CARCINOGENIC ANALYSIS OF HETEROCYCLIC AMINES 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*b*]PYRIDINE (PhIP) AND 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-*f*]QUINOXALINE (MeIQX) USING BHAS 42 CELL LINE

THAYVEE GEETHA BHARATHI A/P SILVARAGI

UNIVERSITI SAINS MALAYSIA

2025

CARCINOGENIC ANALYSIS OF HETEROCYCLIC AMINES 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*b*]PYRIDINE (PhIP) AND 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-*f*]QUINOXALINE (MeIQX) USING BHAS 42 CELL LINE

by

THAYVEE GEETHA BHARATHI A/P SILVARAGI

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

September 2025

ACKNOWLEDGEMENT

Om Sairam. Above all, I express my gratitude to Shri Sai Baba for His benevolence and direction, which has been my driving force and motivation during this journey.

My special thanks, love and respect to my supervisor, Dr. Siti Nazmin Binti Saifuddin, who has provided me support, guidance, and encouragement throughout my Master's program. Her expertise, patience, guidance, and positive criticism undoubtedly made this thesis what it is. I would like to express my immense love to my family for their unwavering love and encouragement throughout my academic journey. Their presence in my life has made all the difference. This milestone is as much theirs as mine, and I dedicate this work to them with all my heart and appreciation. I would also like to extend my heartfelt gratitude to the Mybrain Science Scholarship for their generous financial support towards my academic journey. It is with great honour I thank the Advanced Medical and Dental Institute (AMDI) as well for playing a major role in my studies.

I also wish to appreciate my best friends, Jevitha Panicker, Sarminiyy Lenga Gururuloo as they were there for me during my difficult times. Their kindness and love have been an invaluable driving force, and my heartfelt gratitude for their constant support.

This accomplishment is an outcome of collective efforts and support of all those who have been part of this incredible journey. Thank you for being there every step of the way.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ..... **ii**

TABLE OF CONTENTS ..... **iii**

LIST OF TABLES ..... **vii**

LIST OF FIGURES ..... **viii**

LIST OF SYMBOLS ..... **xii**

LIST OF ABBREVIATIONS ..... **xiii**

LIST OF APPENDICES ..... **xviii**

ABSTRAK ..... **xix**

ABSTRACT ..... **xxi**

CHAPTER 1 INTRODUCTION **1**

1.1 Research Background 1

1.2 Problem Statement 3

1.3 General Objective 4

1.4 Specific Objectives 4

1.5 Research Hypothesis 5

CHAPTER 2 LITERATURE REVIEW **6**

2.1 Cancer Prevalence 6

2.1.1 Cancer in Malaysia 7

2.1.2 Diet-related Cancers 8

2.2 Carcinogens 9

2.2.1 Food-borne Carcinogens 10

2.3 Heterocyclic Aromatic Amines (HCAs) 10

2.3.1 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) 14

2.3.2 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) 15

2.3.3 Mechanism of Action of PhIP and MeIQx 15

2.4 Carcinogenesis 16
2.4.1 The Hallmarks of Carcinogenesis 17
2.4.2 Stages of Carcinogenesis 20
2.5 In Vitro Oncology Research 22
2.5.1 Cell Transformation Assays 23
2.5.2 Bhas 42 Cell Transformation Assay (Bhas 42 CTA) 26
2.6 Proteomic Technologies 29
2.6.1 Mass Spectrometry (MS) 30
2.6.1(a) Liquid Chromatography (LC) Coupled with Tandem MS (LC-MS/MS) 32
2.6.2 Bioinformatics Tools for Protein Analysis 34
2.6.3 Cancer Proteomics and Biomarkers 36
CHAPTER 3 METHODOLOGY 38
3.1 Materials 38
3.1.1 Cell Lines 38
3.1.2 Chemicals and Reagents 38
3.1.3 Kits and Consumables 39
3.1.4 Laboratory Equipments 39
3.2 Cell Culture 42
3.2.1 Preparation of Reagent, Buffer and Stock Solutions 42
3.2.1(a) Complete Growth Medium 42
3.2.1(b) Phosphate-buffered Saline (PBS) 43
3.2.1(c) PhIP Stock Solutions 43
3.2.1(d) MeIQx Stock Solutions 44
3.2.2 Thawing Frozen Cells 44
3.2.3 Sub-culturing of Cells 45
3.2.4 Cryopreservation of Cells 46
3.2.5 Cell Counting Using Haemocytometer 46

3.2.6 Determination of Seeding Density 47
3.3 Cytotoxicity and Cell Proliferation Tests 48
3.4 Bhas 42 Cell Transformation Assay (Bhas 42 CTA) 49
3.5 Protein Extraction 50
3.5.1 Preparation of Total Cell Lysates. 50
3.5.2 Total Protein Concentration. 51
3.6 In-Solution Digestion 51
3.7 Liquid Chromatography (LC) Tandem Mass-Spectrometry (LC-MS/MS) ... 52
3.7.1 Preparation of Samples. 52
3.7.2 LC-MS/MS Analysis. 52
3.8 Proteomic Analysis 53
3.8.1 Protein Identification. 53
3.8.2 Protein Annotation. 54
3.9 Statistical Analysis 54
CHAPTER 4 RESULTS 56
4.1 Cytotoxicity Assay 56
4.1.1 Cytotoxicity findings for PhIP treatment on Bhas 42 cells 56
4.1.2 Cytotoxicity findings for MeIQx treatment on Bhas 42 cells 58
4.2 Cell Proliferation Assay 60
4.2.1 Cell proliferation effects of PhIP on Bhas 42 cells 60
4.2.2 Cell proliferation effects of MeIQx on Bhas 42 cells 62
4.3 Bhas 42 Cell Transformation Assay (Bhas 42 CTA) 64
4.3.1 Morphological changes of vehicle-treated (control) Bhas 42 cells 64
4.3.2 Morphological changes of PhIP-treated Bhas 42 cells 66
4.3.3 Morphological changes of MeIQx-treated Bhas 42 cells 79
4.3.4 Bhas 42 Cell Transformation Assay 92
4.4 Proteomics 96

4.4.1 Confirmation of Proteins. 96

4.4.2 Protein Identification and Annotation 96

4.4.3 Overlapping DEPs and Cancer Potential of Exclusive Proteins. 106

CHAPTER 5 DISCUSSION 112

5.1 The effects of PhIP and MeIQx on cell cytotoxicity and cell proliferation of Bhas 42 cell line 112

5.2 Neoplastic changes of PhIP- and MeIQx-treated Bhas 42 cell line 118

5.3 Functional annotation of proteins expressed in PhIP- and MeIQx-treated Bhas 42 cell line 123

5.4 PhIP- and MeIQx-induced cancer-related pathways 128

5.5 Proteins exclusively associated with PhIP 134

5.6 Proteins exclusively associated with MeIQx 139

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS 144

6.1 Conclusion 144

6.2 Limitations and Recommendations for Future Research 146

REFERENCES 148

APPENDICES

LIST OF TABLES

Page

Table 2.1 List of heterocyclic amines and their classification according to International Agency for Research on Cancer (IARC) 12

Table 3.1 List of drugs and chemicals 38

Table 3.2 List of kits and consumables 39

Table 3.3 List of laboratory equipments 39

Table 3.4 Preparation of PhIP stock solutions 44

Table 3.5 Preparation of MeIQx stock solutions 44

Table 4.1 Results of Bhas 42 cell transformation assay of control, PhIP and MeIQx concentrations in 0.003% DMSO 93

Table 4.2 Results of Bhas 42 cell transformation assay of control, PhIP and MeIQx concentrations in 0.03% DMSO 94

Table 4.3 Results of Bhas 42 cell transformation assay of control, PhIP and MeIQx concentrations in 0.1% DMSO 95

Table 4.4 Total protein concentration of the cell lysates from all the samples in 0.003% DMSO 96

Table 4.5 Number of peptide-spectrum matches (PSM), peptide groups, protein groups, and proteins from all samples. 98

Table 4.6 Cancer-associated pathways and hallmarks of cancer. 105

Table 4.7 Proteins exclusively found in PhIP samples 109

Table 4.8 Proteins exclusively found in MeIQx samples 111

LIST OF FIGURES

Page

Figure 2.1 The former biological hallmarks of cancer 18

Figure 2.2 Hierarchical depiction (from molecular to organismal levels) of the original former hallmarks of cancer 19

Figure 2.3 The recent biological hallmarks of cancer 19

Figure 2.4 Multistage process of cancer 21

Figure 2.5 Timeline of Rodent Cell Transformation Assay 26

Figure 2.6 Timeline for the promotion test component of Bhas 42 CTA 29

Figure 2.7 Schematic representations of parts of a mass spectrometer 31

Figure 2.8 Depiction of a liquid chromatography-tandem mass spectrometry system 33

Figure 3.1 Flowchart of study 41

Figure 4.1 Effects of all PhIP concentrations in A) 0.003% DMSO, B) 0.03% DMSO, and C) 0.1% DMSO in comparison with vehicle treated control on Bhas 42 cell lines using Alamar Blue Assay 57

Figure 4.2 Effects of all MeIQx concentrations in A) 0.003% DMSO, B) 0.03% DMSO, and C) 0.1% DMSO in comparison with vehicle treated control on Bhas 42 cell lines using Alamar Blue Assay 59

Figure 4.3 Effects of all PhIP concentrations in A) 0.003% DMSO, B) 0.03% DMSO, and C) 0.1% DMSO in comparison with vehicle treated control on Bhas 42 cell line proliferation using Alamar Blue Assay 61

Figure 4.4 Effects of all MeIQx concentrations in A) 0.003% DMSO, B) 0.03% DMSO, and C) 0.1% DMSO in comparison with vehicle treated control on Bhas 42 cell line proliferation using Alamar Blue Assay 63

Figure 4.5 Morphology of Bhas 42 cells treated with vehicle, 0.003%, 0.03% and 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 65

Figure 4.6 Morphology of Bhas 42 cells treated with 10^{-7} M PhIP in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 67

Figure 4.7 Morphology of Bhas 42 cells treated with 10^{-8} M PhIP in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 68

Figure 4.8 Morphology of Bhas 42 cells treated with 10^{-9} M PhIP in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 69

Figure 4.9 Morphology of Bhas 42 cells treated with 10^{-10} M PhIP in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 70

Figure 4.10 Morphology of Bhas 42 cells treated with 10^{-7} M PhIP in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 71

Figure 4.11 Morphology of Bhas 42 cells treated with 10^{-8} M PhIP in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 72

Figure 4.12 Morphology of Bhas 42 cells treated with 10^{-9} M PhIP in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 73

Figure 4.13 Morphology of Bhas 42 cells treated with 10^{-10} M PhIP in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 74

Figure 4.14 Morphology of Bhas 42 cells treated with 10^{-7} M PhIP in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21 75

Figure 4.15 Morphology of Bhas 42 cells treated with 10^{-8} M PhIP in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....76

Figure 4.16 Morphology of Bhas 42 cells treated with 10^{-9} M PhIP in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....77

Figure 4.17 Morphology of Bhas 42 cells treated with 10^{-10} M PhIP in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....78

Figure 4.18 Morphology of Bhas 42 cells treated with 10^{-7} M MeIQx in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....80

Figure 4.19 Morphology of Bhas 42 cells treated with 10^{-8} M MeIQx in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....81

Figure 4.20 Morphology of Bhas 42 cells treated with 10^{-9} M MeIQx in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....82

Figure 4.21 Morphology of Bhas 42 cells treated with 10^{-10} M MeIQx in 0.003% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21. 83

Figure 4.22 Morphology of Bhas 42 cells treated with 10^{-7} M MeIQx in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....84

Figure 4.23 Morphology of Bhas 42 cells treated with 10^{-8} M MeIQx in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....85

Figure 4.24 Morphology of Bhas 42 cells treated with 10^{-9} M MeIQx in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....86

Figure 4.25 Morphology of Bhas 42 cells treated with 10^{-10} M MeIQx in 0.03% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....87

Figure 4.26 Morphology of Bhas 42 cells treated with 10^{-7} M MeIQx in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....88

Figure 4.27 Morphology of Bhas 42 cells treated with 10^{-8} M MeIQx in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....89

Figure 4.28 Morphology of Bhas 42 cells treated with 10^{-9} M MeIQx in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....90

Figure 4.29 Morphology of Bhas 42 cells treated with 10^{-10} M MeIQx in 0.1% DMSO. A) Day 12, B) Day 14, C) Day 17, D) Day 19, and E) Day 21.....91

Figure 4.30 List of proteins identified using Proteome Discoverer software.....99

Figure 4.31 The overall statistics of differentially expressed protein. 100

Figure 4.32 GO enrichment classification map of DEPs in PhIP-treated sample. 102

Figure 4.33 GO enrichment classification map of DEPs in MeIQx-treated sample. 103

Figure 4.34 Comparative analysis of overlapping differentially expressed proteins (DEPs) through A) Venn analysis and B) their expression trends. 107

LIST OF SYMBOLS

%	Percentage
°C	Degrees Celsius
µl	Microliter
A0	Angstroms
g/d	Grams per day
g/L	Grams per liter
g/mol	Grams per mole
M	Molarity
mg	Milligram
mg/ml	Milligrams per milliliter
mm	Millimeter
mM	Millimolar
nL/min	Nanoliters per minute
nm	Nanometer
rpm	Revolutions per minute
µm	Micrometer

LIST OF ABBREVIATIONS

3-AP	3-aminopropanal
ACAT1	Acetyl-CoA acetyltransferase, mitochondrial
ACAT2	Acetyl-CoA acetyltransferase, cytosolic
ACATs	Acyl-coenzyme A: cholesterol acyltransferases
ALYREF	THO complex subunit 4
ALYREF2	Aly/REF export factor 2
ANXA4	Annexin A4
APA	Alternative polyadenylation
APP	Antigen processing and presentation pathway
ARF1	ADP-ribosylation factor 1
ARF2	ADP-ribosylation factor 2
ARF4	ADP-ribosylation factor 4
ARF5	ADP-ribosylation factor 5
ARFs	ADP-Ribosylation Factors
ATP	Adenosine triphosphate
B	Basophioic
Caco-2	Human colorectal adenocarcinoma cells
CBX3	Chromobox protein homolog 3
CDI	Chronic Daily Intake
CDIN1	CDAN1-interacting nuclease 1
CDK5	Cyclin-dependent kinase 5
CDKs	Cyclin dependent kinases
CEL	Chronic eosinophilic leukemia
CIN1	CDAN1-interacting nuclease 1
CLPP	ATP-dependent Clp protease proteolytic subunit, mitochondrial
CO2	Carbon dioxide
CRC	Colorectal Cancer
CTA	Cell Transformation Assay
CUL4B	Cullin 4B
Cxs	Connexin proteins
CYP	Cytochrome P450

DAVID	Database for Annotation, Visualization and Integrated Discovery
dbDEPs	Database of DEPs in human cancers
DEPs	Differentially expressed proteins
DF5F	Dulbecco's Modified Eagle's Medium/F12, augmented with 5% FBS and 1% Penicillin/Streptomycin solution
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EIF5A	Eukaryotic translation initiation factor 5A-1
EMT	Epithelial-to-mesenchymal transition
ESI	Electrospray ionization
FBS	Fetal bovine serum
FDR	False discovery rate
GC	Gas Chromatography
GLOBOCAN	Global Cancer Observatory
GO	Gene Ontology
Group 2A	Probable carcinogens
Group 2B	Potential human carcinogens
GSTP1	Glutathione S-transferase P
H1-3	Histone H1.3
H1-4	Histone H1.4
H1-6	Histone H1t
H2O2	Hydrogen peroxide
HCAs	Heterocyclic amines
HCT116	Human colon cancer cell line
HDI	Human Development Index
hESCs	Human embryonic stem cells
HF-MSC	Human foreskin-derived mesenchymal stem cells
HIF	Hypoxia-Inducible Factor
HL-60	Acute promyelocytic leukemia
HMGN1	Non-histone chromosomal protein HMG-14
hnRNP	Heterogeneous nuclear ribonucleoprotein
HNRNPU	Heterogeneous nuclear ribonucleoprotein U

HRAS	GTPase Hras
I	Invasive
IARC	International Agency for Research on Cancer
IIC	infiltrating immune cells (IIC)
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LC-MS	Liquid chromatography (LC)-tandem mass spectrometry (MS)
M	Multilayer
m/z	Mass-to-charge ratio
M10F	Minimum Essential Medium containing 2.2 g/L NaHCO3, enriched with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin solution
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
MEM	Minimum essential medium
METTL3	Methyltransferase-like 3
MIF	Migration inhibitory factor
MMPs	Matrix metalloproteinases
MNCR	Malaysia National Cancer Registry
mRNA	Messenger RNA
MS	Mass spectrometry
MS1	Mass-to-charge ratio values of peptide molecular ions
MS2	Mass-to-charge ratio values of fragment ions formed through fragmentation
MYBBP1A	Myb-binding protein 1A
MYH11	Myosin-11
MYH14	Myosin-14
NAD	Nicotinamide adenine dinucleotide
NCBP1	Nuclear cap-binding protein subunit 1
NET	Neutrophil extracellular trap formation
NIBM	National Institutes of Biotechnology Malaysia
NRAS	GTPase Nras
OECD	Organization for Economic Cooperation and Development
OXCT1	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial
PA2G4	Proliferation-associated protein 2G4
PALM	Paralemmin-1

PAs	Polyamines
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PDC	Pyruvate dehydrogenase complex
PDGFRA	Platelet-derived growth factor receptor alpha
PDHA1	Pyruvate dehydrogenase
PDIA4	Protein disulfide-isomerase A4
PDIs	Protein disulfide isomerases
PDP1	Pyruvate dehydrogenase phosphatase
PhIP	2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine
PPIases	Peptidyl-prolyl cis-trans isomerases
PPP	Pentose phosphate pathway
Pre-mRNAs	Precursor mRNAs
PSMs	Peptide-spectrum matches
PTBP1	Polypyrimidine tract-binding protein 1
Q3V132	ADP/ATP translocase 4
R	Random orientated
RAD18	E3 ubiquitin-protein ligase RAD18
RAN	GTP-binding nuclear protein Ran
RASL2-9	GTP-binding nuclear protein Ran, testis-specific isoform
RhoC	Rho-related GTP-binding protein
RIP	Receptor-interacting protein
RNF213	E3 ubiquitin-protein ligase RNF213
ROS	Reactive oxygen species
RPL6	Large ribosomal subunit protein El6
RPS24	Small ribosomal subunit protein eS24
RR	Relative risk
S	Spindle-shaped
SARS1	Serine--tRNA ligase, cytoplasmic
SD	Standard Deviation
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
SEC13	Protein SEC13 homolog
SEPTIN7	Septin-7
SHE	Syrian Hamster Embryo primary diploid cells

SPSS	Statistical Package for the Social Sciences
TFA	Trifluoroacetic acid
TIMM13	Mitochondrial import inner membrane translocase subunit Tim13
TLS	Translesion DNA synthesis
TOMM6	Mitochondrial import receptor subunit TOM6 homolog
TPA	12-O-tetradecanoylphorbol-13-acetate
UAP1L1	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1
UV	Ultraviolet
VCL	Vinculin
VCPIP1	Deubiquitinating protein VCPIP1
WHO	World Health Organization

LIST OF APPENDICES

Appendix A	Protein Identification from LC-MS/MS Analysis
Appendix B	Differential Protein Expression Analysis
Appendix C	Functional Annotations of Differential Expression Proteins
Appendix D	Pathway Enrichment Analysis
Appendix E	Overlapping Differential Expression Proteins Analysis
Appendix F	List of Publications and Presentations

ANALISIS KARSINOGENIK HETEROSIKLIK AMIN 2-AMINO-1-METIL-6-FENILIMIDAZO[4,5-*B*]PIRIDIN (PHIP) DAN 2-AMINO-3,8-DIMETILIMIDAZO[4,5-*F*]QUINOXALIN (MEIQX) MENGGUNAKAN TITISAN SEL BHAS 42

ABSTRAK

Amina heterosiklik (HCAs) merupakan karsinogen makanan yang terbentuk apabila makanan berprotein dimasak pada suhu tinggi. Kajian ini meneliti kesan karsinogenik dua HCAs, iaitu 2-amino-1-metil-6-fenilimidazo[4-5-*b*]piridin (PhIP) dan 2-amino-3,8-dimetilimidazo[4,5-*f*]quinoxalin (MeIQx) pada dos yang relevan dengan fisiologi manusia terhadap sel Bhas 42, klon yang diperoleh daripada sel fibroblas tikus BALB/c3T3 A31-1-1, satu talian sel yang amat disyorkan oleh OECD untuk kajian kanser kerana sensitivitinya yang tinggi terhadap rangsangan onkogenik. Sel Bhas 42 tersebut diterdedahkan kepada PhIP atau MeIQx pada dos fisiologi manusia 10^{-7}, 10^{-8}, 10^{-9}, dan 10^{-10} M yang jarang dikaji; dilarutkan dalam DMSO pada kepekatan 0.003%, 0.03%, atau 0.1%. Kajian ini menggunakan ujian Alamar Blue untuk sitotoksisiti dan proliferasi sel, ujian transformasi sel Bhas 42, serta analisis proteomik shotgun. Dalam ujian sitotoksisiti, PhIP dan MeIQx tidak menunjukkan kesan sitotoksik, kecuali PhIP pada 10^{-9}M dalam DMSO 0.03%. Manakala, dalam ujian proliferasi, hanya PhIP pada 10^{-7}M dan 10^{-10}M dalam DMSO 0.1% yang menunjukkan kesan signifikan terhadap sel Bhas 42 pada hari ke-8. MeIQx pada 10^{-9}M dalam DMSO 0.03% meningkatkan proliferasi sel kepada 109.26%. Perubahan morfologi sebagai indikasi bahawa sel memperoleh ciri neoplastik, menunjukkan pembentukan foci yang jelas bagi PhIP dan MeIQx pada 10^{-7}M walaupun pada kepekatan DMSO yang paling rendah. Transformasi positif hanya diperhatikan untuk

MeIQx yang diuji pada kepekatan DMSO tertinggi, manakala semua kepekatan yang diuji dalam 0.003% dan 0.03% DMSO menunjukkan transformasi negatif. Analisis proteomik menunjukkan lebih banyak protein diatur naik berbanding turun dalam kedua-dua kumpulan pendedahan, dengan anotasi protein lebih tinggi dalam pendedahan PhIP berbanding dengan pendedahan MeIQx. Analisis pengayaan jalur menggunakan KEGG dalam perisian DAVID menunjukkan beberapa laluan berkaitan kanser. Kesimpulannya, keputusan kajian menunjukkan bahawa, sel Bhas 42 yang diterdedahkan pada PhIP atau MeIQx pada 10^{-7}M dalam DMSO 0.003% mengalami transformasi neoplastik awal pada peringkat selular dan protein. Kajian ini memberikan pemahaman baru mengenai kesan pendedahan PhIP dan MeIQx pada dos yang berkaitan fisiologi manusia pada Bhas 42 talian sel dan mendedahkan peranan beberapa protein tertentu yang berkaitan dengan PhIP dan MeIQx, serta potensi mereka sebagai sasaran terapeutik/diagnostik dalam pencegahan karsinogenesis akibat HCAs.

CARCINOGENIC ANALYSIS OF HETEROCYCLIC AMINES 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*B*]PYRIDINE (PHIP) AND 2-AMINO-3,8-DIMETHYLIMIDAZO[4,5-*F*]QUINOXALINE (MEIQX) USING BHAS 42 CELL LINE

ABSTRACT

Heterocyclic amines (HCAs) are common food carcinogens that are produced in cooked foods rich in protein at a high temperature. The present study investigates the carcinogenic effects of physiologically relevant concentrations of the two most commonly occurring HCAs, 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), on Bhas 42 cells, a clone established from BALB/c3T3 A31-1-1 mouse fibroblast cells, a cell line strongly recommended by OECD for cancer studies due to its greater sensitivity towards oncogenic stimuli. The cells were treated with PhIP or MeIQx at human physiologically relevant concentrations of 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}M which are not widely investigated; diluted in DMSO at concentrations of 0.003%, 0.03%, or 0.1%. This study utilized Alamar Blue assays for cytotoxicity and proliferation, Bhas 42 CTA, and shotgun proteomics. The cytotoxicity results revealed that PhIP and MeIQx had no significant toxic effects on Bhas 42 cells except for 10^{-9}M PhIP in 0.03% DMSO. In the cell proliferation assay, only 10^{-10}M and 10^{-7}M PhIP in 0.1% DMSO showed significant cytotoxicity at the final time point. For MeIQx, no substantial changes were observed in all groups at the final time point, except for 10^{-9}M MeIQx in 0.03% DMSO, which increased cell proliferation to 109.26%. Morphological observations revealed foci formation at 10^{-7}M of both PhIP and MeIQx, even at the lowest DMSO concentration. A positive transformation was observed only for MeIQx

tested in the highest DMSO condition. Proteomic analysis showed a higher number of up-regulated proteins than down-regulated proteins in both groups; PhIP and MeIQx. Interestingly, functional enrichment analysis revealed more annotated proteins for PhIP sample compared to that of MeIQx, aligning with the observed trend of increased differentially expressed proteins (DEPs). KEGG pathway enrichment analysis (via DAVID software) identified some significant pathways related to cancer. The association between cancer-related pathways and cancer hallmarks was further explored. In conclusion, results indicate that Bhas 42 cells treated with 10^{-7}M PhIP or MeIQx in 0.003% DMSO exhibit early neoplastic transformation. This study provides valuable insights into into the effects of PhIP and MeIQx exposures at human physiologically relevant dose on Bhas 42 cell line and unveiling the role of several exclusive proteins associated with PhIP or MeIQx, emphasizing their potential as therapeutic/diagnostic targets in HCA-induced carcinogenesis

CHAPTER 1

INTRODUCTION

1.1 Research Background

Dietary foods which refer to specific types of food chosen for a particular purpose such as weight management or addressing health conditions have been consistently recommended for both preventing and slowing down the advancement of fully developed illnesses. Typically, foods possess therapeutic capabilities against disease progression because of the nutrients they carry, including fatty acids, minerals, and vitamins (Wang et al., 2024). Nevertheless, it is irrefutable that dietary food is also viewed as a major pathway for harmful substances, such as heterocyclic amines (HCAs), to enter the human diet or system (Seidel & Pfau, 2017). Consequently, toxicity assessments, such as mutagenicity, genotoxicity, neurotoxicity, and carcinogenicity, are vital in recognizing food-derived toxicants' toxicological impacts. To our current knowledge, 33 HCAs have been recognized so far, with some of these classified by the International Agency for Research on Cancer (IARC) as probable carcinogens (Group 2A) and potential human carcinogens (Group 2B) (Alaejos & Afonso, 2011). Cooking duration, method, temperature, and precursor concentration significantly affect HCA formation (Li et al., 2021). The two most prevalent HCAs that have appeared in the majority of studies conducted on HCAs, as well as been categorized as Group 2B by the IARC, are 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) Li et al., 2021). These carcinogenic food-borne HCAs have attracted considerable attention from researchers globally, leading to extensive investigations over the past two decades aimed at uncovering their potential mechanisms of action to reduce their

carcinogenic effects. As reported by the World Health Organization (WHO), cancer encompasses a diverse array of diseases that can impact any area of the body. According to latest report, it has been stated that cancer cases were estimated to result in around 20 million new cases and 9.7 million deaths in the year 2022 (World Health Organization, 2024). Furthermore, cancer incidence is expected to increase two-fold by 2040 in Malaysia based on the latest report released by the Malaysia National Cancer Registry (MNCR) (Azizah et al., 2019). However, currently, there is no official data at global scale that directly evaluates the cancer burden specifically related to food-borne carcinogens. Whereas, WHO mainly provides estimates on food-related diseases such as infections and toxins, instead of long-term cancer outcomes (World Health Organization, 2025). Global Burden of Disease (GBD) studies the cancer burden from dietary factors along with specific data/monographs on certain food borne carcinogens by IARC explains their role in specific cancers (Institute for Health Metrics and Evaluation, 2025; International Agency for Research on Cancer, 2025). Currently, the evidence on food-borne carcinogens is generally obtained from relative risk studies instead of global burden statistics. In brief, for the time being, there is information about diet and particular carcinogens, but not about food-borne carcinogens as a whole.

Despite extensive experimental and epidemiological research conducted on PhIP and MeIQx, only a number of studies have employed human physiologically relevant concentrations of these compounds. This study aims to investigate the carcinogenic effects of PhIP and MeIQx at human physiologically relevant concentrations using Bhas 42 cell line, a cell line strongly recommended by Organization for Economic Cooperation and Development (OECD) for cancer studies.

1.2 Problem Statement

Many studies have investigated the relationship between these HCAs and their carcinogenic potential at relatively higher concentrations. Specifically, *in vivo* studies conducted on these compounds have used concentrations from the range of 10 mg/kg to 200 mg/kg which is similar to the concentration range of 10^{-5} M to 10^{-4} M (Chen et al., 2016; Cheungy et al., 2011; Li et al., 2012; Machida & Imai, 2021; Nakai et al., 2007; Wang et al., 2015). Whereas, *in vitro* studies have used concentrations from the range of 10^{-7} M to 10^{-4} M (Glass-Holmes et al., 2015; Malik et al., 2018; Wang et al., 1999). The carcinogenic potential of these HCAs at human exposure dosage, however, has not been thoroughly studied. The dosage of HCAs to which humans are exposed is frequently 3 to 6 orders of magnitudes lower than the levels generally employed in experimental studies which falls in the range of 10^{-11} M to 10^{-7} M (Lauber & Gooderham, 2011). This has become an important concern in toxicology and cancer research, as higher concentrations are often utilized in studies to yield a robust carcinogenic effect or to hasten the manifestation of cellular damage (Yang et al., 2021). Thus, assessing the dosage levels of PhIP and MeIQx to which humans are subjected is a crucial component in evaluating their potential carcinogenicity in humans.

According to the Organization for Economic Cooperation and Development (OECD), Bhas 42 cells, a clone established from BALB/c3T3 A31-1-1 mouse fibroblast cells, are regarded as an exceptional *in vitro* model for cancer research especially in a cell transformation assay (CTA) compared to previous models like primary diploid cell Syrian Hamster Embryo (SHE), immortalized BALB/c3T3 and C3H10T1/2 mouse fibroblast cells due to being an immortalized cell line that additionally exhibits the typical morphology and contact inhibition of a normal cell yet

can readily undergo a morphological change when subjected to a tumour promoter/oncogenic stimuli. The previously conducted CTAs with various cells had limitations such as limited life span (SHE) and laborious and time-intensive nature (BALB/c3T3 and C3H10T1/2). The unique 'initiated' characteristic of Bhas 42 enables scientists to investigate the later phases of carcinogenesis instead of solely concentrating on the initial stages, as is the case with other cell lines aforementioned such as SHE, BALB/c3T3 and C3H10T1/2 cells (Organisation for Economic Co-operation and Development, 2017). Hence, Bhas 42 cells were used as an *in vitro* model in this study to determine the carcinogenicity of PhIP and MeIQx, providing significant advantages in efficiency, cost, and ethical considerations. It is anticipated that this research will enable a carcinogenic analysis of the heterocyclic amines PhIP and MeIQx.

1.3 General Objective

The general objective of this research is to examine the carcinogenic effects of PhIP and MeIQx at human physiologically relevant doses on the Bhas 42 cell line, focusing on both cellular and protein level of carcinogenesis.

1.4 Specific Objectives

1.4.1 Objective 1

To assess the effects of PhIP and MeIQx on cytotoxicity and proliferative activity of Bhas 42 cell line at levels physiologically relevant to human.

1.4.2 Objective 2

To evaluate the morphological transformation acquired by Bhas 42 cells and transformation frequency when exposed to varying concentrations of PhIP and MeIQx at levels physiologically relevant to human.

1.4.3 Objective 3

To identify the proteins expressed in Bhas 42 cells following exposure to PhIP and MeIQx and to characterize their expression profiles.

1.5 Research Hypothesis

1.5.1 Null Hypothesis

There is no significant difference in the carcinogenic effects on Bhas 42 cells treated with PhIP and MeIQx compared to untreated controls.

1.5.2 Alternative Hypothesis

Treatment of Bhas 42 cells with PhIP and MeIQx shows significant carcinogenic effects at the cellular and protein level.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer Prevalence

Cancer is a type of non-communicable disease that is characterized primarily by the loss of genetic control over cell growth and proliferation (Ubago-Guisado et al., 2021). It is undeniably a growing public health concern that puts financial, physical, and emotional strain on every social unit like individuals, families, communities, and healthcare systems (Organisation for Economic Co-operation and Development, 2024). As per the recent report released by WHO, approximately 20 million new cancer cases and 9.7 million deaths were estimated in 2022 where, an unequal progress in cancer control has also been addressed between high-income countries and middle-income countries. Cancer incidence rates differ by country due to the uneven distribution of cancer risk factors around the world, with the majority of risk factors being closely related to the 'development' element, which is frequently examined or evaluated using the Human Development Index (HDI). Individuals are less likely to be diagnosed in lower HDI countries compared to high HDI countries. However, they do possess greater risk of dying due to factors like insufficient access to quality treatment and late diagnosis. Interestingly, lung, prostate and colorectal cancers among males and breasts, lung and colorectal among females were the three most prevalent cancers recorded in the year 2022 (World Health Organization, 2024).

2.1.1 Cancer in Malaysia

Some cancers seem to be more prevalent in Asians. However, there is still insufficient evidence on common cancers in Asians, and simultaneously survival for cancers common in Asians is also reportedly lower than survival for cancers common in Caucasians (Cancer Research Malaysia, 2024). In Malaysia specifically, a total of 51 650 new cancer cases were recorded in 2022 alone (Global Cancer Observatory, 2022) and cancer incidence is expected to increase two-fold by 2040 according to the latest report released by the MNCR (Azizah et al., 2019). Malaysia's involvement in cancer management can be traced back to around 20 years ago, with the implementation of Malaysia's National Cancer Control Plan, which imposed policies on a variety of subjects such as cancer prevention, screening, early detection, treatment, and palliative care across the country (Lim, 2002).

Regarding treatment resources, Malaysia currently owns six Ministry of Health hospitals, three university centres, and not less than 20 private hospitals offering cancer services (Ministry of Health Malaysia, 2017). Given that Malaysia is a multi-racial country, if the incidence of cancer is broken down by ethnicity, the Chinese are categorized as the most affected, followed by the Malays and Indians. Age-standardised incidence rates (ASR), on the other hand, are 102 cancer cases per 100,000 women and 86 cancer cases per 100,000 men with the same population ratio, indicating females are at greater risk of having cancer compared the males in our nation (Azizah et al., 2019). According to MNCR 2012-2016, the percentage of cancer cases identified in late stages 3 and 4 rose from 58.7% between 2007 and 2011 to 63.7% between 2012 and 2016. The rise in cancer incidences, despite various efforts, could be attributed to citizens' limited knowledge and beliefs about cancer, according to a 2015 study involving Malaysian adolescents (Al-Naggar et al., 2015). According to

the WHO, breast cancer, colorectal cancer, lung cancer, liver cancer, and prostate cancer are the cancers listed as most common in Malaysia (Global Cancer Observatory, 2022). The latest report for 2025 is yet to be released. It is worth acknowledging that all of the aforementioned cancers are, to some extent, related to diet, raising the question of whether Malaysians, as previously stated, indeed have insufficient knowledge and limited belief when it comes to the link between dietary habits and cancer

2.1.2 Diet-related Cancers

For years, scientists have been inclined to believe that nutrition has a significant impact on the likelihood of developing cancer (Key et al., 2020). Unlike other cancer risk factors such as genetics, lifestyle, which often includes tobacco, alcohol and even unhealthy diet, is one of the recognized cancer risk factors that can be controlled (Otsuka et al., 2023). As a matter of fact, an increased risk of cancer is often linked to dietary exposures such as westernised diets described by elevated levels of saturated fats, red meat, and ultra-processed foods, but low levels of fruits, vegetables, and whole grains (Otsuka et al., 2023).

Colorectal cancer (CRC) is one of the cancers strongly linked to a high intake of processed and red meat. Unprocessed red meat consumption was associated with CRC risk in a study comprising 18 cohorts and one nested case-control in Europe, 13 cohorts in North America, five cohorts in Asia, and one cohort in Australia (Lescinsky et al., 2022). Unprocessed red meat consumption, on the contrary, was weakly associated with the risk of developing breast cancer (another diet-related cancer. Similarly, an increased risk was observed concerning developing gastric cancer (Kim et al., 2019) and renal cell carcinoma (kidney cancer) (Zhang et al., 2017) upon the

consumption of red meat or processed meat. Based on recent research, high-fat diet is also reported to have a strong positive association with the onset of breast cancer (Uhomoibhi et al., 2022). In conjunction with this matter, obesity shows a significant positive association with higher death rates for the following cancers: oesophagus, colon and rectum, liver, gallbladder, pancreas, kidney, stomach (in men), prostate, breast, uterus, cervix, and ovary (Donaldson, 2004). However, a 60 to 70 percent reduction in breast, colorectal, and prostate cancers is possibly attained with the aid of a guideline-based diet (Donaldson, 2004). Aside from processed and red meat consumption, alcohol consumption is another important dietary risk factor for a variety of diet-related cancers. According to a systematic review and meta-analysis, the Relative Risks (RRs) for CRC, breast cancer, and upper aerodigestive tract cancer for the highest and lowest relative intake levels over a lifetime were 1.49 (1.27-1.74), 1.28 (1.07-1.52), and 2.83 (1.73-4.62), respectively, indicating the effects of alcohol consumption on the development of certain diet-related cancers (Jayasekara et al., 2016). Myriads of carcinogens are generally involved in the development of these various diet-related cancers.

2.2 Carcinogens

A carcinogen is any agent that, when exposed to it, increases the risk of malignant neoplasia (Fishbein et al., 2021). According to WHO, carcinogens are classified into three types: physical, chemical, and biological carcinogens. Ultraviolet (UV) and ionising radiation are examples of the former category, while asbestos, tobacco smoke components, alcohol, and arsenic (a drinking water contaminant) are examples of chemical carcinogens, and infections from specific viruses, bacteria, or parasites are examples of biological carcinogens (World Health Organization, 2021).

Carcinogens can also be further classified as exogenous and endogenous carcinogens. In this regard, food-borne carcinogens are said to be from external sources (exogenous) but has the tendency to form endogenously (Kobets et al., 2022).

2.2.1 Food-borne Carcinogens

Meat products are a crucial source of food in our daily diet, offering us with necessary nutrients, comprising fat, protein, vitamins, essential amino acids and some trace elements, including iron (Jinap et al., 2016). Meat, whether satay or grilled, steamed, roasted, or fried, is a popular dish in Southeast Asia, including Malaysia, Indonesia, Thailand, and Singapore (Jinap et al., 2016). However, overheating, or improper meat cooking methods have the potential to produce several toxic and harmful substances, such as nitrosamines, polycyclic aromatic hydrocarbons, and heterocyclic aromatic amines (HCAs), which can also be identified as food-borne carcinogens (Flores et al., 2019). HCAs, one of the aforementioned harmful substances, could also be found in other food items such as coffee and alcohol-beverages (Teng et al., 2023). Thus, HCAs have been extensively studied over the last 30 years after previous studies revealed their presence in proteinaceous foods cooked at high temperatures for an extended period of time.

2.3 Heterocyclic Amines (HCAs)

According to Chemistry LibreTexts, heterocyclic compounds are compounds that contain nitrogen, oxygen, sulfur, or some other atom in the ring instead of just carbon and hydrogen, with “hetero” meaning other in Greek (Chemistry LibreTexts, 2024). The discovery of heterocyclic aromatic amines (HCAs) dates back to the year 1977, when a scientist named Nagao and his team discovered the mutagenicity test results for tar and tobacco smoke are close to those for the particles produced when

fish are burnt in smoke (Nagao et al., 1977). HCAs are formed by reducing precursors such as sugars, free amino acids, and creatinine (Kang et al., 2022). Strecker degradation by a Maillard reaction involving precursors, as mentioned above, is the process by which HCAs are formed (Mirsadeghi et al., 2019). Cooking time, temperature, fat content, moisture content, type of meat, pH, and water activity all have an impact on the formation of HCAs. HCAs can be classified into two main groups based on the temperature at which they form; thermic (100°C - 300°C) and pyrolytic (above 300°C) HCAs (Kang et al., 2022). Even trace levels of HCAs are capable of inducing cancer in humans and animals, increasing the risk of colon, liver, pancreatic, prostate, stomach, lung and breast cancers (Nadeem et al., 2021). Intake per day (ID) of HCAs has been set at 1 μg maximum by The European Council (Puangsombat et al., 2011). A recent study reported that there is a significant difference between the sexes in terms of exposure to HCAs where the exposure to HCAs in the male population was higher than in the female population in all age groups. Besides, a recent study showed that the levels of chronic daily intake (CDI) of HCAs were enhanced with age (Mohammadi et al., 2023).

To date, thirty-three HCAs have been successfully identified, with some of them having classified as probable carcinogens (Group 2A) and possible human carcinogens (Group 2B) by the IARC. The IARC classification of the thirty-three HCAs has been compiled as depicted in Table 2.1. Among these, 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) are listed as the most abundant HCAs based on the results obtained from previous research. It is critical to note that these two HCAs are also classified as Group 2B carcinogens, indicating that they may cause cancer in humans,

as there is still a scarcity of scientific reports on their significant carcinogenic effect on humans.

Table 2.1 List of heterocyclic amines and their classification according to International Agency for Research on Cancer (IARC) (extracted from Alaejos, 2011)

Number	Heterocyclic aromatic amine (HCA)	Abbreviated name	IARC Class
Thermic HCAs			
1	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine	PhIP	Group 2B
2	2-amino-1-methyl-6-(4'-hydroxyphenyl)imidazo[4,5-b]pyridine	4'-OH-PhIP	-
3	2-amino-1,6-dimethylimidazo[4,5-b]pyridine	DMIP	-
4	2-amino-1,5,6-trimethylimidazo[4,5-b]pyridine	1,5,6-TMIP	-
5	2-amino-3,5,6-trimethylimidazo[4,5-b]pyridine	3,5,6-TMIP	-
6	2-amino-1,6-dimethyl-furo[3,2- e]imidazo[4,5-b]pyridine	IFP	-
7	2-amino-1-methylimidazo[4,5-f]quinoline	iso-IQ	-
8	2-amino-3-methylimidazo[4,5-f]quinoline	IQ	Group 2A
9	2-amino-3,4-dimethylimidazo[4,5-f]quinoline	MeIQ	Group 2B
10	2-amino-1-methylimidazo[4,5-b]quinoline	IQ[4,5-b]	-
11	2-amino-3-methylimidazo[4,5-f]quinoxaline	IQx	-
12	2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline	4-MeIQx	-
13	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline	MeIQx	Group 2B
14	2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline	7,8-DiMeIQx	-
15	2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline	4,8-DiMeIQx	-

Table 2.1 Continued

16	2-amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-f]quinoxaline	4-CH_2OH-8-MeIQx	-
17	2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline	TriMeIQx	-
18	2-amino-1-methylimidazo[4,5-g]quinoxaline	IgQx	-
19	2-amino-1,7-dimethylimidazo[4,5-g]quinoxaline	7-MeIgQx	-
20	2-amino-1,6,7-trimethylimidazo[4,5-g]quinoxaline	6,7-DiMeIgQx	-
21	2-amino-1,7,9-trimethylimidazo[4,5-g]quinoxaline	7,9-DiMeIgQx	-
Pyrolitic HCAs			
22	2-aminodipyrido[1,2-a:3',2'-d]imidazole	Glu-P-2	Group 2B
23	2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole	Glu-P-1	Group 2B
24	3-amino-1-methyl-5H-pyrido[4,3-b]indole	Trp-P-2	Group 2B
25	3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole	Trp-P-1	Group 2B
26	1-methyl-9H-pyrido[3,4-b]indole	Harman	-
27	9H-pyrido[3,4-b]indole	Norharman	-
28	2-amino-5-phenylpyridine	Phe-P-1	-
29	2-amino-9H-pyrido[2,3-b]indole	AαC	Group 2B
30	2-amino-3-methyl-9H-pyrido[2,3-b]indole	MeAαC	Group 2B
31	4-amino-6-methyl-1H-2,5,10,10b- tetraazafluoranthen	Orn-P-1	-
32	4-amino-1,6-dimethyl-2-methylamino- 1H,6H-pyrrolo-[3,4-f]benzimidazole-5,7-dione	Cre-P-1	-
33	3,4-cyclopenteno-pyrido[3,2-a]carbazole	Lys-P-1	-

2.3.1 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)

PhIP, like many HCAs, is derived from cooking muscle meats that are a major part of the human diet (Rogers et al., 2016). PhIP has a chemical formula of $C_{13}H_{12}N_4$ and a molecular weight of 224.26 g/mol (National Center for Biotechnology Information, 2024a). It is formed at high temperatures, for example, during grilling, frying or barbecuing and as a result from the Maillard reaction between substances in the muscle meat such as creatinine, amino acids and sugars when subjected to high temperatures (Gibis & Loeffer, 2019; Iwasaki & Tsugane, 2021). PhIP has been classified as "possibly carcinogenic to humans" (Group 2B) by the International IARC (Chen et al., 2021). Both epidemiological and animal studies indicate that long-term exposure leads to higher risks of various diet-related cancers such as prostate (Glass-Holmes et al., 2015; Li et al., 2012; Nakai et al., 2007; Sfanos et al., 2015), breast (Lauber & Gooderham, 2011; Machida & Imai, 2021; Malik et al., 2018, 2019) and colon cancer (Chen et al., 2016; Cheungy et al., 2011; Igarashi et al., 2014; Jamin et al., 2013; Wang et al., 2015). There are several ways by which PhIP can be reduced: (1) lowering cooking temperature - cooking meat at lower temperatures (e.g., baking, steaming) reduces HCA formation (Chu et al., 2024), (2) shorter cooking times - avoid prolonged high-heat cooking like grilling or pan-frying (Reartes et al., 2016), (3) marination - using marinades (especially with acidic ingredients like lemon or vinegar) can decrease PhIP production (Sepahpour et al., 2018) and (4) pre-cooking methods - pre-cooking meats in the microwave before grilling reduces time at high temperatures, lowering PhIP formation (Chang et al., 2018). Concerns about the toxicity of PhIP and other HCAs are frequently raised in the context of dietary recommendations, particularly among those who consume large amounts of well-cooked meats.

2.3.2 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx)

MeIQx is a heterocyclic amine characterized by the chemical formula $C_{11}H_{11}N_5$ and a molecular weight of 213.24 g/mol (National Center for Biotechnology Information, 2024b). Like PhIP, MeIQx is produced during the cooking of meats, particularly through grilling or frying, as these methods create conditions conducive to the generation of heterocyclic aminesworl. The formation occurs via the Maillard reaction, similar to PhIP, but involves different amino acids (such as glycine and alanine) along with sugars (Sugimura et al., 2004).

Although it is found in considerable amounts, the levels of MeIQx are generally lower than those of PhIP. PhIP is often the focus of human epidemiological research because of its widespread presence and elevated levels in commonly eaten cooked meats. While it is significant, the carcinogenic potential of MeIQx is not as frequently explored in human populations; however, it continues to demonstrate strong carcinogenic effects in controlled experimental environments (Silvaragi et al., 2023).

2.3.3 Mechanism of Action of PhIP and MeIQx

A key metabolic pathway of PhIP and MeIQx includes cytochrome P450 activation (CYP), which is essential for their bioactivation to its mutagenic and carcinogenic derivatives. The role of CYP1A2 in the bioactivation of HCAs is to catalyze the formation and subsequent transformation of N^2-hydroxylated intermediates into deoxyribonucleic acids (DNA) reactive metabolites (DNA adducts) by reacting with DNA that all contribute to its carcinogenicity. The identification of this enzyme has been described as a milestone in the activation of procarcinogens like HCAs (He & Feng, 2015; Stipp & Acco, 2021). Additionally, recent research also emphasized the involvement of enzymes, CYP1A1 and CYP1B1 in the bioactivation

of PhIP as well as other related compounds. Not only do these metabolic pathways provide insight into cancer risk, but genetic variations such as polymorphisms in these enzymes can play a significant role in an individual's susceptibility to cancers such as colon and breast cancer (Iori et al., 2024). It has been suggested that such mutations may be enough to initiate the carcinogenesis process. PhIP was also shown to modify gene expressions through other mechanisms besides DNA damage and mutations. It alters gene expression via interaction with E-α (genomic pathway) and activating cell-signalling pathway (non-genomic pathway) like ERK phosphorylation (Oz et al., 2023). Similar to PhIP, MeIQx may either be bioactivated or detoxified based on its concentration and the activity of various enzymes like CYP1A2 and UGT, as it has shown varying biotransformation capabilities in different experimental setup. MeIQx generally forms two DNA adducts of which one is the major, N-(deoxyguanosin-8-yl) MeIQx (dG-C8-MeIQx) and another the minor 5 (deoxyguanosin-N2-yl)-MeIQx (dG-N2-MeIQx). Interestingly, these adducts could also be removed via nucleotide excision repair (NER), specifically global genome repair (GG-NER) and transcription-coupled repair (TC-NER) (Silvaragi et al., 2023).

2.4 Carcinogenesis

Every carcinogen including PhIP and MeIQx must undergo a complex process known as carcinogenesis in order to manifest their carcinogenic potential. Carcinogenesis is a multi-step process caused by the accumulation of injuries within cells at various biological levels, which includes genetic and biochemical changes (Rowles & Erdman, 2020). The changes mentioned above, in turn, cause alterations in two different broad categories of genes with opposing roles, namely oncogenes and tumour suppressor genes where the oncogenes acquire dominant function and tumour

suppressor genes lose their function (Hanahan & Weinberg, 2000; Dupré & Malik, 2018). Tumour suppressors exert their inhibitory effects against carcinogenesis by mediating cell cycle progression, apoptosis and DNA damage responses induced by endogenous or exogenous substances (Liu et al., 2024). Currently, 14 cancer hallmarks explain the transition of a normal cell into a cancerous cell.

2.4.1 The Hallmarks of Carcinogenesis

Cancer hallmarks are common characteristics that facilitate the transformation of normal cells into cancer cells. In the year 2000, only six hallmarks were proposed: (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan & Weinberg, 2000). In 2011, four additional hallmarks were added to the list: (1) abnormal metabolic pathways, (2) immune system evasion, (3) genome instability, and (4) inflammation (Hanahan & Weinberg, 2011). Abnormal metabolic pathways and immune system evasion were recently classified as "emerging hallmarks" in the most recent advancement of this concept. However, after almost ten years, around 2022, it was clear that they, similar to the initial six, are core hallmarks of cancer and are now included in the current representation. Genome instability and inflammation, referred to as the "enabling characteristics" or can also be addressed as key facilitators, are included in the list to better capture cancer's complete cellular and molecular processes, as the previous eight functional hallmarks were not comprehensive enough. Even though Hanahan and Weinberg illustrated the hallmarks in a circular manner as shown in Figure 2.1, there is also a hierarchical representation of cancer hallmarks suggested by Blagosklonny (2005), from molecular levels to the organism, that separates them into three different

categories namely, enabling, driver and derived hallmarks as shown in Figure 2.2 (Blagosklonny, 2022). However, even after that, discoveries in cancer research still have identified areas with room for enhancement. The most recent advancement in the concept of cancer hallmarks explores how phenotypic plasticity and disrupted differentiation serve as unique hallmark features. At the same time, non-mutational epigenetic reprogramming and polymorphic microbiomes act as enabling characteristics that facilitate the development of other hallmark traits, and how senescent cells from various origins may be considered the predominant cell types in the tumour environment as shown in Figure 2.3 (Hanahan, 2022).

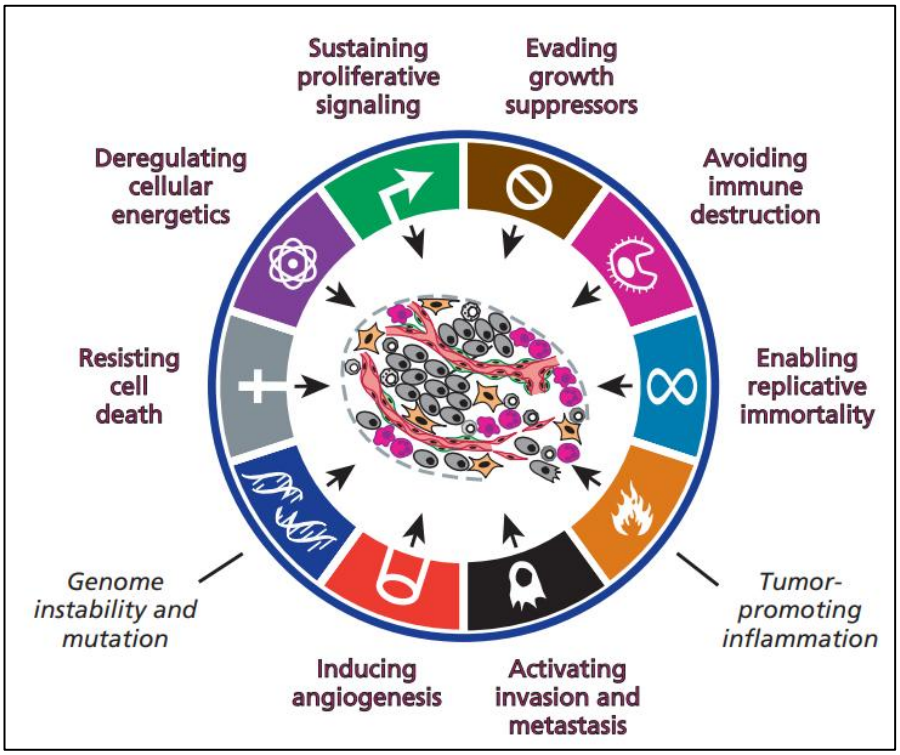

Figure 2.1 The former biological hallmarks of cancer (extracted from Hanahan and Weinberg, 2017).

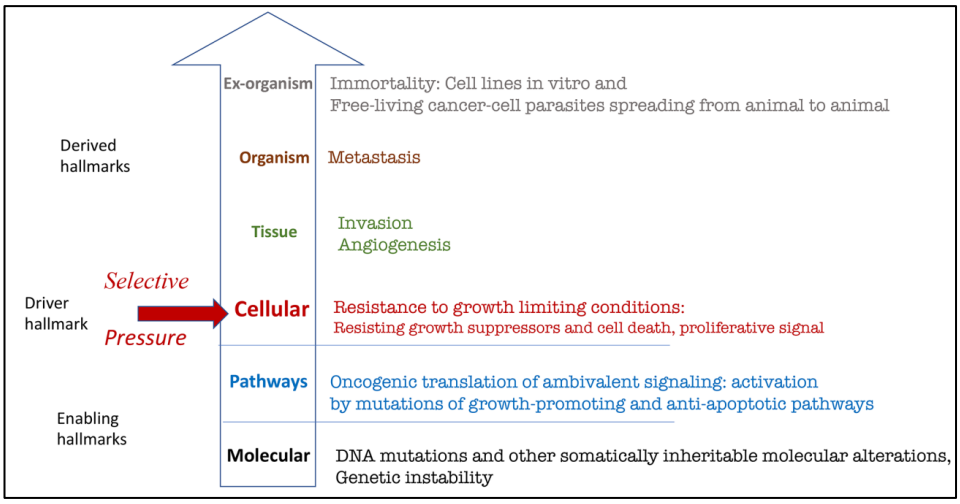

Figure 2.2 Hierarchical depiction (from molecular to organismal levels) of the original former hallmarks of cancer (extracted from Blagosklonny, 2022).

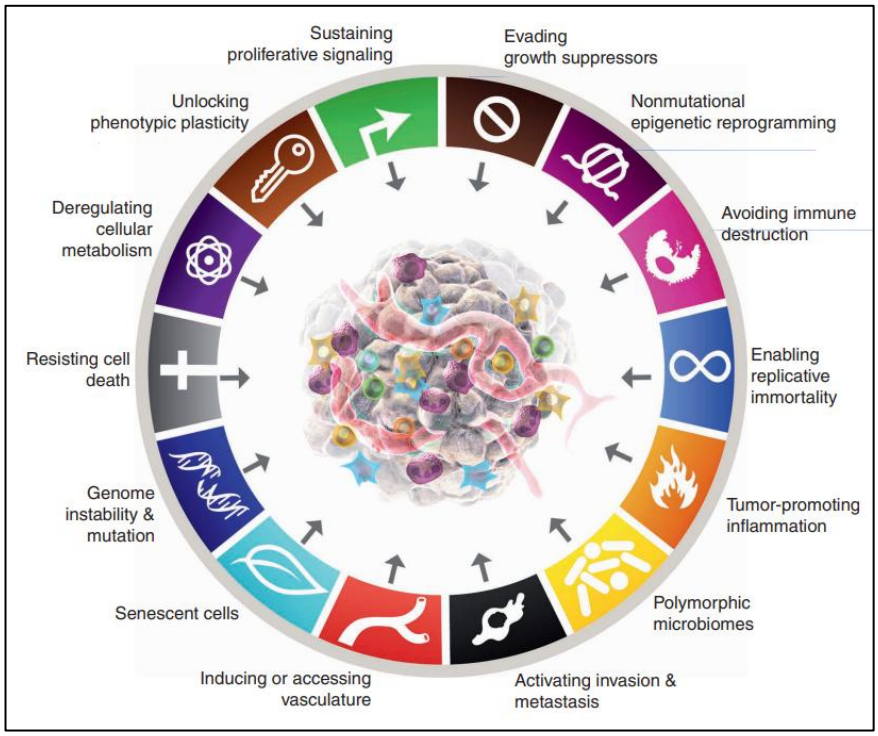

Figure 2.3 The recent biological hallmarks of cancer (extracted from Hanahan., 2022).

Although genome instability was added to the list of cancer hallmarks relatively late, it is still seen as the primary mechanism through which cancer hallmark traits are obtained. Studies show that nearly all types of human cancer contain cancer cells with mutated genomes due to chromosomal rearrangements or intragenic mutations, or both. It is also vital to note that cancer cells contain two categories of mutations: the drivers and passengers. Identifying both the driver mutations and the less common yet functionally significant mutations for tumour growth and progression is equally crucial as identifying the frequent mutations in carcinogenesis (Hanahan & Weinberg, 2017).

2.4.2 Stages of Carcinogenesis

Cancer development or commonly known as carcinogenesis, involves three primary phases: initiation, promotion, and progression in order to change a healthy cell into a cancerous cell (Lambert et al., 2017). Similar to the refinement made to the original list of hallmarks of cancer, the process of carcinogenesis has also been further elucidated once since its first recognition due to the increasing amount of evidence on its functionality. The two-stage "initiation-promotion model" of carcinogenesis was originally introduced by Isaac Berenblum and Philippe Shubik in 1947 (Berenblum & Shubik, 1947). The concept ‘progression’ was then added as the third key stage by Leslie Foulds, although the idea of progression had already been expressed by Peyton Rous (Foulds, 1954).

Initiation, the first phase, is a rapid and irreversible event in the development of cancer. It is considered to be a permanent occurrence due to the inability to eliminate the formation of covalent bonds (adducts) formed by chemicals/carcinogens with DNA (Chahar et al., 2020). The cell is classified as initiated only when the damaged

cell does not undergo apoptosis and the damage becomes permanent in the cell's genome (Choudhuri et al., 2018). In contrast to initiation, the promotion phase can be reversed and is linked to mitogenic signalling, which stimulates the proliferation of initiated cells. Hence, the clonal expansion of mutated cells allows them to avoid cell death, creating chances for more genetic or epigenetic modifications (Choudhuri et al., 2018). Nevertheless, it is important to understand that not every cell exposed to a tumour promoter will undergo promotion, only those cells that are stimulated to divide and avoid programmed cell death will advance to the next phase known as 'progression' (Chahar et al., 2020). Progression is the phase in which a neoplastic phenotype is attained due to the occurrence of more genetic or epigenetic modifications mentioned earlier. This is also the phase that usually exhibits a few cancer-related hallmarks such as genetic instability, invasion and metastasis (Chahar et al., 2020). Figure 2.4 shows the multistage process of cancer. Although further research has revealed that carcinogenesis is more complex than this, however, the three-stage process (initiation, promotion and progression) remains helpful in understanding how modifiers of cancer function (Klaunig & Wang, 2018).

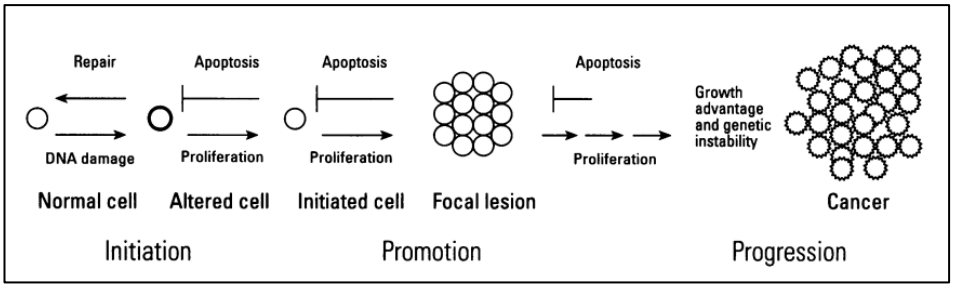

Figure 2.4 Multistage process of cancer (extracted from Klaunig et al., 1998).

2.5 In Vitro Oncology Research

In cancer studies, both *in vitro* and *in vivo* animal models are highly important for identifying carcinogens, developing cancer treatments, testing drugs, and studying the molecular processes of tumour growth and metastases. While in *vivo* models are an effective choice for examining metastasis complexity within a living system, they are inadequate for observing specific steps of metastases. Additionally, using a larger number of animals, extended timeframes, costly expenses, and ethical issues are also considered some of the downsides of conducting carcinogenicity studies with *in vivo* models (Colacci et al., 2023). On the other hand, even though *in vitro* models lack physiological relevance as they only focus on specific aspects of tumour micro-environment, they, however, lay the foundation for studying individual variables by controlling other experimental factors (Katt et al., 2016). Pertaining to this *in vitro* vs *in vivo* issue, the European Union supports the development of alternative methods to meet the objectives of replacing, reducing, and refining animal experiments, in accordance with the 3Rs principles. Likewise, the United States has also stressed the importance of incorporating mechanistic-based *in vitro* and high-throughput chemical testing into the toxicology vision for the 21st century (Mascolo et al., 2018). Therefore, it is recommended to make full use of *in vitro* models or tests first before proceeding with *in vivo* tests, taking into account the other additional factors like costs, complexity in protocols, and the need for skilled professionals for *in vivo* tests (Edmondson et al., 2014; Rodrigues et al., 2021). The existing *in vitro* models for solid tumours vary from cell lines originating from tumours to 3D models of the tumour microenvironment, and the decision on which model to utilize mostly relies on a study's objective (Katt et al., 2016). Interestingly, among the *in vitro* tests being used to study carcinogenesis, the CTA is considered a potential substitute or option to replace animal models, given

there are some experimental evidences that indicate the similarities between the cellular and molecular processes involved in *in vitro* cell transformation and those sustaining *in vivo* carcinogenesis which attributes to the thorough cellular response to both direct and indirect damage to DNA (Mascolo et al., 2018).

2.5.1 Cell Transformation Assays

CTA was developed to study the differences in phenotype between normal and cancerous mammalian cells after being exposed to genotoxic and non-genotoxic carcinogens and CTAs were developed in such a way that they are able to mimic the multi-stage process of carcinogenesis as detailed in section 2.4 (Hayrapetyan et al., 2023). The morphological transformation of cells can be determined by spotting the transformed colonies or malignant foci which ultimately indicate a chemical's carcinogenic potential. The experiments displaying the concept of "transformation" can be traced back to 1950s when initial studies showed that regular cells could turn into cancer cells after being exposed to various viruses like Rous sarcoma, avian myeloblastosis, and SE polyoma viruses which led to fibroblastic transformation in vulnerable cells, followed by research indicating that transformation could also be triggered by chemical and X-ray exposure (Colacci et al., 2023). Preliminarily, CTAs were developed by employing Syrian Hamster Embryo primary diploid cells (SHE) to investigate chemical-induced transformation, previously a method used to research virus-induced transformation. SHE cells, when cultured under normal conditions, are similar to fibroblast-like cells of human origin, shows high genetic stability, and only rarely undergo spontaneous transformation. Similar to other assays, the SHE assay has both its importance and limitations in risk assessment.

In respect to its significance, SHE cells are considered a metabolically competent cell that do not need an exogenous activation system to convert chemicals into their carcinogenic form (Ahmadzai et al., 2012). Moreover, the feature of SHE cells of not becoming "established cells" which by definition are cells that have developed the capability to proliferate, despite having their mitotic rate lessen and their morphology alter over time, allows us to research the initial stages of carcinogenesis effortlessly (Colacci et al., 2023). However, as previously stated, having a “primary diploid cell” status also signifies that SHE cells have a relatively limited lifespan (Poburski & Thierbach, 2016). Hence, this aspect substantially restrains their applicability in researching carcinogenesis since carcinogenesis is a complex process that involves oncogenic chemicals needing a longer timeframe to manifest their carcinogenic effects (Breheny et al., 2011).

Taking into account the mentioned constraint of the original SHE assay, a CTA was created utilizing BALB/c3T3 cells, which are a cloned (A31-1-1) immortalized mouse fibroblast cell line, to investigate carcinogenesis (Ponti et al., 2007). BALB/c3T3 cells typically form a monolayer culture and experience contact inhibition upon reaching confluency, however, they acquire transformation following exposure to chemicals by evading contact inhibition and piling up in a random manner (Mascolo et al., 2018; Sakai, 2007). Among all the cloned cell lines of A31, only BALB/c 3T3, A31-1-13 and A31-1-1 clones displayed increased sensitivity to transforming agents, hence justifying variant A31-1-1’s choice as the target cells for the transformation system (Colacci et al., 2023; Kakunaga & Crow, 1980). The BALB/c 3T3 CTA is a two-stage model that involves or represents carcinogenesis initiation and promotion steps. The protocol involves exposing the cells mainly to a tumour initiator for 72 hours after seeding them the day before. After 4 days of regular medium exposure,