Agriculture Organization of the United Nations. 2006, Rome. 47.
4. IPCS, *Principles and methods for the risk assessment of chemicals in food. Chapter 2: Risk Assessment and its Role in Risk Analysis*. Environmental Health Criteria ed. World Health Organization. 2009, Geneva: Food and Agriculture Organization of the United Nations/World Health Organization.
5. IPCS, *Principles for the Assessment of Risks to Human Health from Exposure to Chemicals*. Environmental Health Criteria 210, ed. World Health Organization. 1999, Geneva: World Health Organization.
6. IPCS, ed. (*International Programme on Chemical Safety*) *Environmental Health Criteria (240): Principles and methods for the risk assessment of chemicals in food*. (Environmental health criteria ; 240), ed. World Health Organization. 2009.
7. Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment. 2004. Guidance on a strategy for the risk assessment of chemical carcinogens. Department of Health, London. Available at <http://www.dh.gov.uk/prod_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_4091207.pdf>.

8. O'Brien, J., Renwick, A. G., Constable, A., Dybing, E., Muller, D. J., Schlatter, J., Slob, W., Tueting, W., van, Benthem J., Williams, G. M., and Wolfreys, A., *Approaches to the risk assessment of genotoxic carcinogens in food: a critical appraisal*. Food Chem.Toxicol., 2006. **44**(10): p. 1613-1635.
9. Boobis, A. R., Cohen, S. M., Dellarco, V., McGregor, D., Meek, M. E., Vickers, C., Willcocks, D., and Farland, W., *IPCS framework for analyzing the relevance of a cancer mode of action for humans*. Crit Rev.Toxicol., 2006. **36**(10): p. 781-792.
10. Clewell, H., *Use of mode of action in risk assessment: past, present, and future*. Regul.Toxicol.Pharmacol., 2005. **42**(1): p. 3-14.
11. Doak, Shareen H., Jenkins, Gareth J. S., Johnson, George E., Quick, Emma, Parry, Elizabeth M., and Parry, James M., *Mechanistic Influences for Mutation Induction Curves after Exposure to DNA-Reactive Carcinogens*. Cancer Research, 2007. **67**(8): p. 3904-3911.
12. Kroes, R., Renwick, A. G., Cheeseman, M., Kleiner, J., Mangelsdorf, I., Piersma, A., Schilter, B., Schlatter, J., van Schothorst, F., Vos, J. G., and Würtzen, G., *Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet*. Food and Chemical Toxicology, 2004. **42**(1): p. 65-83.
13. Barlow, S., Renwick, A. G., Kleiner, J., Bridges, J. W., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., Knaap, A., Kroes, R., Liem, D., Muller, D. J., Page, S., Rolland, V., Schlatter, J., Tritscher, A., Tueting, W., and Wurtzen, G., *Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe*. Food Chem Toxicol, 2006. **44**(10): p. 1636-50.

14. Pratt, I., Barlow, S., Kleiner, J., and Larsen, J. C., *The influence of thresholds on the risk assessment of carcinogens in food*. *Mutat Res*, 2009. **678**(2): p. 113-7.
15. Dybing, Erik, Tore, Sanner, Henk, Roelfzema, Dinant, Kroese, and Raymond, W. Tennant, *T25: A Simplified Carcinogenic Potency Index: Description of the System and Study of Correlations between Carcinogenic Potency and Species/Site Specificity and Mutagenicity*. *Pharmacology & Toxicology*, 1997. **80**(6): p. 272-279.
16. O'Brien, J., Renwick, A. G., Constable, A., Dybing, E., Müller, D. J. G., Schlatter, J., Slob, W., Tueting, W., van Benthem, J., Williams, G. M., and Wolfreys, A., *Approaches to the risk assessment of genotoxic carcinogens in food: A critical appraisal*. *Food and Chemical Toxicology*, 2006. **44**(10): p. 1613-1635.
17. Peto, R., Pike, M. C., Bernstein, L., Gold, L. S., and Ames, B. N., *The TD50: a proposed general convention for the numerical description of the carcinogenic potency of chemicals in chronic-exposure animal experiments*. *Environ Health Perspect*, 1984. **58**: p. 1-8.
18. EFSA, (European Food Safety Authority) *Opinion of the Scientific Committee on a request from EFSA related to a Harmonised Approach for Risk Assessment of Substances Which are both Genotoxic and Carcinogenic (Request No EFSA-Q-2004-020)*. *The EFSA Journal*, 2005(282): p. 1-31.
19. IPCS. (International Programme on Chemical Safety) *IPCS WORKSHOP ON ISSUES IN CANCER RISK ASSESSMENT*. (World Health Organization, Geneva, 1998). Available at:
https://apps.who.int/pcs/harmon_site/harmonize/docs/Issues_Cancer%20RA.pdf

20. UNCED. Agenda 21: Earth Summit - The United Nations Programme of Action from Rio. Report No. 13: 9789211005097, (United Nations Division for Sustainable Development, 1993). Available at:
<http://www.un.org/esa/sustdev/documents/agenda21/english/agenda21toc.htm>
21. IPCS. (International Programme on Chemical Safety) IPCS Workshop on Developing a Conceptual Framework for Cancer Risk Assessment. (World Health Organization, Geneva, 1999). Available at:
http://www.who.int/ipcs/publications/methods/harmonization/en/framework_cancer_ra.pdf
22. IPCS. International Programme on Chemical Safety Harmonization Project Strategic Plan 2005-2009. (World Health Organization, Geneva, 2005). Available at:
http://www.who.int/ipcs/methods/harmonization/strategic_plan_rev.pdf
23. IPCS. (International Programme on Chemical Safety) IPCS Workshop on Developing a Conceptual Framework for Cancer Risk Assessment. Lyon, France, 16-18 February 1999. (World Health Organization, Geneva, 2007). Available at:
http://www.who.int/ipcs/publications/methods/harmonization/en/framework_cancer_ra.pdf
24. Sonich-Mullin, C., Fielder, R., Wiltse, J., Baetcke, K., Dempsey, J., Fenner-Crisp, P., Grant, D., Hartley, M., Knaap, A., Kroese, D., Mangelsdorf, I., Meek, E., Rice, J. M., and Younes, M., *IPCS Conceptual Framework for Evaluating a Mode of Action for Chemical Carcinogenesis*. Regulatory Toxicology and Pharmacology, 2001. **34**(2): p. 146-152.
25. Dellarco, V. L. and Baetcke, K., *A risk assessment perspective: application of mode of action and human relevance frameworks to the analysis of rodent tumor data*. Toxicological Sciences, 2005. **86**(1): p. 1-3.

26. Meek, M. E., Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., Longfellow, D. G., Pastoor, T., Seed, J., and Patton, D. E., *A framework for human relevance analysis of information on carcinogenic modes of action*. Crit Rev.Toxicol., 2003. **33**(6): p. 591-653.
27. Ashby, J., Kier, L., Wilson, Age, Green, T., Lefevre, PA, Tinwell, H., Willis, GA, Heydens, WF, and Clapp, Mjl, *Evaluation of the potential carcinogenicity and genetic toxicity to humans of the herbicide acetochlor*. Human & Experimental Toxicology, 1996. **15**(9): p. 702-735.
28. Williams, Gary M., *Mechanisms of chemical carcinogenesis and application to human cancer risk assessment*. Toxicology, 2001. **166**(1-2): p. 3-10.
29. Hill, B. A., *The Environment and Disease: Association or Causation?* Proc R Soc Med., 1965. **58**: p. 295-300.
30. Cohen, S. M., Meek, M. E., Klaunig, J. E., Patton, D. E., and Fenner-Crisp, P. A., *The human relevance of information on carcinogenic modes of action: overview*. Crit Rev.Toxicol., 2003. **33**(6): p. 581-589.
31. IPCS. (International Programme on Chemical Safety) Workshop Report: Toxicogenomics and the Risk Assessment of Chemicals for the Protection of Human Health (Summary). Held at the Federal Institute for Risk Assessment, Berlin, Germany 17-19 November 2003., (World Health Organization, Geneva, 2003). Available at:
<http://www.who.int/ipcs/methods/en/toxicogenomicssummaryreport.pdf>
32. USEPA. 2005. (United States Environmental Protection Agency) Guidelines for Carcinogenic Risk Assessment. EPA/630/P-03/001B. Washington, DC. Available at
<http://www.epa.gov/raf/publications/pdfs/CANCER_GUIDELINES_FINAL_3-25-05.PDF>.

33. EFSA, *Opinion of the Scientific Panel (PPR) on the scientific principles in the assessment and guidance provided in the field of human toxicology between 2003 and 2006. (EFSA-Q-2005-255)*. The EFSA Journal, 2006. **346**: p. 1-13.
34. IARC. (International Agency for Research on Cancer) IARC Scientific Publications No. 147: Species Differences in Thyroid, Kidney and Urinary Bladder Carcinogenesis. (World Health Organization, Lyon, France, 1997). Available at:
<http://monographs.iarc.fr/ENG/Publications/pub147/IARCpub147.pdf>
35. IARC. (International Agency for Research on Cancer) IARC Technical Publication No. 24: Peroxisome Proliferation and its Role in Carcinogenesis. (World Health Organization, Lyon, France, 1995). Available at:
<http://monographs.iarc.fr/ENG/Publications/techrep24/IARCrep24.pdf>
36. RATSC. (Risk Assessment and Toxicology Steering Committee) Risk assessment approaches used by UK Government for evaluating human health effects of chemicals: Report prepared for the Risk Assessment and Toxicology Steering Committee. (Institute for Environment and Health, Leicester, 1999). Available at: <http://ieh.cranfield.ac.uk/ighrc/cr2.pdf>
37. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. Guidance on a strategy for testing of chemicals for mutagenicity., (Department of Health, London, 2000). Available at:
<http://iacom.org.uk/guidstate/documents/guidance.pdf>
38. JMPR. (Joint FAO/WHO Meeting on Pesticide Residues) Pesticide residues in food 2006. (World Health Organization/ Food and Agriculture Organization of the United Nations, Rome, 2006). Available at:
http://www.fao.org/ag/AGP/AGPP/Pesticid/JMPR/DOWNLOAD/2006_rep/report2006jmp.pdf

39. WHO, (*World Health Organization*) *Environmental Health Criteria 240: Principles and Methods for the Risk Assessment of Chemicals in Food* Environmental Health Criteria, ed. the United Nations and the World Health Organization Joint publication of the Food and Agriculture Organization. 2009, Geneva: World Health Organization.
40. Schreider, Jay, Barrow, Craig, Birchfield, Norman, Dearfield, Kerry, Devlin, Dennis, Henry, Sara, Kramer, Melissa, Schappelle, Seema, Solomon, Keith, Weed, Douglas L., and Embry, Michelle R., *Enhancing the Credibility of Decisions Based on Scientific Conclusions: Transparency Is Imperative*. Toxicol. Sci., 2010. **116**(1): p. 5-7.
41. WHO. (World Health Organization) IPCS harmonization project document ; no. 4. Report No. ISBN 978 92 4 156349 9, (World Health Organization, Geneva, 2007). Available at:
http://www.who.int/ipcs/methods/harmonization/areas/cancer_mode.pdf
42. EFSA, (*European Food Safety Authority*) *Application of systematic review methodology to food and feed safety assessments to support decision making. EFSA Guidance for those carrying out systematic reviews*. EFSA Journal, 2010. **8**(6): p. 1637.
43. CRD. (Centres for Reviews and Dissemination) Undertaking systematic reviews of research on effectiveness: CRD's guidance for those carrying out or commissioning reviews Report No. 4, (NHS Centre for Reviews and Dissemination, York, 2001). Available at:
http://www.york.ac.uk/inst/crd/pdf/Systematic_Reviews.pdf
44. Dix, D. J., Gallagher, K., Benson, W. H., Groskinsky, B. L., McClintock, J. T., Dearfield, K. L., and Farland, W. H., *A framework for the use of genomics data at the EPA*. Nat Biotechnol, 2006. **24**(9): p. 1108-11.

45. Benson, W. H., Gallagher, K., and McClintock, J. T., *U.S. Environmental Protection Agency's activities to prepare for regulatory and risk assessment applications of genomics information*. Environ Mol Mutagen, 2007. **48**(5): p. 359-62.
46. Boverhof, Darrell R. and Zacharewski, Timothy R., *Toxicogenomics in Risk Assessment: Applications and Needs*. Toxicol. Sci., 2006. **89**(2): p. 352-360.
47. Lesko, Lawrence J. and Woodcock, Janet, *Translation of pharmacogenomics and pharmacogenetics: a regulatory perspective*. Nat Rev Drug Discov, 2004. **3**(9): p. 763-769.
48. NRC, *Toxicogenomics Technologies and Risk Assessment of Environmental Carcinogens: A Workshop Summary. (Committee on How Toxicogenomics Could Inform Critical Issues in Carcinogenic Risk Assessment of Environmental Chemicals, Committee on Emerging Issues on Emerging Issues on Data on Environmental Contaminants, National Research Council)*. 2005, Washington: THE NATIONAL ACADEMIES PRESS.
49. Tennant, R. W., *The National Center for Toxicogenomics: using new technologies to inform mechanistic toxicology*. Environ Health Perspect, 2002. **110**(1): p. A8-10.
50. Pettit, Syril, des Etages, Shelley Ann, Mylecraine, Louis, Snyder, Ronald, Foster, Jennifer, Dunn, Robert T., II, Haymes, Kenneth, Duval, Manuel, Stevens, James, Afshari, Cynthia, and Vickers, Alison, *Current and Future Applications of Toxicogenomics: Results Summary of a Survey from the HESI Genomics State of Science Subcommittee*. Environ Health Perspect, 2010. **118**(7).
51. USEPA, Science Policy Council. (United States Environmental Protection Agency) Interim Policy on Genomics. (EPA, Washington, DC, 2002). Available at: <http://www.epa.gov/spc/genomics.htm>

52. USEPA. (United States Environmental Protection Agency) Potential Implications of Genomics for Regulatory and Risk Assessment at EPA. Publication No EPA 100/B-04/002. (EPA, Washington, DC, 2004). Available at: <http://www.epa.gov/osa/genomics.htm>
53. IPCS. (International Programme on Chemical Safety) Workshop Report: Toxicogenomics and the Risk Assessment of Chemicals for the Protection of Human Health (summary). (World Health Organization Geneva, 2003). Available at: <http://www.who.int/ipcs/methods/en/toxicogenomicssummaryreport.pdf>
54. IGHRC. (The Intergovernmental Group on Health Risks from Chemicals) Assessment of Chemical Carcinogens: Background to General Principles of a Weight of Evidence Approach., (MRC Institute for Environment and Health, Leicester, 2002). Available at: <http://ieh.cranfield.ac.uk/ighrc/cr8.pdf>
55. Singh, G., Driever, P. H., and Sander, J. W., *Cancer risk in people with epilepsy: the role of antiepileptic drugs*. Brain, 2005. **128**(Pt 1): p. 7-17.
56. Holsapple, M. P., Pitot, H. C., Cohen, S. M., Boobis, A. R., Klaunig, J. E., Pastoor, T., Dellarco, V. L., and Dragan, Y. P., *Mode of action in relevance of rodent liver tumors to human cancer risk*. Toxicological Sciences, 2006. **89**(1): p. 51-56.
57. McClain, R. Michael, *Mouse Liver Tumors and Microsomal Enzyme Inducing Drugs: Experimental and Clinical Perspectives with Phenobarbital*. Mouse Liver Carcinogenesis: Mechanisms and Species Comparisons, Wiley-Liss, New York 1990: p. pp. 345–365.
58. IARC. (International Agency for Research on Cancer) Phenobarbital and its sodium salt. IARC Monographs Volume 79: Some Thyrotropic Agents. 161-288 (IARC, Lyon, France, 2001). Available at: <http://monographs.iarc.fr/ENG/Monographs/vol79/mono79-11.pdf>

59. Whysner, J., Ross, P. M., and Williams, G. M., *Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion*. Pharmacol.Ther., 1996. **71**(1-2): p. 153-191.
60. Scholz, Werner, SchÄ¼tze, Karin, Kunz, Werner, and Schwarz, Michael, *Phenobarbital Enhances the Formation of Reactive Oxygen in Neoplastic Rat Liver Nodules*. Cancer Research, 1990. **50**(21): p. 7015-7022.
61. Ward, J. M., *Increased susceptibility of livers of aged F344/NCr rats to the effects of phenobarbital on the incidence, morphology, and histochemistry of hepatocellular foci and neoplasms*. J Natl Cancer Inst, 1983. **71**(4): p. 815-23.
62. Benet, L.Z; Sheiner, L.B, *Design and optimization of dosage regimens:pharmacokinetic data*. British Journal of Clinical Pharmacology, 1985(15): p. 71-76.
63. Clemmesen, J. and Hjalgrim-Jensen, S., *Is phenobarbital carcinogenic? A follow-up of 8078 epileptics*. Ecotoxicol.Environ.Saf, 1978. **1**(4): p. 457-470.
64. Wei, Ping, Zhang, Jun, Egan-Hafley, Margarete, Liang, Shuguang, and Moore, David D., *The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism*. Nature, 2000. **407**(6806): p. 920-923.
65. Yamamoto, Y., Moore, R., Goldsworthy, T. L., Negishi, M., and Maronpot, R. R., *The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice*. Cancer Res., 2004. **64**(20): p. 7197-7200.
66. Waxman, D. J. and Azaroff, L., *Phenobarbital induction of cytochrome P-450 gene expression*. Biochem J, 1992. **281 (Pt 3)**: p. 577-92.

67. Ueda, Akiko, Hamadeh, Hisham K., Webb, Heather K., Yamamoto, Yukio, Sueyoshi, Tatsuya, Afshari, Cynthia A., Lehmann, JÃ¼rgen M., and Negishi, Masahiko, *Diverse Roles of the Nuclear Orphan Receptor CAR in Regulating Hepatic Genes in Response to Phenobarbital*. *Molecular Pharmacology*, 2002. **61**(1): p. 1-6.
68. Honkakoski, P. and Negishi, M., *Regulation of cytochrome P450 (CYP) genes by nuclear receptors*. *Biochem.J.*, 2000. **347**(Pt 2): p. 321-337.
69. Kawamoto, Takeshi, Sueyoshi, Tatsuya, Zelko, Igor, Moore, Rick, Washburn, Kimberly, and Negishi, Masahiko, *Phenobarbital-Responsive Nuclear Translocation of the Receptor CAR in Induction of the CYP2B Gene*. *Molecular and Cellular Biology*, 1999. **19**(9): p. 6318-6322.
70. Zelko, I. and Negishi, M., *Phenobarbital-elicited activation of nuclear receptor CAR in induction of cytochrome P450 genes*. *Biochem.Biophys.Res.Comm.*, 2000. **277**(1): p. 1-6.
71. Kodama, S. and Negishi, M., *Phenobarbital confers its diverse effects by activating the orphan nuclear receptor car*. *Drug Metab Rev.*, 2006. **38**(1-2): p. 75-87.
72. Yamamoto, Yukio, Kawamoto, Takeshi, and Negishi, Masahiko, *The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity*. *Archives of Biochemistry and Biophysics*, 2003. **409**(1): p. 207-211.
73. Cunninghame, M. E., Evans, J. G., and Butler, W. H., *An ultrastructural study of spontaneous and phenobarbitone-induced nodules in the mouse liver*. *Int J Exp Pathol*, 1991. **72**(6): p. 695-703.
74. Wolff, George L., Morrissey, Robert L., and Chen, James J., *Amplified response to phenobarbital promotion of hepatotumorigenesis in obese yellow Avy/A (C3H x VY) F-1 hybrid mice*. *Carcinogenesis*, 1986. **7**(11): p. 1895-1898.

75. Carthew, P., Edwards, R. E., and Nolan, B. M., *The quantitative distinction of hyperplasia from hypertrophy in hepatomegaly induced in the rat liver by phenobarbital*. Toxicol Sci, 1998. **44**(1): p. 46-51.
76. Carthew, Philip, Edwards, Richard E., and Nolan, Barbara M., *The Quantitative Distinction of Hyperplasia from Hypertrophy in Hepatomegaly Induced in the Rat Liver by Phenobarbital*. Toxicological Sciences, 1998. **44**(1): p. 46-51.
77. Bursch, W., Lauer, B., Timmermann-Trosiener, I., Barthel, G., Schuppler, J., and Schulte-Hermann, R., *Controlled death (apoptosis) of normal and putative preneoplastic cells in rat liver following withdrawal of tumor promoters*. Carcinogenesis, 1984. **5**(4): p. 453-458.
78. Ross, Jillian, Plummer, Simon M., Rode, Anja, Scheer, Nico, Bower, Conrad C., Vogel, Ortwin, Henderson, Colin J., Wolf, C. Roland, and Elcombe, Clifford R., *Human Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) Support the Hypertrophic but not the Hyperplastic Response to the Murine Nongenotoxic Hepatocarcinogens Phenobarbital and Chlordane In Vivo*. Toxicological Sciences, 2010. **116**(2): p. 452-466.
79. Japundzic, M., Knezevic, B., Djordjevic-Camba, V., and Japundzic, I., *The influence of phenobarbital-NA on the mitotic activity of parenchymal liver cells during rat liver regeneration*. Experimental Cell Research, 1967. **48**(1): p. 163-167.
80. Plant, N. J., Horley, N. J., Dickins, M., Hasmall, S., Elcombe, C. R., and Bell, D. R., *The coordinate regulation of DNA synthesis and suppression of apoptosis is differentially regulated by the liver growth agents, phenobarbital and methylclofenapate*. Carcinogenesis, 1998. **19**(9): p. 1521-1527.
81. Schulte-Hermann, R., *Initiation and promotion in hepatocarcinogenesis*. Arch Toxicol, 1987. **60**(1-3): p. 179-81.

82. Peraino, Carl, Michael Fry, R. J., and Staffeldt, Everett, *Reduction and Enhancement by Phenobarbital of Hepatocarcinogenesis Induced in the Rat by 2-Acetylaminofluorene*. Cancer Research, 1971. **31**(10): p. 1506-1512.
83. Schulte-Hermann, R., Timmermann-Trosiener, I., Barthel, G., and Bursch, W., *DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion*. Cancer Res., 1990. **50**(16): p. 5127-5135.
84. Goldsworthy, Thomas L. and Pitot, Henry C., *The quantitative analysis and stability of histochemical markers of altered hepatic foci in rat liver following initiation by diethylnitrosamine administration and promotion with phenobarbital*. Carcinogenesis, 1985. **6**(9): p. 1261-1269.
85. Pitot, H. C., Dragan, Y., Sargent, L., and Xu, Y. H., *Biochemical Markers Associated with the Stages of Promotion and Progression during Hepatocarcinogenesis in the Rat*. Environmental Health Perspectives, 1991. **93**: p. 181-189.
86. Santos, N. A. G., Medina, W. S. G., Martins, N. M., Rodrigues, M. A. Carvalho, Curti, C., and Santos, A. C., *Involvement of oxidative stress in the hepatotoxicity induced by aromatic antiepileptic drugs*. Toxicology in Vitro, 2008. **22**(8): p. 1820-1824.
87. Klaunig, James E. and Kamendulis, Lisa M., *The Role of Oxidative Stress in Carcinogenesis*. Annual Review of Pharmacology and Toxicology, 2004. **44**(1): p. 239-267.
88. Klaunig, J. E., Xu, Y., Bachowski, S., Ketcham, C. A., Isenberg, J. S., Kolaja, K. L., Baker, T. K., Walborg, E. F., Jr., and Stevenson, D. E., *Oxidative stress in nongenotoxic carcinogenesis*. Toxicol Lett, 1995. **82-83**: p. 683-91.
89. Parke, D. V., *The cytochromes P450 and mechanisms of chemical carcinogenesis*. Environ Health Perspect, 1994. **102**(10): p. 852-3.

90. Dostalek, Miroslav, Hardy, Klarissa D., Milne, Ginger L., Morrow, Jason D., Chen, Chi, Gonzalez, Frank J., Gu, Jun, Ding, Xinxin, Johnson, Delinda A., Johnson, Jeffrey A., Martin, Martha V., and Guengerich, F. Peter, *Development of Oxidative Stress by Cytochrome P450 Induction in Rodents Is Selective for Barbiturates and Related to Loss of Pyridine Nucleotide-dependent Protective Systems*. Journal of Biological Chemistry, 2008. **283**(25): p. 17147-17157.

91. Dostalek, Miroslav, Brooks, Joshua D., Hardy, Klarissa D., Milne, Ginger L., Moore, Megan M., Sharma, Sameer, Morrow, Jason D., and Guengerich, F. Peter, *In Vivo Oxidative Damage in Rats Is Associated with Barbiturate Response but Not Other Cytochrome P450 Inducers*. Molecular Pharmacology, 2007. **72**(6): p. 1419-1424.

92. Kinoshita, Anna, Wanibuchi, Hideki, Imaoka, Susumu, Ogawa, Motome, Masuda, Chikayoshi, Morimura, Keiichirou, Funae, Yoshihiko, and Fukushima, Shoji, *Formation of 8-hydroxydeoxyguanosine and cell-cycle arrest in the rat liver via generation of oxidative stress by phenobarbital: association with expression profiles of p21WAF1/Cip1, cyclin D1 and Ogg1*. Carcinogenesis, 2002. **23**(2): p. 341-349.

93. Kinoshita, Anna, Wanibuchi, Hideki, Morimura, Keiichirou, Wei, Min, Shen, Jun, Imaoka, Susumu, Funae, Yoshihiko, and Fukushima, Shoji, *Phenobarbital at low dose exerts hormesis in rat hepatocarcinogenesis by reducing oxidative DNA damage, altering cell proliferation, apoptosis and gene expression*. Carcinogenesis, 2003. **24**(8): p. 1389-1399.

94. Kolaja, Kyle L., Engelken, Dustin T., and Klaassen, Curtis D., *Inhibition of gap-junctional-intercellular communication in intact rat liver by nongenotoxic hepatocarcinogens*. Toxicology, 2000. **146**(1): p. 15-22.

95. Ruch, R. J. and Trosko, J. E., *The role of oval cells and gap junctional intercellular communication in hepatocarcinogenesis*. Anticancer Res, 1999. **19**(6A): p. 4831-8.

96. Loewenstein, Werner R., *The cell-to-cell channel of gap junctions*. Cell, 1987. **48**(5): p. 725-726.
97. Klaunig, J. E., Ruch, R. J., and Weghorst, C. M., *Comparative effects of phenobarbital, DDT, and lindane on mouse hepatocyte gap junctional intercellular communication*. Toxicol Appl Pharmacol, 1990. **102**(3): p. 553-63.
98. Guppy, Martin J., Wilton, Joanne C., Sharma, Raj, Coleman, Roger, and Chipman, J. Kevin, *Modulation of phenobarbitone-induced loss of gap junctional intercellular communication in hepatocyte couplets*. Carcinogenesis, 1994. **15**(9): p. 1917-1921.
99. Jones, Huw B., Orton, Terry C., and Lake, Brian G., *Effect of chronic phenobarbitone administration on liver tumour formation in the C57BL/10J mouse*. Food and Chemical Toxicology, 2009. **47**(6): p. 1333-1340.
100. Diwan, Bhalchandra A., Ward, Jerrold M., Anderson, Lucy M., Hagiwara, Akihiro, and Rice, Jerry M., *Lack of effect of phenobarbital on hepatocellular carcinogenesis initiated by N-nitrosodiethylamine or methylazoxymethanol acetate in male Syrian golden hamsters*. Toxicology and Applied Pharmacology, 1986. **86**(2): p. 298-307.
101. Peters, Jaime L., Sutton, Alex J., Jones, David R., Rushton, Lesley, and Abrams, Keith R., *A Systematic Review of Systematic Reviews and Meta-Analyses of Animal Experiments with Guidelines for Reporting*. Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 2006. **41**(7): p. 1245 - 1258.
102. Sandercock, Peter and Roberts, Ian, *Systematic reviews of animal experiments*. The Lancet, 2002. **360**(9333): p. 586-586.
103. Weed, Douglas L., *Methodologic Guidelines for Review Papers*. J. Natl. Cancer Inst., 1997. **89**(1): p. 6-7.

104. Egger, M., Smith, G. D., O'Rourke, K., Egger, M., Smith, G. D., and Altman, D. G., *Rationale, potentials, and promise of systematic reviews*. Systematic reviews in health care Meta-analysis in context, 2001. **2**: p. 3 - 19.
105. Stroup, Donna F., Berlin, Jesse A., Morton, Sally C., Olkin, Ingram, Williamson, G. David, Rennie, Drummond, Moher, David, Becker, Betsy J., Sipe, Theresa Ann, Thacker, Stephen B., and for the Meta-analysis Of Observational Studies in Epidemiology, Group, *Meta-analysis of Observational Studies in Epidemiology: A Proposal for Reporting*. JAMA, 2000. **283**(15): p. 2008-2012.
106. Key, J., Hodgson, S., Omar, R. Z., Jensen, T. K., Thompson, S. G., Boobis, A. R., Davies, D. S., and Elliott, P., *Meta-analysis of studies of alcohol and breast cancer with consideration of the methodological issues*. Cancer Causes Control, 2006. **17**(6): p. 759-770.
107. Clemmesen, J., *Letter: Phenobarbitone, liver tumours, and thorotrast*. Lancet, 1975. **1**(7897): p. 37-38.
108. Olsen, J. H., Boice, J. D., Jr., Jensen, J. P., and Fraumeni, J. F., Jr., *Cancer among epileptic patients exposed to anticonvulsant drugs*. J.Natl.Cancer Inst., 1989. **81**(10): p. 803-808.
109. Olsen, J. H., Schulgen, G., Boice, J. D., Jr., Whysner, J., Travis, L. B., Williams, G. M., Johnson, F. B., and McGee, J. O., *Antiepileptic treatment and risk for hepatobiliary cancer and malignant lymphoma*. Cancer Res., 1995. **55**(2): p. 294-297.
110. McLean, A. E., Driver, H. E., and Sutherland, I. A., *Liver tumour promotion by phenobarbital: comparison of rat and human studies*. Prog.Clin.Biol.Res., 1992. **374**: p. 251-259.

111. White, S. J., McLean, A. E., and Howland, C., *Anticonvulsant drugs and cancer. A cohort study in patients with severe epilepsy.* Lancet, 1979. **2**(8140): p. 458-461.
112. Lamminpaa, A., Pukkala, E., Teppo, L., and Neuvonen, P. J., *Cancer incidence among patients using antiepileptic drugs: a long-term follow-up of 28,000 patients.* Eur.J.Clin.Pharmacol., 2002. **58**(2): p. 137-141.
113. Shirts, S. B., Annegers, J. F., Hauser, W. A., and Kurland, L. T., *Cancer incidence in a cohort of patients with seizure disorders.* J.Natl.Cancer Inst., 1986. **77**(1): p. 83-87.
114. Clemmesen, J., Fuglsang-Frederiksen, V., and Plum, C. M., *Are anticonvulsants oncogenic?* Lancet, 1974. **1**(7860): p. 705-7.
115. Marchal, *Alcohol and epilepsy.* La Prevue du Praticien, 1999. **49** (4): p. 383-6.
116. Clemmesen, J. and Hjalgrim-Jensen, S., *Does phenobarbital cause intracranial tumors? A follow-up through 35 years.* Ecotoxicol.Environ.Saf, 1981. **5**(2): p. 255-260.
117. Loiseau, P. J., *Clinical experience with new antiepileptic drugs: antiepileptic drugs in Europe.* Epilepsia, 1999. **40 Suppl 6**: p. S3-S8.
118. Andersson, M. and Storm, H. H., *Cancer incidence among Danish Thorotrast-exposed patients.* J.Natl.Cancer Inst., 1992. **84**(17): p. 1318-1325.
119. Nyberg, U., Nilsson, B., Travis, L. B., Holm, L. E., and Hall, P., *Cancer incidence among Swedish patients exposed to radioactive thorotrast: a forty-year follow-up survey.* Radiat.Res., 2002. **157**(4): p. 419-425.

120. Travis, L. B., Hauptmann, M., Gaul, L. K., Storm, H. H., Goldman, M. B., Nyberg, U., Berger, E., Janower, M. L., Hall, P., Monson, R. R., Holm, L. E., Land, C. E., Schottenfeld, D., Boice, J. D., Jr., and Andersson, M., *Site-specific cancer incidence and mortality after cerebral angiography with radioactive thorotrast*. Radiat.Res., 2003. **160**(6): p. 691-706.
121. Lander, ES (and others included) *Initial sequencing and analysis of the human genome*. Nature, 2001. **409**(6822): p. 860-921.
122. Venter, J. Craig, Adams, Mark D., Myers, Eugene W., Li, Peter W., Mural, Richard J., Sutton, Granger G., Smith, Hamilton O., Yandell, Mark, Evans, Cheryl A., Holt, Robert A., Gocayne, Jeannine D., Amanatides, Peter, Ballew, Richard M., Huson, Daniel H., Wortman, Jennifer Russo, Zhang, Qing, Kodira, Chinnappa D., Zheng, Xiangqun H., Chen, Lin, Skupski, Marian, Subramanian, Gangadharan, Thomas, Paul D., Zhang, Jinghui, Gabor Miklos, George L., Nelson, Catherine, Broder, Samuel, Clark, Andrew G., Nadeau, Joe, McKusick, Victor A., Zinder, Norton, Levine, Arnold J., Roberts, Richard J., Simon, Mel, Slayman, Carolyn, Hunkapiller, Michael, Bolanos, Randall, Delcher, Arthur, Dew, Ian, Fasulo, Daniel, Flanigan, Michael, Florea, Liliana, Halpern, Aaron, Hannenhalli, Sridhar, Kravitz, Saul, Levy, Samuel, Mobarry, Clark, Reinert, Knut, Remington, Karin, Abu-Threideh, Jane, Beasley, Ellen, Biddick, Kendra, Bonazzi, Vivien, Brandon, Rhonda, Cargill, Michele, Chandramouliswaran, Ishwar, Charlab, Rosane, Chaturvedi, Kabir, Deng, Zuoming, Francesco, Valentina Di, Dunn, Patrick, Eilbeck, Karen, Evangelista, Carlos, Gabrielian, Andrei E., Gan, Weiniu, Ge, Wangmao, Gong, Fangcheng, Gu, Zhiping, Guan, Ping, Heiman, Thomas J., Higgins, Maureen E., Ji, Rui-Ru, Ke, Zhaoxi, Ketchum, Karen A., Lai, Zhongwu, Lei, Yiding, Li, Zhenya, Li, Jiayin, Liang, Yong, Lin, Xiaoying, Lu, Fu, Merkulov, Gennady V., Milshina, Natalia, Moore, Helen M., Naik, Ashwinikumar K., Narayan, Vaibhav A., Neelam, Beena, Nusskern, Deborah, Rusch, Douglas B., Salzberg, Steven, Shao, Wei, Shue, Bixiong, Sun, Jingtao, Wang, Zhen Yuan, Wang, Aihui, Wang, Xin, Wang, Jian, Wei, Ming-Hui, Wides, Ron, Xiao, Chunlin, Yan, Chunhua, Yao, Alison, Ye, Jane, Zhan, Ming, Zhang, Weiqing, Zhang, Hongyu, Zhao, Qi, Zheng, Liansheng, Zhong, Fei, Zhong, Wenyan, Zhu,

Shiaoping C., Zhao, Shaying, Gilbert, Dennis, Baumhueter, Suzanna, Spier,
 Gene, Carter, Christine, Cravchik, Anibal, Woodage, Trevor, Ali, Feroze, An,
 Huijin, Awe, Aderonke, Baldwin, Danita, Baden, Holly, Barnstead, Mary,
 Barrow, Ian, Beeson, Karen, Busam, Dana, Carver, Amy, Center, Angela,
 Cheng, Ming Lai, Curry, Liz, Danaher, Steve, Davenport, Lionel, Desilets,
 Raymond, Dietz, Susanne, Dodson, Kristina, Doup, Lisa, Ferriera, Steven,
 Garg, Neha, Gluecksmann, Andres, Hart, Brit, Haynes, Jason, Haynes, Charles,
 Heiner, Cheryl, Hladun, Suzanne, Hostin, Damon, Houck, Jarrett, Howland,
 Timothy, Ibegwam, Chinyere, Johnson, Jeffery, Kalush, Francis, Kline, Lesley,
 Koduru, Shashi, Love, Amy, Mann, Felecia, May, David, McCawley, Steven,
 McIntosh, Tina, McMullen, Ivy, Moy, Mee, Moy, Linda, Murphy, Brian,
 Nelson, Keith, Pfannkoch, Cynthia, Pratts, Eric, Puri, Vinita, Qureshi, Hina,
 Reardon, Matthew, Rodriguez, Robert, Rogers, Yu-Hui, Romblad, Deanna,
 Ruhfel, Bob, Scott, Richard, Sitter, Cynthia, Smallwood, Michelle, Stewart,
 Erin, Strong, Renee, Suh, Ellen, Thomas, Reginald, Tint, Ni Ni, Tse, Sukyee,
 Vech, Claire, Wang, Gary, Wetter, Jeremy, Williams, Sherita, Williams,
 Monica, Windsor, Sandra, Winn-Deen, Emily, Wolfe, Keriellen, Zaveri,
 Jayshree, Zaveri, Karena, Abril, Josep F., Guigo, Roderic, Campbell, Michael
 J., Sjolander, Kimmen V., Karlak, Brian, Kejariwal, Anish, Mi, Huaiyu,
 Lazareva, Betty, Hatton, Thomas, Narechania, Apurva, Diemer, Karen,
 Muruganujan, Anushya, Guo, Nan, Sato, Shinji, Bafna, Vineet, Istrail, Sorin,
 Lippert, Ross, Schwartz, Russell, Walenz, Brian, Yooseph, Shibu, Allen,
 David, Basu, Anand, Baxendale, James, Blick, Louis, Caminha, Marcelo,
 Carnes-Stine, John, Caulk, Parris, Chiang, Yen-Hui, Coyne, My, Dahlke, Carl,
 Mays, Anne Deslattes, Dombroski, Maria, Donnelly, Michael, Ely, Dale,
 Esparham, Shiva, Fosler, Carl, Gire, Harold, Glanowski, Stephen, Glasser,
 Kenneth, Glodek, Anna, Gorokhov, Mark, Graham, Ken, Gropman, Barry,
 Harris, Michael, Heil, Jeremy, Henderson, Scott, Hoover, Jeffrey, Jennings,
 Donald, Jordan, Catherine, Jordan, James, Kasha, John, Kagan, Leonid, Kraft,
 Cheryl, Levitsky, Alexander, Lewis, Mark, Liu, Xiangjun, Lopez, John, Ma,
 Daniel, Majoros, William, McDaniel, Joe, Murphy, Sean, Newman, Matthew,
 Nguyen, Trung, Nguyen, Ngoc, Nodell, Marc, Pan, Sue, Peck, Jim, Peterson,
 Marshall, Rowe, William, Sanders, Robert, Scott, John, Simpson, Michael,
 Smith, Thomas, Sprague, Arlan, Stockwell, Timothy, Turner, Russell, Venter,

- Eli, Wang, Mei, Wen, Meiyuan, Wu, David, Wu, Mitchell, Xia, Ashley, Zandieh, Ali and Zhu, Xiaohong, *The Sequence of the Human Genome*. Science, 2001. **291**(5507): p. 1304-1351.
123. Nuwaysir, E. F., Bittner, M., Trent, J., Barrett, J. C., and Afshari, C. A., *Microarrays and toxicology: The advent of toxicogenomics*. Molecular Carcinogenesis, 1999. **24**(3): p. 153-159.
 124. Heijne, Wilbert H. M., Kienhuis, Anne S., van Ommen, Ben, Stierum, Rob H., and Groten, John P., *Systems toxicology: applications of toxicogenomics, transcriptomics, proteomics and metabolomics in toxicology*. Expert Review of Proteomics, 2005. **2**(5): p. 767-780.
 125. Hamadeh, Hisham K., Bushel, Pierre R., Jayadev, Supriya, Martin, Karla, DiSorbo, Olimpia, Sieber, Stella, Bennett, Lee, Tennant, Raymond, Stoll, Raymond, Barrett, J. Carl, Blanchard, Kerry, Paules, Richard S., and Afshari, Cynthia A., *Gene Expression Analysis Reveals Chemical-Specific Profiles*. Toxicological Sciences, 2002. **67**(2): p. 219-231.
 126. Ulrich, Roger G., *The toxicogenomics of nuclear receptor agonists*. Current Opinion in Chemical Biology, 2003. **7**(4): p. 505-510.
 127. Hisham K. Hamadeh, Eupesh P. Amin, Richard S. Paules, Cynthia A. Afshari, *An Overview of Toxicogenomics*. Current Issues in Molecular Biology, 2002. **4**: p. 45-56.
 128. NRC, *Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment*. Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment, National Research Council, ed. National Academy of Sciences. 2007, Washington, DC: The National Academies Press.
 129. Kennedy, Robert M., *Hydrophobic-Interaction Chromatography*. Current Protocols in Protein Science. 2001: John Wiley & Sons, Inc.

130. Nicholson, J. K., Lindon, J. C., and Holmes, E., '*Metabonomics*': *understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data*. *Xenobiotica*, 1999. **29**(11): p. 1181-1189.
131. Olson, N. Eric, *The Microarray Data Analysis Process: From Raw Data to Biological Significance*. *NeuroRX*, 2006. **3**(3): p. 373-383.
132. Draghici, Sorin, *Statistical intelligence: effective analysis of high-density microarray data*. *Drug Discovery Today*, 2002. **7**(11): p. S55-S63.
133. Tan, Patric. Experimental Validation of Microarray Data. (National Cancer Centre/Defence Medical and Environmental Research Institute, Singapore, 2004). Available at: <http://www2.ims.nus.edu.sg/preprints/2004-27.pdf>
134. Storey, John D. and Tibshirani, Robert, *Statistical significance for genomewide studies*. *Proceedings of the National Academy of Sciences of the United States of America*, 2003. **100**(16): p. 9440-9445.
135. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and Sherlock, G., *Gene ontology: tool for the unification of biology*. *The Gene Ontology Consortium*. *Nat Genet*, 2000. **25**(1): p. 25-9.
136. Kelso, J., Visagie, J., Theiler, G., Christoffels, A., Bardien, S., Smedley, D., Otgaar, D., Greyling, G., Jongeneel, C. V., McCarthy, M. I., Hide, T., and Hide, W., *eVOC: a controlled vocabulary for unifying gene expression data*. *Genome Res*, 2003. **13**(6A): p. 1222-30.
137. Mao, Xizeng, Cai, Tao, Olyarchuk, John G., and Wei, Liping, *Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary*. *Bioinformatics*, 2005. **21**(19): p. 3787-3793.

138. Werner, Thomas, *Bioinformatics applications for pathway analysis of microarray data*. Current Opinion in Biotechnology, 2008. **19**(1): p. 50-54.
139. Curtis, R. Keira, Oresic, Matej, and Vidal-Puig, Antonio, *Pathways to the analysis of microarray data*. Trends in Biotechnology, 2005. **23**(8): p. 429-435.
140. Brazma, Alvis, Hingamp, Pascal, Quackenbush, John, Sherlock, Gavin, Spellman, Paul, Stoeckert, Chris, Aach, John, Ansorge, Wilhelm, Ball, Catherine A., Causton, Helen C., Gaasterland, Terry, Glenisson, Patrick, Holstege, Frank C. P., Kim, Irene F., Markowitz, Victor, Matese, John C., Parkinson, Helen, Robinson, Alan, Sarkans, Ugis, Schulze-Kremer, Steffen, Stewart, Jason, Taylor, Ronald, Vilo, Jaak, and Vingron, Martin, *Minimum information about a microarray experiment (MIAME)[mdash]toward standards for microarray data*. Nat Genet, 2001. **29**(4): p. 365-371.
141. Hester, Susan, (*Genomics Expert, USEPA*) *Personal Communication: Advice on Statistical Strategy for Microarray Analysis*. 2010.
142. Weng, Lee, Dai, Hongyue, Zhan, Yihui, He, Yudong, Stepaniants, Sergey B., and Bassett, Douglas E., *Rosetta error model for gene expression analysis*. Bioinformatics, 2006. **22**(9): p. 1111-1121.
143. Rezen, Tadeja, Juvan, Peter, Fon Tacer, Klementina, Kuzman, Drago, Roth, Adrian, Pompon, Denis, Aggerbeck, Lawrence, Meyer, Urs, and Rozman, Damjana, *The Sterolgene v0 cDNA microarray: a systemic approach to studies of cholesterol homeostasis and drug metabolism*. BMC Genomics, 2008. **9**(1): p. 76.
144. Nesnow, Stephen, Ward, William, Moore, Tanya, Ren, Hongzu, and Hester, Susan D., *Discrimination of Tumorigenic Triazole Conazoles from Phenobarbital by Transcriptional Analyses of Mouse Liver Gene Expression*. Toxicol. Sci., 2009. **110**(1): p. 68-83.

145. Ren, Hongzu, Vallanat, Beena, Nelson, David M., Yeung, Leo W. Y., Guruge, Keerthi S., Lam, Paul K. S., Lehman-McKeeman, Lois D., and Corton, J. Christopher, *Evidence for the involvement of xenobiotic-responsive nuclear receptors in transcriptional effects upon perfluoroalkyl acid exposure in diverse species*. Reproductive Toxicology, 2009. **27**(3-4): p. 266-277.
146. Goetz, Amber K. and Dix, David J., *Toxicogenomic effects common to triazole antifungals and conserved between rats and humans*. Toxicology and Applied Pharmacology, 2009. **238**(1): p. 80-89.
147. Tamasi, Viola, Juvan, Peter, Beer, Markus, Rozman, Damjana, and Meyer, Urs A., *Transcriptional Activation of PPAR α by Phenobarbital in the Absence of CAR and PXR*. Molecular Pharmaceutics, 2009. **6**(5): p. 1573-1581.
148. Kanehisa, Minoru and Goto, Susumu, *KEGG: Kyoto Encyclopedia of Genes and Genomes*. Nucl. Acids Res., 2000. **28**(1): p. 27-30.
149. Shi, L., Reid, L. H., Jones, W. D., Shippy, R., Warrington, J. A., Baker, S. C., Collins, P. J., de, Longueville F., Kawasaki, E. S., Lee, K. Y., Luo, Y., Sun, Y. A., Willey, J. C., Setterquist, R. A., Fischer, G. M., Tong, W., Dragan, Y. P., Dix, D. J., Frueh, F. W., Goodsaid, F. M., Herman, D., Jensen, R. V., Johnson, C. D., Lobenhofer, E. K., Puri, R. K., Schrf, U., Thierry-Mieg, J., Wang, C., Wilson, M., Wolber, P. K., Zhang, L., Amur, S., Bao, W., Barbacioru, C. C., Lucas, A. B., Bertholet, V., Boysen, C., Bromley, B., Brown, D., Brunner, A., Canales, R., Cao, X. M., Cebula, T. A., Chen, J. J., Cheng, J., Chu, T. M., Chudin, E., Corson, J., Corton, J. C., Croner, L. J., Davies, C., Davison, T. S., Delenstarr, G., Deng, X., Dorris, D., Eklund, A. C., Fan, X. H., Fang, H., Fulmer-Smentek, S., Fuscoe, J. C., Gallagher, K., Ge, W., Guo, L., Guo, X., Hager, J., Haje, P. K., Han, J., Han, T., Harbottle, H. C., Harris, S. C., Hatchwell, E., Hauser, C. A., Hester, S., Hong, H., Hurban, P., Jackson, S. A., Ji, H., Knight, C. R., Kuo, W. P., LeClerc, J. E., Levy, S., Li, Q. Z., Liu, C., Liu, Y., Lombardi, M. J., Ma, Y., Magnuson, S. R., Maqsodi, B., McDaniel, T., Mei, N., Myklebost, O., Ning, B., Novoradovskaya, N., Orr, M. S., Osborn, T. W., Papallo, A., Patterson, T. A., Perkins, R. G., Peters, E. H., Peterson, R.,

- Philips, K. L., Pine, P. S., Pusztai, L., Qian, F., Ren, H., Rosen, M., Rosenzweig, B. A., Samaha, R. R., Schena, M., Schroth, G. P., Shchegrova, S., Smith, D. D., Staedtler, F., Su, Z., Sun, H., Szallasi, Z., Tezak, Z., Thierry-Mieg, D., Thompson, K. L., Tikhonova, I., Turpaz, Y., Vallanat, B., Van, C., Walker, S. J., Wang, S. J., Wang, Y., Wolfinger, R., Wong, A., Wu, J., Xiao, C., Xie, Q., Xu, J., Yang, W., Zhang, L., Zhong, S., Zong, Y. and Slikker, W., Jr., *The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements*. Nat Biotechnol., 2006. **24**(9): p. 1151-1161.
150. Werner, Thomas, *Bioinformatics applications for pathway analysis of microarray data*. Current Opinion in Biotechnology. **In Press, Corrected Proof**.
 151. Gold, David L., Coombes, Kevin R., Wang, Jing, and Mallick, Bani, *Enrichment analysis in high-throughput genomics--accounting for dependency in the NULL*. Brief Bioinform, 2007. **8**(2): p. 71-77.
 152. Huang, Da Wei, Sherman, Brad T., and Lempicki, Richard A., *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat. Protocols, 2008. **4**(1): p. 44-57.
 153. IPA®, Ingenuity® Systems, www.ingenuity.com (Version 8.1). 2010. p. Pathway Analysis Software.
 154. Fielden, M. R., Brennan, R., and Gollub, J., *A gene expression biomarker provides early prediction and mechanistic assessment of hepatic tumor induction by nongenotoxic chemicals*. Toxicological Sciences, 2007. **99**(1): p. 90-100.
 155. Nie, A. Y., McMillian, M., Parker, J. B., Leone, A., Bryant, S., Yieh, L., Bittner, A., Nelson, J., Carmen, A., Wan, J., and Lord, P. G., *Predictive toxicogenomics approaches reveal underlying molecular mechanisms of nongenotoxic carcinogenicity*. Mol Carcinog., 2006. **45**(12): p. 914-933.

156. Pei, Yunfei, Zhang, Ting, Renault, Victor, and Zhang, Xuegong, *An overview of hepatocellular carcinoma study by omics-based methods*. Acta Biochim Biophys Sin, 2009. **41**(1): p. 1-15.
157. Guo, Dongsheng, Sarkar, Joy, Suino-Powell, Kelly, Xu, Yong, Matsumoto, Kojiro, Jia, Yuzhi, Yu, Songtao, Khare, Sonal, Haldar, Kasturi, Rao, M. Sambasiva, Foreman, Jennifer E., Monga, Satdarshan P. S., Peters, Jeffrey M., Xu, H. Eric, and Reddy, Janardan K., *Induction of Nuclear Translocation of Constitutive Androstane Receptor by Peroxisome Proliferator-activated Receptor $\hat{\pm}$ Synthetic Ligands in Mouse Liver*. Journal of Biological Chemistry, 2007. **282**(50): p. 36766-36776.
158. Timberll, J. *Toxic Responses to Foreign Compounds in Book* 184-5 (Taylor & Francis, 2000).
159. Nguyen, T., Nioi, P., and Pickett, C. B., *The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress*. Journal of Biological Chemistry, 2009. **284**(20): p. 13291-13295.
160. Li, Wenge, Yu, Siwang, Liu, Tong, Kim, Jung Hwan, Blank, Volker, Li, Hong, and Kong, A. N. Tony, *Heterodimerization with small Maf proteins enhances nuclear retention of Nrf2 via masking the NESzip motif*. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2008. **1783**(10): p. 1847-1856.
161. Marlowe, J. L. and Puga, A., *Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis*. J.Cell Biochem., 2005. **96**(6): p. 1174-1184.
162. Nelson, David M., Bhaskaran, Vasanthi, Foster, William R., and Lehman-McKeeman, Lois D., *p53-Independent Induction of Rat Hepatic Mdm2 following Administration of Phenobarbital and Pregnenolone 16{alpha}-Carbonitrile*. Toxicol. Sci., 2006. **94**(2): p. 272-280.

163. DiPaola, Robert S., *To Arrest or Not To G2-M Cell-Cycle Arrest*. Clinical Cancer Research, 2002. **8**(11): p. 3311-3314.
164. Murphy, M. P., *How mitochondria produce reactive oxygen species*. Biochem J, 2009. **417**(1): p. 1-13.
165. Kusters, Astrid and Karpen, Saul J., *The Role of Inflammation in Cholestasis: Clinical and Basic Aspects*. Semin Liver Dis. **30**(02): p. 186,194.
166. Hennige, Anita M., Staiger, Harald, Wicke, Corinna, Machicao, Fausto, Fritsche, Andreas, Häring, Hans-Ulrich, and Stefan, Norbert, *Fetuin-A Induces Cytokine Expression and Suppresses Adiponectin Production*. PLoS ONE, 2008. **3**(3): p. e1765.
167. Yauk, C. L. and Berndt, M. L., *Review of the literature examining the correlation among DNA microarray technologies*. Environ.Mol Mutagen., 2007. **48**(5): p. 380-394.
168. Waters, Michael D., Jackson, Marcus, and Lea, Isabel, *Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods*. Mutation Research/Reviews in Mutation Research, 2010. **In Press, Uncorrected Proof**.
169. Boobis, A. R., Doe, J. E., Heinrich-Hirsch, B., Meek, M. E., Munn, S., Ruchirawat, M., Schlatter, J., Seed, J., and Vickers, C., *IPCS Framework for Analyzing the Relevance of a Noncancer Mode of Action for Humans*. Crit Rev.Toxicol., 2008. **38**(2): p. 87-96.

170. Baker, S. C., Bauer, S. R., Beyer, R. P., Brenton, J. D., Bromley, B., Burrill, J., Causton, H., Conley, M. P., Elespuru, R., Fero, M., Foy, C., Fuscoe, J., Gao, X., Gerhold, D. L., Gilles, P., Goodsaid, F., Guo, X., Hackett, J., Hockett, R. D., Ikonomi, P., Irizarry, R. A., Kawasaki, E. S., Kaysser-Kranich, T., Kerr, K., Kiser, G., Koch, W. H., Lee, K. Y., Liu, C., Liu, Z. L., Lucas, A., Manohar, C. F., Miyada, G., Modrusan, Z., Parkes, H., Puri, R. K., Reid, L., Ryder, T. B., Salit, M., Samaha, R. R., Scherf, U., Sendera, T. J., Setterquist, R. A., Shi, L., Shippy, R., Soriano, J. V., Wagar, E. A., Warrington, J. A., Williams, M., Wilmer, F., Wilson, M., Wolber, P. K., Wu, X., and Zadro, R., *The External RNA Controls Consortium: a progress report*. Nat Methods, 2005. **2**(10): p. 731-734.

171. Moreau, Am ©lie, Vilarem, Marie Jos ©, Maurel, Patrick, and Pascussi, Jean Marc, *Xenoreceptors CAR and PXR Activation and Consequences on Lipid Metabolism, Glucose Homeostasis, and Inflammatory Response*. Molecular Pharmaceutics, 2007. **5**(1): p. 35-41.

172. Wada, Taira, Gao, Jie, and Xie, Wen, *PXR and CAR in energy metabolism*. Trends in Endocrinology & Metabolism, 2009. **20**(6): p. 273-279.

173. Wei, P., Zhang, J., Dowhan, D. H., Han, Y., and Moore, D. D., *Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response*. Pharmacogenomics J, 2002. **2**(2): p. 117-26.

174. Moore, John T., Moore, Linda B., Maglich, Jodi M., and Kliewer, Steve A., *Functional and structural comparison of PXR and CAR*. Biochimica et Biophysica Acta (BBA) - General Subjects, 2003. **1619**(3): p. 235-238.

175. Hariparsad, Nireesh, Nallani, Srikanth C., Sane, Rucha S., Buckley, Donna J., Buckley, Arthur R., and Desai, Pankaj B., *Induction of CYP3A4 by Efavirenz in Primary Human Hepatocytes: Comparison With Rifampin and Phenobarbital*. The Journal of Clinical Pharmacology, 2004. **44**(11): p. 1273-1281.

176. Ejiri, Noriko, Katayama, Kei-ichi, Kiyosawa, Naoki, Baba, Yasuko, and Doi, Kunio, *Microarray analysis on CYPs expression in pregnant rats after treatment with pregnenolone-16[alpha]-carbonitrile and phenobarbital*. Experimental and Molecular Pathology, 2005. **78**(1): p. 71-77.
177. Kanno, Y., Otsuka, S., Hiromasa, T., Nakahama, T., and Inouye, Y., *Diurnal difference in CAR mRNA expression*. Nucl Recept, 2004. **2**(1): p. 6.
178. Bell, Aaron, W. and George, K. Michalopoulos, *Phenobarbital regulates nuclear expression of HNF-4alpha in mouse and rat hepatocytes independent of CAR and PXR*. Hepatology, 2006. **44**(1): p. 186-194.
179. Herzig, Stephan, Long, Fanxin, Jhala, Ulupi S., Hedrick, Susan, Quinn, Rebecca, Bauer, Anton, Rudolph, Dorothea, Schutz, Gunther, Yoon, Cliff, Puigserver, Pere, Spiegelman, Bruce, and Montminy, Marc, *CREB regulates hepatic gluconeogenesis through the coactivator PGC-1*. Nature, 2001. **413**(6852): p. 179-183.
180. Konno, Y., Negishi, M., and Kodama, S., *The roles of nuclear receptors CAR and PXR in hepatic energy metabolism*. Drug Metab Pharmacokinet., 2008. **23**(1): p. 8-13.
181. Argaud, D., Halimi, S., Catelloni, F., and Leverve, X. M., *Inhibition of gluconeogenesis in isolated rat hepatocytes after chronic treatment with phenobarbital*. Biochem J, 1991. **280 (Pt 3)**: p. 663-9.
182. Manenti, G., Dragani, T. A., and Della Porta, G., *Effects of phenobarbital and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene on differentiated functions in mouse liver*. Chem Biol Interact, 1987. **64**(1-2): p. 83-92.
183. Kurukulasuriya, R., Link, J. T., Madar, D. J., Pei, Z., Richards, S. J., Rohde, J. J., Souers, A. J., and Szczepankiewicz, B. G., *Potential drug targets and progress towards pharmacologic inhibition of hepatic glucose production*. Curr Med Chem, 2003. **10**(2): p. 123-53.

184. Nakae, Jun, Kitamura, Tadahiro, Silver, David L., and Accili, Domenico, *The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression*. The Journal of Clinical Investigation, 2001. **108**(9): p. 1359-1367.
185. Kodama, Susumu, Koike, Chika, Negishi, Masahiko, and Yamamoto, Yukio, *Nuclear Receptors CAR and PXR Cross Talk with FOXO1 To Regulate Genes That Encode Drug-Metabolizing and Gluconeogenic Enzymes*. Mol. Cell. Biol., 2004. **24**(18): p. 7931-7940.
186. Kodama, Susumu, Moore, Rick, Yamamoto, Yukio, and Negishi, Masahiko, *Human nuclear pregnane X receptor cross-talk with CREB to repress cAMP activation of the glucose-6-phosphatase gene*. Biochem J, 2007. **407**(3): p. 373-381.
187. Miao, Ji, Fang, Sungsoon, Bae, Yangjin, and Kemper, Jongsook Kim, *Functional Inhibitory Cross-talk between Constitutive Androstane Receptor and Hepatic Nuclear Factor-4 in Hepatic Lipid/Glucose Metabolism Is Mediated by Competition for Binding to the DR1 Motif and to the Common Coactivators, GRIP-1 and PGC-1 β* . Journal of Biological Chemistry, 2006. **281**(21): p. 14537-14546.
188. Sidhu, Jaspreet, S. and Curtis, J. Omiecinski, *Insulin-mediated modulation of cytochrome P450 gene induction profiles in primary rat hepatocyte cultures*. Journal of Biochemical and Molecular Toxicology, 1999. **13**(1): p. 1-9.
189. Yamazoe, Y., Murayama, N., Shimada, M., Yamauchi, K., and Kato, R., *Cytochrome P450 in livers of diabetic rats: regulation by growth hormone and insulin*. Arch Biochem Biophys, 1989. **268**(2): p. 567-75.
190. Triantafilou, Martha and Triantafilou, Kathy, *Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster*. Trends in Immunology, 2002. **23**(6): p. 301-304.

191. Beigneux, Anne P., Moser, Arthur H., Shigenaga, Judy K., Grunfeld, Carl, and Feingold, Kenneth R., *Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response.* Biochemical and Biophysical Research Communications, 2002. **293**(1): p. 145-149.
192. Zimmerman, Tracy L., Thevananther, Sundararajah, Ghose, Romi, Burns, Alan R., and Karpen, Saul J., *Nuclear export of retinoid X receptor alpha in response to interleukin-1beta-mediated cell signaling: roles for JNK and SER260.* Journal of Biological Chemistry, 2006. **281**(22): p. 15434-15440.
193. Dyson, Nicholas, *The regulation of E2F by pRB-family of Proteins.* Genes & Development, 1998. **12**(15): p. 2245-2262.
194. Sears, Rosalie C. and Nevins, Joseph R., *Signaling Networks That Link Cell Proliferation and Cell Fate.* Journal of Biological Chemistry, 2002. **277**(14): p. 11617-11620.
195. Sherr, C. J., *Cancer cell cycles.* Science, 1996. **274**(5293): p. 1672-1677.
196. Pajarvi, Gerd, Viluksela, Matti, Pohjanvirta, Raimo, Stenius, Ulla, and Hogberg, Johan, *TCDD activates Mdm2 and attenuates the p53 response to DNA damaging agents.* Carcinogenesis, 2005. **26**(1): p. 201-208.
197. Corcos, Laurent, Marc, Nathalie, Wein, Sharon, Fautrel, Alain, Guillouzo, André, and Pineau, Thierry, *Phenobarbital induces cytochrome P4501A2 hnRNA, mRNA and protein in the liver of C57BL/6J wild type and aryl hydrocarbon receptor knock-out mice.* FEBS Letters, 1998. **425**(2): p. 293-297.
198. Jansen-Durr, P., Meichle, A., Steiner, P., Pagano, M., Finke, K., Botz, J., Wessbecher, J., Draetta, G., and Eilers, M., *Differential modulation of cyclin gene expression by MYC.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(8): p. 3685-3689.

199. Millet, C. and Zhang, Y. E., *Roles of Smad3 in TGF-beta signaling during carcinogenesis*. Crit Rev Eukaryot. Gene Expr., 2007. **17**(4): p. 281-293.
200. Grisham, J. W., *Interspecies comparison of liver carcinogenesis: implications for cancer risk assessment*. Carcinogenesis, 1997. **18**(1): p. 59-81.
201. Sanders, S. and Thorgeirsson, S. S., *Promotion of hepatocarcinogenesis by phenobarbital in c-myc/TGF-alpha transgenic mice*. Mol. Carcinog., 2000. **28**(3): p. 168-173.
202. Santoni-Rugiu, Eric, Jensen, Michael, Rugaard, and Thorgeirsson, Snorri S., *Disruption of the pRb/E2F Pathway and Inhibition of Apoptosis Are Major Oncogenic Events in Liver Constitutively Expressing c-myc and Transforming Growth Factor + γ* . Cancer Research, 1998. **58**(1): p. 123-134.
203. Santoni-Rugiu, E., Preisegger, K. H., Kiss, A., Audolfsson, T., Shiota, G., Schmidt, E. V., and Thorgeirsson, S. S., *Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model*. Proc. Natl. Acad. Sci. U.S.A., 1996. **93**(18): p. 9577-9582.
204. Dinkova-Kostova, Albena T., Holtzclaw, W. David, and Kensler, Thomas W., *The Role of Keap1 in Cellular Protective Responses*. Chemical Research in Toxicology, 2005. **18**(12): p. 1779-1791.
205. McMahon, Michael, Itoh, Ken, Yamamoto, Masayuki, and Hayes, John D., *Keap1-dependent Proteasomal Degradation of Transcription Factor Nrf2 Contributes to the Negative Regulation of Antioxidant Response Element-driven Gene Expression*. Journal of Biological Chemistry, 2003. **278**(24): p. 21592-21600.
206. Katsuoka, F., Motohashi, H., Engel, J. D., and Yamamoto, M., *Nrf2 transcriptionally activates the mafG gene through an antioxidant response element*. Journal of Biological Chemistry, 2005. **280**(6): p. 4483-4490.

207. Pi, Jingbo, Qu, Wei, Reece, Jeffrey M., Kumagai, Yoshito, and Waalkes, Michael P., *Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: involvement of hydrogen peroxide*. Experimental Cell Research, 2003. **290**(2): p. 234-245.
208. Lambert, Carine B., Spire, Catherine, Claude, Nancy, and Guillouzo, Andre, *Dose- and time-dependent effects of phenobarbital on gene expression profiling in human hepatoma HepaRG cells*. Toxicology and Applied Pharmacology, 2009. **234**(3): p. 345-360.
209. Guzelian, Philip S., Victoroff, Michael S., Halmes, N. Christine, James, Robert C., and Guzelian, Christopher P., *Evidence-based toxicology: a comprehensive framework for causation*. Human & Experimental Toxicology, 2005. **24**(4): p. 161-201.
210. Rushton, Lesley, *The use of Systematic Review Techniques in Risk Assessment*. 2010: London. p. Discussion on the Issue of the Use of Systematic Review Techniques in Risk Assessment.
211. Klimisch, H. J., Andreae, M., and Tillmann, U., *A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data*. Regulatory Toxicology and Pharmacology, 1997. **25**(1): p. 1-5.
212. Edwards, S. W. and Preston, R. J., *Systems biology and mode of action based risk assessment*. Toxicol Sci, 2008. **106**(2): p. 312-8.
213. Barlow, T., Battershill, J., Jeffery, B. R., Pollitt, F. D., and Tahourdin, C. S. M., *Report of a symposium on the use of genomics and proteomics in toxicology*. Mutagenesis, 2003. **18**(3): p. 311-317.
214. Dix, David J., Houck, Keith A., Martin, Matthew T., Richard, Ann M., Setzer, R. Woodrow, and Kavlock, Robert J., *The ToxCast Program for Prioritizing Toxicity Testing of Environmental Chemicals*. Toxicol. Sci., 2007. **95**(1): p. 5-12.

215. Gibb, Steven, *Toxicity testing in the 21st century: A vision and a strategy*. Reproductive Toxicology, 2008. **25**(1): p. 136-138.
216. NRC, (*National Research Council*) *Toxicity Testing in the 21st Century: A Vision and A Strategy* 2007, Washington, DC: National Academy Press.
217. Committees on Toxicity, Mutagenicity and Carcinogenicity of Chemicals in Food, Consumer Products and the Environment. Joint statement of the use of toxicogenomics in toxicology. (Food Standards Agency & Department of Health, London, 2004). Available at:
<http://cot.food.gov.uk/cotstatements/cotstatementsyrs/cotstatements2004/toxicogenomics>
218. Corvi, R., Ahr, H. J., Albertini, S., Blakey, D. H., Clerici, L., Coecke, S., Douglas, G. R., Gribaldo, L., Groten, J. P., Haase, B., Hamernik, K., Hartung, T., Inoue, T., Indans, I., Maurici, D., Orphanides, G., Rembges, D., Sansone, S. A., Snape, J. R., Toda, E., Tong, W., van Delft, J. H., Weis, B., and Schechtman, L. M., *Meeting report: Validation of toxicogenomics-based test systems: ECVAM-ICCVAM/NICEATM considerations for regulatory use*. Environ Health Perspect, 2006. **114**(3): p. 420-9.
219. Krewski, Daniel, Acosta, Daniel, Andersen, Melvin, Anderson, Henry, Bailar, John C., Boekelheide, Kim, Brent, Robert, Charnley, Gail, Cheung, Vivian G., Green, Sidney, Kelsey, Karl T., Kerkvliet, Nancy I., Li, Abby A., McCray, Lawrence, Meyer, Otto, Patterson, Reid D., Pennie, William, Scala, Robert A., Solomon, Gina M., Stephens, Martin, Yager, James, Zeise, Lauren, and Staff of Committee on Toxicity Testing and Assessment of Environmental, Agents, *Toxicity Testing in the 21st Century: A Vision and a Strategy*. Journal of Toxicology and Environmental Health, Part B: Critical Reviews, 2010. **13**(2): p. 51 - 138.
220. Hartung, Thomas, *Lessons Learned from Alternative Methods and their Validation for a New Toxicology in the 21st Century*. Journal of Toxicology and Environmental Health, Part B: Critical Reviews, 2010. **13**(2): p. 277 - 290.

221. Hudson, M., *Why do the numbers of laboratory animal procedures conducted continue to rise? An analysis of the Home Office Statistics of Scientific Procedures on Living Animals: Great Britain 2005*. Altern Lab Anim, 2007. **35**(1): p. 177-87.
222. Schmidt, C. W., *TOX 21: new dimensions of toxicity testing*. Environ Health Perspect, 2009. **117**(8): p. A348-53.
223. Ankley, Gerald T., Daston, George P., Degitz, Sigmund J., Denslow, Nancy D., Hoke, Robert A., Kennedy, Sean W., Miracle, Ann L., Perkins, Edward J., Snape, Jason, Tillitt, Donald E., Tyler, Charles R., and Versteeg, Donald, *Toxicogenomics in Regulatory Ecotoxicology*. Environmental Science & Technology, 2006. **40**(13): p. 4055-4065.
224. Freeman, K., *Toxicogenomics data: the road to acceptance*. Environmental health perspectives, 2004. **112**(12): p. A678-85.