



Methyl Picolinic Acid Plays a Role in Epigenetic Modifications in Human HepG2 Liver Cells

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DECLARATION

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The research described in this study was carried out in the Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Science, College of Health Sciences, University of Kwa-Zulu Natal, under the supervision of Professor A.A. Chuturgoon and Miss T. Ghazi

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Date

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TABLE OF CONTENT

DECLARATION.....	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	xiii
INTRODUCTION.....	1
1.1. Aim:.....	3
1.2. Hypothesis:	3
1.3. Objectives:	3
CHAPTER 2:.....	4
LITERATURE REVIEW	4
2.1. Picolinic Acid.....	4
2.1.1. Biological Synthesis of Picolinic Acid.....	5
2.1.2. <i>In vitro</i> and <i>In vivo</i> experimental action of Picolinic Acid.....	6
2.2 Distribution of methylated cytosines and CpG Islands	7
2.3. DNA Methylation and DNA Methyltransferases	8
2.4. DNA Methylation and Repression of Transcription.....	10
2.5. Methyl-CpG Binding Domain Protein	11
2.6. Histone Modification	13
2.7. Histone Methylation.....	13
2.8. The Histone Methyltransferase SUV39H1	14
2.9. Trimethylated Lysine 9 on Histone H3 (H3K9me3)	15
2.10. Linking DNA Methylation and Histone Modifications.....	16
CHAPTER 3:.....	18
MATERIALS AND METHODS	18
3.1. Materials:.....	18
3.2. Cell Culture:	18
3.2.1. Introduction.....	18
3.2.2. Cell Culture Conditions.....	18

3.3. WST-1 Assay:	19
3.3.1. Introduction	19
3.3.2. Protocol	20
3.4. DNA Isolation and Quantification of DNA Methylation:	21
3.4.1. Introduction	21
3.4.2. Protocol	21
3.5. mRNA Isolation and Quantitative Polymerase Chain Reaction:	22
3.5.1. Introduction	22
3.5.2. Protocol	23
3.5.2.1 RNA Isolation	23
3.5.2.2. cDNA Synthesis	24
3.5.2.3. qPCR	24
3.6. Western Blot:	25
3.6.1. Introduction	25
3.6.2. Protein Isolation	25
3.6.2.1 Introduction	25
3.6.2.2. Protocol	26
3.6.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	27
3.6.3.1. Introduction	27
3.6.3.2. Protocol	28
3.6.4. Transfer of Proteins	29
3.6.4.1. Introduction	29
3.6.4.2. Protocol	29
3.6.5. Blocking, Washing and Antibody Incubation	30
3.6.5.1. Introduction	30
3.6.5.2. Protocol	30
3.6.6. Imaging and Quenching	31
3.7. Statistical Analysis	32
CHAPTER 4	33

RESULTS	33
4.1. WST1 Assay:	33
4.2. DNA Methylation Assay:.....	34
4.3. qPCR:.....	35
4.4. Western Blot:.....	36
4.4.1. Methyl-CpG binding domain 2 (MBD2).....	36
4.4.2. SUV39H1	37
4.4.3. H3K9me3	38
CHAPTER 5.....	39
DISCUSSION	39
CHAPTER 6.....	42
CONCLUSSION	42
REFERENCES.....	43
APPENDIX A	58
APPENDIX B	59
APPENDIX C	62

LIST OF FIGURES

Chapter 2 – Literature Review:

Figure 2.1: Chemical Structure of (a) Picolinic Acid (Grant et al., 2009) and (b) Methyl Picolinic Acid (prepared by author)..... 5

Figure 2.2: The in vitro synthesis of Picolinic Acid via the Kynurenine Pathway (Grant et al., 2009).. 6

Figure 2.3: A representation of DNA methylation, which converts cytosine to 5'methyl-cytosine via the actions of DNA methyltransferase (DNMT) (prepared by author)..... 9

Figure 2.4: (a) The de novo methylation activity of DNMT3a and 3b, introducing new methylation patterns in DNA sequences and (b) the maintenance methylation activity of DNMT1, conserving established methylation patterns in DNA sequences (Moore et al., 2013). 10

Figure 2.5: Representation of heterochromatin spreading. HP1 and SUV39H1 are recruited by H3K9me3 through its chromo domain (CD). The latter catalyses the S-adenosylmethionine (SAM)-dependent methylation of H3K9 via its SET domain (Müller et al., 2016). 16

Figure 2.6: The link between DNA methylation and Histone Modifications. Methylation is regulated by DNMTs which suppress gene expression by targeting CpG sites and actively methylate DNA. The catalytic activity of some DNMTs are enhanced by associating with histone tails. DNA methylation is also recognized by MBDs which work with DNMTs to recruit enzymes that modify the histone tail, such as histone methyltransferase (HMTs). HMTs methylate histones and in combination with DNA methylation serve to repress gene expression (Moore et al., 2013). 17

Chapter 3 – Materials and Methods:

Figure 3.1: Schematic mechanism of the WST-1 reduction (prepared by author). 20

Figure 3.2: Overview of steps involved in PCR resulting in synthesis of DNA (prepared by author).	23
Figure 3.3: Principle Scheme of the Bicinchoninic Acid (BCA) Assay (prepared by author).	26
Figure 3.4: Separation of Proteins, according to size, by SDS-PAGE (prepared by author).....	28
Figure 3.5: Transfer of Proteins from the SDS-PAGE Gel to a nitrocellulose membrane (prepared by author).....	29
Figure 3.6: Signal Emission associated with antigen – antibody interactions (prepared by author). ...	30

Chapter 4 – Results:

Figure 4.1: The effect of PA and MPA on HepG2 cell viability.	33
Figure 4.2: The effect of PA and MPA on global DNA methylation in HepG2 cells. PA and MPA decreased 5-methylcytosine content in HepG2 cells. 5-aza-2-DC is used as a negative control as it is a DNA methylation inhibitor (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).	34
Figure 4.3: The effect of PA and MPA on MBD2 expression in HepG2 cells. PA and MPA alter MBD2 expression in HepG2 cells (** $p < 0.001$, *** $p < 0.0001$).	35
Figure 4.4: The effects of PA and MPA on MBD2 protein expression in HepG2 cells (** $p < 0.001$, *** $p < 0.0001$).	36
Figure 4.5: : The effect of PA and MPA on SUV39H1 protein expression, in HepG2 cells (** $p < 0.001$, *** $p < 0.0001$).	37
Figure 4.6: The effects of PA and MPA on protein H3K9me3 in HepG2 cells (*** $p < 0.0001$).	38

Appendix A:

Figure 5: Standard curve used to calculate the concentration of proteins in samples, from known concentrations of Bovine Serum Albumin (BSA).....59

Appendix B:

Figure 6.1: Gene Expression of DNMT1 was increased at MPA concentrations 3mM and 10mM but decreased at MPA concentrations 13 mM and 15 mM (**p<0.01, ***p<0.0001)..... 59

Figure 6.2: Gene Expression of DNMT3A increased at MPA concentration 3 mM but decreased for all remaining MPA concentrations (**p<0.01, ***p<0.0001). 60

Figure 6.3: Gene expression of DNMT3B decreased for all concentrations of MPA (*p<0.05). 60

Figure 6.4: Gene expression for miR29b decreased at MPA concentrations 3 mM and 15 mM, increased at 10mM and remained relatively constant at 13 mM (**p<0.001, ***p<0.0001)..... 61

Appendix C:

Figure 7: KDM5B showed significant decrease in protein expression for all concentrations of MPA in HepG2 cells (***p<0.0001).....62

LIST OF TABLES

Chapter 3 – Materials and Methods:

Table 3.1: Primer sequences for MBD2 and GAPDH genes used in qPCR	24
---	----

Table 3.2: Antibodies and the dilutions used in Western Blotting	31
--	----

Appendix B:

Table 3.3: Primer sequences for DNMT1, DNMT3A, DNMT3B, miR29b and RNU6 genes used in qPCR.....	61
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Appendix C:

Table 3.4: Antibody and dilution used for KDM5 in Western Blotting	62
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ABBREVIATIONS

5-aza-2-DC	5-aza-2'-deoxycytidine
5mC	5-methylcytosine
ACMSD	amino- β -carboxymuconate-semialdehyde-decarboxylase
ACMSDase	α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase
AMSDHase	α -aminomuconate- ϵ -semialdehyde dehydrogenase
APS	Ammonium persulphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CCM	Complete culture media
CD	Chromo Domain
cDNA	Complementary deoxyribonucleic acid
CH₃	Methyl group
CREB	cyclic AMP-response element binding protein
Ct	Comparative Threshold
C_T	Cycle Threshold
Cu	Copper
Cu¹⁺	Cuprous ions
Cu²⁺	Cupric ions
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxynucleoside triphosphates
EMEM	Eagle's Minimum Essentials Medium
gDNA	Genomic deoxyribonucleic acid
H₂O₂	Hydrogen peroxide

H3K27	Lysine 27 on Histone H3
H3K36	Lysine 36 on Histone H3
H3K4	Lysine 4 on Histone H3
H3K79	Lysine 79 on Histone H3
H3K9	Lysine 9 on Histone H3
H4K20	Lysine 20 on Histone H4
HCC	Hepatocellular carcinoma
HepG2	Hepatocellular carcinoma
HMTs	Histone methyltransferases
HP1	Heterochromatin Protein 1
HRP	Horse Radish Peroxidase
IC₅₀	Inhibitory concentration of 50%
IDO	indoleamine 2,3-dioxygenase
IFN- γ	Interferon- γ
K	Lysine
KP	Kynurenine Pathway
L-TRP	L-Tryptophan
MAC	Mycobacterium Avium Complex
MBD2/3	Methyl-CpG Binding Domain 2/3
MDA	Malondialdehyde
me1	Monomethylated
me2	Dimethylated
me3	Trimethylated
MeCP1/2	Methyl Cytosine Binding Protein ½
MPA	Methyl Picolinic Acid
mPMS	1-methoxy-5-methyl-phenazinium methyl sulphate

NaCl	Sodium chloride
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa beta
NO	Nitric Oxide
PA	Picolinic Acid
PBS	Phosphate buffered saline
PTM	Post Transcriptional Modification
qPCR	Quantitative Polymerase Chain Reaction
R	Arginine
RB	Retinoblastoma
RBD	Relative Band Density
RNA	Ribonucleic acid
RT	Room temperature
SAM	S-adenosyl methionine
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEMED	Tetramethyl ethylenediamine
TRD	Transcriptional repression domain
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloric acid
TTBS	Tween 20-Tris buffered saline
WST-1	Water Soluble Tetrazolium salt 1

ABSTRACT

Picolinic Acid (PA) is an endogenous catabolite synthesized from L-Tryptophan via the kynurenine pathway. PA has been reported to possess immunological, neuroprotective, and anti-proliferative properties; however, its most widely researched function is its efficient chelating properties. Despite these findings, the physiological function of PA is yet to be discovered. Many PA derivatives exist, with its methyl derivative being of interest in this study. Studies have shown that Picolinic Acid derivatives such as Fusaric Acid are involved in epigenetic regulation by inducing global DNA hypomethylation in HepG2 liver cells. Therefore, the aim of the study was to investigate the epigenetic properties of Picolinic Acid (parent molecule of Fusaric Acid) and Methyl Picolinic Acid in human HepG2 Liver cells.

DNA methylation and Histone modifications are epigenetic phenomena that modify accessibility to DNA and chromatin structure, which results in regulation of gene expressions. While DNA methylation results in long-term repression, Histone methylation leads to formation of heterochromatin. The methyl-CpG binding domain 2 (MBD2) binds to methylated CpG dinucleotides and negatively regulates DNA methylation. MBD2 also associates with histone lysine methyltransferases, such as SUV39H1, which is involved in pericentric heterochromatin silencing and is responsible for methylating Histone 3 on lysine 9 (H3K9).

The cytotoxicity of Picolinic Acid and Methyl Picolinic Acid in HepG2 liver cells was assessed by the WST-1 assay. The DNA methylation ELISA kit was used to quantify 5-methylcytosine in HepG2 cells. Gene expression for the DNA demethylase MBD2 was determined by qPCR. Protein expression for MBD2, trimethylated H3K9 (H3K9me3) and its methyltransferase, SUV39H1, was assessed by Western Blotting.

The IC₅₀ values for Picolinic Acid (7.5 mM) and Methyl Picolinic Acid (13 mM) were obtained from the WST-1 assay. DNA methylation was significantly decreased in Methyl Picolinic Acid, Picolinic Acid and 5-aza-2-DC treated cells ($p < 0.0001$). In Methyl Picolinic Acid and Picolinic Acid treated cells, MBD2 gene expression was downregulated ($p < 0.0001$) followed by an increase in its protein expression ($p < 0.0001$). MPA increased protein expression of SUV39H1 ($p < 0.0001$) leading to an increase in H3K9me3 ($p < 0.0001$), while Picolinic Acid induced a decrease in SUV39H1 and H3K9me3 protein expression ($p < 0.0001$).

Methyl Picolinic Acid induced DNA hypomethylation as a result of increased MBD2 protein expression, despite the decrease in MBD2 gene expression. The upregulation of MBD2 protein expression promoted the activity of SUV39H1 which subsequently enhanced expression of H3K9me3. Since DNA hypomethylation can restore expression of aberrantly expressed genes, this study suggests a possible role for MPA in ameliorating carcinogenesis.

CHAPTER 1:

INTRODUCTION

The study of genetics refers to heritable changes in gene expression due to changes in the DNA sequence. These changes can be insertions, deletions, translocations or point mutations (Das and Singal, 2004). In contrast, the study of epigenetics refers to changes in gene expression that takes place during development and cell proliferation but does not affect the DNA sequence (Moore et al., 2013). Increasing interest in epigenetics has led to large scale epigenome studies, allowing the mapping of epigenetic marks, such as DNA methylation, Histone modification and nucleosome positioning, which are vital for the regulation of gene and noncoding RNA expressions (Portela and Esteller, 2010). Epigenetics has evolved rapidly with studies showing its importance in cancer biology (Singal and Ginder, 1999 and Jones and Baylin, 2002), viral infections (Baylin, 1997), somatic gene therapy, cloning, genomic imprinting, developmental anomalies, X-inactivation and mental health (Laird, 2003 and Amir et al., 1999).

DNA methylation is a commonly occurring epigenetic event that takes place in the mammalian genome at clustered CpG dinucleotide regions known as CpG islands. This covalent chemical modification involves the addition of a methyl (-CH₃) group from S-adenosyl methionine (SAM) to the carbon at position 5 of the cytosine ring leading to the formation of 5-methylcytosine (5mC) (Das and Singal, 2004). DNA methylation is catalysed by a family of DNA methyltransferases (DNMTs) namely; DNMT1, DNMT 3A and DNMT 3B. The methyl-CpG binding domain 2 (MBD2) is a DNA demethylase which binds to methylated DNA and initiates transcription of methylated genes. 5-aza-2'-deoxycytidine (5-aza-2-DC) is a DNA methyltransferase inhibitor which leads to DNA hypomethylation.

Chromatin is a complex of DNA and histone proteins in the nucleus. DNA in chromatin is wrapped around an octamer of histone proteins and referred to as the nucleosome. The nucleosome is formed by DNA wrapping around a histone octamer. This histone octamer is made up of 2 copies each of 4 core histones: H2A, H2B, H3 and H4 (Portela and Esteller, 2010). Histone H1 is located between nucleosomes where it binds linker DNA and is referred to as the linker histone (Handy et al., 2011). The space between nucleosomes determines chromatin structure, which can either be heterochromatin or euchromatin (Handy et al., 2011). Post transcriptional modifications (PTMs) of histones can affect gene expression by altering chromatin structure or by recruiting histone modifiers.

Apart from playing an important role in regulating gene expression, histones also function in DNA repair, DNA replication and recombination (Lennartsson and Ekwall, 2009). All histones are subjected to PTMs and are covalently modified by phosphorylation, ubiquitination, acetylation and methylation. Some modifications disturb histone-DNA interactions, resulting in the unwinding of the nucleosome to an open chromatin structure, known as euchromatin. This modification makes DNA accessible to transcription factors, followed by gene activation (Handy et al., 2011). Other modifications strengthen histone-DNA interactions, packing chromatin tightly in a structure called heterochromatin. This modification silences genes by preventing transcription factors from accessing DNA (Handy et al., 2011).

Histone-modifying enzymes, such as acetylases and deacetylases, cannot access their histone substrate unless they are targeted by DNA bound repressors or activators (Kouzarides, 2002). Recently numerous histone methyltransferases and proteins, which read the methyl-lysine code, have been discovered (Kouzarides, 2002). Histone methylation is a process which involves the addition of methyl groups (CH_3) to lysine or arginine, by SAM-dependent histone lysine methyltransferases or protein arginine methyltransferases, respectively. Methylation of lysine residues K4, K9 and K27 occurs on histone H3 while methylation of lysine residue K20 occurs on H4 (Kouzarides, 2002). The first histone methyltransferase discovered was the Suppressor of Variegation 3-9 (SUV39) protein which directs its activity to lysine 9 of H3 (H3K9) (Kouzarides, 2002).

Picolinic acid (PA) is a six-membered ring structure synthesized from L-tryptophan by a side branch of the kynurenine pathway (Grant et al., 2009). The formation of PA is controlled by two enzymes: α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase (ACMSDase) and α -aminomuconate- ϵ -semialdehyde dehydrogenase (AMSDHase) (Shibata and Fukuwarati, 2014). PA is found in human milk, intestines and pancreatic juice (Evans, 1980). Apart from its chelating properties, PA displays immunomodulatory properties involving activation of mononuclear phagocyte effector functions (Varesio, 1994). PA is also a co-stimulus for induction of macrophage mediated cytotoxicity. Methyl Picolinic Acid (MPA) is a derivative of PA (Figure 2.1) that has a methyl group attached to its ring structure. The epigenetic effects of MPA are currently unknown.

The mycotoxin, Fusaric Acid (FA), is a PA derivative isolated from the *Fusarium* species. FA plays a role in plant pathogenesis and is potentially toxic to animals (Bacon et al., 1996). FA treated cells induce global DNA hypomethylation by regulating DNMTs and MBD2 expression (Ghazi et al., 2019). Ghazi et al (2019) showed that FA-induced MBD2 expression may contribute to global DNA hypomethylation

in human liver cells, displaying an alternative mechanism for FA toxicity at an epigenetic level. Since FA is a PA derivative, it was deemed that PA, more specifically its methyl derivative, could also play a role in epigenetic modifications.

The human hepatocellular carcinoma (HepG2) cell line produces and releases many major human plasma proteins, including plasminogen, fibrinogen and α 2-macroglobulin (Knowles et al., 1980). HepG2 cells demonstrate many cellular features of normal human hepatocytes (Bouma et al., 1989) and fetal hepatocytes as it expresses both albumin and α -fetoprotein (Kunnath and Locker, 1983). The molecular pathogenesis of liver cancer involves genetic changes, such as mutations or chromosomal abnormalities, and epigenetic changes, such as modifications in DNA and in the histones wrapped around DNA that form chromosomes (Wong et al., 2010). Epigenetic anomalies, in the liver during precancerous stages, alters hepatocyte differentiation and survival, resulting in tumorigenesis (Farazi and DePinho, 2006). Aberrant DNA methylation in liver cells is usually responsible for epigenetic silencing of various genes (Mann, 2014; Nishida and Kudo, 2014; Revill et al., 2013). The liver is an organ which constantly adapts to highly variable conditions, such as metabolic processes, changes in microbiota, circadian cues, viral infections and xenobiotics (Wilson et al., 2017). Therefore, it requires constant repair and regeneration (Toh et al., 2019). The liver epigenome is a good model for epigenetic studies due to its high sensitivity to various environmental conditions.

1.1. Aim:

To investigate the epigenetic effects of Methylated Picolinic Acid (MPA) in Human HepG2 Liver cells

1.2. Hypothesis:

Methyl Picolinic Acid promotes DNA methylation and leads to altered regulation of gene expression in human HepG2 Liver cells

1.3. Objectives:

- * To determine the cytotoxicity of Picolinic Acid and MPA in HepG2 liver cells
- * To determine the effects of Picolinic Acid and MPA on the production of 5-methylcytosine by DNA methylation
- * To determine the effects of Picolinic Acid and MPA on SUV39H1-mediated H3K9me3

CHAPTER 2:

LITERATURE REVIEW

2.1. Picolinic Acid

PA (Figure 2.1) is a naturally occurring product of tryptophan degradation. It is a chelating agent for metal ions like Zinc (Zn^{2+}) and Iron (Fe^{2+}). Chromium Picolinate is commonly used as a dietary supplement to aid weight loss in overweight patients (Evans and Johnson, 1980; Vincent, 2003). With a lethal dose, 50% (LD-50) of 140 grams in a 70-kilogram subject, PA is known to up-regulate host immune responses (Ruffmann et al., 1984 and Varesio et al., 1990) and is readily water soluble (Vincent, 2003). PA possesses regulatory effects on cell cycle while it shows inhibitory effects of bacterial growth (Johnson and Fernandez-Pol, 1977; Collins et al., 1979; Leuthauser et al., 1982). It has shown altered intramacrophage growth of *Mycobacterium avium* complex organisms, which is dependent on host macrophage apoptosis (Pais and Appelberg, 2000).

L-Tryptophan (L-TRP) catabolism initiated by indoleamine 2,3-dioxygenase (IDO) or hepatic enzymes, result in the production of metabolites, among which, PA is an end-product of L-TRP degradation (Mehler, 1956). Enhanced L-TRP breakdown has been seen in inflammatory responses and implicated in T cell tolerance (Taylor and Gensheng, 1991), antimicrobial activity (Murray et al., 1989), some biological effects of Interferon- γ (IFN- γ) (Munn et al., 1998) and increased IDO expression (Brown et al., 1991). PA possess vital immunomodulatory properties associated with activation of mononuclear phagocyte effector functions (Varesio et al., 1994). PA is an effective co-stimulus of macrophage induced cytotoxicity, it may play a role in *in vivo* macrophage microbial activity and can inhibit tumor growth by macrophage tumoricidal activity, in tumor-bearing mice (Leuthauser et al., 1982 and Ruffman et al., 1987). Intracerebral and intraperitoneal administration of PA protects mice from a lethal intracerebral challenge associated with the *Candida albicans* pathogen (Blasi et al., 1994). Furthermore, PA combined with IFN- γ , both *in vitro* and *in vivo*, inhibits macrophage retrovirus expression and stimulates nitric oxide (NO) production by inducing transcriptional activity of NO synthase (Melillo et al., 1994).

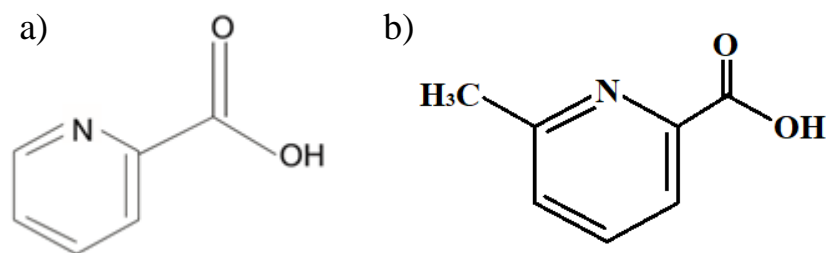


Figure 2.1: Chemical Structure of (a) Picolinic Acid (Grant et al., 2009) and (b) Methyl Picolinic Acid (<https://www.sigmaaldrich.com/south-africa.html>).

2.1.1. Biological Synthesis of Picolinic Acid

L-TRP can be metabolised by oxidative degradation by the kynurenine pathway (KP), to three end products; Kynurenic acid, nicotinamide adenine dinucleotide (NAD) or Picolinic acid (PA). During PA synthesis (Figure 2.2), tryptophan is catabolised to 3-hydroxyanthranilic acid which is subsequently acted upon by 3-hydroxyanthranilic acid oxygenase to form 2-amino-3-carboxymuconic semialdehyde (Grant et al., 2009). This intermediate is then converted to 2-aminomuconic semialdehyde by the rate limiting enzyme of PA synthesis, amino- β -carboxymuconate-semialdehyde-decarboxylase (ACMSD), followed by non-enzymatic conversion to PA (Ikeda et al., 1965). PA is found in blood serum (Dazzi et al., 2001), cerebrospinal fluid (Smythe et al., 2002), pancreatic juice, human milk and intestinal homogenates (Rebello et al., 1982). Alan Mehler (Mehler, 1956) was the first to suggest that PA is a metabolic product of KP. He questioned whether PA is a metabolic TRP product with physiological function and if PA production has any influence on NAD synthesis. Although research has shown that PA is a KP metabolite, the role of PA remains elusive. The most common researched characteristic of PA is its efficient chelator properties, PA-metal complexes are widely used as a medium to incorporate bioactive metals into biological systems (Grant et al., 2009). Dietary supplementation particularly with chromium picolinate has been implemented in type 2 diabetes (Broadhurst and Domenico, 2006), as chromium plays a role in lipid and carbohydrate metabolism.

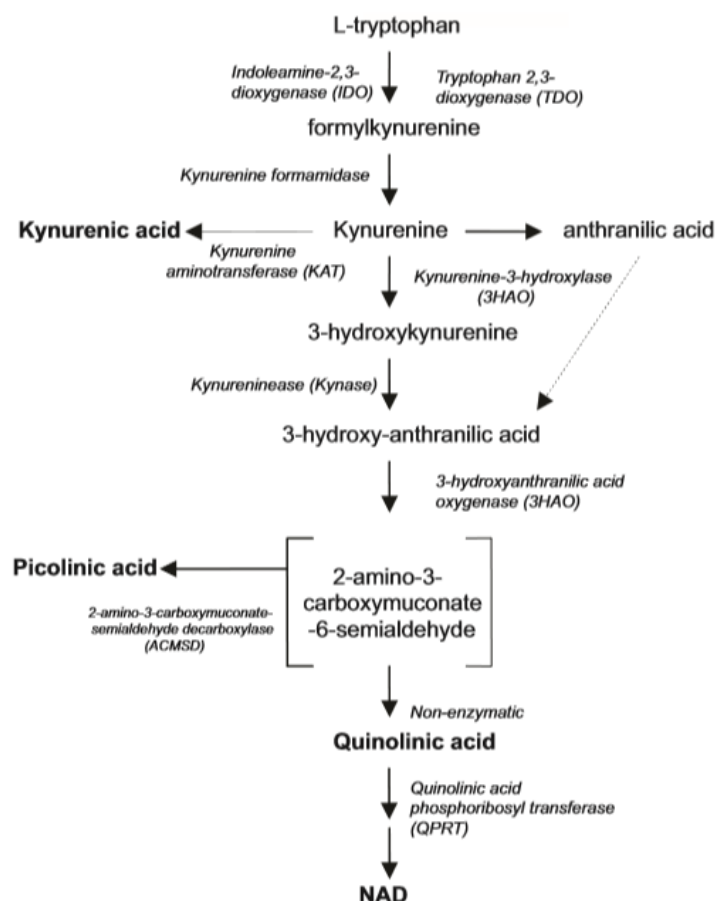


Figure 2.2: The *in vitro* synthesis of Picolinic Acid via the Kynurenine Pathway (Grant et al., 2009).

2.1.2. *In vitro* and *In vivo* experimental action of Picolinic Acid

The naturally occurring compound, Picolinic Acid, has no known function. In experiments, PA has shown many potential effects within the body, especially in immune function and antimicrobial activity (Grant et al., 2009). Studies conducted *in vitro* propose PA may promote macrophage effector functions by enhanced IFN- γ -dependent NO synthase gene expression (Melillo et al., 1994 and Varesio et al., 1990). These studies also suggest that PA can induce macrophage expression of inflammatory proteins 1 α and 1 β (Bosco et al., 2000), through a process dependent on iron chelation. High PA concentrations (1-4mM) has been reported to inhibit a range of viruses in culture. These include Human Immunodeficiency virus, Simian virus and Herpes Simplex virus (Fernandez-Pol and Johnson, 1997 and Fernandez-Pol et al., 2001). The antiviral activity of PA appears to be induced by cytotoxic action, increasing apoptosis of infected cells and reducing viral replication (Fernandez-Pol et al., 2001). Anti-microbial effects of PA against Mycobacterium avium complex (MAC) has been demonstrated, with significant increase of the antimicrobial action of rifampicin, clarithromycin and several fluoroquinolones (Cai et al., 2006 and Shimizu et al., 2006). Since PA is an efficient chelator of metal

ions, it is likely that the antimicrobial action of PA against MAC organisms is due to its chelating properties (Cai et al., 2006). A kinetic study revealed that PA inhibited iron uptake in normal rat kidney epithelial cells (Fernandez-Pol, 1977). Experiments in radioiron labelled cells showed that PA removed iron from the cells in a dose-dependent manner (Fernandez-Pol, 1977). This suggests that PA may induce growth inhibition of cells by selectively depleting iron levels (Fernandez-Pol, 1977).

In vivo studies have been done to assess the effects of PA on tumor growth, on mice inoculated with Mannose-binding lectin -2 (MBL-2) lymphoma cells. These results showed significant increase in the lifespan of mice injected with PA combined with activated macrophages, compared to the control (Ruffman et al., 1987). This effect may be due to IFN- γ mediated macrophage activation mechanisms (Ruffman et al., 1987). A study on insulin mimetic agents against type 2 diabetes suggested that the addition of a methyl group into the Zn (II) picolinate ligand provides a more effective way to develop active insulin mimetics, in *in vivo* and *in vitro* experiments (Yoshikawa et al., 2001). In rats, 2-PA enhances Zn dietary absorption in intestinal sacs (Seal and Heaton, 1983) while labelled PA administered into rats is excreted via urine as a glycine conjugate, with no other metabolites detected (Fernandez-Pol et al., 1977).

2.2 Distribution of methylated cytosines and CpG Islands

The eukaryotic genome is not uniformly methylated but contains interspersed methylated regions with unmethylated domains (Bird, 1986). During evolution in eukaryotes, CpG dinucleotides have been depleted from the genome and is only present at 5 to 10% of its predicted frequency (Antequera and Bird, 1993). This loss of CpG dinucleotides seems to be due to cytosine methylation since most of the lost CpG sites were influenced by conversion of methylcytosine to thymine via deamination (Singal and Ginder, 1999). Approximately 70 to 80% of the remaining CpG sites in vertebrates consist of methylated cytosine which are characterized by late DNA replication and are relatively inaccessible to transcription factors (Tazi and Bird, 1990). The rest of the genome contains smaller DNA regions known as CpG islands. CpG islands have a higher CpG density than the rest of the genome (Bird et al., 1985) and occur approximately every 100kb, ranging from 0.5kb to 5kb. They are unmethylated, GC rich (60% to 70%) and have a CpG to GpC ratio of at least 0.6, therefore does not suppress CpG dinucleotide frequency (Antequera and Bird, 1993 and Cross and Bird, 1995). CpG islands in chromatin are usually heavily acetylated, lack histone H1 and contain a nucleosome free region (Tazi and Bird, 1990). CpG islands in promoter regions of normal tissue are usually unmethylated, regardless of the gene's transcriptional activity (Singal and Ginder, 1999), apart from imprinted autosomal genes where 1 parental allele may be methylated and non-transcribed genes in the inactive X chromosome (Baylin,

1997). Tissue specific genes which lack CpG islands are variably methylated and methylation is usually inversely proportional to the gene's transcriptional status (Singal and Ginder, 1999). Majority of gene promoters, particularly those of housekeeping genes, are located within CpG islands (Saxonov et al., 2006). CpG islands associated with promoter regions are highly preserved in humans and mice (Illingworth et al., 2010). This preservation of CpG islands emphasises its functional importance in promoting gene expression by regulating chromatin structure and binding of transcription factors (Moore et al., 2013). The methylation of CpG islands, during gametogenesis and early embryonic development (Wutz et al., 1997), leads to silencing of gene expression (Mohn et al., 2008). Methylated CpG regions can silence gene expression, impair binding of transcription factors and take on repressive methyl-binding proteins (Moore et al., 2013).

2.3. DNA Methylation and DNA Methyltransferases

Rollin Hotchkiss discovered modified cytosines in 1948 and hypothesized that this naturally occurring modification in DNA was 5-methylcytosine (Hotchkiss, 1948). However, it was only around the 1980s when researchers demonstrated the involvement of DNA methylation in gene regulation and cell differentiation (Holliday and Pugh, 1975 and Compere and Palmiter, 1981). DNA methylation is now well recognized, in conjunction with other regulators, as a major epigenetic modification affecting gene activity (Moore et al., 2013). Methylation of cytosine residues is a covalent modification that occurs commonly at 5'-CpG-3' dinucleotides. It involves the enzymatic transfer of a methyl group from S-adenosylmethionine (SAM), to position 5 of cytosines, to generate 5-methylcytosine (5-mC) in genomic DNA (Krista et al., 2012) (Figure 2.3). This enzymatic transfer is catalysed by DNA methyltransferases (DNMTs) (Jaenisch and Bird, 2003). DNA methylation regulates gene expression and maintains genome integrity by associating with proteins that alter nucleosomes (Jaenisch and Bird, 2003 and Wolffe and Matzke, 1999).

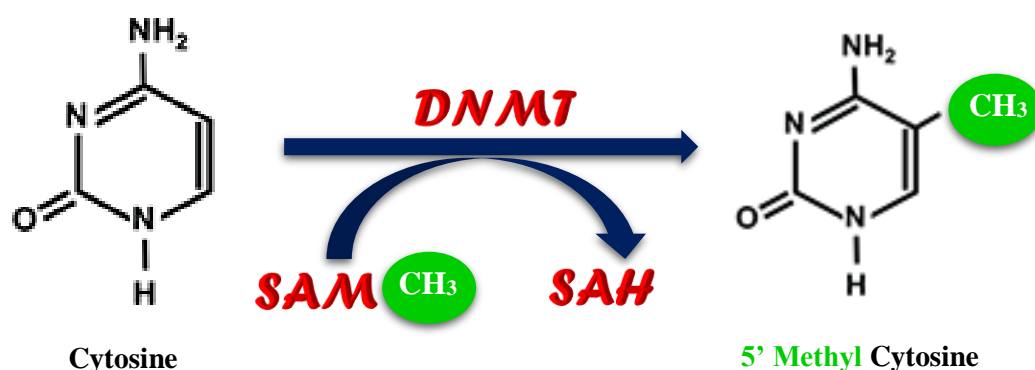


Figure 2.3: A representation of DNA methylation, which converts cytosine to 5'methyl-cytosine via the actions of DNA methyltransferase (DNMT) (prepared by author).

The addition of methyl groups, during DNA methylation, is catalysed by three members of the DNMT family; DNMT1, DNMT 3a and DNMT 3b. These three enzymes share similar structures consisting of a C-terminal catalytic domain and a large N-terminal regulatory domain; however each enzyme has unique expression patterns and functions (Yen et al., 1992 and Xie et al., 1999). DNMT1, the most extensively studied of the three, is highly expressed in mammalian tissue (Goto et al., 1994). During DNA replication DNMT1 is located at the replication fork where it binds to newly synthesized DNA (Leonhardt et al., 1992). It then methylates the newly synthesized DNA to conserve the original methylation pattern present in DNA prior to replication (Hermann et al., 2004). Since DNMT1 maintains the original DNA methylation pattern, it is referred to as the maintenance methyltransferase (Figure 2.4). DNMT1 is also able to repair DNA methylation (Mortusewicz et al., 2005).

Mammalian DNMT1 is highly attracted to hemimethylated substrates but can also perform *in vitro de novo* methylation of unmethylated regions (Singal and Ginder, 1999). This has been stimulated in aberrant DNA structures (Laayoun and Smith, 1995) and 5mC of DNA substrates that can be single or double stranded (Tollefsbol and Hutchison, 1997). Pradhan et al (1997) demonstrated that DNMT1 synthesized in Baculovirus was equally activated in hemimethylated and unmethylated DNA, due to an inhibitory effect of the additional N-terminal amino acids on hemimethylated DNA. This indicates that DNMT1 has an inherent *de novo* methylating activity, which can be altered by protein-protein interactions and enhanced by abnormal structures or 5mC residues in DNA (Pradhan et al., 1997). Lei et al (1996) generated a null mutation on the DNMT1 gene, in mouse embryonic stem cells, via homologous recombination. These null embryonic stem cells were viable with low but stable levels of methylcytosine and methyltransferase activity (Lei et al., 1996).

DNMT-3a and -3b share common structure and functions. When overexpressed, DNMT-3a and -3b can methylate native and synthetic DNA with no preference for hemimethylated DNA (Okano et al., 1999). DNMT-3a and -3b are known as the *de novo* methyltransferases as they introduce methylation into DNA (Figure 2.4). DNMT 3a is distinguished from 3b by its gene expression pattern (Moore et al., 2013). DNMT 3a is universally expressed while DNMT 3b is poorly expressed in most differentiated tissue, except thyroid, bone marrow and testes (Xie et al., 1999). Knockout of DNMT 3b in mice is embryonic lethal (Okano et al., 1999) while DNMT 3a knockout mice are stunted but survive to approximately 4 weeks after birth (Okano et al., 1999). These results imply that DNMT 3b is needed during early development where as DNMT 3a is needed for normal cellular differentiation (Moore et al., 2013).

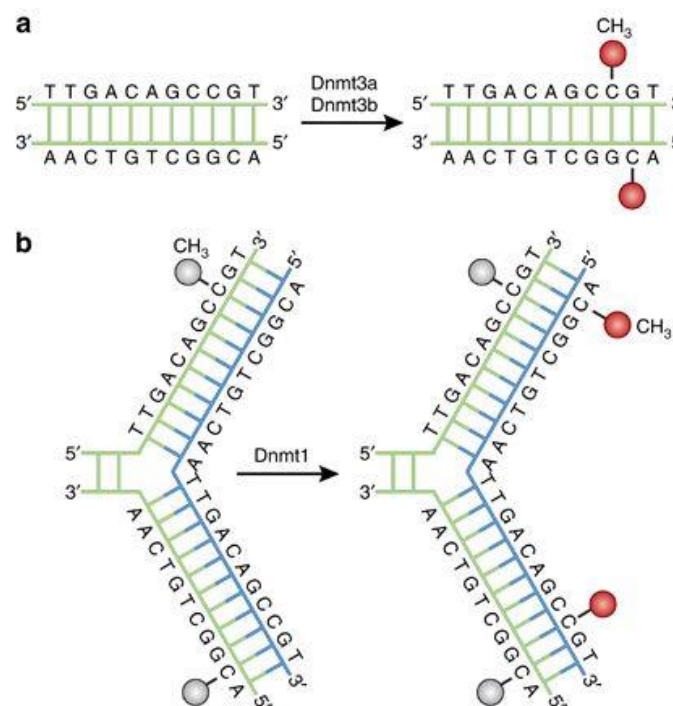


Figure 2.4: (a) The *de novo* methylation activity of DNMT3a and 3b, introducing new methylation patterns in DNA sequences and (b) the maintenance methylation activity of DNMT1, conserving established methylation patterns in DNA sequences (Moore et al., 2013).

2.4. DNA Methylation and Repression of Transcription

The role of DNA methylation in regulation of gene expression was hypothesized many years ago (Riggs, 1975). Many early observations suggested a potential mechanism where site-specific cytosine methylation within genes or adjacent to genes correlated with transcriptional repression (Ginder and McGhee, 1981). Numerous studies on *in vitro* transfection assays have shown inhibition of transcription by promoter DNA methylation (Singal and Ginder, 1999). Three possible pathways have been proposed

to account for transcriptional repression. The first pathway involves the binding of specific transcription factors directly to their respective promoters at its recognition sites (Singal and Ginder, 1999). Transcriptional factors, such as activating protein 2 (AP-2), c-Myc/Myn, E2F, cyclic AMP-response element binding protein (CREB) and nuclear factor kappa beta (NF- κ B), identify sequences with CpG residues and methylation prevents binding to these residues (Singal and Ginder, 1999). Other transcription factors, such as Sp1 and CAAT box-binding transcription factor (CTF), lack CpG dinucleotides in their binding sites (Singal and Ginder, 1999) and are therefore not sensitive to methylation at their binding sites (Tate and Bird, 1993). The second potential pathway induces silencing by binding of specific transcriptional repressors to methylated DNA (Singal and Ginder, 1999). Methyl cytosine binding protein 1 and 2 (MeCP-1 and MeCP-2) are two such factors which bind to methylated CpG residues in any sequence context (Singal and Ginder, 1999). Although DNA methylation in vertebrates is associated with preventing transcriptional activation, methylation in *Neurospora* blocks transcription elongation by mechanisms possibly mediated MeCP-1 and MeCP-2 (Rountree and Selker, 1999). The third mechanism involves the repression of transcription by altering chromatin structure (Singal and Ginder, 1999). Experiments conducted by microinjecting certain methylated and unmethylated genes into nuclei showed inhibition of transcription by methylation, only after assembly of chromatin (Kass et al., 1997). Once GAL4VP16, a strong transcriptional activator, has assumed its inactive state induced by methylation, it is incapable of counteracting the effect of chromatin (Kass et al., 1997). Apart from stabilizing the inactive state, methylation also inhibits activation by preventing access of transcription factors (Kass et al., 1997 and Siegfried and Cedar, 1997).

2.5. Methyl-CpG Binding Domain Protein

The methyl-CpG binding domain (MBD) family contains eleven known proteins, which are key players in transcription. The first MBD protein discovered was the methyl-CpG binding domain protein 2 (MeCP2) which lead to the identification of MBDs 1 to 6 by sequence homology to the MeCP2 MBD domain (Henrich and Bird, 1998; Baymaz and Fournier, 2014). MeCP2 is characterized by a 70 amino acid MBD and a transcriptional repression domain (TRD) (Meehan et al., 1992) and is associated with histone methylation and histone deacetylation. MeCP2 is required for histone remodelling and silencing suggesting its role in formation of heterochromatin and chromatin organization (Fuks et al., 2003). The MBD domain consists of 70 to 80 amino acids and can attach to single symmetrically methylated CpG dinucleotides (Nan et al., 1993; Ohki et al., 2001). Most members of the MBD family have a TRD which mediates protein interactions (Boeke et al., 2000) while other members have unique glycosylase or unmethylated-CpG binding zinc finger domains.

Among the MBD family, MBD2 is of great interest; In vertebrates, invertebrates and plants, MBD2 is the most phylogenetically ancient methyl cytosine binding protein (Hendrich and Tweedie, 2003), it expresses the highest degree of selectivity for methylated sequences (Fraga et al., 2003) and has been proposed as a therapeutic agent by direct implication in silencing of tumour suppressor genes (Jones and Baylin, 2002). MBD2 and MBD3 are closely related and share almost 80% homology. Both, MBD2 and MBD3, are made up of an MBD domain and a C-terminal coiled coil domain (Menafrá and Stunnenberg, 2014) responsible for protein interactions. However, MBD2 also contains an N-terminal glycine-arginine repeat region, exposed to PTMs (Gnanapragasam et al., 2011) and a TRD domain. Unlike other MBD proteins, the MBD and TRD domains of MBD2 overlap in the centre of its protein sequence, signifying its role in methylation binding and transcriptional repression (Boeke et al., 2000). MBD2 consists of 3 units: a full length MBD2a, MBD2b lacking the N-terminal glycine-arginine repeat and MBD2c, a C-terminal deficient testis specific isoform (Menafrá and Stunnenberg, 2014). The major difference between MBD2 and MBD3 is that only MBD2 binds to methylated DNA (Menafrá and Stunnenberg, 2014). The MBD domain of MBD3 lacks four conserved amino acids preventing it from binding to methylated DNA (Menafrá and Stunnenberg, 2014). Each MBD protein performs distinct functional roles as they demonstrate unique phenotypes (Fatemi and Wade, 2006).

MBD2 regulatory complexes have been indicated in silencing of genes in normal tissues such as human and chicken globin genes (Kransdorf et al., 2006), interleukin -4 mouse gene (Berger et al., 2007) mouse gut developing genes (Berger et al., 2007) and multiple abnormally methylated tumour suppressor genes (Scarsdale et al., 2011). The MBD2 gene is subjected to alternative splicing which may produce nonsense transcripts (Hendrich and Bird, 1998). Repressor complexes bind to methylated DNA via MBDs (Razin and Szyf, 1984). MBD2 is an enzyme shown to demethylate DNA, both *in vitro* (Bhattacharya et al., 1999) and *in vivo* (Cervoni and Szyf, 2001). Demethylases activate genes by removing the repressive methyl residues (Detich et al., 2002). Since methylation is associated with the silencing of genes, it is expected that a demethylase would activate transcription. However, MBD2 binds to methylated CpG sites and represses transcription by recruiting inactivated complexes of chromatin containing histone deacetylase (Lui et al., 2011). Therefore, MBD2 induces expression of certain genes while it inactivates other genes (Lui et al., 2011).

2.6. Histone Modification

The nucleosome comprises of DNA wrapped, 1.75 superhelical turns, around the core histones, H2A, H2B, H3 and H4 (Luger et al., 1997) and is the fundamental unit of eukaryotic chromatin structure (Turner, 2007). Given this close association between DNA and histones, it is clear that histones influence most aspects of DNA function. Histones are exposed to the nucleosome surface where they are subjected to a range of enzyme-catalysed modifications (Margueron et al., 2005; Nightingale et al., 2006), as they project through DNA (Turner, 2007). These modifications include lysine acetylation, serine and threonine phosphorylation as well as lysine and arginine methylation (Turner, 2007). Histone modifications are linked to multiple processes within the cell which are continuously occurring, for instance, in transcriptionally active promoters which show increased acetylation and methylation at lysines and arginines (Nightingale et al., 2006). Histones also induce long term effects on genomic functions by defining and maintaining chromatin structure from one cell generation to the next or throughout the cell cycle (Turner, 2007). These long-term effects have mainly been studied through heterochromatin. Constitutive heterochromatin is rich in certain DNA repeat families, gene poor, transcriptionally silent and is marked by histone modifications, including enhancement of both trimethylation of lysine 9 on histone H3 (H3K9me3) and trimethylation of lysine 20 on histone H4 (H4K20me3) and reduction in histone acetylation (Turner, 2007). Facultative heterochromatin is characterised by loss of methylation on lysine 4 on histone H3 (H3K4), increased methylation on lysine 27 on histone H3 (H3K27) and increased macroH2A levels (Heard, 2005).

2.7. Histone Methylation

The chromatin state in eukaryotes is said to contribute to the regulation of gene expression. Histone PTMs, such as acetylation, methylation, phosphorylation and ubiquitylation (Strahl and Allis, 2000, Tan et al., 2011), control gene expression by influencing chromatin compaction or signalling to other protein complexes (Greer and Shi, 2012). Histone methylation has demonstrated its modification power on DNA based functions, regulating gene transcription and DNA repair (Bannister and Kouzarides, 2005). Histones are methylated by histone methyltransferases which use SAM as the donor of the methyl group (Bannister et al., 2002) and occur at the histone tails of the amino acid side chains of arginine (R) and lysine (K) residues (Kouzarides, 2002; Zhang and Reinberg, 2001). Studies on bulk histones have revealed that mammals contain different ratios of methylated arginine and lysine species, depending on the cell type or tissue source (Borun et al., 1972; Byvoet et al., 1972). Lysine residues, *in vivo*, can be monomethylated (me1) (Murray, 1964), dimethylated (me2) (Fischle et al., 2008) or trimethylated (me3) (Paik and Kim, 1967). Methylation may alter chromatin structure by condensing

or relaxing its structure, however, methyl groups are relatively small and when attached to arginine and lysine residues, it does not neutralize their charge. Therefore, it is more likely that the addition of methyl groups will provide binding sites for regulatory proteins, rather than significantly alter chromatin structure (Bannister and Kouzarides, 2005).

The most studied lysine methylation sites are in the amino termini of histones H3 and H4 which are characterized by their presence in a certain type of chromatin, namely heterochromatin or euchromatin (Bannister and Kouzarides, 2005). Heterochromatin is tightly packed and transcriptionally silent while euchromatin is loosely packed and is transcriptionally active (Bannister and Kouzarides, 2005). Not all heterochromatin is the same regarding the methylated histones it contains. Histones H3K9, H3K27, H3K79 and H4K20 contain methylated sites in heterochromatin (Bannister and Kouzarides, 2005). Histone H3K9me3 and H4K20me3 are enriched in pericentric heterochromatin while H3K27me3 is enriched at the inactive X-chromosome (Martens et al., 2005). Likewise, not all euchromatin are the same. Genes within euchromatin can potentially be active and are linked to methylated histones H3K4 and H3K36 (Bannister and Kouzarides, 2005). In yeast, genes in euchromatin appear to be methylated at the genes 5' end, mostly in the trimethylated form (Margueron et al., 2005). This was also observed in mammals in a large-scale analysis of human euchromatin (Bernstein, 2005). Recently histone methylation was shown to be essential for the formation of a checkpoint control, required for cell arrest and DNA repair, following DNA damage (Huyen et al., 2004; Sanders et al., 2004).

2.8. The Histone Methyltransferase SUV39H1

The first histone methyltransferase discovered was the Suppressor of Variegation 3-9 Homolog 1 (SUV39H1) protein whose activity is directed against H3K9 and its highly catalytic SET (Suppressor of variegation, Enhancer of Zeste, Trithorax) domain (Kouzarides, 2002). Methylation by SUV39H1 occurs when two flanking cysteine-rich sequences, the PRE-SET and POST SET sequences, are merged to the SET domain (Kouzarides, 2002). The PRE-SET domain is needed for enzymatic activity and provides the specificity required by the SET domain to methylate H3K9 (Kouzarides, 2002). Trimethylated H3K9 (H3K9me3) was first studied as a marker of epigenetic silencing and constitutive heterochromatin (Schotta et al., 2004). Functional analysis on H3K9me3 revealed that it is tightly regulated in a cell cycle dependent manner (Heit et al., 2009).

H3K9me2/3 recruit Heterochromatin protein 1 (HP1) to mediated downstream silencing of PTM affected chromatin domains (Müller et al., 2016). HP1 co-localises with SUV39 at heterochromatin

sites (Aagaard et al., 1999) and binds to histone H3 when SUV39H1 methylates H3K9 (Lachner et al., 2001). In fission yeast, HP1 was shown to recruit to centromeric heterochromatin by the SUV39H1 homologue CLR4 (Bannister et al., 2001; Nakayama et al., 2001). HP1 contains two highly conserved regions known as chromo and chromo-shadow domains (Jones et al., 2000). The chromo domain recognizes the methyl mark left by SUV39H1, while the chromo-shadow domain is responsible for interactions between HP1 and other proteins, including SUV39H1 (Jones et al., 2000; Eissenberg and Elgin, 2002). Evidence of the SUV39/HP1 complex in transcriptional repression at euchromatic loci is seen by recruitment of SUV39/HP1 to the cell cycle by retinoblastoma (RB) co-repressor proteins, controlling genes such as *cyclin E* (Nielsen et al., 2001). At lysine 9, of RB null cells, the *cyclin E* promoter is under methylated and HP1 does not associate with this promoter, emphasising that methylation and subsequent HP1 binding are targeted events which must be delivered by a protein repressor, like RB.

2.9. Trimethylated Lysine 9 on Histone H3 (H3K9me3)

Gene poor regions are dimethylated or trimethylated at H3K9 by SUV39H1. H3K9me3 plays a role in normal cell development as a repressor of lineage-inappropriate genes and maintains early cell integrity as well as genomic stability (Monaghan et al., 2019). Several groups have demonstrated the importance of H3K9me3 in interacting with the conserved amino terminal chromo domain of HP1, thereby recruiting it to specific chromatin loci (Lachner et al., 2001). HP1 may cause deposition of H3K9me3 by recruiting methyltransferase SUV39H1 (Muramatsu et al., 2016), resulting in propagation of H3K9me3 across DNA and allowing large domains of heterochromatin to be established (Bannister et al., 2001). By associating with the HP1 protein, H3K9me3 recruits epigenetic modifications that play a role in heterochromatin maintenance (Becker et al., 2016). Constitutive heterochromatin, facilitated by H3K9me3, preserves regulatory factors and repetitive gene clusters and inhibits recombination and mutations (Monaghan et al., 2019). The spreading mode of H3K9me3 by SUV39H1 in an *in vitro* synthetic chromatin model, which is much like natural chromatin, revealed that SUV39H1 first attaches to its target H3K9, then interacts with chromatin via a second zinc finger like domain that enhances its catalytic activity (Müller et al., 2016). Mouse models with reduced or knock out of SUV39H1 is embryonic lethal at different stages of development (Kim and Kim, 2012). Thus far, H3K9me3 has been discovered to play roles in regulation of apoptosis (Lu et al., 2018), autophagy (Biga et al., 2017), aging (Mendelsohn et al., 2015), cell identity (Koide et al., 2016), DNA repair (Sun et al., 2009), imprinting (Fukuda et al., 2014), splicing (Saint-Andre et al., 2011), self-renewal (Pederson et al., 2016), transcriptional elongation (Vakoc et al., 2005) and viral latency (Maksakova et al., 2011).

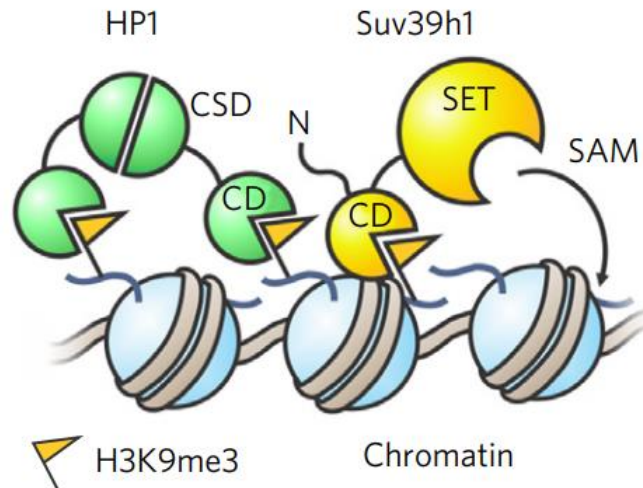


Figure 2.5: Representation of heterochromatin spreading. HP1 and SUV39H1 are recruited by H3K9me3 through its chromo domain (CD). The latter catalyses the S-adenosylmethionine (SAM)-dependent methylation of H3K9 via its SET domain (Müller et al., 2016).

2.10. Linking DNA Methylation and Histone Modifications

DNA methylation regulates transcription by working with histone modifications and microRNAs (Figure 2.6) (Moore et al., 2013). In eukaryotes, histone proteins assist in packing long strands of DNA into small nuclear compartments. Chemical modifications (methylation, acetylation, phosphorylation and ubiquitination) influences how DNA strands are packaged and their transcriptional activity. Histone modifications can loosen or tighten DNA associations with histones, resulting in a permissive environment for transcription or repression of gene expression, respectively (Moore et al., 2013). DNMTs interact with enzymes, typically involved in gene repression, which regulate histone modifications (Figure 2.6). During methylation of H3K9, DNMT-1 and -3a are known to bind to SUV39H1 to restrict gene expression (Fuks et al., 2003). In general, DNMTs associate with histone-modifying enzymes that add and/or remove histone markers to impose a repressive state in a gene region (Moore et al., 2013). Histone modifications may also influence DNA methylation (Figure 2.6). The trimethylation of H3K36 sometimes facilitates binding of DNMT3a to the histone H3 tail and stimulates its methyltransferase activity (Dhayalan et al., 2010). In contrast, in the presence of H3K4me3, the binding of DNMT-3a and -3b to the histone H3 tail is impaired, preventing methylation (Ooi et al., 2007). An observation in *Neurospora* has led to the proposal that DNA methylation may require a specific chromatin structure involving methylated histones to occur (Tamaru and Selker, 2001). It was found that dim-5, a H3K9 methyltransferase in *Neurospora*, is necessary for DNA methylation (Moore et al., 2013). Methyl binding proteins are the strongest link between DNA methylation and histone

modifications as they interact with methylated DNA and histones to enhance gene repression (Ng et al., 1999). MeCP2 removes histone modifications by recruiting histone deacetylases (Fuks et al., 2003) and enhances the repressive state of chromatin by recruiting histone methyltransferases involved in repressive H3K9 methylation (Fuks et al., 2003).

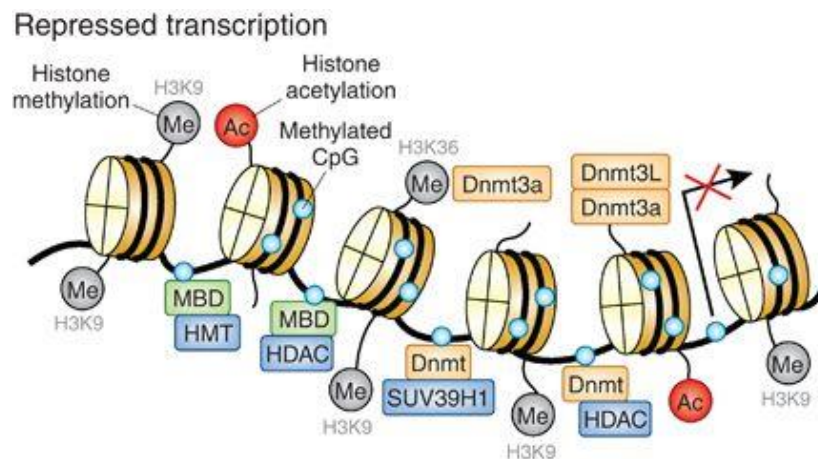


Figure 2.6: The link between DNA methylation and Histone Modifications. Methylation is regulated by DNMTs which suppress gene expression by targeting CpG sites and actively methylate DNA. The catalytic activity of some DNMTs are enhanced by associating with histone tails. DNA methylation is also recognized by MBDs which work with DNMTs to recruit enzymes that modify the histone tail, such as histone methyltransferase (HMTs). HMTs methylate histones and in combination with DNA methylation serve to repress gene expression (Moore et al., 2013).

CHAPTER 3:

MATERIALS AND METHODS

3.1. Materials

The human hepatocellular carcinoma (HepG2) cell line was purchased from American Type Culture Collection (ATCC; HB-8065). Cell culture equipment and reagents were purchased from Lonza Biotechnology (Basel, Switzerland). Western Blot reagents were purchased from Bio-Rad (Hercules, CA, USA). qPCR primers were obtained from Inqaba Biotech (Johannesburg, SA). All other reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

3.2. Cell Culture

3.2.1. Introduction

The HepG2 cell line were derived from a 15-year-old Caucasian male with a well differentiated hepatocellular carcinoma. These cells are adherent and epithelial in morphology. The HepG2 cell line is a good model for toxicity and epigenetic testing as it produces and secretes numerous major human plasma proteins and display many cellular characteristics of normal human hepatocytes. These cells show low levels of alcohol dehydrogenase – 1B and -1C genes, both of which are regulated by epigenetic mechanisms in HepG2 cells.

3.2.2. Cell Culture Conditions

HepG2 cells were cultured in complete culture media [CCM; Eagle's Minimum Essentials Medium (EMEM) supplemented with 10% foetal calf serum, 1% penicillin-streptomycin-fungizone and 1% L-glutamine]. Cultures were maintained in 25 cm³ cell culture flasks at 37 °C with 5% CO₂ in a humidified incubator, until approximately 90% confluent. Thereafter, 50 mM stocks of Picolinic Acid and Methyl Picolinic acid were prepared and the cells were incubated (37°C, 5% CO₂, 24 hrs) with a range of Methyl Picolinic Acid Concentrations (3, 10, 13, 15 mM) and Picolinic Acid (7.5 mM). Untreated cells, containing CCM only, were cultured under the same conditions and were used as controls. Cell viability was determined using the trypan blue cell exclusion assay.

3.3. Water Soluble Tetrazolium salt 1 Assay

3.3.1. Introduction

The reduction of tetrazolium salt to a purple coloured formazan compound by succinate-tetrazolium reductase, found in viable cells, is a suitable and accurate method used to measure cell viability and proliferation (Ishiyama et al., 1993). Succinate-tetrazolium reductase belongs to the mitochondrial respiratory chain and is only active in metabolically intact cells. Water Soluble Tetrazolium salt 1 (WST-1) is a negatively charged desulphonated inner salt with an iodine residue. It is reduced extracellularly to water soluble formazan by electron transport across the membrane of dividing cells. With the help of the 1-methoxy-5-methyl-phenazinium methyl sulphate (mPMS), an intermediate electron acceptor, WST-1 is cleaved to formazan by a complex cellular mechanism that occurs at the cell surface (Ngamwongsatit et al., 2008) (Figure 3.1). This reduction is dependent on the glycolytic production of Nicotinamide adenine dinucleotide phosphate (NAD(P)H) in viable cells. The amount of formazan produced directly correlates to the number of metabolically active cells.

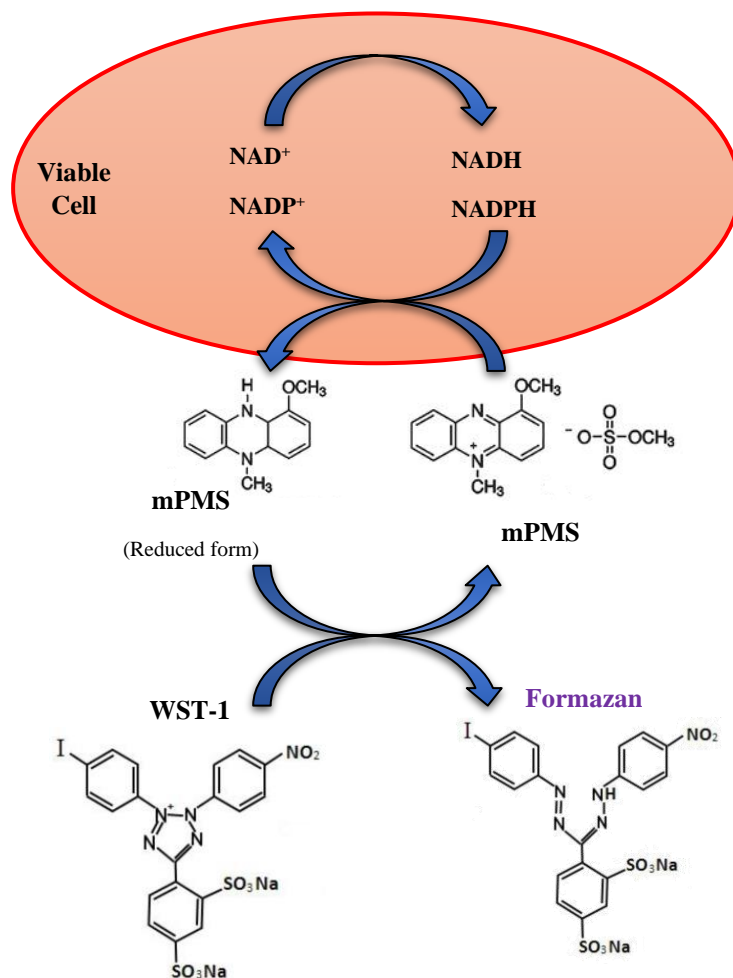


Figure 3.1: Schematic mechanism of the WST-1 reduction (prepared by author).

3.3.2. Protocol

The WST-1 Assay was used to determine the cytotoxicity of Picolinic Acid and Methyl Picolinic Acid on HepG2 cells. Approximately 20 000 cells/well were seeded, in triplicate, and treated for 24 hours with Picolinic Acid and Methyl Picolinic Acid (0, 1, 3, 5, 7, 10, 15, 20, 25 mM). Thereafter, the supernatant was removed, the cells were washed once with 0.1 M PBS, and subsequently incubated with WST-1 reagent (10 μl WST-1 reagent in 100 μl CCM per well) for 2 hrs at 37°C. The optical density of the formazan produced was measured using a spectrophotometer (Bio-Tek μQuant) at a wavelength of 450 nm and a reference wavelength of 620 nm. The percentage cell viabilities were calculated per concentration and used to construct a dose-response curve from which the IC_{50} values of Picolinic Acid and Methyl Picolinic Acid were obtained.

3.4. DNA Isolation and Quantification of DNA Methylation

3.4.1. Introduction

DNA methylation occurs by the enzymatic conversion of cytosine residues to 5-methylcytosine residues (5-mC) via methyl transfer from S - adenosylmethionine (SAM) to position 5 of cytosine (Razin and Riggs, 1980). In the DNA methylation assay, the binding solution enables DNA to attach to the microtiter plate wells which are specifically treated to have a high DNA affinity. Methylated DNA is detected using capture and detection antibodies. The developer solution is used to detect methylated DNA as it turns blue in the presence of methylated DNA. The stop solution halts the enzyme reaction and changes the blue to a yellow colour. Samples are then quantified colorimetrically by reading the absorbance in a spectrophotometer.

3.4.2. Protocol

Genomic DNA (gDNA) was isolated from Picolinic Acid and Methyl Picolinic Acid treated HepG2 cells. Briefly, control, PA and MPA-treated HepG2 cells were incubated with cell lysis solution (EDTA, Tris-Cl, 0.1% SDS, (600 μ l, 15 mins, RT)) and Potassium acetate (5 M potassium Acetate, Glacial acetic acid dH₂O, (600 μ l, 8 mins, RT)) followed by centrifugation (Eppendorf Centrifuge 5804 R; 13000 rpm, 5 mins, 24 °C). Subsequently 100% isopropanol (600 μ l) was added to the supernatant containing gDNA. DNA was precipitated by centrifugation (Eppendorf Centrifuge 5804 R; 13000 rpm, 5 mins, 24 °C) and washed in 100% ethanol (300 μ l) followed by centrifugation (Eppendorf Centrifuge 5804 R; 13000 rpm, 5 mins, 24 °C). The DNA pellets were then air dried (15 mins, RT), resuspended in Hydration Solution (10 mM EDTA (pH 8), 100mM Tris-Cl (pH 7.4), dH₂O, (40 μ l)), DNA was heated (15 mins, 65 °C). All DNA samples were then quantified using the Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific) and standardized to 100 ng/ μ l. The A₂₆₀/A₂₈₀ absorbance ratio was used to assess DNA purity. The Colorimetric Methylated DNA Quantification Kit (Abcam, ab117128) was used to quantify global DNA methylation, as per manufacturer's instructions. The positive control, prepared by serial dilution (0.5 – 10 ng/ μ l), control, PA and MPA treated DNA samples were added to a 96 well microtiter plate, containing binding solution (80 μ l) and incubated (37 °C, 90 min). Samples were then incubated in capture antibody (50 μ l, RT, 60 min), detection antibody (50 μ l, RT, 30 min) and enhancer solution (50 μ l, RT, 30 min) followed by removal of antibodies/solutions and washing with 1x wash buffer (150 μ l, 3, 4 and 5 times respectively). Samples were then incubated in developer solution (100 μ l, RT, dark, 10 min) followed by addition of stop

solution (50 µl). Absorbance of the plate was then read at 450 nm. The percent 5-methylcytosine content was determined using a supplied formula and represented as fold change relative to the control.

3.5. mRNA Isolation and Quantitative Polymerase Chain Reaction

3.5.1. Introduction

Polymerase Chain Reaction (PCR) is a widely used molecular biology technique for the amplification of DNA *in vitro*. It relies on thermal cycling, which exposes reactions to repeated cycles of cooling and heating which allows DNA to be synthesized. Two primers, a forward and reverse primer, specific to the gene of interest are used in each reaction. These primers are designed to flank the target region by binding to the 3' end of the single stranded template DNA. DNA Polymerases then extended the primers along the strand by incorporating Deoxynucleoside triphosphates (dNTPs) to the flanked primers. PCR involves 3 steps (Karcher, 1995):

- 1) Denaturation (96 °C): The reaction is heated to denature/separate the double stranded template DNA into 2 single strands of template DNA. Each DNA strand then serves as the template for the synthesis of new DNA strands.
- 2) Annealing (55 °C to 65 °C): The reaction is then cooled to allow the Primers to bind to their complementary single-strand template DNA.
- 3) Extension (72 °C): The reaction temperature is raised to extend the primers, synthesizing new strands of DNA.

These 3 steps make up 1 PCR cycle and each cycle are repeated 30 to 40 times to accomplish optimal amplification (Karcher, 1995). PCR is an effective method of producing multiple copies of DNA as it allows for the exponential growth of the original template DNA (Figure 3.2).

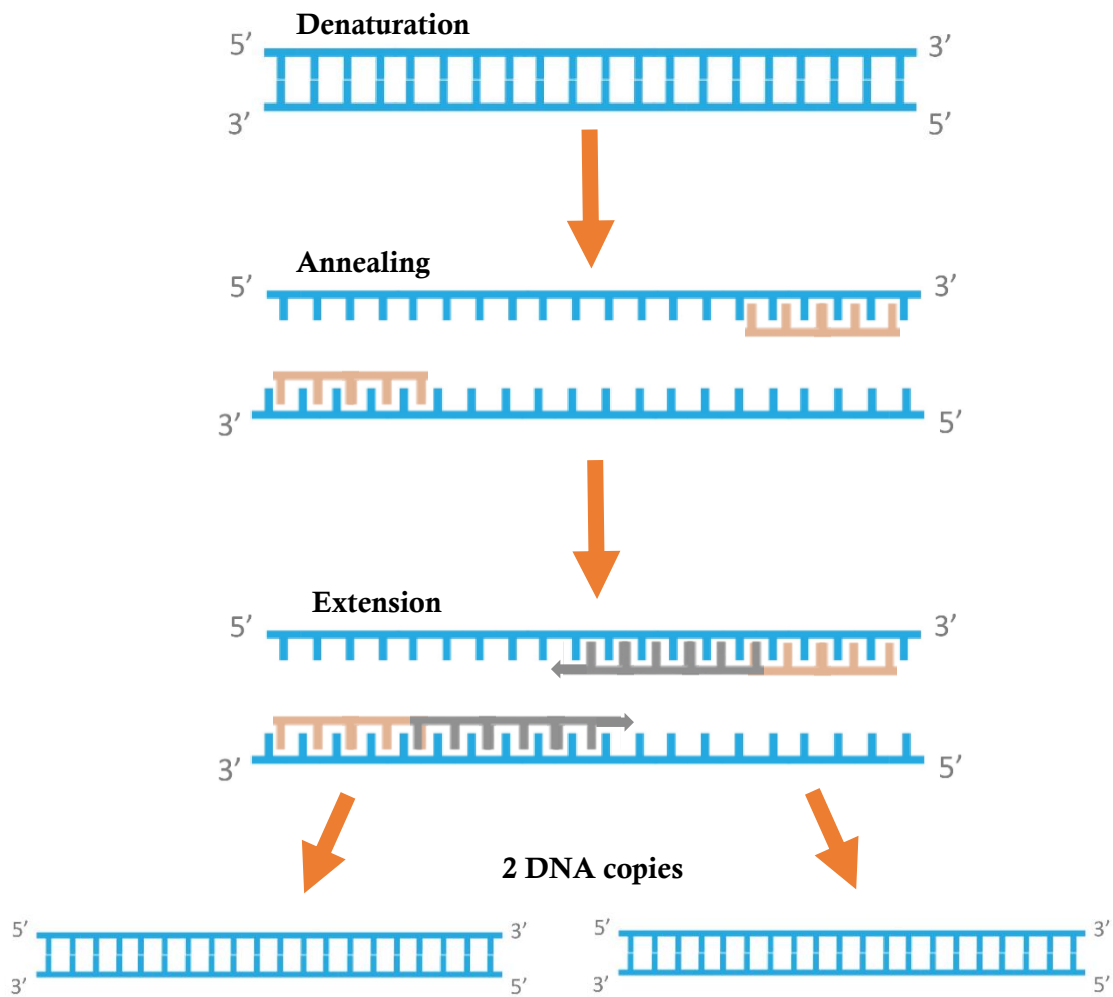


Figure 3.2: Overview of steps involved in PCR resulting in synthesis of DNA (prepared by author).

3.5.2. Protocol

3.5.2.1 RNA Isolation

RNA was isolated from Control, PA and MPA treated cells. Cells were incubated with 500µl Qiazol reagent (Qiagen, Hilden, Germany) and 500 µl 0.1M PBS (5 min, RT). Cells were then lysed using a cell scraper, transferred to 1.5ml micro-centrifuge tubes and stored overnight at -80 °C. Chloroform (100 µl) was added to the thawed samples followed by centrifugation (12 000xg, 15 min, 4 °C). The aqueous phase was transferred to fresh 1.5 ml micro-centrifuge tubes and Isopropanol (250 µl) was added before overnight incubation at -80°C. Thereafter, the samples were centrifuged (12 000 xg, 20 min, 4 °C), the supernatants were removed, and the RNA pellets were washed in 75% cold Ethanol (500 µl). After centrifugation (7400 xg, 15 min, 4 °C), the Ethanol was removed, samples were allowed to air dry (30 min), and RNA pellets were re-suspended in Nuclease Free Water (15 µl). RNA was

quantified using a Nanodrop2000 Spectrophotometer (Thermo-Fischer Scientific) and RNA integrity was assessed using the A260/A280 ratio. RNA samples were then standardised to 1000 ng/μl and used to prepare cDNA.

3.5.2.2. cDNA Synthesis

cDNA was synthesized, using the iScript™ cDNA Synthesis Kit (BioRad) as per manufacturer's instructions. A reaction master mix (20 μl) was prepared containing 1 μl RNA template, 4 μl 5X iScript™ reaction mix, 1 μl iScript reverse transcriptase and nuclease free water per sample which was followed by incubation in a Thermocycler (GeneAmp® PCR System 9700, Applied Bioscience) with the following thermal conditions: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min.

3.5.2.3. qPCR

cDNA was used to determine gene expression of MBD2 (Santa Cruz, sc-271562; 1:500) using the Sso Advanced™ Universal SYBR Green Supermix (Bio-Rad, 1725270), as per manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Each reaction (10 μl) was carried out in triplicate containing SYBR Green (5 μl), Forward Primer (1 μl), Reverse Primer (1 μl), Nuclease Free Water (2 μl) and template cDNA (1 μl). The qPCR experiments were conducted in the CFX Touch™ Real Time PCR Detection System (Bio-Rad) at the following thermal conditions: an initial denaturation at 95 °C (40 min), subsequent denaturation for 37 cycles (95 °C, 15 sec), annealing (56 °C, 40 sec) and extension (72 °C, 30 sec). The data was analysed using the CFX manager software version 3.1 followed by the comparative threshold (Ct) method (Livak and Schmittgen, 2001) and represented as a mean fold change relative to the control.

Table 3.1: Primer sequences for MBD2 and GAPDH genes used in qPCR

Gene:	Accession No.	Primer:	Sequence:
MBD2	NM_003927	Forward	5' -AGGTAGCAATGATGAGACCCTTTTA-3'
		Reverse	5' -TAAGCCAAACAGCAGGGTTCTT-3'

GAPDH (human)	NM_002046	Forward	5'-TCCACCACCCTGTTGCTGTA-3'
		Reverse	5'-ACCACAGTCCATGCCATCAC-3'

3.6. Western Blot

3.6.1. Introduction

Western blotting is a technique used to identify a specific protein in a homogenous mixture of proteins. The technique is based on 3 elements:

- 1) Separation of proteins by size
- 2) Transfer of separated proteins to a solid support
- 3) Detection of proteins using primary and secondary antibodies.

The proteins are separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), according to their molecular weights. Current applied to SDS-PAGE allows the negatively charged protein to migrate through the gel, with the smaller proteins migrating quicker than the large proteins (Mahmood and Yang, 2012). Migrated proteins are then transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System. The membrane is then incubated in blocking buffer and immunoblotted in specific primary and secondary antibodies. Unbound antibody is washed off after which bound antibody is detected by chemiluminescence. Proteins are detected as bands and the band thickness corresponds to the amount of protein present (Mahmood and Yang, 2012).

3.6.2. Protein Isolation

3.6.2.1 Introduction

Control, PA and MPA treated HepG2 cells were mechanically lysed with Cytobuster™ Protein Extraction Reagent supplemented with protease and phosphate inhibitors. Cytobuster has a gentle, non-ionic composition which permits extraction of functionally active expressed proteins. Protein degradation is prevented by working on ice, along with protease inhibitors. Protein samples are then quantified and standardized using the Bicinchoninic Acid (BCA) Assay. The BCA assay is a colorimetric biochemical assay used to determine the concentration of total protein in a solution. The

BCA assay relies on 2 reactions that occur under alkaline conditions. In the first reaction, peptide bonds in protein reduce cupric (Cu^{2+}) ions to cuprous (Cu^+) by a reaction known as the Biuret test. The amount of protein present in samples are directly proportional to the amount of Cu^{2+} ions reduced. In the second reaction, 2 BCA molecules react with each Cu^+ ion, producing an intense purple colour which absorbs light at a maximum wavelength of 562 nm (Figure 3.3).

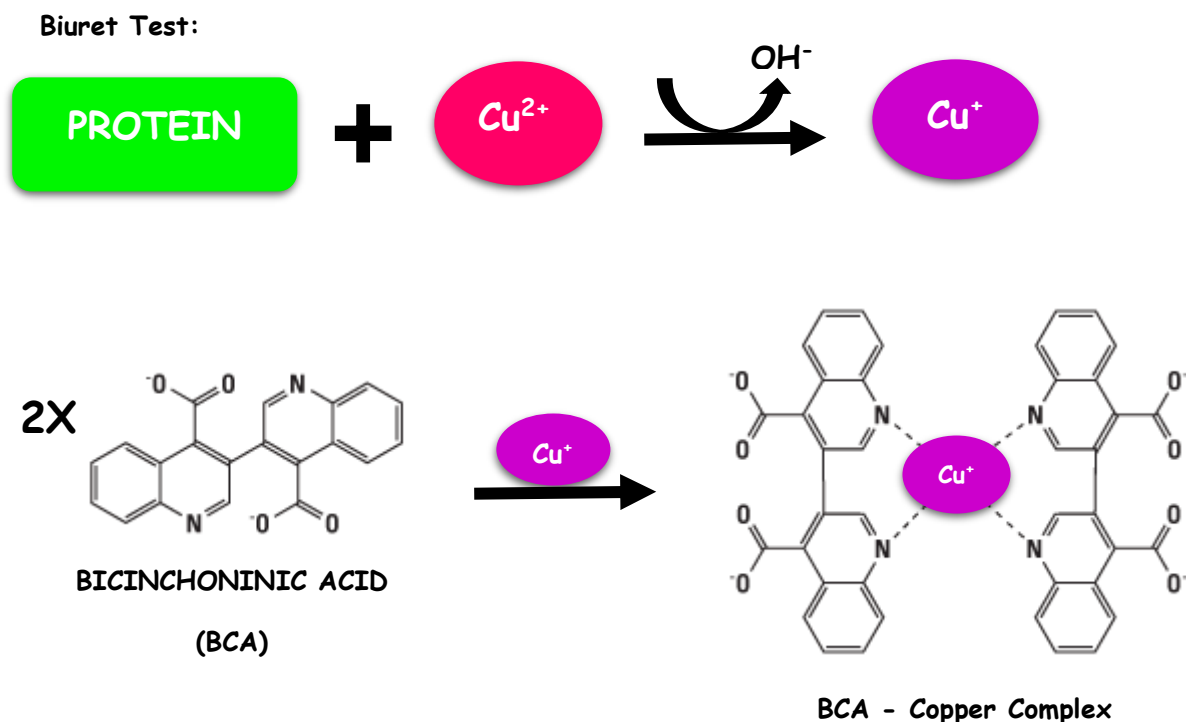


Figure 3.3: Principle Scheme of the Bicinchoninic Acid (BCA) Assay (prepared by author).

3.6.2.2. Protocol

Following treatment with PA and MPA for 24 hrs, HepG2 cells were washed with 0.1M PBS and lysed with Cytobuster™ (Novagen, USA) supplemented with protease inhibitors (Roche, catalogue no. 05892791001) and phosphate inhibitors (Roche, catalogue no. 04906837001) was added to the flask. The flask was kept on ice (30 min) followed by mechanical cell lysing using a cell scraper. Cell lysates were centrifuged (12 000 xg, 10 min, 4 °C) and the crude protein extract was transferred to fresh 1.5 ml micro-centrifuge tubes. Bovine Serum albumin (BSA) standards were prepared (0 – 1 mg/ml) in distilled water. Thereafter, 25 μl of each standard (in triplicate) and protein samples (in duplicate) was added to a 96 well microtiter plate followed by 200 μl working solution (BSA (198 μl) and CuSO_4 (4 μl)) and the plate was incubated (37 °C, 30 min). Absorbance was then measured at 562 nm using a spectrophotometer (Bio-Tek μQuant). A standard curve was constructed from the optical density of the

BSA standards; and the protein concentrations were derived from this standard curve (Appendix). Quantified proteins were then standardised to 1 mg/ml with cytobuster. Once standardised, samples were diluted in 1x Laemmli buffer [dH₂O, 0.5M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol and 1% bromophenol blue] and boiled (5 min). Tris-HCl acts as a buffer and maintains pH levels. Glycerol allows samples to easily sink into the wells, during SDS-PAGE, by adding weight to the protein samples. SDS denatures proteins and neutralizes protein charges ensuring that it is separated by size rather than shape or charge. β-mercaptoethanol reduces disulphide bonds in proteins enabling unfolding of proteins. Bromophenol blue acts as a tracking dye during migration in SDS-PAGE.

3.6.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.6.3.1. Introduction

SDS-PAGE is an analytical technique used to separate charged molecules based on their molecular weights, in an electric field. Negatively charged proteins migrate towards the positively charged electrode through the gel, with the high molecular weight proteins migrating slower and remaining towards the top of the gel (Mahmood and Yang, 2012). The low molecular weight proteins migrate faster and are located at the bottom of the gel (Figure 3.4). When preparing gels, for the separation of proteins by size, acrylamide is the compound of choice. Acrylamide in combination with bis-acrylamide forms a crosslinked polymer network when mixed with ammonium persulphate (APS). This reaction is polymerized by Tetramethyl ethylenediamine (TEMED) which promotes the production of free radicals by APS (Towbin, 1998). SDS-PAGE uses 2 types of gels: resolving and stacking gel. The resolving gel with pH 8.8, has a high acrylamide concentration with smaller pores compared to the stacking gel which has a low acrylamide concentration with pH 6.8. The stacking gel allows proteins to form into a tight band before entering the resolving gel where proteins are separated by size.

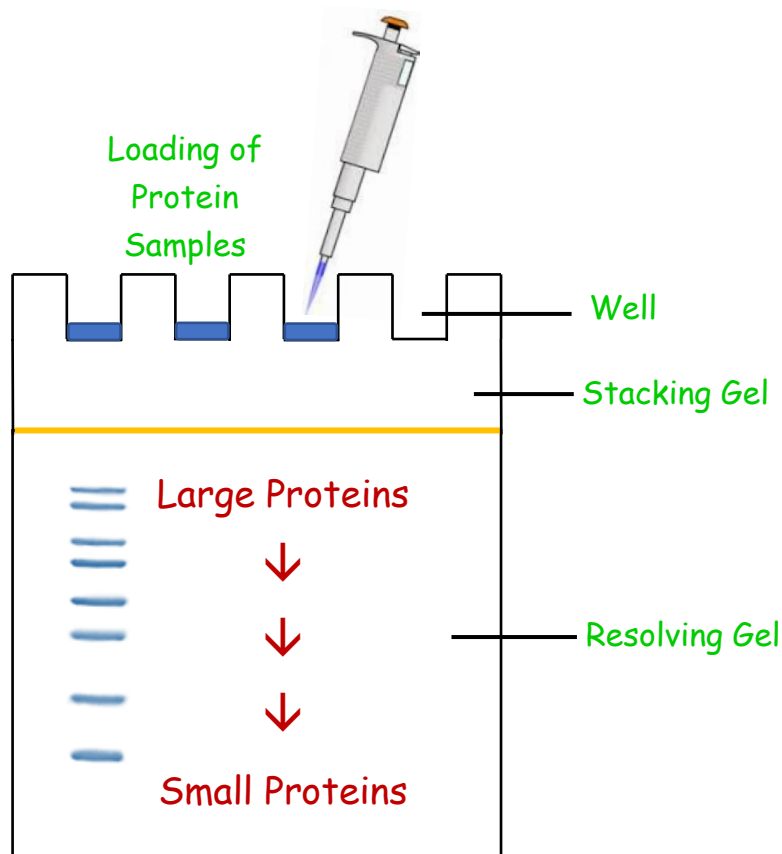


Figure 3.4: Separation of Proteins, according to size, by SDS-PAGE (prepared by author).

3.6.3.2. Protocol

The gels for SDS-PAGE were prepared using the Mini-PROTEAN Tetra Cell casting frame (BioRad). The 10% Resolving Gel (dH₂O, Tris (1.5 M, pH 8.8), SDS, Bis-acrylamide, APS, TEMED) was cast first and allowed to polymerize (RT, 1 hr). The 4% Stacking Gel (dH₂O, Tris (0.5 M, pH 6.8)), SDS, Bis-acrylamide, APS, TEMED) was then cast above the Resolving Gel. A plastic comb was placed into the Stacking Gel, between the glass plates, to allow wells to form and was allowed to set (RT, 40 min). Once set, the gels were transferred to the electrode assembly and placed in the electrode tank (Mini-PROTEAN Tetra Cell System, Bio-Rad). The tank was then filled with Running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS), the comb was removed and the protein samples (25 µl) and a molecular weight marker (5 µl) (Precision Plus Protein All Blue Standards, catalogue no. #161-0373, Bio-Rad) were loaded into the wells. The samples were then electrophoresed (150 volts, 1 hr) using the Bio-Rad compact power supply.

3.6.4. Transfer of Proteins

3.6.4.1. Introduction

After separating the proteins, it is transferred to a membrane (Figure 3.5). This transfer takes place using an electric field perpendicular to the gel surface. A nitrocellulose membrane is used for its high affinity for proteins and its retention abilities (Mahmood and Yang, 2012). The electrophoresed gel and nitrocellulose membrane are sandwiched between 2 fibre pads and placed in the transfer apparatus (Bio-Rad Trans-Blot Turbo Transfer System), where the proteins are electro-transferred from the gel to the nitrocellulose membrane.

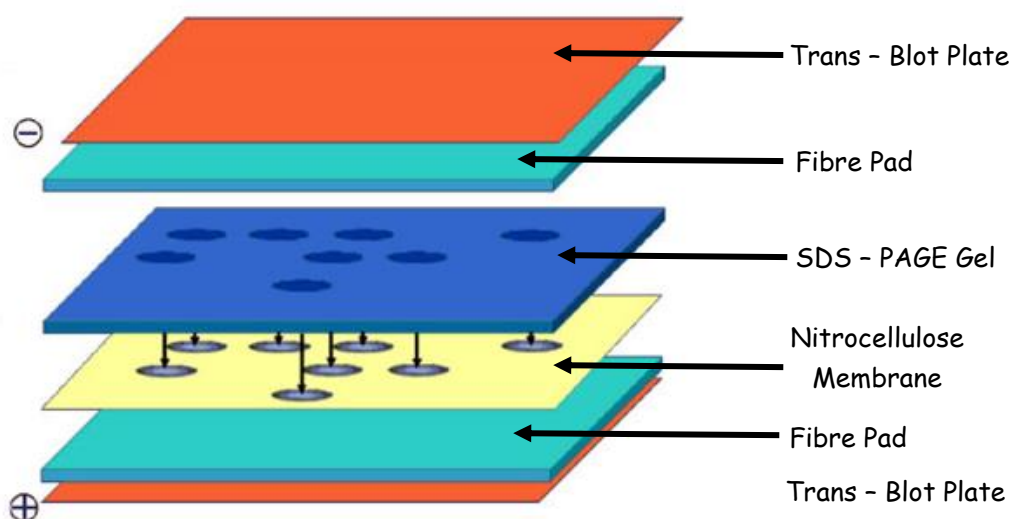


Figure 3.5: Transfer of Proteins from the SDS-PAGE Gel to a nitrocellulose membrane (prepared by author).

3.6.4.2. Protocol

The electrophoresed gel, nitrocellulose membrane and fibre pads were equilibrated individually in Transfer buffer (25 mM Tris, 191.8 mM glycine and 20% methanol) (10 min). A gel sandwich consisting of a fibre pad, nitrocellulose membrane, gel and a second fibre pad was assembled and placed between the electrode plates of the transfer apparatus. The proteins were then transferred from the gel onto nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer System (30 min, 20 V).

3.6.5. Blocking, Washing and Antibody Incubation

3.6.5.1. Introduction

Blocking is an important step in Western Blotting as it prevents the non-specific binding of antibody to the membrane. Blocking solution is made up of 5% BSA, diluted in TTBS (Tris-buffered saline with Tween 20), to reduce the background. The membrane is then incubated with antibodies specific to the protein of interest. Unbound antibody is washed off, leaving only the bound antibody. The protein of interest is then detected using an enzyme such as Horse Radish Peroxidase (HRP)-conjugate secondary antibody (Figure 3.6).

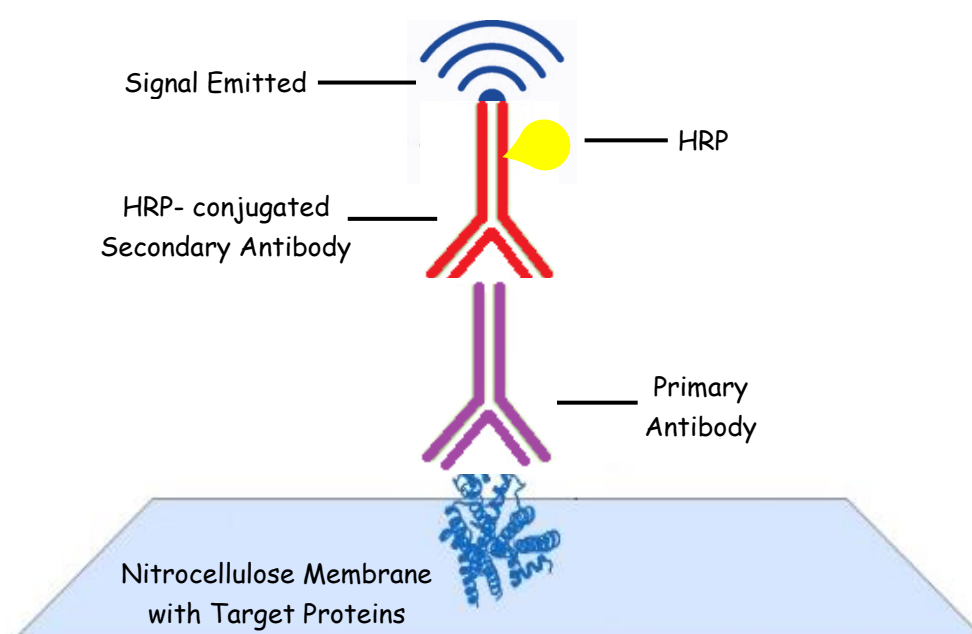


Figure 3.6: Signal Emission associated with antigen – antibody interactions (prepared by author).

3.6.5.2. Protocol

Following transfer, the membrane was blocked in blocking solution made up of either 5% BSA in tween 20-Tris buffered saline (TTBS: 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH₂O, pH 7.5) (1 hr, RT), simultaneously with gentle shaking to prevent non-specific binding of proteins. This was followed by incubating the membrane with the specific primary antibody (Table 3.2) for 1 hr at RT, with gentle shaking, and overnight at 4 °C. Following overnight incubation, the primary antibody is removed, and the membrane is washed with TTBS (5 times, 10 min). The membrane is then probed

with an HRP-conjugated secondary antibody (Table 3.2) (2 hrs, RT) with gentle shaking. The membrane is washed again with TTBS (5 times, 10 min).

3.6.6. Imaging and Quenching

The Clarity™ Western ECL Substrate Kit (catalogue no. #170-5060, Bio-Rad) was used for visualization of the protein bands. Images were detected using the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). After detection, the membrane is quenched in 5% hydrogen peroxide (30 min, 37°C), washed once in TTBS, incubated in blocking solution and probed with HRP-conjugated anti β -actin (Sigma). β -actin is the housekeeping protein used to normalise protein expression. Protein expression was analysed using the Image Lab Software version 5.0 (Bio-Rad) and protein expression was normalised by dividing the relative band density (RBD) of the target protein by the RBD of β -actin. Results were expressed as RBD and fold-change relative to the control.

Table 3.2: Antibodies and the dilutions used in Western Blotting

	Antibody:	Dilution:	Catalogue Number:
Primary Antibodies	Rabbit Anti-MBD2	1:1000 in 5% BSA	ab38646 (Abcam)
	Rabbit Anti – SUV39H1	1:1000 in 5% BSA	ab155164 (Abcam)
	Mouse Anti-Histone H3 (tri methyl K9)	1:1000 in 5% BSA	ab8898 (Abcam)
Secondary Antibody	Goat Anti-rabbit IgG HRP	1:3000 in 5% BSA	Sc-2004 (Santa Cruz)
	Goat Anti-mouse IgG HRP	1:3000 in 5% BSA	Sc-2005 (Santa Cruz)
Housekeeping Antibody	Anti- β -actin	1:5000 in 5% BSA	A3854 (Sigma-Aldrich)

3.7. Statistical Analysis

Microsoft Excel 2013 and GraphPad Prism version 5.0 (GraphPad Software Inc., California) were used to perform all statistical analyses. The one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple group comparison was used to determine statistical significance for all assays. A value of $p < 0.05$ was considered statistically significant.

CHAPTER 4

RESULTS

4.1. Water Soluble Tetrazolium salt 1 Assay

The WST-1 Assay was conducted to determine the cytotoxicity of PA and MPA in HepG2 cells (Figure 4.1). HepG2 cells were incubated for 24 hrs with a range of concentrations (0, 1, 3, 5, 7, 10, 15, 20, 25 mM) of PA and MPA. A dose response curve was obtained showing a decrease in cell activity by both PA and MPA (Figure 4.1). The IC_{50} values for PA (7.5 mM) and MPA (13 mM) were determined from the curve. Both PA and MPA displayed different dose response curves (Figure 4.1). PA initiated a decrease at a log concentration of 0.5 log while MPA initiated a decrease at a log concentration of 1.0. A plateau is seen from 0 to approximately 0.8 MPA log concentration, before dose-dependently decreasing. For all experiments the IC_{50} for PA was used whilst a range of concentrations (3, 10, 13, 15 mM) for MPA was selected, based on the IC_{50} value.

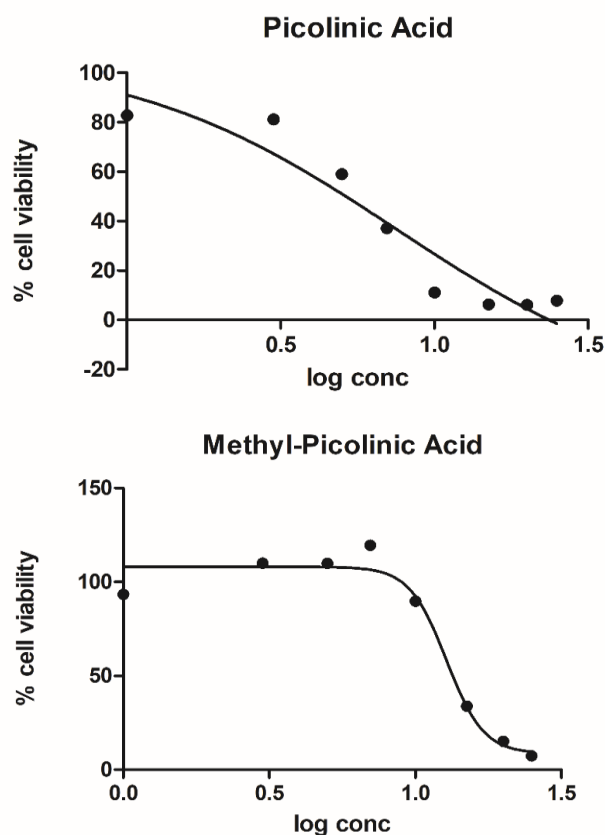


Figure 4.1: The effect of PA and MPA on HepG2 cell viability.

4.2. DNA Methylation Assay

5mC in PA and MPA treated HepG2 cells were assessed by the DNA methylation assay. Methylated DNA is detected using capture and detection antibodies and by colorimetric quantification. The percentage 5mC is proportional to the OD intensity measured. PA showed a slight decrease (not significantly) in 5mC compared to the control (Figure 4.2). All concentrations of MPA significantly decreased 5mC as compared to the controls ($p<0.0001$; Figure 4.2). Although MPA shows an overall decrease in DNA methylation, 5mC in MPA treated cells increased in a dose-dependent manner. 5-aza-2-DC (DNA methylation inhibitor) used as a negative control, also significantly inhibited formation of 5mC ($p<0.0001$; Figure 4.2).

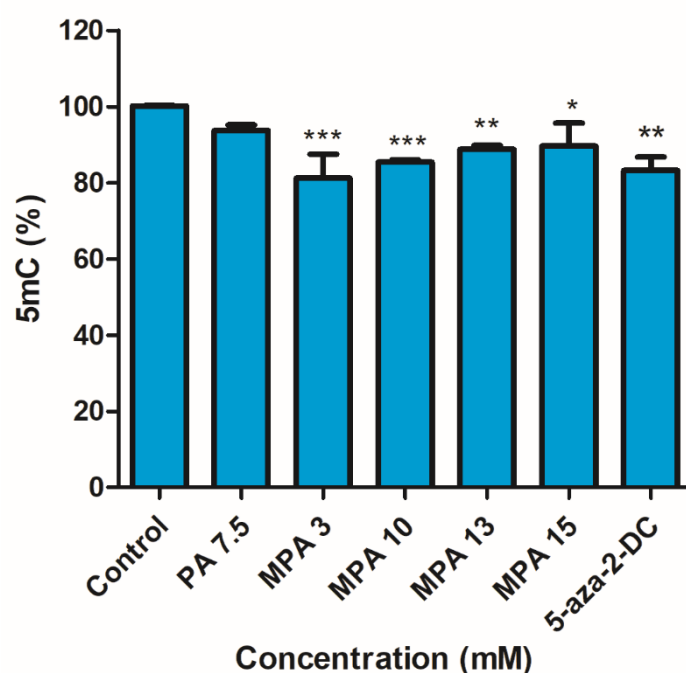


Figure 4.2: The effect of PA and MPA on global DNA methylation in HepG2 cells. PA and MPA decreased 5-methylcytosine content in HepG2 cells. 5-aza-2-DC is used as a negative control as it is a DNA methylation inhibitor (* $p<0.05$, ** $p<0.001$, *** $p<0.0001$).

4.3. Quantitative Polymerase Chain Reaction

Gene expression of *MBD2* was measured by qPCR. *MBD2* binds to methylated DNA and plays a role in gene transcription. It acts as a transcriptional repressor and a DNA demethylase. *MBD2* gene expression was significantly decreased in 10, 13 and 15 mM MPA, however, at 3 mM MPA the expression of *MBD2* was increased compared to the control ($p<0.0001$; Figure 4.3). The expression of *MBD2* in the PA treated cells was decreased compared to the control ($p<0.0001$; Figure 4.3).

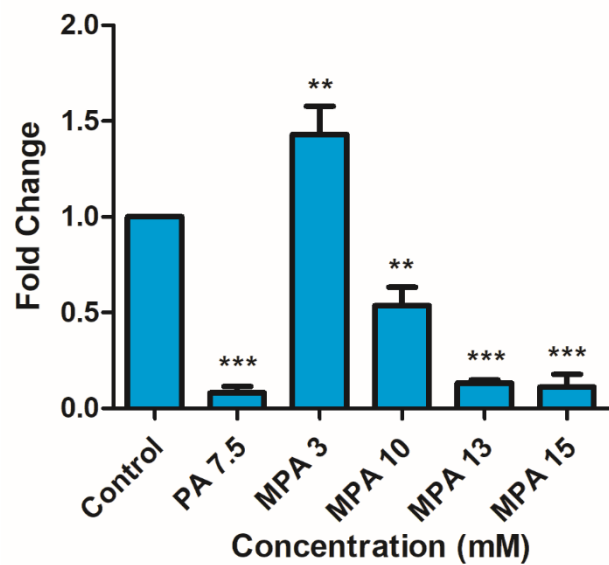


Figure 4.3: The effect of PA and MPA on *MBD2* expression in HepG2 cells. PA and MPA alter *MBD2* expression in HepG2 cells (** $p<0.001$, *** $p<0.0001$).

4.4. Western Blot

Protein expression of MBD2, SUV39H1 and H3K9me3 were assessed by Western Blot. The methyltransferase, SUV39H1, methylates lysine residues on histone H3. The expressions of MBD2, SUV39H1 and H3K9me3 were normalised against the housekeeping protein β - Actin and results were expressed as Relative Band Density to the control.

4.4.1. Methyl-CpG binding domain 2 (MBD2)

MBD2, binds to methylated DNA in CpG islands at position 5 of cytosines and plays a role in regulating DNA methylation. Protein expression was significantly upregulated for all concentrations of MPA, compared to the control (Figure 4.4) ($p < 0.0001$). MBD2 protein expression in PA treated cells was increased compared to the control (Figure 4.4) ($p < 0.0001$).

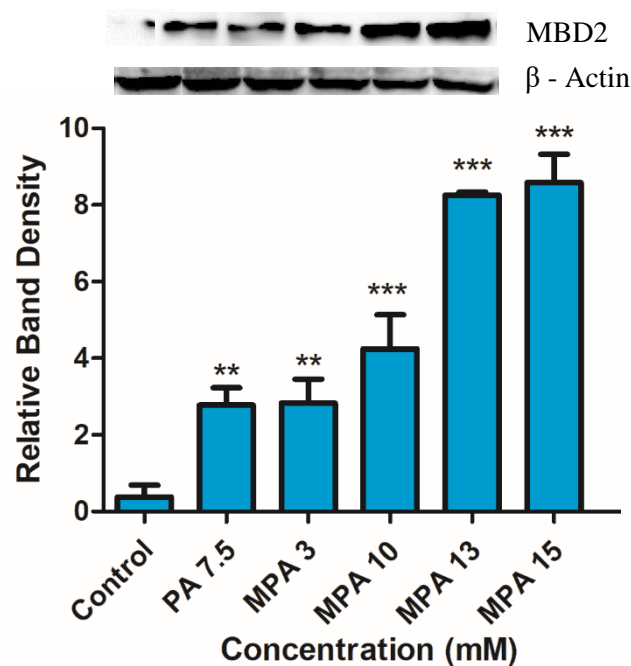


Figure 4.4: The effects of PA and MPA on MBD2 protein expression in HepG2 cells (** $p < 0.001$, *** $p < 0.0001$).

4.4.2. SUV39H1

SUV39H1, a methyltransferase, trimethylates H3K9. SUV39H1 protein expression in HepG2 cells increased in response to MPA treatment as compared to the control; however, at 3 mM MPA there was no change in protein expression as compared to the control (Figure 4.5) ($p < 0.0001$). PA also reduced protein expression (not significantly) of SUV39H1 when compared to the control (Figure 4.5) ($p < 0.0001$).

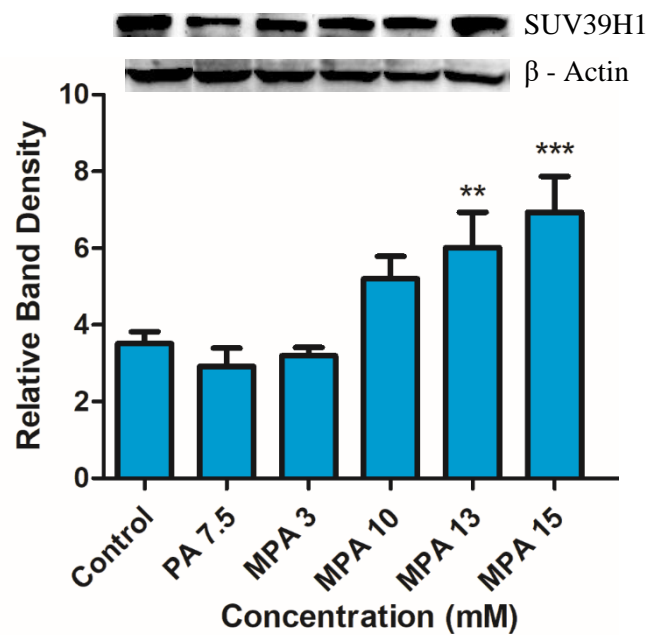


Figure 4.5: The effect of PA and MPA on SUV39H1 protein expression in HepG2 cells (** $p < 0.001$, *** $p < 0.0001$).

4.4.3. H3K9me3

Methylation of lysine residues occur on histone H3. Histone H3K9 can be methylated by SUV39H1 methyltransferase to its di-methylated (H3K9me2) and tri-methylated (H3K9me3) forms. The expression of H3K9me3 in HepG2 cells was increased by 10, 13 and 15 mM MPA; however, at 3 mM MPA, the expression was decreased (Figure 4.6) ($p < 0.0001$). PA also reduced protein expression (not significantly) of H3K9me3 when compared to the control (Figure 4.6) ($p < 0.0001$).

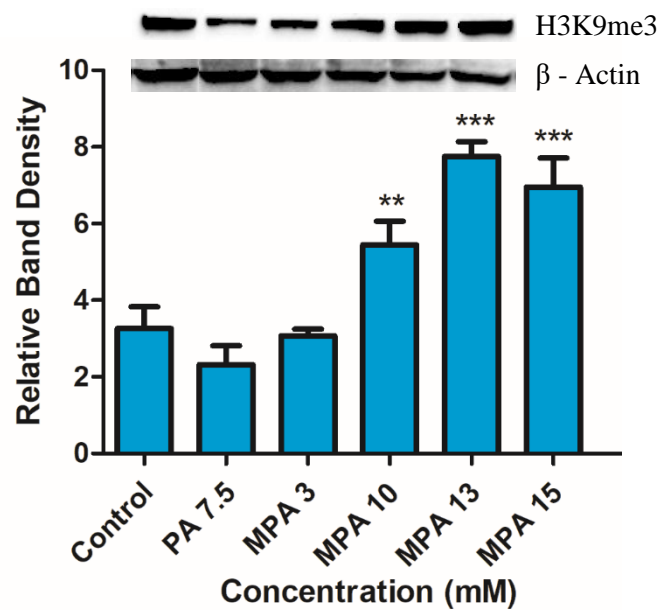


Figure 4.6: The effect of PA and MPA on of H3K9me3 in HepG2 cells (** $p < 0.001$, *** $p < 0.0001$).

CHAPTER 5

DISCUSSION

PA is a prime natural chelator for minerals such as zinc, iron, copper, manganese, chromium and molybdenum in the body. PA is produced in the liver and kidney, with quantities identified in human milk and the brain, and is transported to the pancreas from which it is secreted into the intestine during digestion. Studies in normal rat kidney cells have shown that PA reversibly halts growth of cultured cells (Fernandez et al., 1977). In an HIV study by Fernandez et al. (2001), cultured cells treated with PA showed cytotoxicity which leads to apoptosis. In contrast, PA was shown to inhibit neurotoxicity induced by quinolinic acid (Cockhill et al., 1992). For this study, cytotoxicity of PA and MPA in HepG2 cells was conducted using the WST-1 Assay. In this assay, WST-1 salt is cleaved to a purple coloured formazan and the amount of formazan produced directly correlates to the amount of metabolically active cells present. The average optical density results were used to construct a dose response curve (Figure 4.1) from which IC_{50} values for PA and MPA were obtained. The IC_{50} value for PA was 7.5 mM while the IC_{50} value for MPA was 13 mM. The two dose response curves have different shapes as PA induces toxicity from a log concentration of 0.5 mM while MPA induces toxicity from a log concentration of 1 mM (Figure 4.1). MPA seems to initially induce cell proliferation before displaying toxic effects at 1 mM log concentration. PA has a lower IC_{50} value (7.5 mM) compared to MPA (13 mM). MPA is a derivative of PA and indicates that PA is almost twice as potent to HepG2 cells compared to MPA.

DNA methylation is a process in which a methyl group is attached to carbon 5 of cytosine residues in a SAM mediated reaction. In contrast, DNA hypomethylation involves the removal of a methyl group and is used to describe the unmethylated state of CpG sites. Aberrant DNA methylation is a hallmark of cancer. DNA demethylation, caused by DNMT inhibitors, is extensive in cancer and is believed to mainly be involved in hypomethylation of repetitive sequences (Stefanska et al., 2011). DNA demethylation leads to induction of pro-metastatic genes and metastasis, suggesting a role for hypomethylation in cancer metastasis (Pakneshan et al., 2004; Shteper et al., 2003). Pharmacological and genetic evidence confirm that global DNA hypomethylation may compromise chromosomal and genomic instability (Chen et al., 1998; Gaudet et al., 2003). In this study, 5mC in HepG2 cells was measured after treatment with PA and MPA using the DNA methylation assay kit. In this assay, colorimetric quantification allowed the percent 5mC in samples to be determined. 5-aza-2-DC was used as a negative control and showed a decrease in DNA methylation compared to the control. The 5mC content seen in 5-aza-2-DC and MPA treated cells were quite similar. This could possibly indicate that 5-aza-2-DC and MPA share a similar mechanism of action. 5-aza-2-DC is a well-known DNA hypomethylating agent that incorporates directly into DNA and inhibits DNMT1 leading to inhibition

in DNA synthesis and cytotoxicity (Gowher and Jeltsch, 2004). DNA methylation is inhibited by 5-aza-2-DC as its acceptor carbon atom, from the methyl group, is replaced with a nitrogen atom at position 5 (Gowher and Jeltsch, 2004). Overall, PA and MPA both induced DNA hypomethylation in HepG2 cells compared to the control (Figure 4.2). A study by Li et al. (2013) showed that nicotinamide, which is structurally similar to PA, also decreased hepatic global DNA methylation. A study by Ghazi et al. (2019) showed that Fusaric Acid, a PA derivative, also induces global DNA hypomethylation in HepG2 cells. Although MPA showed reduced 5mC content, it seems to show increasing 5mC content with increasing concentration albeit still lower than the untreated control. This increase in 5mC content could be due to the methyl functional group present in MPA. As the MPA concentration increased, the methyl functional group may have contributed to the methylation status of MPA. This suggests that MPA treated HepG2 cells could possibly show increased methylation at a concentration higher than 15 mM. Looking at the cytotoxicity test conducted, HepG2 cells may barely survive in any MPA concentration greater than 15 mM (Figure 4.1). For this study it was hypothesized that MPA would promote DNA methylation, as it is the methyl derivative of PA, and lead to altered regulation of gene expression in human HepG2 liver cells. MPA alters regulation of gene expression; however, it induced DNA hypomethylation rather than hypermethylation in HepG2 cells.

MBD2 expression is linked to DNA hypomethylation, and hence the decrease in DNA methylation in PA and MPA treated HepG2 cells led to the investigation of the MBD2 transcript (Figure 4.3) and protein levels (Figure 4.4). MBD2, a DNA demethylase, removes methyl groups from nucleotides in DNA. MBD2 binds specifically to CpG islands in methylated DNA at position 5 of cytosines within CpG dinucleotides. Gene expression of MBD2 in PA and MPA treated HepG2 cells was evaluated by qPCR (Figure 4.3). PA showed very low *MBD2* gene expression compared to the control. MPA 3 mM displayed increased *MBD2* gene expression, while the other concentrations (10, 13 and 15 mM) of MPA downregulated *MBD2* expression, compared to the control. This could be explained by a process known as hormesis, where a low dosage of a toxin shows a favourable biological response (Mattson, 2008). Since the DNA hypomethylation observed in PA and MPA treated cells cannot be attributed to the decrease in MBD2 gene expression, MBD2 protein expression in PA and MPA treated HepG2 cells (Figure 4.3) was measured to determine whether DNA hypomethylation was induced as a result of MBD2 protein expression. In contrast to its gene expressions, PA and MPA both demonstrated increased MBD2 protein expression, compared to the control. Similarly, in the study conducted by Ghazi et al. (2019), FA decreased *MBD2* gene expression but increased MBD2 protein expression. Increased MBD2 protein expression by PA and MPA resulted in the removal of methyl groups from PA and MPA treated cells, which reduced 5mC in these cells and led to DNA hypomethylation in PA and MPA treated cells. These results suggest that PA and MPA induced global DNA hypomethylation by targeting MBD2 at its protein level. Depletion of MBD2 by antisense oligonucleotides lead to the

silencing of pro-metastatic genes as well as inhibition of invasiveness and metastasis of prostate and breast cancer cell lines (Pakneshann et al., 2004). To investigate the role of hypomethylation and its mechanism in cancer, Stefanska et al. (2011) created a knock down model of *MBD2* in HepG2 cells and SkHep1 cells. This model displayed that liver cancer hypomethylation targets promoters of specific genes encoding functional pathways essential for invasion and cell growth while partial reversal of this process leads to reversal of invasiveness and tumour growth (Stefanska et al., 2011).

Methylation of H3K9, especially in *Neurospora crassa*, requires DNA methylation to occur as numerous DNA methylation proteins interact directly with histone methylating enzymes (Freitag and Selker, 2005). Trimethylated H3K9 (H3K9me3) is an important repressive histone mark that plays a role in gene silencing. H3K9me3 is established by SUV39H1 (Dodge et al., 2004). SUV39H1 regulates H3K9me3 at pericentric heterochromatin (Fritsch et al., 2010). Aberrant histone modifications are associated with cancer development and progression (Zeng et al., 2010); however, the role of SUV39H1 and H3K9me3 in hepatocellular carcinoma (HCC) is yet to be determined. Apart from its association with methylated CpG sites, methyl-CpG binding proteins also bind to various chromatin modifying enzymes, such as histone deacetylases and histone lysine methyltransferases (Rose and Klose, 2014). Therefore, to determine further interactions of MBD2 by PA and MPA, the protein expressions of SUV39H1 (a histone lysine methyltransferase) and H3K9me3 were assessed by Western Blotting (Figure 4.5 and 4.6, respectively). Since H3K9me3 is regulated by SUV39H1, expression of these proteins should be directly proportional to each other, with H3K9me3 activity being dependent on SUV39H1 expression. PA showed a corresponding decrease in protein expression of H3K9me3 (Figure 4.6) with SUV39H1 (Figure 4.5). Protein expression of H3K9me3 in MPA treated cells, except at 3 mM, was increased (Figure 4.6), following a corresponding increase in SUV39H1 protein expression, except at 3 mM (Figure 4.5). SUV39H1 and H3K9me3, in MPA treated cells showed a dose-dependent increase in protein expressions. Since MBD proteins recruit histone modifiers, the increase in MBD2 protein expression in MPA treated cells resulted in recruitment of the histone lysine methyltransferase, SUV39H1, which also showed increased protein expression, except at 3 mM MPA. H3K9me3 is regulated by SUV39H1. Therefore, H3K9me3 also showed increased protein expression in MPA treated cells, except at 3 mM. In methyl deficient mice H3K9me3 and SUV39H1 expressions simultaneously increased with the progression from pre-neoplastic nodules to establish tumours of rat hepatocarcinogenesis (Pogribny et al., 2006).

CHAPTER 6

CONCLUSION

This study provides a mechanism for MPA induced epigenetic modifications in the human liver HepG2 cell line. MPA induces DNA hypomethylation in HepG2 cells. When bound to methylated DNA, methyl CpG binding proteins recruit histone deacetylases, methyltransferases and other proteins involved in chromatin remodelling. Increased MBD2 protein expressions stimulated its interaction with the methyltransferase SUV39H1. Upregulation of SUV39H1 promoted H3K9me3. These findings suggest a role for MPA in cancer development and progression. Interestingly, PA showed similar DNA methylation results (DNA hypomethylation, decreased *MBD2* gene and increased MBD2 protein expression) to its methyl derivative. However, PA displayed differing Histone methylation results (decreased SUV39H1 and H3K9me3 expression) to its methyl derivative. It is also notable that although there was an overall decrease in DNA methylation, DNA methylation in MPA treated cells did increase in a dose dependent manner. This suggests that MPA treated cells may show DNA hypermethylation at concentrations higher than 15mM, however according to the cell viability test, such high concentrations may prove detrimental to the liver cells.

Limitations of this study include the use of an *in vitro* liver model, where cells are removed from their natural environment, and the 24hr time exposure of cells to MPA treatment. In future it may be beneficial to test this study in an *in vivo* model to get a better understanding of the epigenetic modifications induced by MPA. It would also be interesting to determine the effects of MPA under 6hr, 48hr and 72hr exposure periods to investigate its effects on acute, sub-chronic and chronic conditions, respectively. Future work could also include effects of MPA on promoter methylation of DNMTs and MBD2.

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APPENDIX A

Standard curve for Protein Isolation

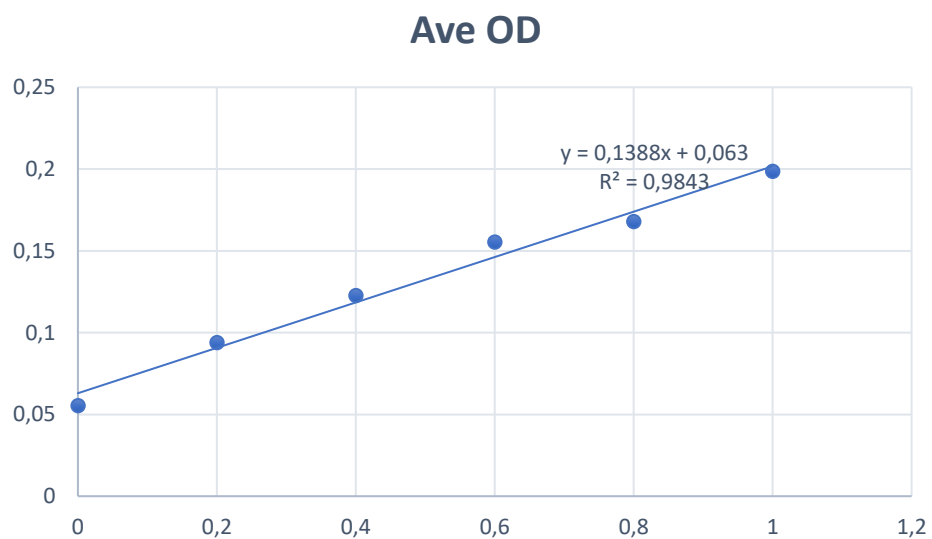


Figure 5: Standard curve used to calculate the concentration of proteins in samples, from known concentrations of Bovine Serum Albumin (BSA).

APPENDIX B

qPCR results obtained for DNA methyltransferases (DNMTs) and miR29b

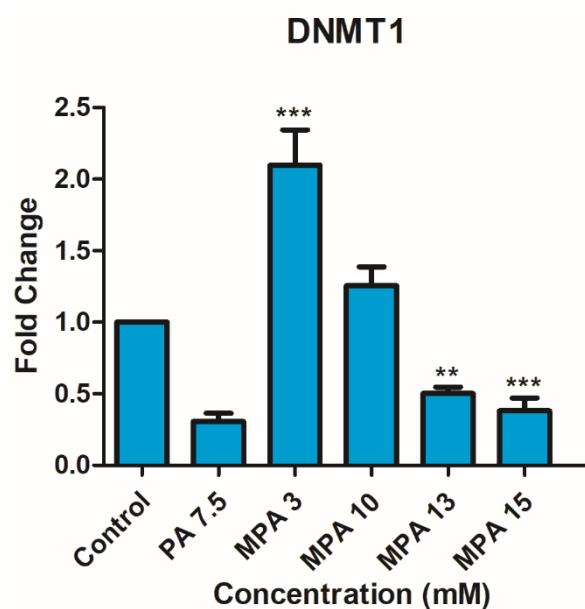


Figure 6.1: Gene Expression of DNMT1 was increased at MPA concentrations 3mM and 10mM but decreased at MPA concentrations 13mM and 15mM (** $p < 0.01$, *** $p < 0.0001$).

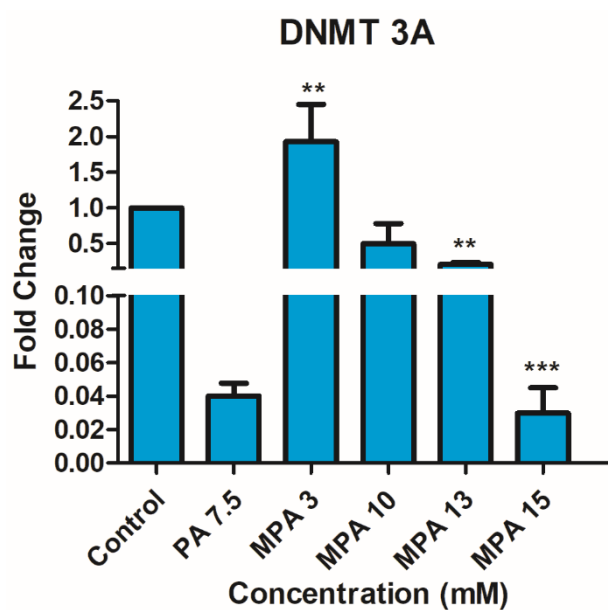


Figure 6.2: Gene Expression of DNMT3A increased at MPA concentration 3mM but decreased for all remaining MPA concentrations (** $p < 0.01$, *** $p < 0.0001$).

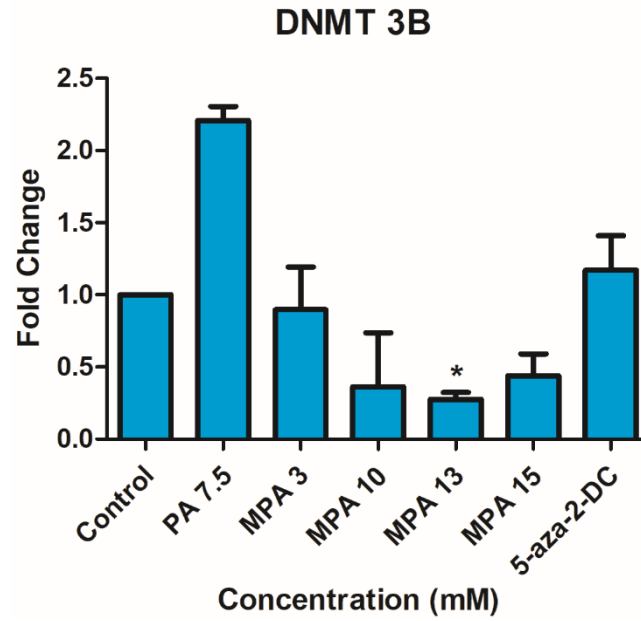


Figure 6.3: Gene expression of DNMT3B decreased for all concentrations of MPA (* $p < 0.05$).

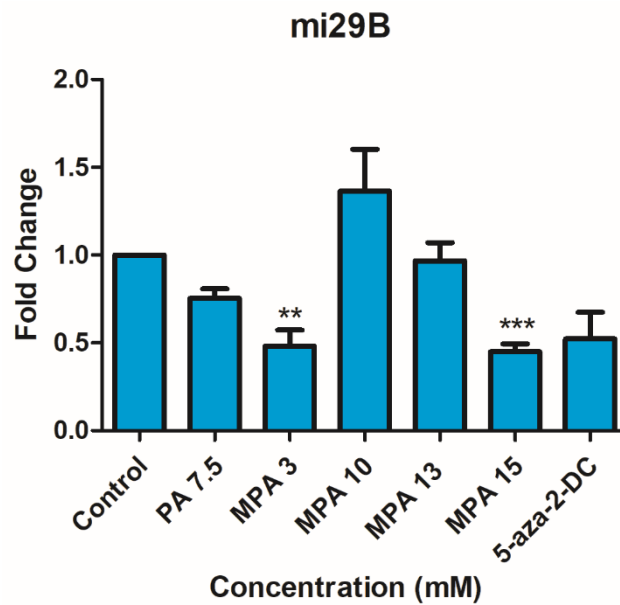


Figure 6.4: Gene expression for miR29b decreased at MPA concentrations 3mM and 15mM, increased at 10mM and remained relatively constant at 13mM (** $p < 0.001$, *** $p < 0.0001$).

Table 3.3: Primer sequences for DNMT1, DNMT3A, DNMT3B, miR29b and RNU6 genes used in qPCR

Gene	Accession No.	Annealing Temperature	Primer	Sequence
DNMT1	NM_001130823	60°C	Forward	5'-ACCGCTTCTACTTCCTCGAGGCCTA-3'
			Reverse	5'-GTTGCAGTCCTCTGTGAACACTGTGG-3'
DNMT3A	NM_175629	58°C	Forward	5'-GGGGACGTCCGCAGCGTCACAC-3'
			Reverse	5'-CAGGGTTGGACTCGAGAAATCGC-3'
DNMT3B	NM_006892	60°C	Forward	5'-CCTGCTGAATTACTCACGCCCC-3'
			Reverse	5'-TAAGCCAAACAGCAGGGTTCTT-3'
miR29b		55°C		Universal primer sequences
RNU6 (human)				

APPENDIX C

Protein Expression of KDM5B

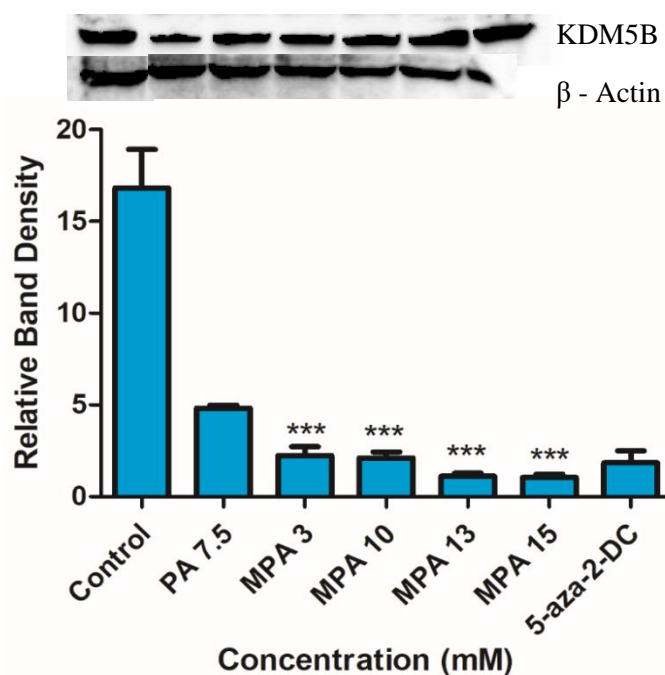


Figure 7: KDM5B showed significant decrease in protein expression for all concentrations of MPA in HepG2 cells (***) $p < 0.0001$).

Lysine specific demethylase 5B (KDM5B) is a histone demethylase which specifically demethylates lysine 4 of histone H3 (H3K4) causing gene repression. It demethylates trimethylated, dimethylated and monomethylated H3K4 and plays a major role in defining the histone code.

Table 3.4: Antibody and dilution used for KDM5 in Western Blotting

	Antibody	Dilution	Catalogue Number
Primary Antibody	Rabbit Anti-KDM5B	1:1000 in 5% BSA	ab198884
Secondary Antibody	Goat Anti-rabbit IgG HRP	1:3000 in 5% BSA	Sc-2004 (Santa Cruz)
Housekeeping Gene	Anti- β -actin	1:5000 in 5% BSA	A3854 (Sigma-Aldrich)