AN INVESTIGATION INTO THE BIOCHEMICAL, MOLECULAR AND EPIGENETIC EFFECTS OF FUMONISIN B_1 IN LIVER (HEPG2) CELLS

By

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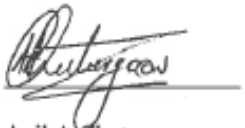

Anil A Chuturgoon

13/03/2015

Date

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Dedication

I am deeply indebted to my Spritual Master and Guru– Shree Ramakrishna for always guiding me on the path of Righteousness and Truth.

“Our duty is to encourage every one in his struggle to live up to his highest idea, and strive at the same time to make the ideal as near as possible to the Truth.”

Swami Vivekananda

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My parents

Although you did not have an opportunity to educate yourself – you instilled in me the value of good education and I appreciate all the sacrifices you made to allow me to pursue my dreams.

PUBLICATIONS

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3. Chuturgoon, A.A., Phulukdaree, A., and Moodley, D. (2014). Fumonisin B_1 inhibits apoptosis in HepG2 cells by inducing Birc-8/ILP-2. Toxicology Letters (*In press*, February 2015)
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PRESENTATIONS

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ABSTRACT

Fumonisins are carcinogenic mycotoxins that occur world wide in maize and maize-based products intended for human consumption. Consumption of fumonisin-contaminated maize as a staple diet has been associated with oesophageal and liver cancer in South Africa and China. Fumonisin B_1 (FB_1) inhibits sphingolipid biosynthesis and has been implicated in cancer promoting activity in animals and humans. FB_1 disrupts DNA methylation and induces chromatin modifications in human hepatoma (HepG2) cells. In this study FB_1 (IC_{50}=200μM) altered liver enzyme expression of DNA methyltransferases and demethylases. DNA methyltransferase activities of DNMT1, 3a and 3b were significantly decreased, whilst both DNA methylase (MBD2) activity and expression was significantly up-regulated resulting in global DNA hypomethylation. In addition the histone demethylases, *KDM5B* and *KDM5C,* expression was increased. FACS data confirmed FB_1 significantly increased global DNA hypomethylation – a process that causes chromatin instability. Next the effect of FB_1 on miRNA expression was evaluated; FB_1 significantly down-regulated (11 fold) expression of miR-27b. MiR-27b modulates expression of human cytochrome P_{450} (CYP1B1) that catalyzes the metabolic activation of many procarcinogens. In order to directly assess the effect of miR-27b on *CYP1B1* mRNA levels, liver cells were transfected with the mimic to miR-27b. CYP1B1 mRNA and protein expression was significantly up-regulated by 1.8- fold and 2.6- fold respectively. CYP1B1 is post-transcriptionally regulated by miR-27b suggesting that FB_1- induced modulation of miR-27b in hepatic cells may be an additional mode of hepatic neoplastic transformation. Finally, the effect of FB_1 on the apoptotic pathway in HepG2 cells was investigated using an mRNA expression array panel of pro- and anti- apoptotic molecules. FB_1 significantly increased an AIP family member - BIRC-

8/ILP-2 (8-fold) in an apoptosis array. In addition, ILP2 protein expression was increased (2.3-fold) with a corresponding decrease in Smac/DIABLO protein levels (1.7-fold). Further analysis showed an FB_1 (0μM, 50μM, 100μM, 200μM) dose-dependent increase in BIRC-8/ILP-2 mRNA and protein expression in HepG2 cells. This data suggests that FB_1 modulates apoptosis in a complex dose-dependent regulation of pro- and anti-apoptotic molecules – and it is not a matter of simply switching on or off.

In conclusion, the data shows that FB_1 possess epigenetic properties by inducing global DNA hypomethylation, modulating miRNA expression, and increasing expression of the AIP protein family (BIRC8/ILP-2) that may lead to liver tumourigenesis.

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ABBREVIATIONS

AIF	Apoptosis-inducing factor
AIP	Apoptosis-inducing protein
APAF	Apoptotic protease activating factor
Bid	BH3 interacting domain death
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
BIRC8	Baculoviral IAP repeat-containing protein3
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domains
CCM	Complete culture medium
CDK	Cyclin-dependent kinase
cDNA	copy DNA
CER	Ceramide
CpG	-Cytosine-phosphate-Guanine-
CSL	Complex sphingolipid
Ct	Comparative threshold cycle
CYT	Cytochrome
DAPK1	Death-associated protein kinase 1
DDCt	Delta delta comparative threshold cycle
DED	Death effector domain
DGCR8	DiGeorge syndrome critical gene 8
DICER	Endonuclease DICER

DISC	Death inducing signalling complex
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DROSHA	Rnase III enzyme
DRs	Death receptors
EDTA	Ethylenediaminetetraacetic acid
ELEM	Equine leucoencephalomalacia
ELISA	Enzyme -linked immusorbent assay
EXP5	Exportin5
FACS	Fluorescent activated cell sorting
FADD	Fas associated protein with death domain
FB_1	Fumonisin B_1
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
H3K4	Histone 3 lysine 4
H3K4Me2	Histone 3 lysine 4 dimethyl
H3K4Me3	Histone 3 lysine 4 trimethyl
HCC	Hepatocellular carcinoma
Hr	Hour
IAP	Inibitor of apoptosis protein
IARC	International Agency for Research on Cancer
ILP	Inhibitor of apoptosis-like protein
JmJC	Jumonji C

KDM	Histone lysine demethylase
LMPA	Low melting point agarose
MBD2	Methyl-CpG-binding domain 2/DNA methylase
5-MeCyt	5-Methyl Cytosine
Min	Minute
miRNA	MicroRNA
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PPO	Pulmonary porcine oedema
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real time PCR
RAN-GTP	Ras related nuclear binding protein-guanine triphosphate
RISC	RNA-induced silencing complex
RT	Room temperature
Sa	Sphinganine
SAM	Significance of analysis of microarray
SDS	Sodium dodecyl sulphate
Smac/DABLO	Second mitochonria-derived activator of caspases/ Direct inhibitor of apoptosis binding protein with low pI

So	Sphingosine
TAR	Trans-activation response element
tBid	Truncated BH3 interacting domain death
TCA	1,2,3-Tricarboxylic acid
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF	TNF receptor associated factor
TRBP	Trans-activation response RNA binding protein
TTBS	Tris-buffered saline
USFDA	United States Food and Drug Adminsitration
UTR	Untranslated region
WHO	World Health Organisation

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CHAPTER 1

INTRODUCTION

Fusarium verticillioides is a ubiquitous soil fungus that infects agricultural crops worldwide especially maize. Fumonisins (series A, B and C) are the major class of mycotoxins produced by this fungus, and they all resemble sphingolipids (Merrill et al., 1993). Fumonisin B_1 (FB_1) inhibits sphingosine N-acetyltransferase and blocks sphingolipid biosynthesis. FB_1 has toxicological significance as it is present in high concentrations in contaminated crops such as healthy maize plants (Gelderblom et al., 2001).

Human populations ingesting maize that is contaminated by *F. verticillioides* display a higher incidence of primary liver cancer (Ueno et al., 1997) and in South Africa and China such populations also display a higher incidence of oesophageal cancer (Marasas, 1996). In laboratory experiments, rats developed primary hepatocellular carcinoma after being fed a diet containing 50 ppm FB_1 (Gelderblom et al., 1992, Gelderblom et al., 1994). Further, FB_1 was found to be a potent tumour promoter in rat liver after initiation with diethylnitrosamine (Gelderblom et al., 1988). FB_1 cancer promoting activity occurred at levels that did not induce liver or kidney toxicity and did not initiate cancer. In summary, these results demonstrate that FB_1 possesses carcinogenic properties; a US Food and Drug Adminstration (USFDA) study showed that FB_1 was a kidney carcinogen in rats and a liver carcinogen in mice, respectively (Howard et al., 2001).

Several *in vivo* studies demonstrated that FB_1 induced apoptosis in rat kidney and liver (Tolleson et al., 1996, Sharma et al., 1997). In addition, FB_1 induced apoptosis in several mammalian cell lines (Tolleson et al., 1996; Wang et al., 1996; Schmelz et

al., 1998, Ciacci-Zanella and Jones, 1999). However, neoplastic African green monkey kidney cells (COS-7) were resistant to apoptosis by FB_1 as compared to normal African green monkey kidney cells (CV-1) (Wang et al., 1996; Ciacci-Zanella et al., 1998). FB_1 failed to induce apoptosis in a transformed human leukaemia cell line (Ciacci-Zanella et al., 1998) suggesting that transformed cells may be resistant to its apoptotic effects. The ability of cells to escape apoptosis is important in the development of cancer, whilst the ability of FB_1 to induce apoptosis may select for cells resistant to apoptosis.

In a study on FB_1-induced apoptosis in CV-1 and normal MEF mice derived cells, cyclin-dependent kinase (cdk) activity increased (not significantly) after three hours, but then declined (Ciacci-Zanella et al., 1998). During the early stages of apoptosis a transient increase in cdk activity occurs and this was demonstrated *in vitro* after FB_1 treatment (Zhou et al., 1998).

Several pathways resulting in apoptosis have been identified (White, 1996). A study on FB_1 induced apoptosis in CV-1 cells demonstrated that a baculovirus gene (CpIAP) inhibited apoptosis (Jones et al., 2001). CpIAP blocks apoptosis induced by the tumour necrosis (TNF) pathway. Further support for the TNF signal transduction pathway in FB_1 -induced apoptosis was the cleavage of caspase-8. CpIAP inhibits caspase-8 cleavage and thus prevents FB_1 -induced apoptosis (Jones et al., 2001).

Fumonisin B_1 exerts its toxic effects by disruption of sphingolipid biosynthesis and accumulation of sphinganine which plays a major role in the modulation of apoptosis and cell proliferative pathways related to cancer development (Riley et al., 2001; Gelderblom and Marasas 2012; Muller et al., 2012). FB_1 also disrupts the oxidative status of liver cells that result in lipid peroxidation (Abel and Gelderblom, 1998).

Fumonisin B_1 is considered to be a non-genotoxic (epigenetic) carcinogen in risk assessment studies (Gelderblom et al., 2008; Muller et al., 2012). FB_1 lacked DNA interactive reactivity in different short-term genotoxicity assays using bacteria (Gelderblom and Snyman 1991; Knasmuller et al., 1997; Aranda et al., 2000) and *in vivo* and *in vitro* DNA repair assays in rat liver and primary hepatocytes (Gelderblom et al., 1992; Norred et al., 1992; Domijan et al., 2006; Domijan et al., 2007; Domijan et al., 2008).

Epigenetic mechanisms include promoter DNA methylation, histone modifications and microRNAs play an important role in the modulation of functional pathways that are key to neoplastic development. In general, epigenetic events occur early in neoplasia and many tumour suppressor genes in tumour cells are inactivated by epigenetic mechanisms. DNA methylation, a well-known primary epigenetic regulator of gene expression, is an important event involved in chemical carcinogenesis (Moggs et al., 2004; LeBaron et al., 2010). Several studies showed that folate deficiency by FB_1 can cause disruption of DNA methylation and FB_1-induced carcinogenesis (Stevens and Tang 1997; Abdel Nour et al., 2007).

A study on rat C6 Glioma cells showed that FB_1 (at concentrations of 9 – 18μM) displayed epigenetic properties by inducing hypermethylation of DNA, whilst FB_1 concentrations of 27 and 54μM FB_1 decreased methylation (Mobio et al., 2000). Another study on a human colon cancer cell line (Caco-2) showed that FB_1 (10, 20 and 40μM) induced hypermethylation, with the 10 and 20μM FB_1 concentrations, however,at 5μM FB_1 concentration– a slight hypomethylation effect was observed

(Kouadio et al., 2007). In these studies the observation of hypermethylation was reported and no mechanism was provided.

Hepatocellular carcinoma (HCC) is the 6th most common cancer in the world (Ferlay et al., 2010). In high risk HCC areas, such as sub-Saharan Africa and eastern Asia, major risk factors include chronic hepatitis B virus infection and consumption of foods contaminated with mycotoxins. The carcinogenic potential on FB_1 in humans is a topic of continuing research. The USFDA published guidelines recommending that total fumonisins in human food be limited to 2ppm (Administration, 2001).

It was hypothesised that FB_1 possesses epigenetic properties that disrupts DNA meyhylation; suppresses microRNA expression involved in neoplastic transformation; and inhibits apoptosis in human hepatoma cells.

To test this hypothesis, the study aimed to determine the epigenetic and apoptotic effects of FB_1 on human liver (HepG2) cells. The epigenetic effects of FB_1 in hepatocytes were determined by measuring the global DNA methylation status and microRNA profile changes and the biological effects related to cell death. The specific objectives of the study were to investigate the effects of FB_1 on:

- Methylation, especially DNA methyltransferase and demethylase enzymes involved in chromatin maintenance and gross changes in structural integrity of DNA
- MicroRNA profile(s) induced and their significance to liver cells, especially neoplasia
- The apoptotic pathway and the mechanism of induction/inhibition

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

2.1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by fungi and are capable of causing disease or death in humans and various animal species. Fungi are usually plant and insect pathogens and a small number are classified as disease causing agents in vertebrates. Fungi exert detrimental effects either via mycoses or mycotoxicosis (Bennett and Klich, 2003). Mycosis is regarded as 'superficial' as this is simply the growth of the fungus on a host, e.g., athletes foot. Mycotoxicosis caused by secondary fungal metabolites, however, is more severe and is often regarded as 'poisoning by natural means'. Mycotoxicosis is common in underdeveloped nations as it arises from the exposure and consumption of fungal contaminated food. Although the number of people affected by mycosis and mycotoxicosis is not as overwhelming as compared to viral, protozoan and bacterial infections, it is still regarded as a serious international health problem (Bennett and Klich, 2003).

There are approximately four hundred compounds that have been classified as mycotoxins since 1960 following the outbreak of 'turkey X' syndrome caused by aflatoxin B_1, a secondary metabolite produced by *Aspergillus flavus*. The fumonisins were described and characterised in 1988 and has created controversy about the occurrence and effects of these mycotoxins due to their hydrophilic nature (Bennett and Klich, 2003).

2.1.2. Fumonisin

Fumonisins are naturally occurring secondary metabolites produced predominantly by *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg (Marasas, 2001). These mycotoxins occur world wide in many agricultural grains, especially in maize (*Zea mays* L.) and maize-based products intended for human and animal consumption (Shephard et al., 1996). Poor storage conditions is the leading cause of contamination, where fungi favour moist, humid and dark conditions to grow (Heidtmann-Bemvenuti, 2011). To date, 28 fumonisin analogues have been identified and categorised into A, B, C and P series (Rheeder et al., 2002). Fumonisin B_1, B_2, and B_3 (FB_1, FB_2 and FB_3) are the most abundant naturally occurring fumonisins (Figure 2.1) of which FB_1 is the most significant analogue. FB_1 is also the major contaminant mycotoxin in cereal grains such as maize (Shephard et al., 1992; Rheeder et al., 2002). The FB series have a free amine group at the C-2 position in contrast to the FA series that are acetylated on the amino group at the same position; the FC series have no methyl group at the C-1 position and the FP series have a 3-hydroxypyridinium group at the C-2 position (Bezuidenhout et al., 1988; Musser et al., 1996).

2.1.21. Fumonisin B_1

Fumonisin B_1, the most toxic of the fumonisins, has a wide range of toxic effects in both humans and animals. It has also been classified as a possible class 2B carcinogen by the International Agency for Research on Cancer (IARC). South Africans are continuously exposed to doses of FB_1 with an estimated daily dietary intake of 200µg/kg body weight (Domijan and Abramov; 2011).

2.1.2.2. Chemical structure

The chemical structure of FB_1 is based on a hydroxylated hydrocarbon chain rather than a cyclic structure most commonly associated with mycotoxins (Shephard et al., 1996). FB_1 is a diester of propane-1,2,3-tricarboxylic acid (TCA) and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane, in which the C_{14} and C_{15} hydroxyl groups are esterfied with a terminal carboxyl group of TCA (Figure 2.1A) (Soriano et al., 2005; Heidtmann-Bemvenuti, 2011). The chemical structure of FB_1 resembles the sphingoid bases: sphinganine (Sa) and sphingosine (So) (Figure 2.1 B and C) - important precursors in sphingolipid biosynthesis. The sphingoid bases consist of an amine and two alcohol groups attached to a 3C chain that is attached to a long hydrocarbon chain (Figure 2.1B and C). This structural similarity is critical for FB_1 toxicity because it disrupts sphingolipid metabolism. The free amino group in FB_1 (Figure 2.1A) creates an amino pentol backbone that is important in FB_1 toxic activity where it competes with the sphingoid bases in the biosynthesis of sphingolipids (Soriano et al., 2005).

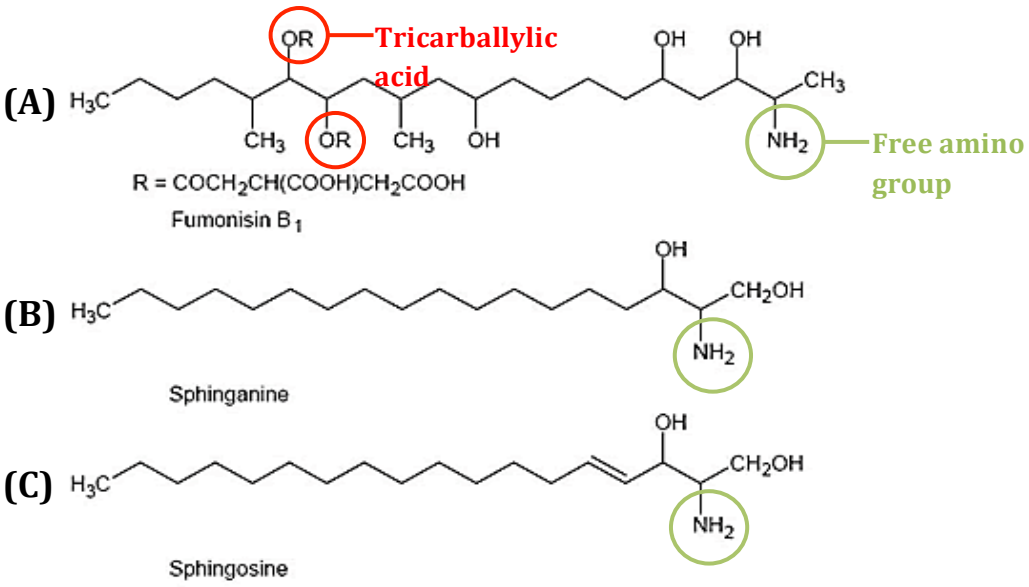

Figure 2.1: Chemical structure of (A) Fumonisin B_1 and its similarities to sphingoid bases (B) Sphinganine ad (C) Sphingosine (Griessler 2008).

2.1.3. Absorption, Metabolism and Distribution of Fumonisin B_1

Fumonisin B_1 is a polar mycotoxin and is a 1, 2, 3-Propanetricarboxylic acid, 1, 1'-[1-(12-amino-4, 9, 11-trihydroxy-2-methyl-tridecyl)-2-(1-methylpentyl)-1, 2-ethane-diyl] ester. The structure of FB_1 was elucidated by Bezuidenhout et al. (1988) (Bezuidenhout et al., 1988). The polarity of the molecule is due to the presence of hydroxyl groups and tricarballylic acid side chains (Figure 2.1) (Carratu et al., 2003). Fumonisin B_1 has not been found to be absorbed via the pulmonary system although the potential exists due to the presence of the toxin in mycelia, spores and conidiophores (Tejada-Simon, 1995). The absorption of FB_1 through skin is negligible because FB_1 is a polar molecule and thus very water soluble, and therefore would be unable to penetrate the lipid bilayer of cells in undamaged skin. The main

site of absorption, however, is in the small intestine following consumption of contaminated maize and maize based products (Shephard et al., 1996).

There is little evidence of FB_1 metabolism, but the minor metabolism of FB_1 involves the partial and complete hydrolysis of the toxin which is thought to occur in the intestine, by intestinal microflora. The hydrolysis of FB_1 results in the removal of either one or two propane tricarboxylic acid residues (Shephard et al., 1994). The hydrolysis metabolites are easily absorbed in the intestine but a major portion of the toxin remains unmetabolised in the feaces and excretory products. Although the presence of FB_1 in the organism results in increased induction of Cytochrome (Cyt) P_{450}-1A1 and Cyt P_{450}-4A1 activities, there is no evidence that the enzyme system is responsible for biotransformation of the toxin (Martinez-Larranaga et al., 1996).

The poor absorption of FB_1, rapid distribution to tissues and elimination is common to several species of animals including rodents, pigs, vervet monkeys, cows, rats and hens.

In rodents, absorption of FB_1 treated with an oral dosage of 10mg/kg body weight was shown to be approximately 3.5% of the dose reaching a maximum plasma concentration of 0.18μg/ml. The distribution of the absorbed dose was matched to a two compartment open model which displayed elimination half-lives of 3.15hrs for plasma, 4.07hrs for the liver and 7.07hrs for kidney (Martinez-Larranaga et al., 1999). This study concluded that FB_1 accumulated in the liver and kidney with ratios of 2.03 and 29.9 compared to plasma concentration. In 2006 Riley and Voss confirmed these findings in an experiment where rodents were fed a fumonisin-spiked diet over three weeks. Using HPLC it was determined that the concentration of the toxin in the kidney was ten-fold greater than that of the liver (Riley and Voss, 2006).

Prelusky et al. (1994, 1996) analysed the bio-distribution of FB_1 in pigs and showed a similar pattern to that determined in the rodent model (Prelusky et al., 1994; Prelusky et al., 1996). The absorption and elimination was rapid and low tissue residues were detected. The pigs in this study were dosed intravenously with 0.4mg/kg body weight of radiolabelled FB_1. Recovery of the toxin from urine was determined to be 0.76 to 0.83 of the dose after 72hrs. Tissue recovery was approximately 0.12 of the dose with highest activities in the liver and kidney. Results from intragastric dosing of 0.5mg radiolabelled FB_1/kg body weight however, demonstrated that the bioavailability was approximately 0.041 of the dose (Prelusky et al., 1996) and 0.005 of the dose was excreted in the urine. The studies by Prelusky concluded that absorption of FB_1 from feed is low, but is retained in tissue over a prolonged period of time, and accumulates in the kidney and the liver preferentially (Prelusky et al., 1996). To date, no such data on human is available.

Studies conducted in ruminants suggest that FB_1 is taken up poorly, with 0.80 of the total dosage recovered unmetabolised in feaces, poorly taken up by tissues and diminutive amounts found in urine (Prelusky et al., 1995; Smith and Thakur, 1996). Similarly, intravenous administration of radiolabelled FB_1 (2mg/kg body weight) to laying hens was rapidly eliminated with very low distribution to tissues, particularly the liver and kidneys.

The small amount of FB_1 which is absorbed in animal systems accumulates in the kidney and liver tissue (Norred et al., 1992; Shephard et al., 1992; Prelusky et al., 1994; Prelusky et al., 1996; Smith et al., 1996) resulting in hepatotoxicity and nephrotoxicity.

2.1.4. Mechanism of Action of Fumonisin B_1

Fumonisin B_1 is a structural analogue of sphinganine and sphingosine, important substrates in sphingolipid metabolism. The biochemical mode of action of FB_1 includes the disruption of sphingolipid metabolism (Wang et al., 1991). In order to understand the mechanism of toxicity of FB_1 it is important to understand the biosynthesis and biological function of sphingolipids.

Sphingolipid biosynthesis occurs *de novo* through a series of reactions (Merrill and Jones, 1990). The condensation of serine with palmitoyl-CoA catalysed by serine palmitoyltransferase results in the formation of 3-ketosphinganine which is reduced to sphinganine (Sa) using NADPH. Sphinganine is then acylated to dihydroceramides by ceramide (CER) synthase by the utilisation of fatty acyl-CoA's, or phosphorylated to form sphinganine-1-phosphate. The desaturation of dihydroceramide results in the production of CER. Further conjugation of phosphocholine or oligosaccharides to ceramide produces complex sphingolipids (CSL). Catalytic reactions of complex sphingolipids during turnover involve the hydrolysis of sphingolipids producing CER, followed by the hydrolysis of CER to produce sphingosine (So) (Figure 2.2).

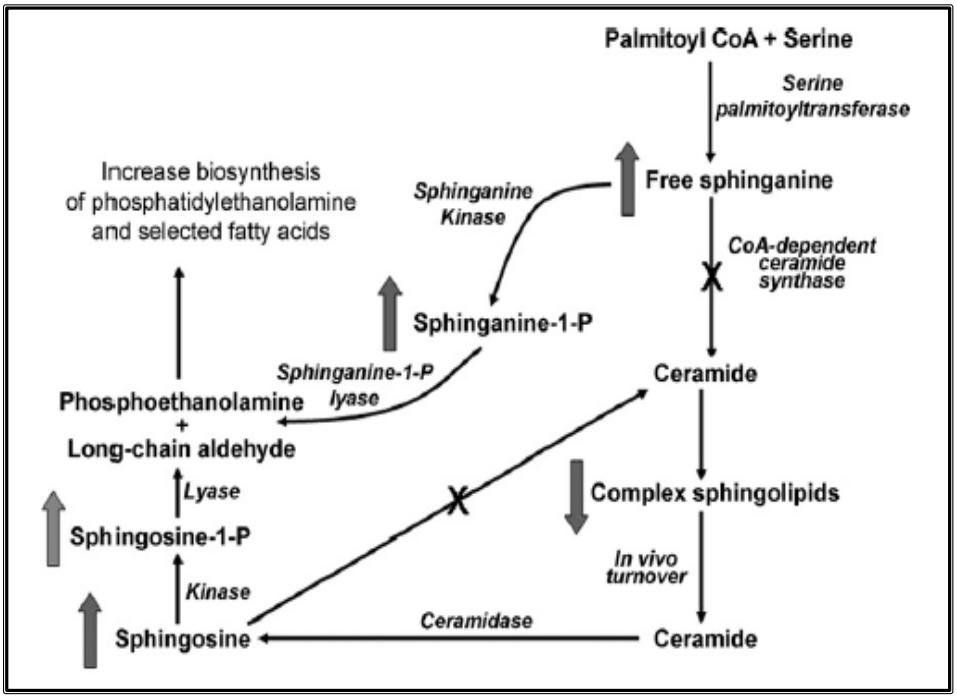

Figure 2.2: The *de novo* synthesis of sphingolipids in animal cells; the points at which Fumonisin B_1 disrupts this process indicated by X (Voss et al., 2006).

Fumonisin B_1 disrupts the metabolism of sphingolipids by competitive inhibition of CER synthase (Figure 2.2). The aminopentol (AP1) backbone of FB_1 competes for the sphingoid base substrate and the anionic tricarballylic acids interfere with fatty acyl-CoA binding. Microflora in primate intestines have the ability to hydrolyse FB_1 to AP1 (Merrill et al., 2001). Ceramide synthase inhibition causes an accumulation of Sa and So, a decrease in CER and concentration-dependant depletion of CSL. Sphingoid bases, CER and sphingolipids are important structural molecules of cell membranes and are regulators of cellular functions.

The consequences of the inhibition of ceramide biosynthesis for cells are:

- Cell death normally induced by CER
- Ceramide mediated apoptosis of oxidant damaged cells to avert accumulation of damaged DNA in cells

- Activation of mitogen activated protein kinases in Swiss 3T3 cells
- Promotion of free sphingoid base accumulation
- Dephosphorylation of retinoblastoma protein, repression of cyclin dependent kinase (CDK) 2 and induction of CDK inhibitors in CV-1 cells
- Apoptosis inhibitor and protease inhibitor protection of CV-1 cells and primary human cells from FB_1 induced apoptosis (Riley et al., 2001).

2.1.5. Elimination of Fumonisin B_1

Excretion of FB_1 after intra peritoneal dosage appeared to be different in rats and pigs. In rats, the initial elimination is rapid with a half-life of 10-20mins (Norred et al., 1993; Shephard et al., 1994). In the pig, the half-life is described to be a three compartment model, with values of 2.5, 10.5 and 183mins. The enterohepatic circulation is important for the removal of FB_1 using bile, as studies show that the absence of this path of elimination increases the half-life of the toxin to 96mins (Marasas et al., 2004).

2.1.6. Toxic effects of Fumonisin B_1 *in viv*o and *in vitro*

Reported animal diseases due to FB_1 exposure include the fatal neurotoxic syndrome, equine leukoencephalomalacia (ELEM) (Marasas et al., 1988), porcine pulmonary oedema (PPO) syndrome (Kriek et al., 1981), hepatotoxicity in non-human primates (Kriek et al., 1981), and hepatotoxicity in mice and nephrotoxicity and hepatoxicity in rats (Tolleson et al., 1996).

The ELEM syndrome has been shown to be specific to equids but there are studies that have been conducted on rabbits which showed brain lesions and haemorrhaging. The characteristic feature of ELEM is the presence of liquefactive necrotic lesions in

the white matter of the cerebrum. Marasas et al. (1988) showed that grey matter may also be affected (Marasas et al., 1988).

Kriek et al. (1981) were the first to report PPO following an experimental trial in which horses, sheep, rats, baboons and pigs were exposed to culture material of *F. verticilloides* (Kriek et al., 1981). The hypothesised mechanism of PPO is by the dysfunction of pulmonary interstitial macrophages resulting in the release of vasoactive mediators (Haschek et al., 1992). Studies conducted to screen maize on different farms on which PPO outbreaks occurred, showed a strong correlation between maize fungal contamination and PPO prevalence (Osweiler et al., 1992). In pigs, the kidney was also a target of fumonisin toxicity (Colvin et al., 1993; Harvey et al., 1995).

During a non-human primate study, three baboons were fed *F. verticilloides* culture material. Two of the baboons died of acute congestive heart failure, while the third survived up to 720 days at which point it was sacrificed. An autopsy of the third baboon revealed liver cirrhosis (Kriek et al., 1981). Vervet Monkeys fed *F. verticilloides* culture material over 180 days showed chronic hepatotoxicity which resulted in an increase in serum cholesterol, plasma fibrinogen and factor VII (Fincham et al., 1992).

Numerous acute and chronic studies on rats and mice demonstrated FB_1 induced hepatoxicity, nephrotoxicity, immunotoxicity, reproductive toxicity, embryotoxicity, teratogenicity, mutagenicity and carcinogenicity.

Voss et al. (1995) showed that FB_1 produced kidney lesions in rats and mice. The proximal tubule located in the outer medulla was found to have lesions in both male

and female rats; however the concentration of FB_1 that resulted in these lesions was different between males and females (Voss et al., 1995).

Balb/C mice exposed to a range of FB_1 concentrations (0.25 – 6.25mg/kg body weight per day) by subcutaneous administration showed a dose-dependent increase in apoptosis in the liver and the kidney (Sharma et al., 1997).

Apoptotic renal tubule epithelial cells in Fischer-344 rats were observed by Tolleson et al. (1996) following exposure to 0, 99, 163, 234 and 484mg FB_1/kg in the diet (Tolleson et al., 1996).

Fumonisin B_1 has also been shown to display genotoxic properties using human derived hepatoma cells, human fibroblasts, rabbit kidney cells (Galvano et al., 2002), rat C6 glioma cell (Rumora et al., 2007) rat astrocytes and p53 null mouse embryo fibroblasts (Mobio et al., 2000).

2.1.7. Carcinogenic potential of Fumonisin B_1

Treatment of rats with FB_1 caused acute and chronic liver toxicity, bile duct proliferation, fibrosis further developing into cirrhosis, cholangiogenesis and hepatocellular carcinoma and/or cholangiocarcinoma (Gelderblom et al., 1988; Voss et al., 1993). Although the findings regarding the FB_1-related tumour genesis in humans are speculative, FB_1 has been associated with the high incidences of oesophageal cancer endemic in Transkei, South Africa (Dutton, 1996) and in China (Li et al., 1980). A different form of tumour, primary liver cancer, was also linked to FB_1 consumption in Haimen province, China (Li et al., 1980). The carcinogenic properties of FB_1 observed in rodents are not associated with its direct genotoxicity. As a potent promoter of apoptosis, FB_1 causes further regeneration that is

characterized by increased cell proliferation in affected tissues. Consequently, the constant increase of DNA replication in exposed tissues may increase the likelihood of cancer induction (Riley et al., 2001). The International Agency for Research on Cancer (IARC) has declared toxins of *F. verticillioides* (including FB_1) as potential human carcinogens graded as class 2B (Vainio et al., 1993).

Studies from South Africa, Iran and China indicate a correlation between the prevalence of oesophageal cancer in humans (Figure 2.3) and the consumption of maize containing high levels of FB_1 (Wilson et al., 1990; Chu and Li 1994; Yoshizawa et al., 1994; Mosavi-Jarrahi and Mohagheghi, 2006; Eslick 2009). South Africans are continuously exposed to low doses of FB_1 with a daily dietary intake of 200µg/kg body weight (Domijan and Abramov, 2011).

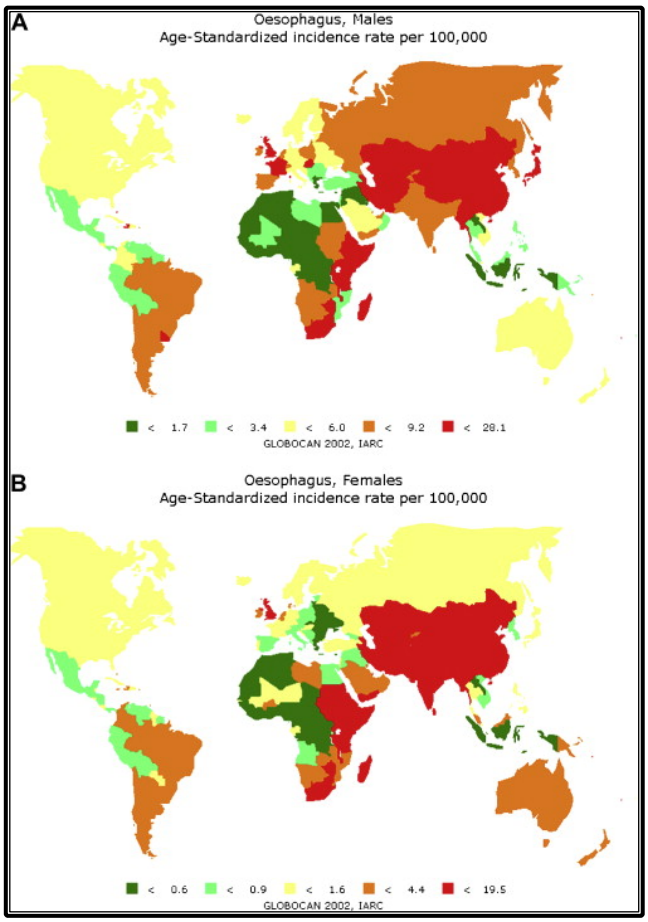

Figure 2.3 The geographical representation of oesophageal cancer incidence between males and females in 2009; A: incidence of oesophageal cancer in males, B: incidence of oesophageal cancer in females (Eslick, 2009).

The challenge in toxicology research is integration of knowledge from diverse fields novel technological developments in the biological and medical sciences.

2.2. Toxicology and Cancer

2.2.1. Epigenetics and Cancer

According to the World Health Organisation (WHO), cancer is the leading cause of death, accounting for approximately 14.1 million newly diagnosed cases and 8.2 million deaths in 2012 (Ferlay et al., 2013). Genetic and epigenetic modifications have been considered as two distinct mechanisms associated with the initiation and

progression of cancer. Cancer has traditionally been seen as a set of diseases that occur due to the accumulation of genetic changes (Hanahan and Weinberg, 2011). Genetic changes include those that alter nucleotide sequences of the genome leading to chromosomal loss or gain, loss of heterozygosity and amplification or mutations of genes. More recently, however, it is recognised that cancer is also a result of abnormal epigenetic events (Sawan et al., 2008; You and Jones 2012). Alterations in the regulation of gene expression that do not involve a change in the DNA sequence is an important key event of carcinogenesis, referred to as "epigenetic'' changes. Epigenetic mechanisms include the methylation of cytosines in DNA, changes of histone and chromatin and variations in the expression of microRNAs (miRNAs) (Kelly et al., 2010; You and Jones 2012; Akhavan-Niaki and Samadani, 2013).

2.2.1.1. DNA Methylation and Cancer

DNA methylation is an important biochemical process involved in normal development of higher organisms. One of the hallmarks of cancer is aberrant DNA methylation. The process involves the addition of a methyl group to the 5' position of the cytosine ring or the nitrogen 6 of the purine ring of adenine. This modification can be inherited through cell division.

In normal organism development and cellular differentiation, DNA metylation constantly changes gene expression patterns in cells. DNA methylation forms the basis of chromatin structure. It also plays a crucial role in the development of many types of cancers (Singal and Ginder, 1999; Docherty et al., 2010). Changes in DNA methylation patterns or in DNA methyltransferase expression may play a role in the onset or course of cancer (Baylin and Bestor, 2002). Methylation is mainly restricted to the palindromic CpG sequence. The methyl-CpG-dinucleotide represents a

mutational hotspot as it can undergo spontaneous deamination to produce thymine (Bird, 2002). DNA methylation is carried out by DNA methyltransferase (DNMT) enzymes (Figure 2.4) (Frémont et al., 1997). The most common DNMTs are DNMT1, DNMT3a and DNMT3b. Methylation can be *de novo* (CpG dinucleotides on both DNA strands are unmethylated) or maintenance (CpG dinucleotides on one DNA strand are methylated). DNMT1 has *de novo* as well as maintenance methyltransferase activity, while DNMT3a and DNMT3b are powerful *de novo* methyltransferases (Monk et al., 1991).

In addition to the DNMTs, other members of the methylation function include demethylases, methylation centres that trigger DNA methylation or methylation protection centres (Cooper and Krawczak, 1990). The enzymes that demthylate DNA include 5-methylcytosine glycosylase, which removes the methylated cytosine from DNA, leaving the deoxyribose intact, and MBD2b, an isoform from the initiation of translation at the second methionine codon of the gene encoding methyl-CpG binding domain 2 (MBD2) protein (Dar et al., 2013). MBD2 lacks glycosylase or nuclease activity and is thought to cause demethylation by hydrolysing 5-methylcytosine to cytosine and methanol (Figure 2.4) (Yen et al., 1992; Vairapandi and Duker 1993; Ehrlich, 2002).

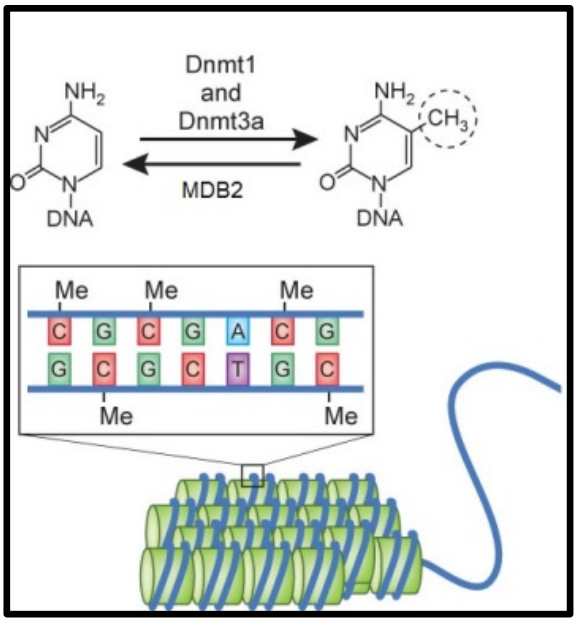

Figure 2.4: The process of methylation and demethylation of cytosine by DNA methyl transferases (DNMT) and methyl domain binding protein (MDB2) (Korzus, 2010).

Hypomethylation, follows hypermethylation in the ranks of epigenetic changes responsible for a wide variety of malignancies (Kim et al., 1994; Jones and Baylin, 2007). Hypomethylation is often seen in solid tumours such as metastatic hepatocellular cancer (Wilson et al., 2007), in cervical cancer (Kim et al., 1994), as well as in B-cell chronic lymphocytic leukaemia (Ehrlich, 2002). The global hypomethylation seen in a number of cancers, such as breast, cervical, and brain, show a progressive intensification as the grade of malignancy worsens (Ehrlich, 2002). Hypomethylation has been thought to contribute to oncogenesis by activation of oncogenes such as *cMYC* and *H-RAS75* (Singer-Sam and Riggs, 1993, Alves et al., 1996) or by chromosome instability (Tuck-Muller et al., 2000; Lujambio and Lowe, 2012).

2.2.1.2. MicroRNAs and Cancer

Cancer is defined as uncontrolled proliferation of functionally compromised/damaged cells, which results in tumour formation. Cells have developed several safeguards to ensure that cell division, differentiation and death occur in a harmonized manner during development and repair of the body. Various regulatory factors switch genes on or off thereby controlling cellular proliferation and differentiation. Damage to the genes involved in proliferation and differentiation are referred to as tumour-suppressor genes and oncogenes when they are affected and results in cancer. Most tumour suppressor genes and oncogenes are first transcribed from DNA into RNA, and are then translated into protein to exert their effects. Recent evidence indicates that small non-protein-coding RNA molecules, called microRNA (miRNA), also function as tumour suppressors and oncogenes and are referred to as 'oncomirs' (Shenouda and Alahari, 2009). Approximately half of the annotated human miRNAs fall within regions of chromosomes, which are areas of the genome that are associated with various human cancers (Esquela-Kerscher and Slack, 2006; Baylin and Jones 2011).

MicroRNAs are important regulators of of gene expression. Alteration of miRNA expression casued by exposure to FB_1 (not well documented) may lead to chemical carcinogenesis. Many miRNAs deregulated by carcinogens are involved in regulation of genes important in chemical carcinogenesis, including xenobiotic metabolism, carcinogen-induced hypomethylation, apoptosis, cell transformation, oncogenesis, etc. The carcinogenic functions of carcinogens may be dependent on the balance between tumour suppressor miRNAs and oncogenic miRNAs.

2.2.1.3. MicroRNAs

MicroRNAs are single-stranded non-coding RNA strands of 19-25 nucleotides in length that are thought to regulate the expression of as many as one-third of all human mRNAs (Marquez and McCaffrey, 2008). The first miRNAs was identified in *C. elegans* in 1993 (Lee et al., 2003). Currently there are 1881 homosapien sequences registered in the miRBase database. Each miRNA can regulate many target genes and several miRNAs may affect a single gene (Di Leva et al., 2014). Recent evidence shows that miRNAs are found to be involved in many regulatory processes such as cellular proliferation, differentiation, cell cycle regulation, angiogenesis metabolism, regulation of the immune response and apoptosis (Kato and Slack 2008; O'Connell et al., 2010).

2.2.1.4. MicroRNA biogenesis

There are two main miRNA biogenesis pathways – a canonical and an alternate pathway. The canonical pathway driven by RNase III enzymes generate the majority of animal miRNAs (Ghildiyal and Zamore, 2009). The biogenesis starts with RNA polymerase II-dependent transcription of a miRNA gene locus, generating a long primary transcript known as pri-miRNA that can fold into a hairpin structure (van Kouwenhove et al., 2011). Pri-miRNAs are cleaved into approximately 70-nucleotide precursor-miRNAs (pre-miRNAs) by the microprocessor complex that contains a RNase III enyme called Drosha and its cofactor DGCR8 (DiGeorge syndrome critical gene 8) (Han et al., 2006). DGCR8 is dsRNA binding protein that recognises the proximal ~10bp stem of the pri-miRNA hairpin, positioning the catalytic sites of the RNase III enzyme Drosha (Di Leva et al., 2014).

Mitrons are a subset of miRNAs which utilise the alternative pathway for miRNA biogenesis (Ruby et al., 2007). These miRNAs are located within short introns, and once splicing is complete, a debranching enzyme generates the pre-miRNA-like hairpin (Figure 2.5) (Baley and Li 2012). The pre-miRNAs are recognised by a complex of exportin-5 (Exp5) and RAN-GTP (van Kouwenhove et al., 2011). The pre-miRNAs are then exported to the cytoplasm by Exp5, where they are cleaved into mature 20-25 nucleotide miRNA duplexes by another RNAse III endonuclease, DICER, and by its double stranded RNA binding cofactor the RNA-binding protein: trans-activation response (TAR) RNA binding protein (TRBP), among other proteins (Figure 2.5) (Winter et al., 2009, Bronze-da-Rocha, 2014). While the passenger strand is typically degraded, the guide strand is incorporated into RNA-induced silencing complex (RISC) and then mediates mRNA degradation or translational inhibition (D'Anzeo et al., 2014) (Figure 2.5).

MicroRNAs regulate gene expression by acting as RNA silencers or post-transcriptional gene regulators (Baylin and Jones, 2011).

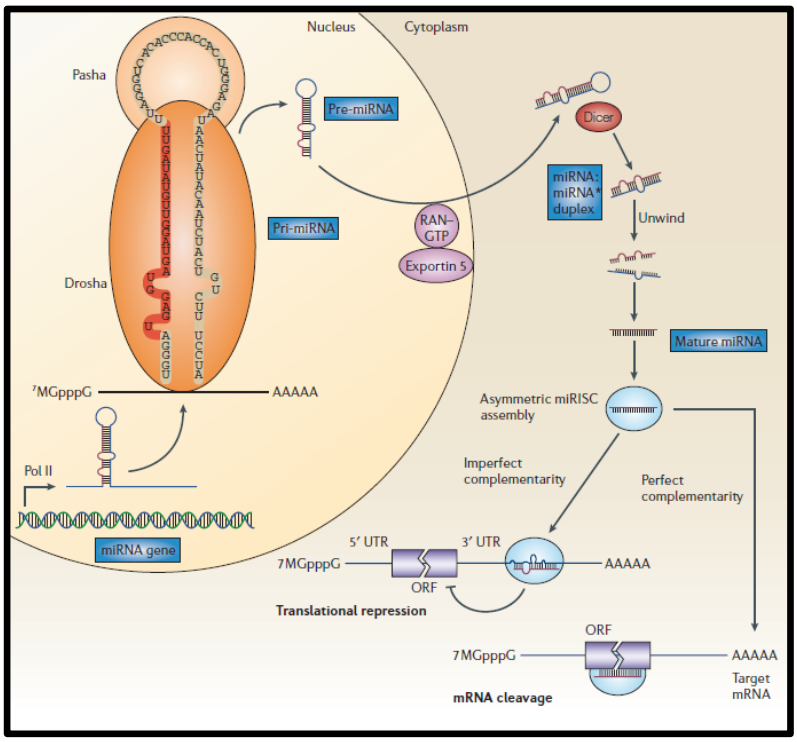

Figure 2.5 The process of miRNA biogenesis, maturation and regulation (Esquela-Kerscher and Slack, 2006).

The potential sites of regulation of gene expression by miRNAs are represented in intronic miRNAs encoded within the same protein code transcript that regulates similar processes of that protein; a single miRNA targets several mRNA targets involved in the same biological process, several miRNAs regulate the same biological process by targeting individual components of a pathway and miRNAs target molecules that are involved in the regulation of a specific process (Baylin and Jones, 2011). As with all cellular processes, abnormal regulation of the expression of miRNAs has been linked to the onset and progression of numerous malignancies (Figure 2.6).

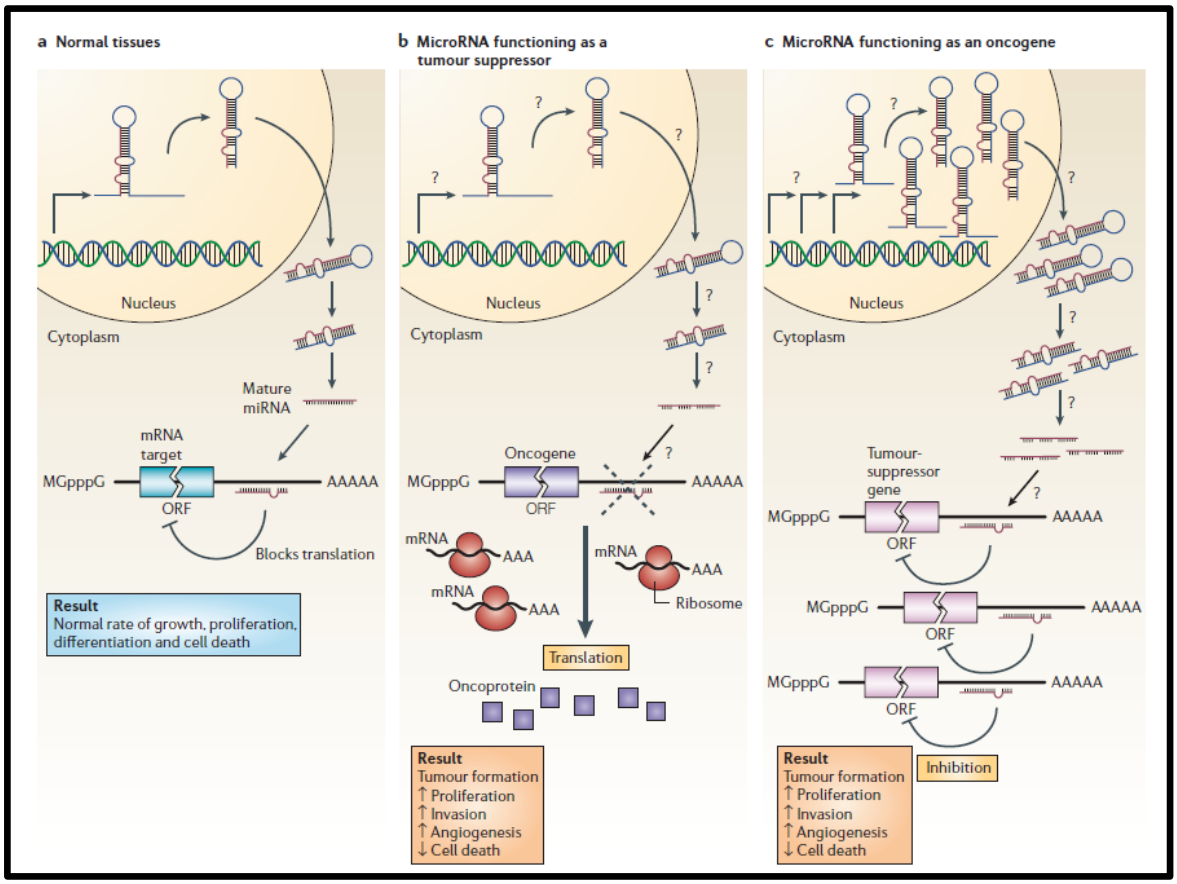

Figure 2.6 MicroRNAs function in A: normal tissue, B: as a tumour suppressor and C: as an oncogene (Esquela-Kerscher and Slack, 2006).

MicroRNAs can function as tumour suppressors and oncogenes by the reduction or complete deletion of a miRNA (that functions as a tumour suppressor) - leading to tumour formation (Figure 2.6b). A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein. The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, leading to tumourigenesis (Esquela-Kerscher and Slack, 2006).

The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumourigenesis. In this condition, increased expression of a miRNA, produced inappropriately, either when not required or in the incorrect tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene and lead to cancer progression (Figure 2.6c). Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (Esquela-Kerscher and Slack, 2006).

The role of toxins (environmental pollutants and mycotoxins) on the aberrant epigenetic function has yet to be elucidated. The link between degree of exposure and epigenetic alterations has not been established and the *in vitro* model of hepatocellular carcinoma provides an ideal platform to interrogate plausible mechanisms of toxicity. The first stage of carcinogenesis is initiation (genetic changes of DNA in a cell). DNA damage can occur due to interactions between genotoxic agents (FB_1) and DNA. If the damaged DNA is not properly repaired, then this damaged DNA could be fixed into mutations that can predispose the cell to become cancerous.

2.3. Toxicology and Apoptosis

2.3.1. Overview

Programmed cell death, also referred to as apoptosis, is an important physiological process regulating homeostasis of cell numbers during development and throughout an organisms life, by facilitating the removal of irreparable damaged or undesirable cells (Orrenius et al., 2011).

During apoptosis, organelle integrity is maintained and minimal leakage of intracellular contents occurs, thereby preventing an inflammatory response (Slater et al., 1995). This distinguishes the process of apoptosis from necrosis.

It has been hypothesised that the dysregulation of the apoptotic process tends to play an integral role in the pathogenesis of human diseases and pathological conditions (Kaufmann and Hengartner, 2001). Cancer for example, is a disease characterised by little or no apoptosis due to mutations that reduce the susceptibility of cells to apoptosis. Therefore, the understanding of the apoptotic mechanisms and its role in cancer development is essential in relation to toxicology, specifically to that of FB_1 which has been shown to possess both pro- and anti-apoptotic activities (Jones et al., 2001; Zimmermann et al., 2001; Voss and Riley 2013).

2.3.2. The extrinsic and intrinsic pathways of apoptosis

During the early stages of apoptosis a family of aspartate-directed cysteine (caspases) proteases, induces the cleavage of important cellular substrates required for normal function. The caspases selectively mediate the cleavage of downstream substrates for the execution of apoptosis (Figure 2.7) (Orrenius et al., 2011).

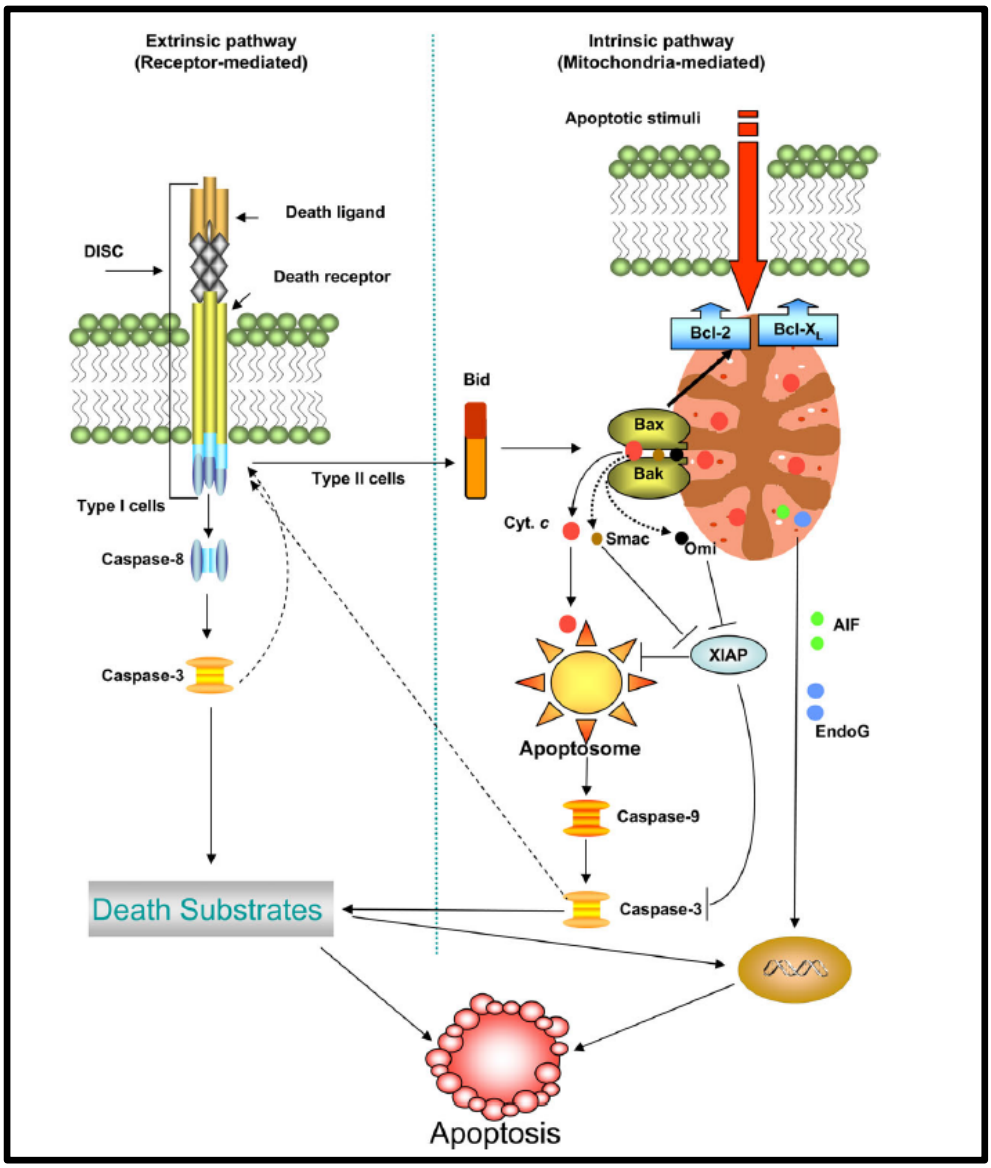

Figure 2.7 The activation of the extrinsic and intrinsic pathways of apoptosis (Orrenius et al., 2011).

The biochemical features of apoptosis include internucleosomal cleavage of DNA which leads to an oligonucleosomal ‘‘ladder’’ and phosphatidylserine (PS) translocation from the inner to the outer cell membrane. These processes induce morphological distinctions that define apoptotic cells such as membrane blebbing, condensation of chromatin, nuclear fragmentation, loss of adhesion and cell shrinkage (Zimmermann et al., 2001).

The extrinsic and intrinsic pathways represent the two major well-studied apoptotic processes (Figure 2.7) (Hassan et al., 2014). Apoptosis may be triggered by various stimuli depending on the intracellular functionality or interactions of cell membrane associated death receptors (DRs) with specific stimuli. Death receptors are cell surface receptors that facilitate the communication of apoptotic signals initiated by ligands. Cell surface DRs belong to the TNF receptor (TNFR) superfamily. The best-characterised family members include Fas (CD95) and TNFR1 (Orrenius et al., 2011). Binding of extracellular ligands, such as Fas to its related receptor induces trimerisation of the receptor (Song and Steller, 1999). After trimerisation and activation of DRs, other proteins are recruited and serve as adaptor molecules in the signal transduction cascade.

During the activation of the extrinsic pathway the adaptor molecule Fas-associated death domain (FADD) associates with Fas which mediates its association with death effector domain (DED) containing protein: pro-caspase-8. The complex of Fas, FADD and pro-caspase-8, known as the death inducing signalling complex (DISC), then releases active caspase-8 into the cytosol (Kaufmann and Hengartner, 2001). Once caspase-8 is activated, the execution phase of apoptosis is triggered. Initiator caspase-8 may either directly activate downstream effector caspases -3 and -7 that execute apoptosis or cleave the pro-apoptotic protein, BH3-interacting-domain death agonist (Bid), to truncated Bid (tBid) resulting in activation of an additional mitochondrial loop (Song and Steller 1999; Elmore, 2007). Truncated Bid then causes the release of the mitochondrial proapoptotic factors: cytochrome c (cyt-c) , Smac (second mitochondrial activator of caspases)/DIABLO, AIF (apoptosis inducing factor) and endonuclease G. Thus Bid through its effect on the mitochondrial membrane potential interacts with the intrinsic pathway (D'Archivio et al., 2008).

The intrinsic signalling pathway (also known as the mitochondrial mediated apoptotic pathway) that initiates apoptosis involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial initiated events (Elmore, 2007). A multitude of diverse stress signals, including cytotoxic stress, oxidative stress, heat shock or DNA damage initiate the inrinsic pathway of apoptosis. These triggers cause the release of cyt-c from the mitochondrial intermembrane space into the cytoplasm. The release of cyt-c causes the activation of downstream molecules responsible for the final execution of apoptosis (Chowdhury et al., 2009).

Under normal cellular conditions, cyt-c is found in the inner mitochondrial membrane bound to cardiolipin (Prétet 2002). Once released, cyt-c interacts with apoptotic protease-activating factor-1 (APAF-1) and pro-caspase-9 forming an apoptosome which is also known as the active form of caspase-9. Caspase-9 can then directly activate caspase-3 and -7, resulting in cell death by tightly regulated proteolytic processing of downstream targets.

During the release of cyt-c, the mitochondrial factor, AIF (a flavoprotein) is also released from the mitochondrion and translocates to the nucleus. In the nucleus, AIF causes DNA fragmentation and chromatin condensation (Kaufmann and Hengartner, 2001). Mitochondria also release endonuclease G, which is involved in DNA degradation associated with apoptosis (Kaufmann and Hengartner, 2001).

2.3.3. Inhibitors of apoptosis (IAP)

The apoptotic cascade is regulated by both initiators and inhibitors and can be activated by diverse stimuli. The inhibitor of apoptosis (IAP) family of proteins plays a central role in apoptotic and inflammatory processes, conferring protection against

cell death. The IAP family members interfere with the transmission of intracellular death signals inhibiting caspase-dependent apoptotic pathways (Figure 2.8). The IAP proteins were initially identified in baculovirus as factors that prevented host cell apoptosis, allowing time for the virus to replicate (Crook et al., 1993; Clem and Miller, 1994). To date, eight IAP-family members have been identified in human cells: NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), XIAP (BIRC4), Survivin (BIRC5), Bruce (BIRC6), Livin (BIRC7) and ILP-2 (BIRC8) (Vucic and Fairbrother, 2007).

The IAP proteins exibit a modular structure characterised by the presence of one or more baculovirus IAP repeat (BIR) domains. The BIR domain has a zinc-binding fold of ~70 amino acid residues that is essential for the anti-apoptotic properties of IAP proteins. The fact that all known IAP members have a BIR domain suggests that this domain plays a pivotal role in mediating cellular protection (De Almagro and Vucic, 2012). Some members of this family (XIAP, c-IAP1, c-IAP2 and Livin) also have a RING domain that allows these proteins to act as E3 ubiquitin ligases (Eckelman et al., 2006). E3 ubiquitin ligase activity gives IAPs the ability to promote the ubiquitination and subsequent proteasomal degradation of caspases, TRAF2, and additional partners (Vucic and Fairbrother, 2007). Several studies indicate that IAPs may play a role in oncogenesis (Hunter et al., 2007; LaCasse et al., 2008).

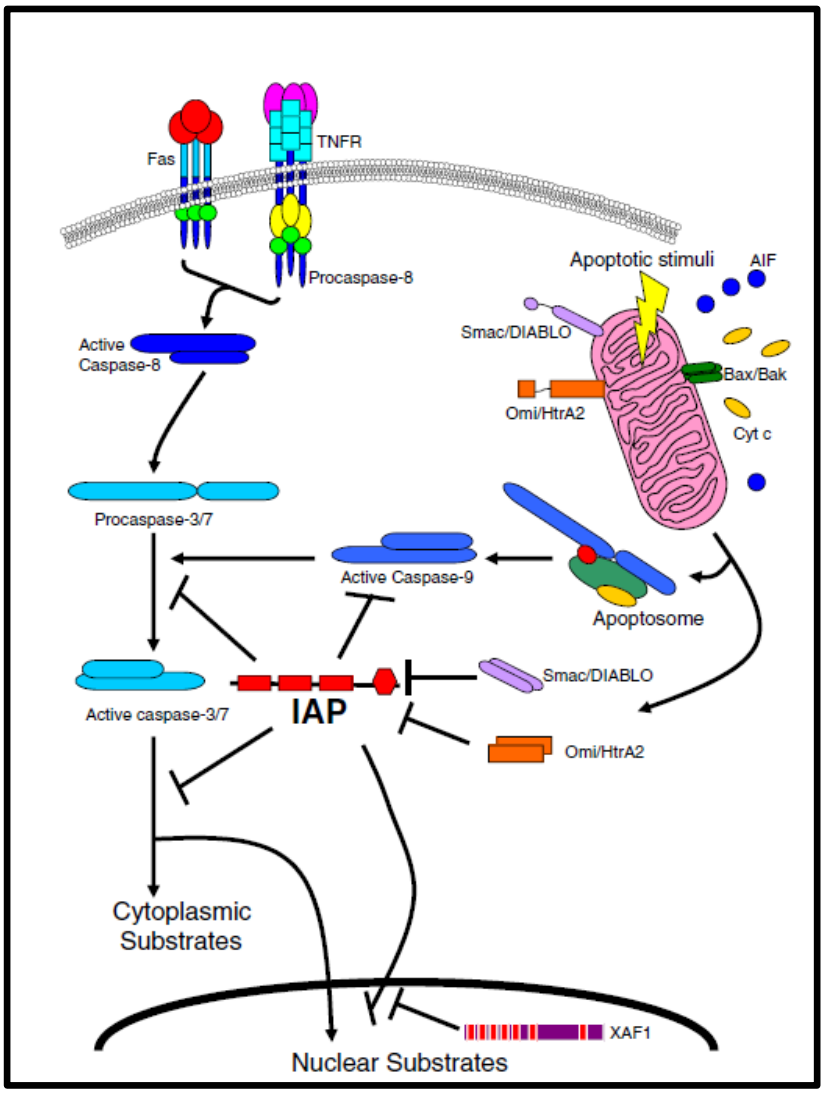

Figure 2.8 The inhibitors of apoptosis (IAP) protein function as caspase inhibitors halting the execution phase of apoptosis (Hunter et al., 2007).

Caspase inhibition occurs mainly by binding to the unique BIR domains of IAP. NAIP, c-IAP1, c-IAP2 and XIAP have three BIR domains, the third BIR domain mediates the binding to, and inhibition of caspase-9, an initiator caspase capable of processing and thereby activating other caspases (Srinivasula et al., 2001). Interaction of caspase-9 with the third BIR domain of IAPs prevents its homodimerisation, inhibiting its activity (Shiozaki et al., 2003). The second BIR domain mediates the interaction of IAPs with caspase-3 and -7, the effector caspases that are activated by

initiator caspases (Figure 2.8). In summary, the binding of IAPs to caspases prevents the interaction between caspases and their cellular substrates, thus inhibiting the proteolytic degradation of the cell that marks the final execution stage of apoptosis (Chai et al., 2001).

2.3.4. Second-mitochondria-derived activator of caspases (Smac)

The mitochondrial protein Smac/DIABLO performs a critical function in apoptosis by eliminating the inhibitory effect of IAPs on caspases. Concurrent with cyt-c release, Smac is also released from the mitochondria into the cytosol. Whereas cyt-c induces multimerisation of Apaf-1 to activate procaspase-9 and -3, Smac eliminates the inhibitory effect of many IAPs (Chai et al., 2001). Thus Smac interacts with all IAPs and appears to be a master regulator of apoptosis in mammals.

There is limited epigenetic data on FB_1 and hepatotoxicity in humans. In addition, there is conflicting data on FB_1 induced apoptosis. An integration of epigenetic and cellular death machanisms is not common in toxicology research. This study aimed to determine epigenetic properties of FB_1 and its influence on hepatocellular death mechanisms.

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CHAPTER 3

Fumonisin B_1 induces global DNA hypomethylation in HepG2 cells – an alternative mechanism of action

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ABSTRACT

Fumonisin B_1 (FB_1), a common mycotoxin contaminant of maize, is known to inhibit sphingolipid biosynthesis and has been implicated in cancer promoting activity in animals and humans. FB_1 disrupts DNA methylation and chromatin modifications in human hepatoma (HepG2) cells. We investigated the effect of FB_1 on enzymes, DNA methyltransferases and demethylases, involved in chromatin maintenance and gross changes in structural integrity of DNA in HepG2 cells. We measured: (i) the expression of 84 key genes encoding enzymes known to modify genomic DNA and histones (super array and qPCR); (ii) protein expression of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) and the major demethylase (MBD2) (western blotting); (iii) degree of DNA methylation by FACS using anti-5-MeCyt and (iv) DNA migration (single cell gel electrophoresis). FB_1 significantly decreased the methyltransferase activities of DNMT1, DNMT3A and DNMT 3B, and significantly up regulated the demethylases (MBD2 expression and activity, and *KDM5B* and *KDM5C* expression). FACS data showed FB_1 significantly increased DNA hypomethylation and resulted in gross changes in structural DNA as evidenced by the Comet assay. We conclude that FB_1 induces global DNA hypomethylation and histone demethylation that causes chromatin instability and may lead to liver tumourigenesis.

Key Words

Fumonisin B_1; DNA hypomethylation; methyltransferase; demethylase; histones; liver cancer

1. Introduction

Fumonisin B_1 (FB_1), a mycotoxin produced by the ubiquitous soil fungus *Fusarium verticilliodes*, is the most common fungal contaminant of maize worldwide (Domijan et al. 2007; Gelderblom and Marasas 2012; Marasas 2001). FB_1 has been implicated in cancer promoting activity in the liver and kidney in animals and humans (Domijan et al. 2007; Gelderblom et al. 2008; Soriano et al. 2005; Stoev et al. 2012; Voss et al. 2006).

Fumonisin B_1 is a diester of propane-1,2,3-tricarboxylic acid (TCA) and 2 amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane, in which the C14 and C15 hydroxyl groups are esterified with a terminal carboxyl group of TCA (Bezuidenhout et al. 1988; Soriano et al. 2005). This toxin has structural similarity to the sphingoid bases sphinganine and sphingosine. FB_1 disrupts sphingolipid biosynthesis by competitively inhibiting ceramide synthase (N-acetyltransferase), resulting in accumulated levels of sphingoid bases (Soriano et al. 2005; Voss et al. 2006). This toxic action of FB_1 affects cellular processes such as cell growth and differentiation, apoptosis and oxidative stress (Soriano et al. 2005). FB_1 has been shown to be carcinogenic in both the liver and kidney due to disruption of sphingolipid biosynthesis (Voss et al. 2002).

Because FB_1 is a highly polar toxin, it traverses the plasma membrane, probably via amino acid transporters, and interacts with subcellular structures and disrupts cellular function (Bezuidenhout et al. 1988). It is resistant to conditions normally used in food processing and therefore poses a significant hazard to human and animal health (Scott 2012). It is estimated that the average daily intake of FB_1 may range between 12-140μg, however in geographical regions where maize is the staple diet (e.g., South

Africa), this may increase up to 2500μg (WHO 2012). Many types of pathologies, which appear to be both species and tissue specific, have been associated with exposure to FB_1 (WHO 2012).

Animal studies have shown that FB_1 is a hepatocarcinogen and epidemiological evidence suggests that it may be an aetiological agent in human oesophageal cancer (Alizadeh et al. 2012; Chu and Li 1994; Sun et al. 2007; Yoshizawa et al. 1994). While the cytotoxic mechanism of FB_1 is attributed to its disruption of sphingolipid metabolism, the underlying mechanisms of its cancer initiating/promoting properties are unknown. Several studies in rodents have shown that FB_1 promotes pre-neoplastic lesions in the liver, suggesting a role for FB_1-induced genotoxicity (Domijan et al. 2008; Gelderblom et al. 2008; Karuna and Rao 2013).

Molecular dynamic simulations of FB_1 show that the toxin adopts multiple long-lived dielectric conformations when in solution, and can therefore interact with macromolecules within the cell (Momany and Dombrink-Kurtzman 2001). It is likely that FB_1 may interact with DNA bases, or structural components of chromatin, thereby promoting a pre-neoplastic phenotype; however there is currently no experimental evidence to support this.

The integrity of chromatin structure is strictly maintained by a repertoire of enzymes, which can directly modify DNA bases by methylation (methylases/demethylases) or structural components such as histones (acetylases/deacetylases). DNA methylation is a chemical modification of DNA involved in regulation of gene expression (Issa et al. 1993). A hallmark of cancer is aberrant DNA methylation: (i) hypermethylation of tumour suppressor genes, (ii) aberrant expression of DNA methyltransferase 1 (*DNMT1* and other *DNMTs*) and methyl binding proteins (e.g., MBD2), as well as (iii) hypomethylation of unique genes (Baylin et al. 2001; Ehrlich 2002; Issa et al.

1993; Stefanska et al. 2013). DNA methylation is an epigenetic mechanism involved in transcriptional silencing of genes. Methyl-CpG binding domain protein 2 (MBD2), a member of the MBD protein family, binds to methylated promoter CpG islands and acts as a methylation-dependent transcriptional repressor (Berger and Bird 2005).

Both DNA methylation and chromatin modifications have been associated with tumourigenesis, few studies have investigated the role of FB_1 in liver cancer progression. This study investigated the effect of FB_1 on enzymes involved in chromatin maintenance and gross changes in structural integrity of DNA in HepG2 cells.

2. Materials and Methods

2.1 Treatment

Approximately 1.5 $x10^6$ HepG2 cells were plated in sterile $25cm^3$ flasks in complete culture media [Eagle's minimum essential medium, 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone] and incubated overnight at 37^oC in a humidified incubator with a supply of 5% CO_2. A stock solution of 5mM FB_1 was prepared in 0.1M phosphate buffered saline (PBS). HepG2 cells were treated with a range (0- 1000µM) of concentrations of FB_1. A dose dependent decline in HepG2 cell viability was observed and an IC_{50} value of 200µM was determined (Addendum A: Table 1 and Figure 1). For all subsequent experiments, cells were treated with 200µM FB_1 in a final volume of 5ml/ $25cm^3$ flasks and corresponding control flasks were maintained for 24hr (37^oC, 5% CO_2). All treatments were done in triplicate. A second set of experiments was performed (in triplicate with another batch of cultured cells) to check for reproducibility.

2.2 Transcriptional profiling of chromatin modifying enzymes

Following FB_1 treatment cells were rinsed (0.1M PBS) and 500µl 0.1M PBS was added to each flask. An aliquot of 500µl Tri Reagent® Solution (Ambion, USA) was added to each flask for isolation of RNA. Total RNA was isolated using the RNeasy Isolation Kit (Qiagen, USA) as per standard methods and quantified spectrophotometrically using a Nanodrop 2000. cDNA was synthesised using the RT^2 First-Stand Kit (Qiagen, USA) and the Epigenetic Chromatin Modification Enzymes PCR array (PAHS-085Z, Qiagen, USA) was used to profile expression of 84 key genes encoding enzymes known or predicted to modify genomic DNA and histones to regulate chromatin accessibility and therefore gene expression.

2.3 Western blot

Isolated protein samples (HepG2 crude protein extract standardized to 1mg/ml) were denatured by boiling for 10min with a 1:1 dilution with 1× Laemmli sample buffer (0.375M Tris–HCl pH 6.8; 10%, w/v SDS; 3%, v/v glycerol; 0.2%, w/v bromophenol blue; 12% β-mercaptoethanol in dH_2O). For each sample, 100µg of total protein was loaded on a 10% polyacrylamide gel, run at 150V. Transfer onto nitrocellulose membrane was conducted at 350mA for 1hr. Membranes were blocked for 1hr with blocking buffer containing 5% non-fat dry milk in Tris buffered saline (TTBS, 25mM Tris pH 7.6, 150mM NaCl, 0.05% Tween 20).

Membranes were probed overnight at 4°C with anti-DNMT1, anti-DNMT3A, anti-DNMT3B, anti-MBD2 (Santa Cruz Biotechnology, USA) and anti-β actin (Sigma, USA) diluted to 1:500 in 1% BSA in TTBS. A horseradish peroxidase-conjugated secondary antibody diluted to 1:10 000 in 1% bovine serum albumin (BSA) in TTBS was used to allow detection of appropriate bands using LumiGLO®

Chemiluminescent Substrate Kit (KPL). Images were captured on the Alliance 2.4 gel documentation system (UViTech, UK). All experiments were conducted at least thrice and blots were analysed using UViBand analysis software (UViTech, UK).

2.4 DNA methylation analysis by Fluorescent-activated cell sorting (FACS)

The degree of DNA methylation in FB_1 treated and control HepG2 cells was assessed via intracellular probing using flow cytometry. Cells ($1x10^6$, in triplicate) were fixed in Fix & Perm solution Medium A (Caltag, Germany) (room temperature (RT), 30min, in the dark) and pelleted (10 000xg, 3min). Cells were resuspended in and permeabilised with Fix and Perm Medium B (Caltag, Germany) which contained the monoclonal anti-5-MeCyt (1:100, RT, 30min, in the dark). A 1ml aliquot of wash buffer (0.1M PBS, 5% foetal bovine serum, 1% sodium azide) was added to each sample and cells were pelleted (10 000xg, 3min). Cells were resuspended in Medium B containing APC-conjugated goat anti-mouse secondary antibody (1:100, 15min, RT, in the dark).

A 1ml aliquot of wash buffer (0.1M PBS, 5% foetal bovine serum, 1% sodium azide) was added to each sample and cells were pelleted (10 000xg, 3min). Cells were resuspended in 300µl sheath fluid and transferred to polystyrene cytometry tubes. All experiments were done in duplicate. Flow cytometry data from stained cells (25 000 events) was obtained using a FACSCalibur (BD Biosciences, USA) flow cytometer with CellQuest PRO v4.02 software (BD Biosciences, USA). Cells were gated to exclude debris using FlowJo v7.1 software (Tree Star, Inc). (Addendum B- Figures 2 and 3).

2.5 Single cell gel electrophoresis assay

The effect of FB_1 on DNA migration/damage was determined with the comet assay. Briefly, following treatment of cells (20 000 cells/well) in a 6-well plate, supernatants were removed and cells were trypsinized. Three slides (for FB_1 treatment and controls) were prepared as the first layer of 1% low melting point agarose (LMPA, 37ºC), second layer of 25µl of cells (20 000) from the treated and control samples with 175µl of 0.5% LMPA (37ºC) and third layer of 0.5% LMPA (37ºC) covered the slides.

After solidification, the slides were then submerged in cold lysing solution [2.5M NaCl, 100mM EDTA, 1% Triton X-100, 10mM Tris (pH 10), 10% DMSO] and incubated (4ºC, 1hr). Following incubation the slides were placed in electrophoresis buffer [300mM NaOH, 1mM Na_2EDTA (pH 13)] for 20min and thereafter subjected to electrophoresis (25V, 35min, RT) using Bio-Rad compact power supply. The slides were then washed 3 times with neutralisation buffer [0.4M Tris (pH 7.4)] for 5min each.

The slides were stained overnight (4ºC) with 40µl ethidium bromide (EtBr) and viewed with a fluorescent microscope (Olympus IXSI inverted microscope with 510-560nm excitation and 590nm emission filters). Images of 50 cells and comets were captured per treatment and the comet tail lengths were measured using Soft imaging system (Life Science - Olympus Soft Imaging Solutions v5) and expressed in µm.

3. Results

3.1 Fumonisin B_1 decreased expression of DNA methyltransferases and concomitantly increased expression of DNA demethylases

We screened human hepatoma (HepG2) cells treated with FB_1 for transcriptomic changes in enzymes known to be involved in chromatin modification using qPCR arrays (Qiagen). Cluster gram analysis showed notable transcriptional changes in enzymes that regulate DNA methyl metabolism in FB_1 treated HepG2 cells compared to controls (Figure 3.1A). Fold regulation analyses showed that FB_1 treatment significantly decreased the expression of DNA methyltransferases specifically, (*DNMT1*, *DNMT3A* and *DNMT3B*, 2 fold, 6 fold and 4 fold decreases respectively) in HepG2 cells. Conversely, expression of DNA demethylases *KDM5B* (2.5 fold increase), *KDM5C* (4 fold increase) and *MBD2* was significantly increased. The most significant FB_1-induced increase in expression was observed for *MBD2* (7.7 fold increase in transcript levels) (Figure 3.1B/C).

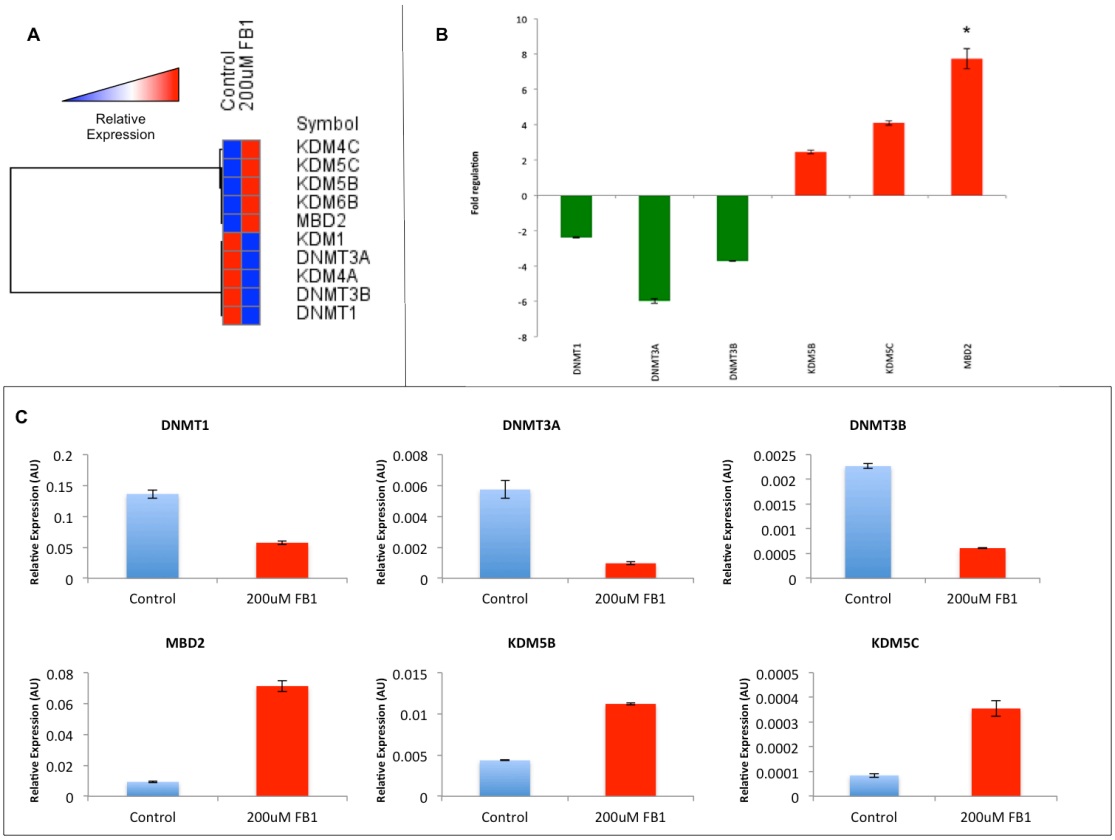

Figure 3.1 Fumonisin B_1 induced changes in expression levels of DNA methyltransferases and DNA demethylases in HepG2 cells. (A) Cluster gram analysis and (B) fold regulation analysis shows down-regulation of DNA methyltransferases and concomitant up-regulation of *MBD2* and DNA demethylases in response to FB_1. *p<0.005 (One way ANOVA). (C) Relative changes in expression in control vs. 200 μM FB_1 treated HepG2 cells.

3.2 Fumonisin B_1 decreased protein expression of DNA methyltransferases and increased expression of MBD2

We next investigated whether the transcriptional fluxes observed in the gene expression studies were related to absolute changes in protein levels. Western-blot experiments and digital densitometry confirmed that levels of DNMT1 (1.6-fold), DNMT3A (2.4-fold) and DNMT3B (1.7-fold) were decreased in HepG2 cells in

response to the toxic insult by FB_1. The data also showed a change in MBD2 protein levels (1.2-fold change) as a result of FB_1 exposure in HepG2 cells (Figure 3.2).

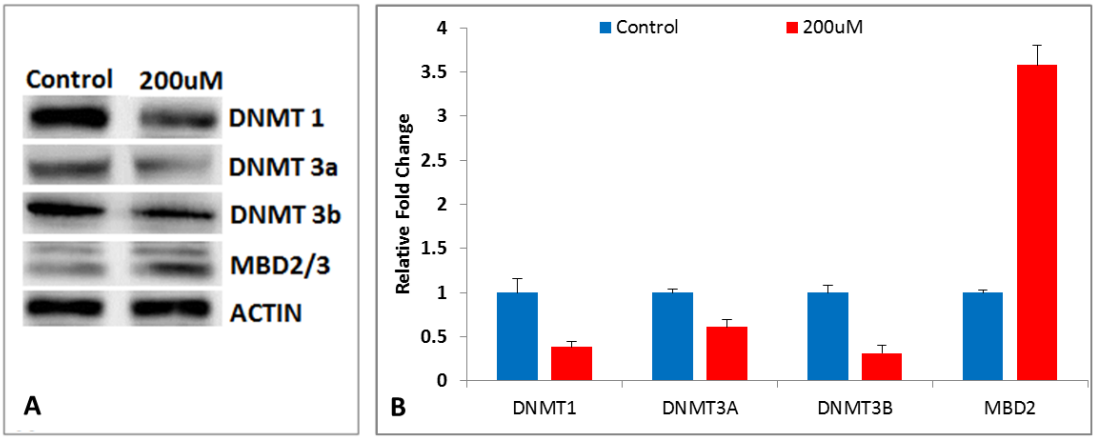

Figure 3.2 Fumonisin B_1 decreased expression of DNA methyltransferases and increased MBD2 protein expression as shown by western blots (A). Data represented as a fold change relative to the control (B). Non-parametric Mann Whitney Test. (DNMT1 - *$p < 0.0286$; DNMT3A- *$p < 0.0286$; DNMT3B - *$p < 0.0286$; *MBD2* - **$p < 0.0079$).

3.3 Fumonisin B_1 induced global DNA hypomethylation in HepG2 cells

After treatment with FB_1 we enumerated the number of HepG2 cells positive for 5-methylcytosine, a marker used to monitor global DNA methylation. The data showed that FB_1 induced global DNA hypomethylation in HepG2 cells at 200µM (Figure 3.3A). There was a significant reduction in the number of FB_1 treated HepG2 cells positive for 5-methylcytosine (Figure 3.3B).

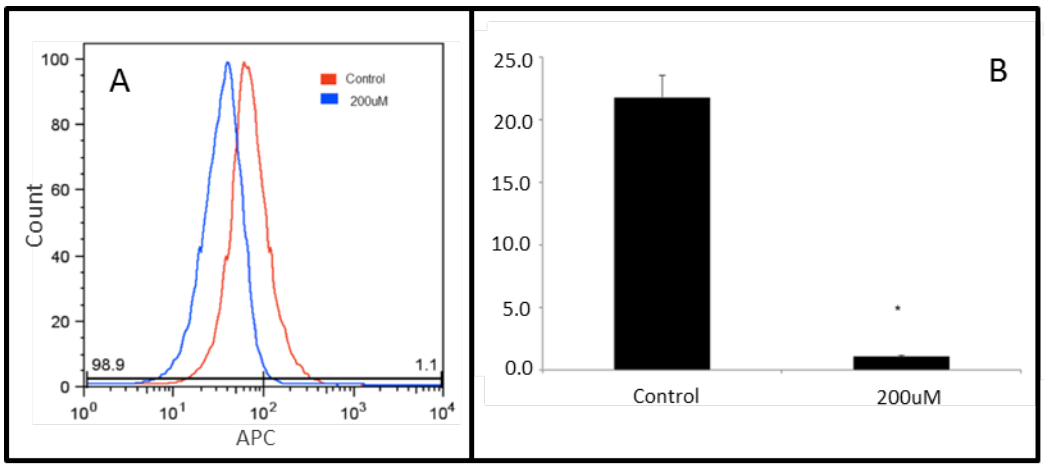

Figure 3.3 Fumonisin B_1 induced global DNA hypomethylation in HepG2 cells as assessed using flow cytometry (A). Data represented as a fold change compared to control (B).

3.4 Fumonisin B_1 increased DNA migration in HepG2 cells

Following treatment with FB_1 (200µM) we assessed DNA migration in HepG2 cells using the comet assay. The data showed that FB_1 induced a significantly higher degree of DNA migration (2.57 µm ± 0.02 µm vs. control: 0.812 µm ± 0.01 µm, p=0.0022, Figure 3.4A/B).

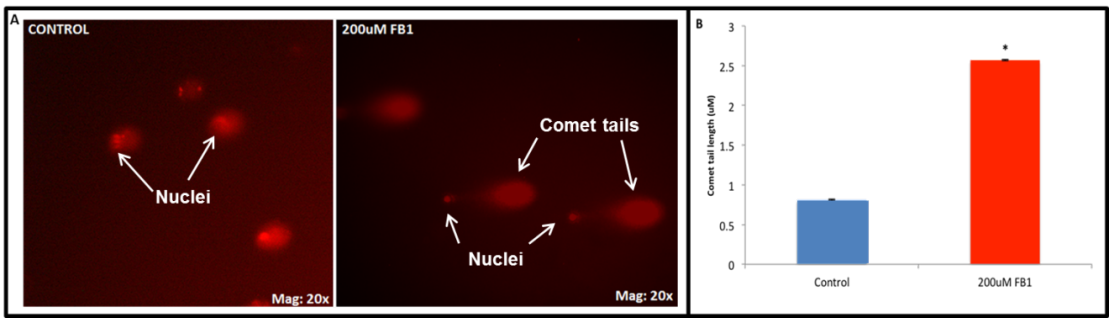

Figure 3.4 Fumonisin B_1 increased DNA comet tails in HepG2 cells (A). Comparison of comet tail lengths (in µM) between control and Fumonisin B_1 treated cells (B).

4. Discussion

The canonical mechanism of action associated with FB_1 is disruption of sphingolipid biosynthesis by inhibition of ceramide synthase. There are various reports that confirm this phenomenon and have established that FB_1 dysregulation of sphingolipid metabolism strongly correlates with both human and veterinary pathogenesis. *In vitro* evidence suggests that a decreased bioavailability of sphingoid bases will tend toward a cytotoxic phenotype. In the context of FB_1 therefore, disruption of sphingolipid metabolism may not fully explain the proliferative and ultimately oncogenic properties of this food borne mycotoxin. In this report, we present evidence that FB_1 induces global DNA hypomethylation in human hepatoma cells and suggests that the mechanism is by altering the balance of DNA methylases/demethylases, thereby disrupting the structural integrity of DNA.

Our data strongly suggests that the mechanism of FB_1-induced DNA hypomethylation is related to modulation of key enzymes critical for the maintenance of an integral epigenetic landscape. This report clearly shows that FB_1 leads to chromatin instability and aberrant transcriptional activation. FB_1 significantly decreased the expression of DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) and concomitantly significantly increased expression of *MBD2* (a demethylase) and the histone demethylases (*KDM5B* and *KDM5C*). Decreased DNA methyltransferase (DNMT1, DNMT3A and DNMT3B) and increased demethylase (MBD2) protein expression was further confirmed with western blot analysis. Demethylation in cancer is common and involves hypomethylation of repetitive sequences, although its role in genes encoding proteins still remains to be elucidated. However, DNMT inhibitors were shown to cause DNA demethylation that led to the induction of prometastatic genes and metastasis suggesting a possible role for hypomethylation in

cancer metastasis (Pakneshan et al. 2004; Shteper et al. 2003). *MBD2*, a major demethylase, is essential for expression and demethylation of the prometastatic genes urokinase-type plasminogen activator (*PLAU*) and matrix metalloproteinase 2 (*MMP2*) (Pakneshan et al. 2004; Shukeir et al. 2006). *MBD2* inhibition by antisense oligonucleotides silenced both these genes and resulted in inhibition of invasiveness and metastasis of breast and prostate cancer cell lines (Pakneshan et al. 2004). Furthermore, Stefanska et al. (2011) demonstrated that knock down of *MBD2* in HepG2, SkHep1 (liver adenocarcinoma cell line) and NorHep (human untransformed hepatocytes) cells confirmed that hypomethylation in liver cancer targets promoters of specific genes encoding functional pathways required for growth and invasion. However, even a partial reversal of this process in the liver cell lines used resulted in tumour growth and invasiveness in the liver cancer cells but not the normal liver cells (Stefanska et al. 2011).

In our study, FB_1 significantly up regulated both *MBD2* transcript and protein expression by 7.7- and 3.5- fold respectively strongly suggesting that FB_1 may play a causal role in cellular transformation and invasiveness in HepG2 cells. *MBD2* was previously shown to act as both a transcriptional repressor and an activator of gene expression by promoting demethylation of its target-activated genes (Detich et al. 2002; Stefanska et al. 2013). Our data shows that FB_1 upsets the balance of enzymes necessary for proper regulation of DNA methylation. While demethylation is necessary for transcription, if it occurs on a global scale it will result in large stretches of "loose" DNA, as observed by the increased comet tail lengths induced by FB_1. This is the first report that shows FB_1 (a type 2B carcinogen) perturbs epigenetic gene regulation and may be involved in tumourigenesis.

A study on depletion of *MBD2* in HepG2 cells not only resulted in the silencing of at least 15 genes induced in liver cancer, but also blocked the transformation and invasive properties of the cell (Stefanska et al. 2011). These authors concluded that *MBD2* was a master regulator of some of the genes activated in liver cancer but also that hypomethylation and activation of this set of genes played a causal role in cancer, particularly cancer metastasis (Stefanska et al. 2011). Our data is in agreement with this study as FB_1 significantly increased the expression of both *MBD2* (7.7 fold) and MBD2 (3.5 fold), implicating FB_1 as a hepatocarcinogen. Further our results confirm that FB_1 induces global DNA hypomethylation in HepG2 cells as evidenced by a decreased number of cells staining for 5-methylcytosine.

FB_1 also significantly increased the expression of two histone demethylase genes *KDM5B* and *KDM5C*. Both demethylases are members of the Jumonji (JmjC) family of proteins that catalyse the demethylation of tri- and dimethylated lysines (Cloos et al. 2008; Cloos et al. 2006). They specifically act on tri- and dimethylated lysine 4 on histone H3 (H3K4) (only found at transcribed genes) and are associated to euchromatin and active transcription (Cloos et al. 2008). Trimethylated H3K4 is highly enriched around transcriptional start sites whilst dimethylated H3K4 is present throughout the coding region of transcribed genes (Bernstein et al. 2005). *KDM5B* is regulated by the oncogene *c-ErbB2* and is highly expressed in ductal breast carcinomas and is associated with the malignant phenotype in breast (Barrett et al. 2002). In addition to its role in promoting cancer, *KDM5B* is needed for the proliferation of MCF-7 breast cancer cell line and tumour growth of mammary carcinoma cells in nude mice (Yamane et al. 2007). *KDM5B* target genes in breast cancer proliferation have been identified as *BRCA 1, CAV 1* and *HOXA5* (Yamane et al. 2007). In our study, FB_1 increased *KDM5B* and *KDM5C* expression in liver

HepG2 cells that will result in increased H3K4Me3/Me2 demethylation. A consequence of this increased H3K4 demethylation will disrupt its role as a transcriptional repressor.

5. Conclusion

In conclusion, our results show that FB_1 induces global DNA hypomethylation (by downregulating DNA methyltransferases and upregulating MBD2) and histone demethylation in human hepatoma cells that causes chromatin instability and may lead to liver tumourigenesis.

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CHAPTER 4

FUMONISIN B_1 MODULATES EXPRESSION OF HUMAN CYTOCHROME P_{450} 1B1 IN HUMAN HEPATOMA (HepG2) CELLS BY REPRESSING miR-27b

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ABSTRACT

Fumonisin B_1 (FB_1), a common mycotoxin contaminant of maize, is known to inhibit sphingolipid biosynthesis and has been implicated in hepatocellular carcinoma promoting activity in humans and animals. MicroRNAs (miRNA) are small noncoding RNAs that regulate gene expression via translational repression. Human cytochrome P_{450} (CYP1B1) is highly expressed in oestrogen target tissues and catalyzes the metabolic activation of many procarcinogens. The aim of our study was to investigate the effect of FB_1 on miR-27b suppression and its effect on CYP1B1 modulation in a human hepatoma cell line (HepG2). MiR27b and *CYP1B1* expressions were evaluated in HepG2 cells by quantitative PCR. In order to directly assess the effect of miR-27b on *CYP1B1* mRNA levels, cells were transfected with the mimic to miR-27b. CYP1B1 protein expression was measured using western blot. FB_1 significantly down-regulated (11 fold) expression of miR-27b in HepG2 cells; whilst CYP1B1 mRNA and protein expression was significantly up-regulated by 1.8 fold and 2.6 fold respectively. *CYP1B1* is post-transcriptionally regulated by miR-27b after HepG2 exposure to FB_1. FB_1- induced modulation of miR-27b in hepatic cells may be an additional mode of hepatic neoplastic transformation.

Key Words

Fumonisin B_1; MicroRNA (miR)-27b; Cytochrome P $_{450}$ (CYP1B1); liver cancer; HepG2 cells

1. INTRODUCTION

Fusarium verticilloides, a ubiquitous soil fungus and common contaminant of corn worldwide, produces the carcinogen fumonisin B_1 (FB_1) that is responsible for a wide range of species-specific toxicoses (Norred et al. 1998). FB_1, a structural analogue of sphingoid bases, is implicated in cancer promotion as it disrupts sphingolipid, phospholipid and fatty acid metabolism, which play a major role in the modulation of apoptosis and cell proliferation pathways (Gelderblom and Marasas 2012). An elevation of free sphingoid bases by FB_1 can induce apoptosis, whereas production of sphingosine-1-phosphate or inhibitors of ceramide biosynthesis can also inhibit apoptosis (Norred et al. 1998). Chronic feeding of animals with FB_1 caused liver cancer in mice and rats (Howard et al. 2001). At present little is known about FB_1 genotoxicity however, it is known that FB_1 induces necrosis as well as apoptosis in the liver (Gelderblom and Marasas 2012).

MicroRNAs (miRNA) are small non-coding RNAs regulating the expression of genes involved in various biological processes including cell proliferation and apoptosis (Ambros 2004). Mature functional miRNAs (approximately 22 nucleotides in length) generated from long primary miRNA transcripts, control gene expression at the post-transcriptional level by either degrading or repressing target mRNAs. MiRNAs are thus selected gene regulatory molecules with each cell type likely to have a specific miRNA milieu to control gene expression (Chen 2005). The functional miRNAs have been predicted to regulate expression of approximately 30% of all human genes (Lewis et al. 2005). MiRNAs play an important role in coordinating many cellular processes such as regulating apoptosis, proliferation, differentiation, development and metabolism (Gusev 2008; Maziere and Enright 2007).

Some miRNAs expressed in cancer were found to regulate the expression of signalling molecules such as cytokines, growth factors, transcription factors and both pro- and anti-apoptotic genes; whilst some miRNAs may function as tumor suppressors or conversely oncogenes in cells (Calin and Croce 2006). MiRNAs may therefore be one of the key regulators of tumourigenesis.

Tsuchiya et al., (2006) found that cytochrome P_{450} (CYP1B1), a superfamily of drug metabolizing enzymes, was a target of miR-27b (Tsuchiya et al. 2006). CYP1B1, a highly expressed enzyme in oestrogen target tissues, catalyzes the activation of various procarcinogens and promutagens, polycyclic aromatic hydrocarbons and aryl amines (Shimada et al. 1996) and 17β-estradiol (Lee et al. 2003). In addition *CYP1B1* is highly expressed in cancerous tissues (e.g. breast cancer) and a near-perfect matching sequence was identified with miR-27b in the 3'-untranslated region (UTR) of CYP1B1 (Tsuchiya et al. 2006). It was concluded that CYP1B1 was post-transcriptionally regulated by miR-27b and that the expression level of miR-27b was decreased accompanied by high levels of CYP1B1 in the cancerous liver tissue (Tsuchiya et al. 2006).

In response to noxious stimulation by FB_1, and as a result of aberrant gene expression, cells may alter their normal miRNA profiles and create a micro-environment which facilitates carcinogenesis. This toxin is a potent liver carcinogen, however there is little consensus on the precise mechanism of neoplastic transformation. The effect of FB_1 on miRNA has not been previously investigated and it is likely that cells may change their miRNA milieu in response to FB_1. In this study we screened for changes in miRNA expression profiles in human hepatoma cells (HepG2) following exposure to FB_1. Interestingly, we show that FB_1 induced significant changes in miR-27b

expression and in turn modulates *CYP1B1* expression. We suggest that FB_1- induced modulation of miR-27b in hepatic cells is an additional mode of hepatic neoplastic transformation.

2. MATERIALS AND METHODS

2.1 Treatment

Approximately 1.5 $x10^6$ HepG2 cells were plated in sterile $25cm^3$ flasks in complete culture media [Eagle's minimum essential medium, 10% foetal calf serum, 1% L-Glutamine and 1% penstrep-fungizone] and incubated overnight at 37^oC in a humidified incubator with a supply of 5% CO_2. A stock solution of 5mM FB_1 was prepared in 0.1M phosphate buffered saline (PBS). HepG2 cells were treated with a range (0- 1000µM) of concentrations of FB_1. A dose dependent decline in HepG2 cell viability was observed and an IC_{50} value of 200µM was determined (data not presented). Cells were then treated with 200µM FB_1 in a final volume of 5ml/ $25cm^3$ flasks and corresponding control flasks were maintained for 24h (37^oC, 5% CO_2). All treatments were done in triplicate.

2.2 Quantitative PCR array based profiling of microRNA

Following FB_1 treatment cells were rinsed (0.1M PBS) and 500µl 0.1M PBS was added to each flask. An aliquot of 500µl Tri Reagent® Solution (Ambion, USA) was added to each flask for isolation of RNA. Total RNA was isolated using the RNeasy Isolation Kit (Qiagen, USA) as per standard methods and quantified using a Nanodrop 2000. Copy DNA (cDNA) was synthesised using the miScript II RT kit (Qiagen, USA) and the miScript miRNA PCR Array Human miFinder (MIHS-001Z, Qiagen, USA) which contains 84 mature miRNA-specific primers.

2.3 Statistical analysis and selection of significant miRNA

Data was normalised using U6 small RNA expression. Difference in miRNA expression was determined by fold change analysis using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). To stringently select miRNAs significantly up- or down-regulated in FB_1 treated cells, a significance of analysis of microarray (SAM) strategy was used. Significant ($p<0.05$) deviation of residuals from normal distribution was used to identify miRNAs of interest. Data was analysed with Microsoft excel (2011) Statplus plug-in and R.

2.4 Computational analysis of miRNA targets

Pathway analysis was conducted by first identifying miRNA targets using the Targetscan and Miranda algorithms and thereafter, scanning for ontological enrichment in the KEGG database.

2.5 Transfection of HepG2 cells with miR-27b mimic

In order to directly assess the effect of miR-27b on *CYP1B1* mRNA levels, cells were transfected with the mimic (Syn-hsa-miR-27b, MYS0000419, Qiagen, USA) to miR-27b. Briefly, HepG2 cells were seeded at a density of 400 000 cells/well in a 6-well plate and allowed to adhere for 24h (37^oC, 5% CO_2) until 80% confluent. Lyophilized miRNA mimic (5nmol) was reconstituted to 20µM in nuclease free water. For the transfection, 10µl miRNA mimic was added to medium without serum, proteins, or antibiotics to a total volume of 60µl to which 2µl of HiPerFect Reagent (Qiagen, USA) was added and mixed by reverse-pipetting. Samples were then incubated for 15min (room temperature) to allow complex formation. During this time CCM (complete culture medium) was gently aspirated from the cells and 1940µl fresh CCM

was added such that the final concentration of mimic per well was 50nM. The transfection complex was added in a drop-wise fashion into the appropriate well with gentle swirling of the plate to ensure uniform distribution. An untreated control and a 200µM FB_1 treatment were included on the plate. All treatments were then incubated for 24h (37^{o}C, 5% CO_2) and utilized for RNA isolation.

2.6 Quantification of CYP1B1 mRNA levels

To quantify CYP1B1 qPCR was performed. Total RNA was first isolated from control and treated cells by adding 500µl Tri reagent (Am9738) as per manufacturer's guidelines. Thereafter, RNA was quantified (Nanodrop 2000) and standardised to 100ng/µl. RNA was reverse transcribed by reverse transcriptase into cDNA using the RT2 First Strand Kit (SABiosciences, C-03, USA) as per manufacturer's instructions. Briefly, a 20µl reaction was prepared by adding 10µl genomic DNA (gDNA) elimination mixture (Total RNA, 5× gDNA elimination buffer, H_2O) to 10µl of RT cocktail (5× RT buffer 3, primer and external control mix, RT enzyme mix, H_2O). The reaction was then subjected to 42°C (15min) and 95°C (5min) (GeneAmp® PCR System 9700, Applied Biosystems, USA) to obtain cDNA. Quantitative PCR was used to determine mRNA expression using RT2 SYBR® Green qPCR Master Mix (SABiosciences, USA). A 25µl reaction consisting of 12.5µl iQ™ SYBR® green supermix (cat. no. 170–8880), 8.5µl nuclease-free water, 2µl cDNA, and 1µl sense and anti-sense primers for CYP1B1 (1B1DSF 5'- GCCACTATCACTGACATCT-3', 1B1DSR 5'-CTTGCCTCTTGCTTCTTATT-3'), 10mM, Inqaba Biotechnical Industries™, South Africa) were used. The mRNA expression was compared and normalised to a housekeeping gene, GAPDH (sense: 3'-CAACAGCCTCAAGATCATCAGC-5'; anti-sense: 3'-TGAGTCCTTCCACGATACCAAAG-5').

The reaction was subjected to an initial denaturation (95°C, 10min). It was followed by 40 cycles of denaturation (95°C, 15s), annealing at 57°C, 40s; and extension (72°C, 30s) (CFX Real-Time PCR detector, Bio-Rad, USA). The data was analyzed using CFX analysis software V3.0, Bio-Rad. The mRNA expression was determined using the method described by Livak and Schmittgen (2001) (Livak and Schmittgen 2001) and is represented as fold changes relative to the control.

2.7 Quantification of CYP1B1 protein levels

Samples (HepG2 crude protein extract standardized to 1mg/ml) were denatured by boiling for 10min with a 1:1 dilution with 1× Laemmli sample buffer (0.375 M Tris–HCl pH 6.8; 10%, w/v SDS; 3%, v/v glycerol; 0.2%, w/v bromophenol blue; 12% β-mercaptoethanol in dH_2O). For each sample, 100μg of total protein was loaded on a 10% polyacrylamide gel, run at 150V. Transfer onto PVDF membrane was conducted at 350mA for 1h. Membranes were blocked for 1h with blocking buffer containing 5% non-fat dry milk TTBS (25mM Tris pH 7.6, 150mM NaCl, 0.05% Tween 20). Membranes were probed overnight at 4°C with anti-CYP1B1 antibody (PA5-28040, Thermo-Scientific, USA) and anti-β actin antibody (A3854, Sigma, USA) diluted to 1:500 in 1% BSA in TTBS. A horseradish peroxidase-conjugated secondary antibody diluted to 1:10 000 in 1% BSA in TTBS was used to allow detection of appropriate bands using LumiGLO® Chemiluminescent Substrate Kit (KPL). Images were captured on the Alliance 2.4 gel documentation system (UviTech, UK). All experiments were conducted at least thrice and blots were analysed using UViBand analysis software (UviTech, UK).

3. RESULTS

3.1 MicroRNA profiles in Fumonisin B_1 treated HepG2 cells

MiRNA profiling showed that FB_1 induced differential regulation of miRNAs in HepG2 cells. Expression profile heat map shows robust signals from control and treated cells (Figure 4.1A). This indicated good data integrity and that dataset is suitable to mine for candidate miRNAs that may be differentially regulated by FB_1 treatment. Each point in the expression profile plot (Figure 4.1B) represents a single miRNA species with total coverage of 84 unique miRNAs. This analysis shows that miRNA expression profiles in both control and FB_1 treated cells track similarly which indicates that the majority of miRNA expression in HepG2 cells remain unchanged upon exposure to FB_1. However, four peaks/miRNAs in FB_1 treated cells and one peak/miRNA in control track with higher intensity, suggesting that these miRNAs may be differentially regulated in the context of FB_1.

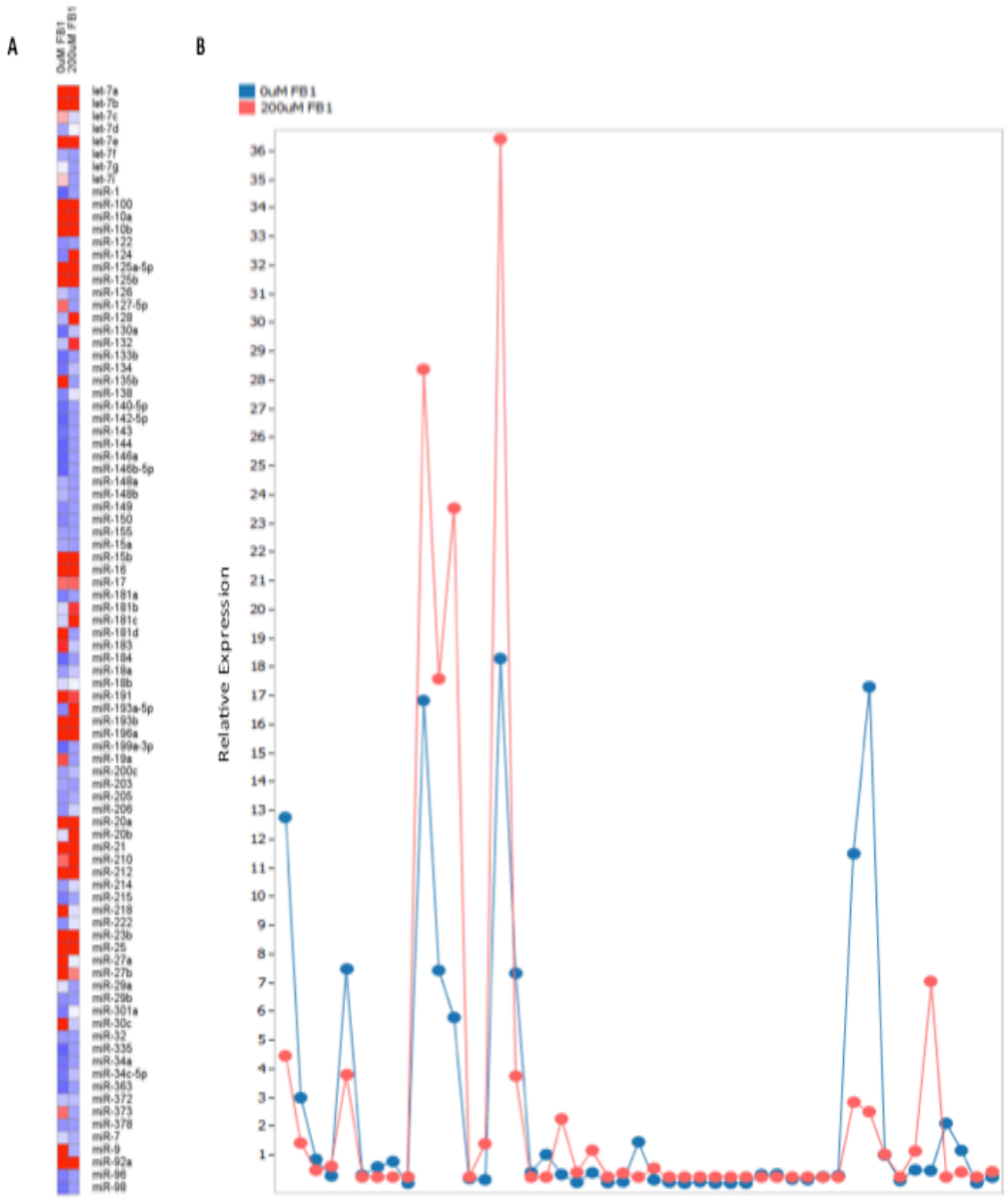

Figure 4.1 **MiRNA profiles in Fumonisin B_1 treated HepG2 cells. A**: Expression profile heat map. **B**: Relative expression profile plot of miRNAs in control and FB_1 treated HepG2 cells.

3.2 Cluster analysis of miRNA expression in Fumonisin B_1 treated HepG2 cells

To further examine discrete differences in miRNA expression induced by FB_1, we used hierarchical cluster analysis. miRNA expression was first clustered by Euclidian distance and then by linkage analysis. We found that five miRNAs were down regulated (miR-135b, miR-181d, miR-27a, miR-27b and miR-30c) (Figure 4.2A). Relative expression profile of top five clustered miRNAs shows that of these, miR-27b is most significantly decreased upon treatment with FB_1 (Figure 4.2B).

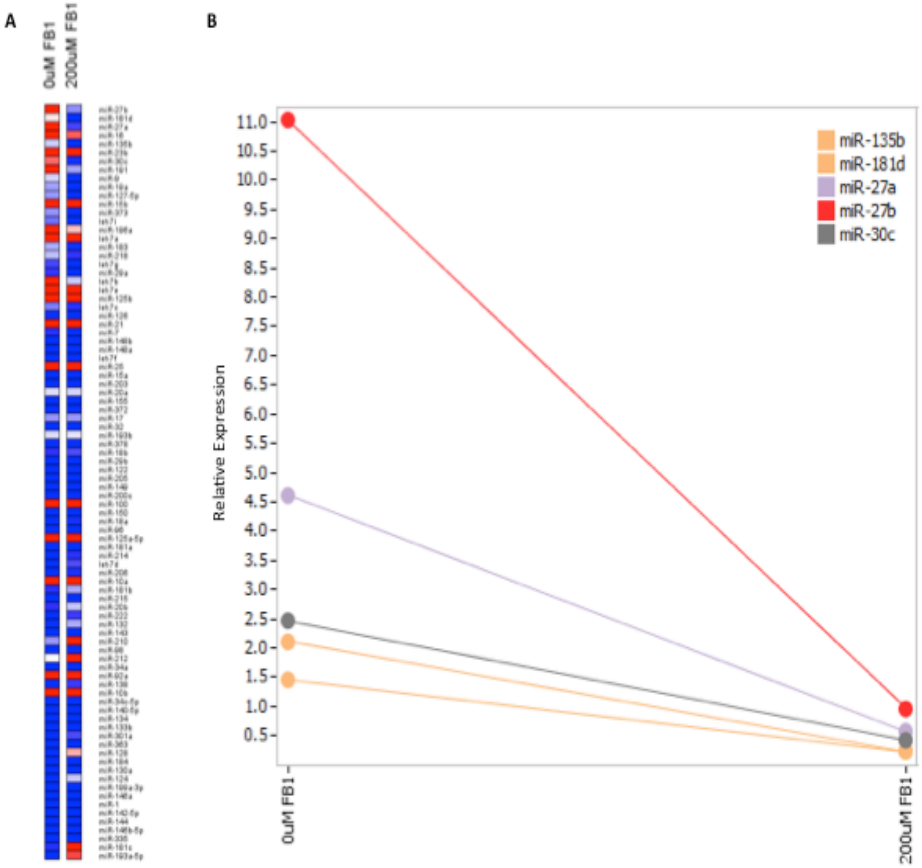

Figure 4.2 **Hierarchical cluster analysis of miRNA expression in Fumonisin B_1 treated HepG2 cells.** **A**: Heat map shows a descending ordered list of significant miR clusters in control cells. **B**: miR-27b expression is dysregulated by FB_1 treated cells.

3.3 Fumonisin B_1 down-regulates miR-27b

Unperturbed HepG2 cells express high levels of miR-27b however, exposure to FB_1 significantly decreased miR-27b by over 10 fold (Figures 4.3A and 4.3B). Independent experiments confirmed that FB_1 represses miR-27b reproducibly and significantly (Figure 4.4).

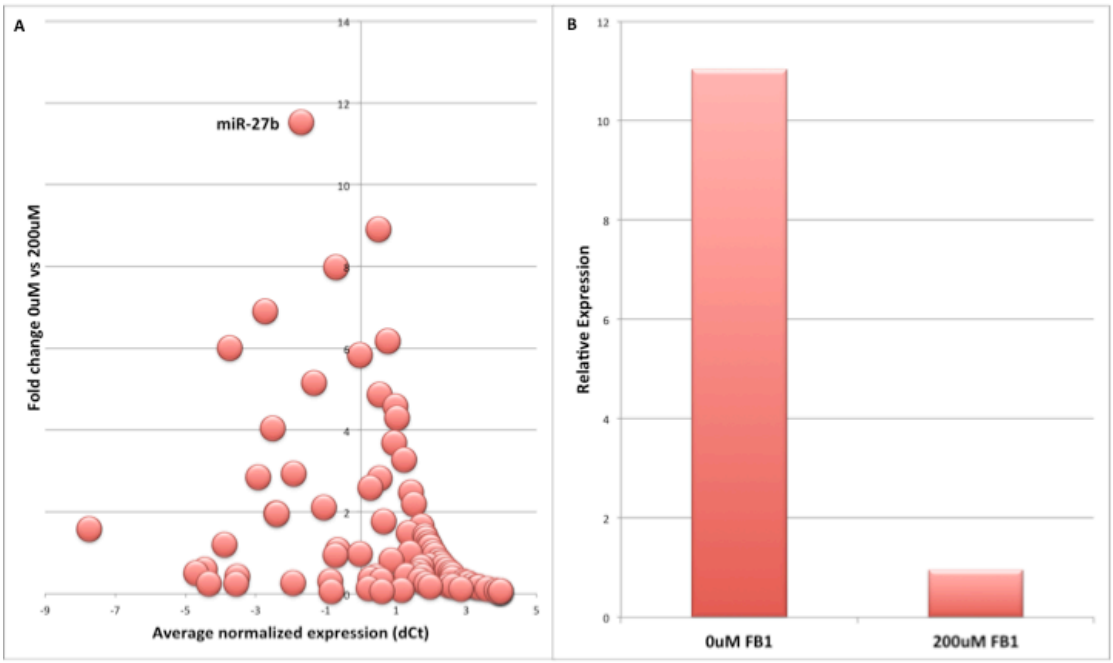

Figure 4.3 **miR-27b expression is dysregulated by Fumonisin B_1. A**: Expression vs fold change analysis shows that miR-27b is expressed at high levels in control HepG2 cells and suggests that FB_1 represses miR-27b. **B**: In depth evaluation of relative expression shows that miR-27b is decreased by more than 10 fold in HepG2 cells upon treatment with FB_1.

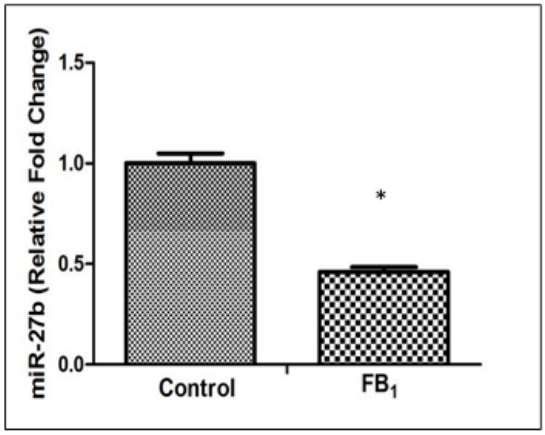

Figure 4.4 **Fumonisin B_1 decreased expression of miRNA-27b**. Data represented as a fold change relative to the control. Non-parametric Mann Whitney Test. (**p < 0.001; ***p < 0.0001).

3.4 Target prediction of miR-27b

The standard miRNA target prediction tools were used to search for potential candidates genes that may be transcriptionally regulated by miR-27b. All tools returned extensive lists (Table 4.1) of possible targets for miR-27b. Of these however, the 3'UTR of *CYP1B1* has a near perfect complementarity to the seed sequence of miR-27b (Figure 4.5A) Furthermore, apart from Notch1, *CYP1B1* remains the only experimentally validated target for miR-27b to date (Biyashev et al. 2012). Taking into account the role of cytochromes in liver metabolism, *CYP1B1* is a likely target in this experimental context.

Table 4.1: **Target prediction metrics for miR-27b**

Prediction tool	Number of miR-27b targets
Pictar	783
Targetscan	1211
Miranda	1100

3.5 Altered CYP1B1 mRNA levels by Fumonisin B_1

We investigated whether down-regulation of miR-27b by FB_1 influenced mRNA levels of *CYP1B1*. Quantitative PCR experiments confirmed that levels of CYP1B1 were increased in HepG2 cells treated with FB_1 (1.80-fold ± 0.03) compared to the control (1.00 ± 0.04). *CYP1B*1 levels were normalised in the presence of a miR-27b mimic confirming that the miR-27b – CYP1B1 axis is operational in this system and furthermore, can be modulated by FB_1 (Figure 4.5B).

3.6 Fumonisin B_1 increases CYP1B1 protein expression

We assessed the effect of FB_1 on levels of CYP1B1 protein in HepG2 cells by western blotting. A significant 2.6-fold increase in CYP1B1 expression was found in FB_1 treated cells following densitometry analysis of protein bands normalised to β-actin ($p<0.0001$) (Figure 4.5C).

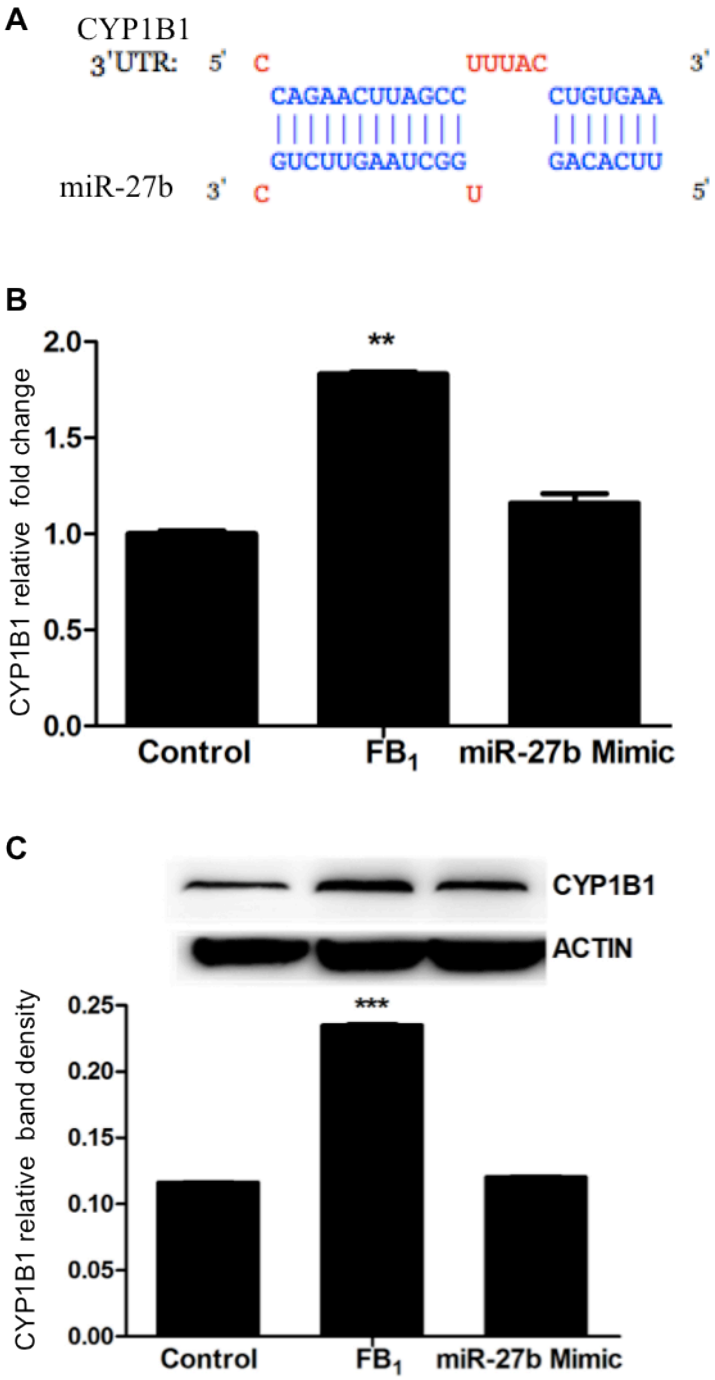

Figure 4.5 **Fumonisin B_1 modulates CYP1B1 expression**. **A**: Sequence homology alignment between CYP1B1 mRNA and predicted target sequence of miR-27b. **B**: FB_1 increased expression of CYP1B1 mRNA. Data represented as a fold change relative to the control (**$p < 0.001$, Mann Whitney Test). **C**: Immuno-blot of CYP1B1. CYP1B1 expression was highest in FB_1 treated HepG2 cells. Data represented as relative band density normalised against β-actin (***$p < 0.0001$, Unpaired t-test).

4. DISCUSSION

The current mechanism of action associated with FB_1 is inhibition of ceramide synthase and the disruption of sphingolipid biosynthesis. The sphingolipid dysregulation by FB_1 strongly correlates with mammalian pathogenesis, including cancer. However, disruption of sphingolipid metabolism alone may not fully explain the proliferative and ultimately oncogenic properties of FB_1. In this report, we present evidence that FB_1 significantly down-regulates miR-27b and concomitantly up-regulates both *CYP1B1* mRNA and its protein expression in human hepatoma cells. This is the first study, to our knowledge, on miRNA and the food-borne hepatotoxin FB_1. CYP1B1 (an enzyme that catalyzes the conversion of procarcinogens and oestradiol) is strongly associated with mutagenesis and is found in high levels in many cancerous tissues (Cheung et al. 1999; Ragavan et al. 2004). Specifically, CYP1B1 metabolises 17β-oestradiol (a known contributor to the growth and development of oestrogen-dependent cancers) (Lee et al. 2003). Further, 4-hydroxyoestradiol, a reactive metabolite formed by CYP1B1, is known to generate free radicals and that can cause DNA damage (Han and Liehr 1994; Newbold and Liehr 2000). We recently found that FB_1 (200μM) induced global DNA hypomethylation in HepG2 cells by significantly decreasing the methyltransferase activities of DNMT1, DNMT3A and DNMT3B, and significantly up-regulated the activity of demthylase methyl binding protein (MBD2) (Chuturgoon AA et al. 2013). We concluded that FB_1 induced global DNA hypomethylation and histone demethylation in human hepatoma cells by causing chromatin instability that leads to liver tumorigenesis. There is still come confusion regarding the selective expression of *CYP1B1* in cancer as its mRNA is expressed in both cancer and normal cells and it was erroneously assumed that CYP1B1 protein expression would be the same

(McFadyen and Murray 2005). McFadyen and Murray (2005) provided evidence for plausible mechanisms to explain the dislocation between CYP1B1 mRNA and protein expression in normal cells (McFadyen and Murray 2005). In 2005 two different mechanisms of CYP1B1 regulation were proposed, with experimental evidence, showing extreme over-expression in cancer cells. One suggestion was the transcriptional regulation by hypermethylation of the promoter/enhancer region of the *CYP1B1* gene resulting in decreased mRNA expression in normal cells (Tokizane et al. 2005); whilst the second suggestion was post-transcriptional regulation by proteosomal degradation of CYP1B1 protein in normal cells, targeted through polyubiquitination, but not phosphorylation (Bandiera et al. 2005). The most convincing post-transcriptional mechanism postulates that the over-expression of CYP1B1 in cancer cells because these cells are largely deficient of miRNA (miR-27b) which in normal cells inhibits the translation of CYP1B1 mRNA into protein (Tsuchiya et al. 2006). It is well known that miRNA expression is globally changed in cancerous tissue.

After seven years since the Tsuchiya et al. (2006) study (Tsuchiya et al. 2006), we provide evidence to support the post-transcriptional modification of *CYP1B1* mRNA and protein expression by miR-27b in hepatoma cells, albeit induced by the hepatotoxin FB_1. There is strong complementarity between 3'-UTR *CYP1B1* mRNA transcript and the seed sequence of miR-27b (Figure 5A). Increased expression and activity of CYP1B1 has been associated with mutagenesis and carcinogenesis. We provide evidence that FB_1 "induces" over-expression of both *CYP1B1* mRNA and protein (Figures 5B and 5C). Placing our evidence in context, the effect of FB_1 on CYP1B1 may not be direct, but instead as a result of decreased repressive activity by miR-27b due to FB_1 related decrease in miR-27b levels (Figure 6).

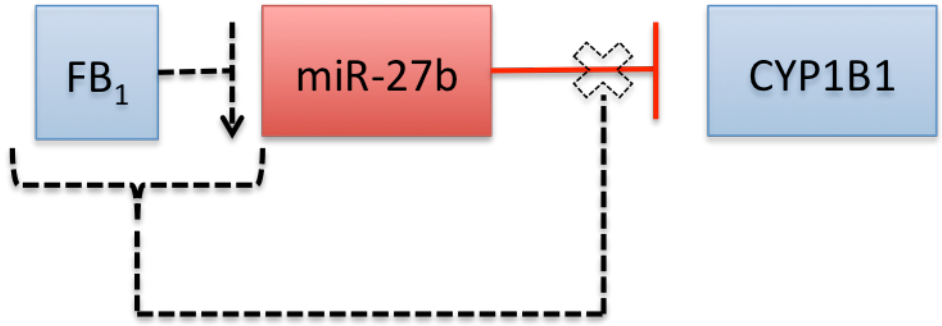

Figure 4.6 **Fumonisin B_1 regulation of CYP1B1 by modulation of miR-27b.**

In conclusion a significant inverse association was observed between the expression levels of miR-27b and CYP1B1 protein in HepG2 cells induced by FB_1. Thus, the decreased expression of miR-27b would be one of the causes of higher CYP1B1 protein expression in cancerous tissues.

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CHAPTER 5

Fumonisin B_1 inhibits apoptosis in HepG2 cells by inducing Birc-8/ILP-2

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Abstract

Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium* sp., a common contaminant of maize. FB_1 inhibits sphingolipid biosynthesis, alters sphingosine/sphinganine ratios and modifies cell survival and cell death processes at varying propensities at both species- and tissue-specific level. We investigated the effect of FB_1 on the apoptotic pathway in human hepatoma (HepG2) cells. We measured: (i) the level of cell proliferation and cell death mechanism of HepG2 cells (MTT assay, Annexin V and Propidium Iodide staining); (ii) initiator and executioner caspase activity (Luminometric enzyme activity assays); (iii) regulation of mRNA expression of pro- and anti- apoptotic molecules using an apoptosis array (qPCR) and (iv) levels of significantly altered apoptosis-related proteins (Western blotting) following a 24hr incubation. FB_1 caused a dose-dependent decrease in cell viability with an inhibitory concentration for 50% of cell growth at 200μM. FACS data showed FB_1 induced a 2.5-fold increase in Annexin V staining, however, caspase activity was not significantly influenced by the toxin. BIRC-8/ILP-2 was most significantly up regulated (8-fold) in the apoptosis array. ILP-2 protein levels were elevated (2.3-fold) with a corresponding decrease in Smac/DIABLO protein levels (1.7-fold). Further analysis showed a dose-dependent increase in BIRC-8/ILP-2 mRNA and protein expression in HepG2 cells. We conclude that FB_1 modulates apoptosis in a complex dose-dependent regulation of pro- and anti-apoptotic molecules.

Keywords:

Fumonisin B_1; Apoptosis; BIRC-8; ILP-2; Liver cancer

1. Introduction

Fumonisin B_1 (FB_1) is a carcinogenic mycotoxin produced by *Fusarium verticilloides* and *F. proliferatum*, a common worldwide fungal contaminant of corn (Marasas, 2001). Contamination of corn by FB_1 is of concern as it causes various animal diseases and is found at high levels in corn-based products intended for human consumption (Shephard et al., 1996). Elevated levels of FB_1 in naturally contaminated corn has been associated with high incidences of human disease globally, namely, liver cancer in Japan (Ueno et al., 1997), oesophageal cancer in South Africa (Rheeder et al., 1992) and gastrointestinal cancers in China (Chu and Li, 1994). FB_1 in itself is not mutagenic (Knasmuller et al., 1997). Rather, its cancer promoting activities in the liver is related to major changes in cellular biochemistry coupled with associated epigenetic changes (Chuturgoon et al., 2014a; Chuturgoon et al., 2014b).

FB_1 disrupts *de novo* sphingolipid biosynthesis by inhibiting ceramide synthase, thus preventing conversion of sphinganine to dihydroceramide and reacylation of sphingosine to ceramide (Wang et al., 1991). This results in increased sphingoid bases and their 1-phosphate derivatives and decreased ceramides and complex sphingolipids (Merrill et al., 2001). Slight variations in levels of sphingolipids can be perceived as cellular stress signals that can induce apoptosis. However, evidence shows that manipulation of ceramide levels by cancer cells render them resistant to cytotoxic agents that induce apoptosis. FB_1 sphingolipid disruption can either induce or prevent apoptosis, depending on the cell type and the relative amounts of bioactive sphingolipid molecules (Desai et al., 2002).

Early reports showed that FB_1 induced apoptosis in several different mammalian cell

types (Ciacci-Zanella and Jones, 1999; Schmelz et al., 1998; Wang et al., 1996). However, studies on neoplastic African green monkey kidney cells (COS-7) demonstrated resistance to the apoptotic effects of FB_1 compared to their normal counterparts, (CV-1) (Ciacci-Zanella et al., 1998; Wang et al., 1996). Additionally, murine or human leukemia cells do not undergo apoptosis when treated with FB_1 (Bose et al., 1995; Ciacci-Zanella et al., 1998). The ability of cells to escape apoptosis is critical in the development of cancer and FB_1 may selectively manipulate apoptotic pathways leading to resistance to apoptosis, thus allowing altered growth capabilities. Interestingly, the cancer promoting effects of FB_1 are closely correlated with increased apoptosis and subsequent regenerative cellular proliferation (Dragan et al., 2001).

The mechanisms underlying the disparate observations with regard to FB_1 and apoptosis are largely unknown. We investigated the effects of FB_1 on apoptosis in HepG2 liver cells by using a quantitative assay for apoptosis identifying specific genes that interfere with FB_1-induced apoptosis

2. Material and Methods

2.1 *Materials*

The HepG2 (hepatocellular carcinoma) cells were purchased from Highveld Biologicals (Johannesburg, South Africa). Cell culture reagents were purchased from Whitehead Scientific (Johannesburg, South Africa). Cell Titre Glo, GSH-Glo, Caspase-8, -9 and -3/7-Glo luminometry assays were purchased from Promega (USA). Annexin V-FLUOS was obtained from Roche (South Africa) and the Human

Apoptosis RT² Profiler PCR Array (PAHS-012A) was purchased from Qiagen (USA). All other reagents and consumables were purchased from Merck (SA), unless otherwise stated.

2.2 Cell culture and viability assay

$HepG_2$ cells were cultured (37^oC, 5% CO_2) to confluency in $25cm^3$ flasks in complete culture media (CCM) [Eagle's minimum essential medium, 10% foetal calf serum, 1% L-Glutamine and 1% penstrepfungizone]. The cytotoxicity of FB_1 in $HepG_2$ cells was measured using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells (20,000/well) were incubated for 24hr with varying FB_1 concentrations (0µM to 1000µM) in triplicate in a 96-well microtitre plate together with the respective control treatments (cells incubated with CCM only). The cells were then incubated with the MTT substrate (5 mg/ml in 0.1M phosphate buffered saline (PBS)) for 4hr. Thereafter all supernatants were aspirated, and DMSO (100 µl/well) was added to the wells. Finally the optical density was measured at 570 nm and a reference wavelength of 690 nm with an ELISA plate reader (Bio-Tek µQuant). The data was translated to percentage cell viability versus concentration of FB_1 treatment from which the half maximal inhibitory concentration (IC_{50}) was determined. This IC_{50} value was used in all experiments, except when a dose dependent (200µM, 100µM, 50µM, 0µM) effect of FB_1 on BIRC-8/ILP-2 induction was evaluated.

2.3 Annexin-V-FLUOS assay

The annexin-V-Fluos assay (Roche, SA) was used to determine phosphatidylserine (PS) translocation. To each flow cytometry tube, 100µl of staining buffer, 100µl of

annexin-V-Fluos labeling solution (annexin-V: propidium iodide (PI): staining buffer (1:1:50 vol/vol/vol)) and 100µl of cell suspension was added, and incubated in the dark (15min, RT). Cells were analyzed on a FACS Calibur (BD Biosciences, USA) flow cytometer. Data were analyzed using CellQuest PRO v4.02 software (BD Biosciences, USA). Cells were gated to exclude cellular debris using FlowJo v7.1 software (Tree Star, Inc, USA). Approximately 50,000 events were analyzed for apoptotic, necrotic and live cells. The results were expressed as a percentage of the total events.

2.4 *Assessment of caspase activity*

Caspase-3/-7, -8 and -9 activities were detected with Caspase-Glo® assays (Promega, Madison, USA). As per manufacturer's protocol, Caspase-Glo®-3/-7, -8 and -9 reagents were reconstituted and added to wells (in six replicates) of an opaque 96-well microtitre plate (40µl of reagent per 100µl of 20,000 cells/well). Samples were mixed and incubated in the dark (30min, RT). The luminescent signal was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). Caspase-3/-7, -8 and -9 activities were expressed as relative light units (RLU).

2.5 *Apoptosis Array*

Following treatment, cells were rinsed (0.1M PBS) and 500µl 0.1M PBS was added to each flask. An aliquot of 500µl Tri Reagent® Solution (Ambion, USA) was added to each flask for isolation of total RNA. Small RNA was isolated using the RNeasy Isolation Kit (Qiagen, USA) as per standard methods and quantified spectrophotometrically using a Nanodrop 2000. cDNA was synthesised using the RT^2 First-Stand Kit (Qiagen, USA) which contained an effective genomic DNA

elimination step and a built-in external RNA control. The Human Apoptosis RT² Profiler PCR Array (PAHS-012A) was used to profile the expression of 84 key genes involved in programmed cell death. Data analysis was performed using SABiosciences' PCR array data analysis software. Relative changes in gene expression were calculated using the comparative threshold cycle (Ct), delta delta (DDCt) method. This method first subtracts the Ct (threshold cycle number) of the gene-average Ct of the housekeeping genes on the array to normalize for the amount of RNA per sample. Finally the DDCt was calculated as the difference between the normalized average Ct of the irradiated group and the normalized average Ct of the untreated control group. This DDCt value was raised to the power of 2 to calculate the degree of change.

2.6 *Validation of Birc-8 expression by quantitative PCR*

Total RNA was extracted using the Tri Reagent® Solution (Ambion) and was reverse transcribed using the iScript cDNA synthesis kit (BioRad, SA). Real-time PCR was performed using the iQ Superscript reagent (BioRad, SA). Levels of mRNA for BIRC 8 (sense: 5' GTGAGCGCTCAGAAAGACACTAC 3', antisense: 5' CACATGGGACATCTGTCAACTG 3') was quantified with standardization of levels to the average of a housekeeping gene: GAPDH (sense: 5' CAACAGCCTCAAGATCATCAGC 3', antisense: 5' TGAGTCCTTCCACGATACCAAAG 3').

The PCR conditions were as follows: 40 cycles of a denaturation step (95^{o}C, for 30s), an annealing step (56^{o}C, for 15s), a plate read and an elongation step at 72^{o}C. This was followed by a melt curve from 60^{o}C to 95^{o}C, with a plate read every 2^{o}C held for 30s. For BIRC 8 and GAPDH, the final concentrations of primers used were 300nM

and 100nM, respectively. The method described by Livak and Schmittgen (2001) was used to calculate a relative fold change of each gene from cycle threshold values (Livak and Schmittgen, 2001). All experiments were conducted in triplicate and repeated at least thrice.

2.7 *Western blot of Birc-8/ILP-2 and Smac/DIABLO protein level*

Isolated protein samples (HepG2 crude protein extract standardized to 1mg/ml) were denatured by boiling for 10min with a 1:1 dilution with 1× Laemmli sample buffer (0.375M Tris–HCl pH 6.8; 10%, w/v SDS; 3%, v/v glycerol; 0.2%, w/v bromophenol blue; 12% β-mercaptoethanol in dH_2O). For each sample, 100μg of total protein was loaded on a 10% polyacrylamide gel, run at 150V. Transfer onto nitrocellulose membrane was conducted at 350mA for 1h. Membranes were blocked for 1h with blocking buffer containing 5% non-fat dry milk in Tris buffered saline (TTBS, 25mM Tris pH 7.6, 150mM NaCl, 0.05% Tween 20).

Membranes were probed overnight at 4°C with anti-ILP2 (ab9664) (Abcam, USA), anti-Smac/DIABLO (ab110291) and anti-β actin (Sigma, USA) diluted to 1:1000 in 3% BSA in TTBS. A horseradish peroxidase-conjugated secondary antibody diluted to 1:5,000 in 1% bovine serum albumin (BSA) in TTBS was used to allow detection of appropriate bands using LumiGLO® Chemiluminescent Substrate Kit (KPL). Images were captured on the Alliance 2.4 gel documentation system (UViTech, UK). All experiments were conducted at least thrice and blots were analysed using UViBand analysis software (UViTech, UK).

2.8 *Statistical evaluation*

Statistical analyses were performed using GraphPad Prism version 5.00 software package (GraphPad PRISM®). Data are expressed as mean ± standard error of the mean (sem). Comparisons were made using Mann Whitney Tests, unpaired t tests with Welch Correction or the One-way analysis of Variance with Dunn's multiple comparisons posttest. Statistical significance was set at 0.05.

3. Results

3.1 *Dose response studies*

A dose response was determined using serially diluted concentrations of FB_1 (0 - 1000μM) in HepG2 cells over 24h. Analysis of the dose response curve showed that 200μM FB_1 was sufficient to cause 50% HepG2 cytotoxicity (IC_{50}) (Figure 5.1A). This concentration of FB_1 was used in all subsequent experiments unless otherwise stated.

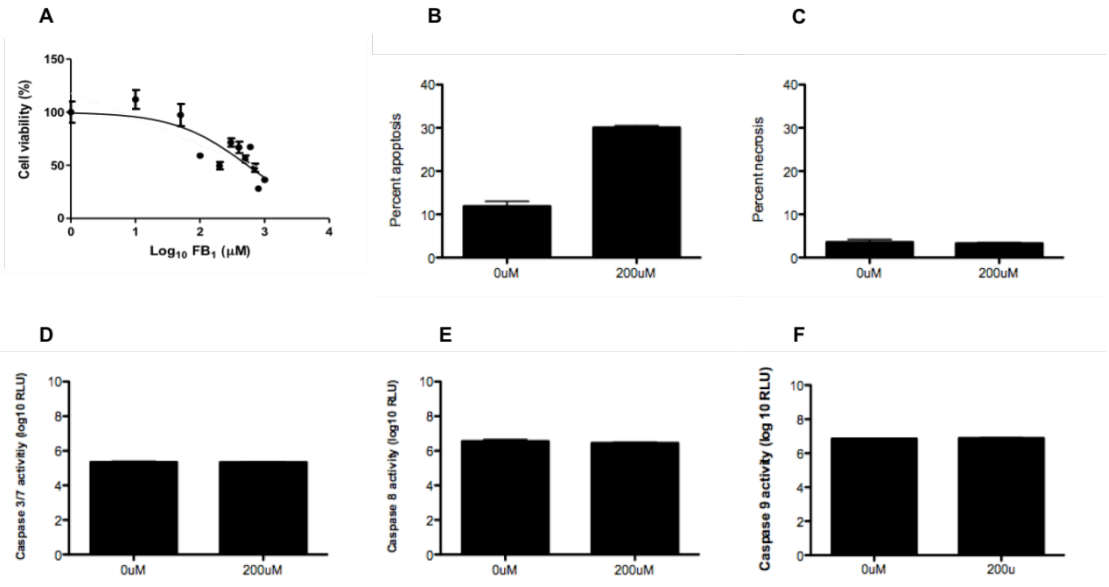

Figure 5.1 A: Dose dependent decline in HepG2 cell viability following treatment for 24hours with a range (0-1000μM) of concentrations of FB_1. Data represented as a percentage of the means from 3 separate experiments. Where *p<0.05, **p<0.01, ***p<0.001 compared to 0μM; **B:** Percentages of apoptotic and **C:** necrotic HepG2 cells after treatment with FB_1 for 24hours (* $p < 0.005$ (-8.841 to -1.359, 95% CI of difference); ** $p < 0.0004$ (-21.94 to -14.46, 95% CI of difference); Mann Whitney Test; **D-F:** Apoptotic caspase activity. Initiator Caspases -8, -9, and executioner caspases C: -3/7. * $p < 0.005$ (5.56×10^6 to 6.63×10^6, 95% CI of difference) ** $p < 0.0004$ (-7.23×10^6 to -6.19×10^6, 95% CI of difference).

3.2 *Apoptosis studies*

Next we investigated the effects of FB_1 on HepG2 cell apoptosis by examining translocation of PS residues on the plasma membrane using the Annexin-V assay. FB_1 (200μM) significantly increased (2.5 fold) the percentage of PS externalization in HepG2 cells – an indication of early apoptotic cell death. Cell death due to necrosis was ruled out by assessing incorporation of PI. Less than 5% necrosis was observed and control cells did not differ significantly from the FB_1 treatment (Figure 5.1B-C).

The caspases are the molecular machinery of apoptosis and the activities of key initiator caspases (-8 and -9) and executioner caspases (-3 and -7) in HepG2 cells after treatment with FB_1 was determined. Interestingly, despite the increase in PS translocation by FB_1, there were differences in caspase activity after treatment (Figure 5.1D-F).

To address this disparity in measurements of conventional markers of apoptosis, we systematically investigated changes in expression of all known apoptosis-associated genes by qPCR arrays. In total we evaluated the expression of 84 apoptosis related genes (Figure 5.2A). Fold change profile plots showed that of all genes evaluated, BIRC-8/ILP-2 was significantly up regulated (more that 8-fold) after treatment with FB_1 (Figure 5.2B). In addition both death-associated protein kinase 1 (DAPK1) and caspase associated recruitment domain 8 (CARD) were also elevated with deceased expression of caspases -9, -3 and -7 (Figure 5.2B)

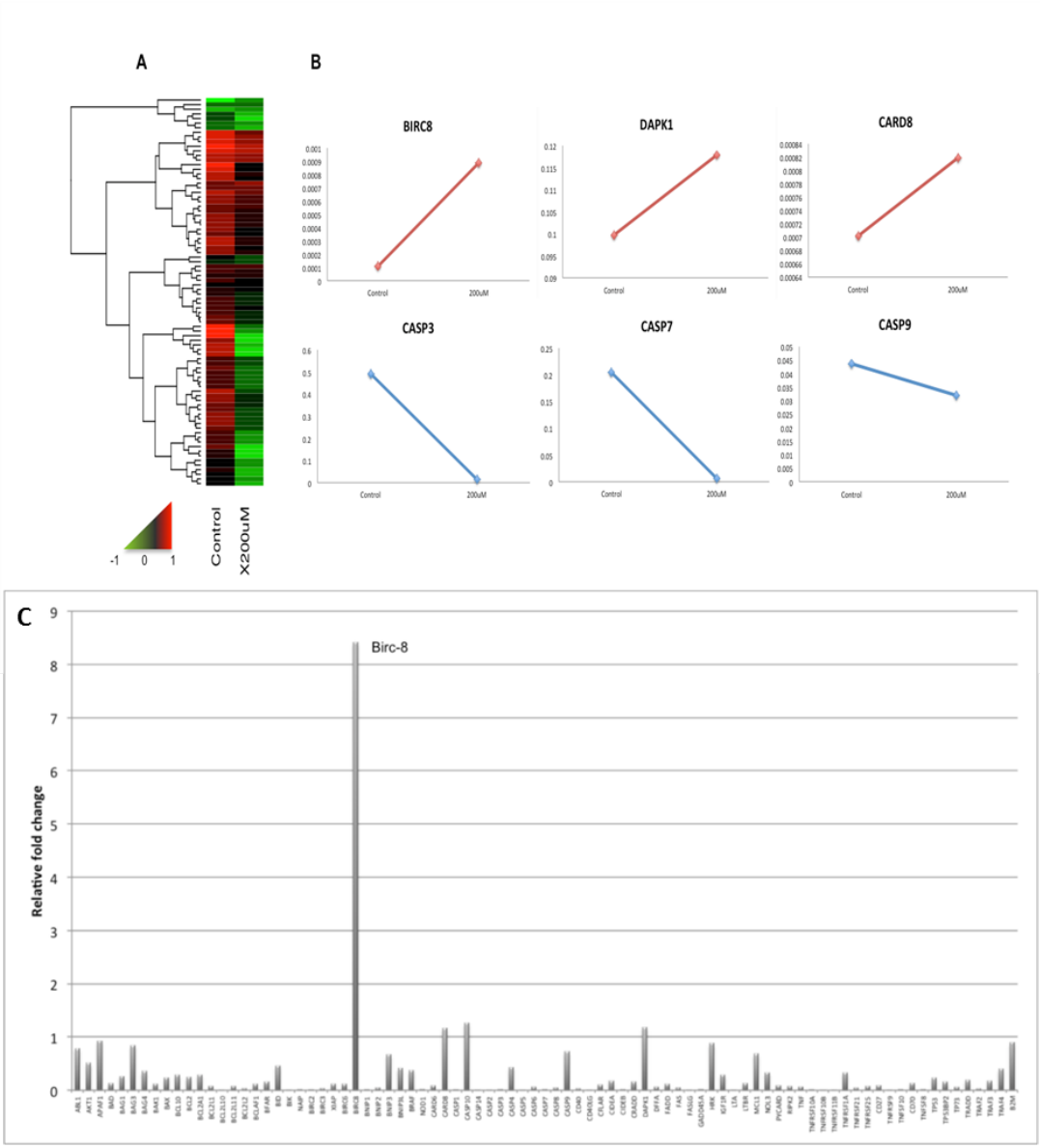

Figure 5.2: Fold change profiles for apoptotis-associated genes in HepG2 cells after FB_1 treatment with 200µM FB_1. (A) Heatmap of relative expression of all 84 genes in apoptosis array. It shows that in general, basal expression of apoptosis relevant genes are higher. Upon treatment, it seems that most of these genes are repressed relative to the control. There are however some clusters of induced genes. (B) Some examples of induced genes include Birc8/ILP2, DAPK1 and CARD8. The usual apoptosis machinery (Caspases -3, -7, -9) seem to be repressed as early as the transcript level. (C) Fold change profile plot shows BIRC-8/ILP-2 was significantly up-regulated by FB_1 p=0.0038; Mann Whitney Test).

3.3 *Evaluation of BIRC-8/ILP-2 induction and Smac/DIABLO expression*

To validate the induction of BIRC-8/ILP-2 by FB_1 we performed independent qPCR experiments. These analyses showed that 200μM FB_1 was indeed able to up-regulate expression of BIRC-8/ILP-2 in HepG2 cells after 24hr of culture by more that 4-fold (Figure 5.3A). Immuno-blot experiments showed that increases observed in the BIRC-8/ILP-2 transcript fully translated to increases in BIRC-8/ILP-2 protein levels (Figure 5.3B). Fumonisin B_1 also caused a 1.7-fold decrease in Smac-DIABLO protein levels (Figure 5.3C).

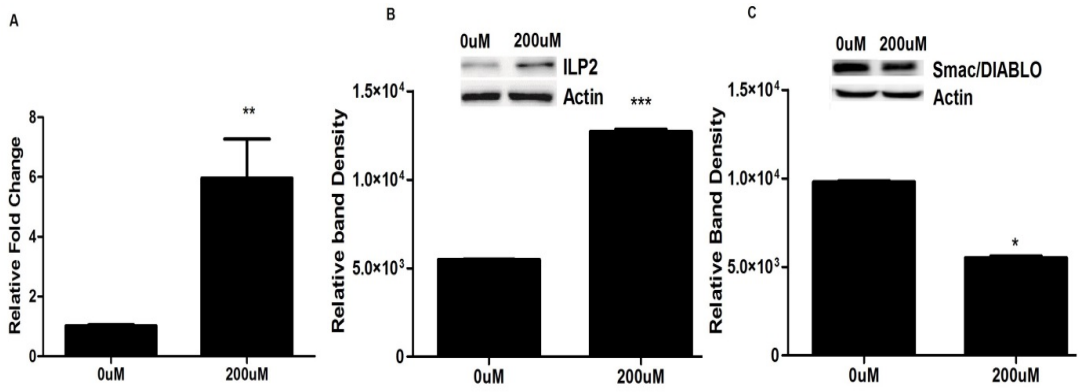

Figure 5.3: BIRC-8/ILP-2 transcript **(A)** and protein **(B)** expression up-regulated and Smac/DIABLO protein expression **(C)** down-regulated in HepG2 cells after FB_1 treatment with 200μM FB_1. The ILP-2 protein levels was significantly up-regulated by FB_1 (2.3 fold vs. control, p=0.003; Mann Whitney Test) and Smac/DIABLO protein expression was decreased by 1.7-fold vs. control, p=0.029; Mann Whitney Test).

3.4 *Dose dependent effects of FB_1*

We next tested whether the effects of FB_1 with respect to BIRC-8/ILP-2 induction were dose related. From the IC_{50} dose, FB_1 concentration was serially decreased by two-fold (200μM, 100μM, 50μM, 0μM) and thereafter HepG2 cells were treated for 24hr. Measurements of BIRC-8/ILP-2 transcripts by qPCR show no difference up to 50μM FB_1. However there is a sharp and significant increase in BIRC-8/ILP-2 expression at 100μM and 200μM FB_1 (Figure 5.4A). Immuno-blots clearly show that ILP-2 protein levels track with a similar trend. There are no significant changes in BIRC-8/ILP-2 protein from baseline up to 50μM FB_1. FB_1 concentrations above 50μM caused significant increases in BIRC-8/ILP-2 protein levels (Figure 5.4B). These data suggest that induction and stabilization of BIRC-8/IL-2 is dependent on dose of FB_1. Interestingly, caspase-8 /-9 activities correspond directly with dose of FB_1. Up to 50μM FB_1 there is a steady and significant increase in caspase-8/-9 activity. However, activity of both caspases diminishes at higher doses of FB_1 as BIRC-8/ILP-2 is induced (Figure 5.4C). Taken together, these data strongly suggest that the switch in apoptosis inducing capability of FB_1 is dose dependent.

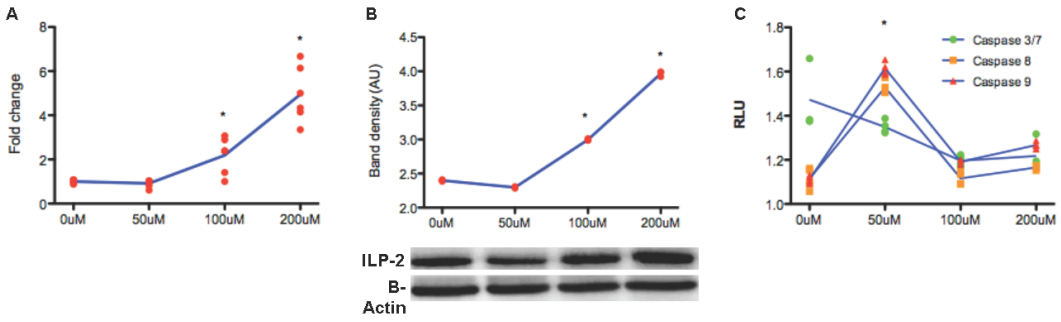

Figure 5.4: Dose dependent induction of BIRC-8/ILP-2 transcript **(A**, n = 6 replicates per dose**)** and dose dependent increase in BIRC8/ILP-2 protein expression **(B**, n = 3 replicates per dose**)** in HepG2 cells after FB_1 treatment. * $p<0.0001$ ANOVA with Bonferroni post test (0µM vs. 100µM and 0µM vs. 200µM) **(C)** Caspase activity in HepG2 cells at different FB_1 concentrations (n = 4 replicates per dose). * $p<0.0001$ ANOVA with Bonferroni post test (0µM vs. 50µM).

4. DISCUSSION

Fumonisin B_1 has been implicated in the nephrotoxicity, hepatotoxicity and carcinogenicity in several animal systems (Bulder et al., 2012; Gelderblom and Marasas, 2012). The contamination of corn by FB_1 results in the inevitable exposure of humans to this mycotoxin. Since early 1970's studies on FB_1 have associated it with mitogenic and apoptotic inducing properties and literature indicates distinct species-specific toxicity. In China, South Africa and Iran, FB_1 has been linked with an increased risk of esophageal cancer (Shephard et al., 2007; Shephard et al., 2002; Sun et al., 2007; Sun et al., 2011) and with stunted growth in Tanzania (Kimanya et al., 2010). The mechanism of action of FB_1 which occurs via competitive inhibition of ceramide synthase has been well described (van der Westhuizen et al., 2004).

There are several conflicting mechanisms of molecular toxicity in different organ systems (Gelineau-van Waes et al., 2005; Marasas et al., 2004; Mobio et al., 2003; Voss et al., 2011). It has been established that following the biological distribution of FB_1, the liver and kidneys retain the highest concentrations (Voss et al., 2001). The current study evaluated the acute toxicity of FB_1 on human derived hepatocellular carcinoma (HepG2) cells.

With the intention of completing the battery of cytotoxicity tests recommended by Miret et al. (2006), an evaluation of programmed cell death and necrosis markers were evaluated (Miret et al., 2006). FB_1 did not cause an increase in necrosis but significantly increased the externalization of PS - an early marker of apoptosis. The initiator caspase-8 involved in the extrinsic activation of the apoptosis pathway and the late activated executioner caspases-3/-7 was not affected by FB_1 at 200µM. In addition FB_1 did not influence HepG2 caspase-9 activity as compared to the control. Programmed cell death is a tightly regulated process that occurs via distinguished activation pathways. Our findings, however, suggests that HepG2 cells treated with FB_1 is able to translocate PS to the external cell membrane in the absence of caspase activation. This process has been previously described as 'caspase-independent PS externalisation' (Ferraro-Peyret et al., 2002; Quignon et al., 1998; Thon et al., 2005). One mechanism described that caspase-dependent cell death via the extrinsic pathway is dependent on ceramide as a mediator of TNF-induced apoptosis, but was still active in the presence of caspase inhibitors and did require a change in mitochondrial membrane permeability or cytochrome C release (Thon et al., 2005).

Following exposure to FB_1, an inhibitor of ceramide synthase, ceramide synthesis decreases but ceramide is then sequestered from complex sphingolipids, altering cell membrane integrity potentially increasing PS exposure. The second mechanism of

caspase-independent cell death suggested that the process occurs via activation of oncogenes and viral associated structural change in nuclear material (Quignon et al., 1998). This is important as we previously reported the localization of FB_1 in the cytoplasm, mitochondria as well as the nuclei of oesophageal cancer cells by immunocytochemistry and transmission electron microscopy (Myburg et al., 2002).

To further verify our results we evaluated the mRNA levels on a panel of 84 apoptosis associated genes. Only one gene, from the inhibitor of apoptosis (IAP) protein family – BIRC 8 was significantly increased (8 fold - 200μM FB_1) relative to the untreated control cells. The AIP's inhibit apoptosis by either inhibiting enzyme activity of the caspases, or via ubiquitin-targeted proteasome degradation (Eckelman and Salvesen, 2006; Eckelman et al., 2006). Birc-8 specifically is referred to as an IAP-like protein 2 (ILP-2) and it contains all of the surface elements required for caspase-9 inhibition. The BIR domain, however, is thought to have an intrinsically unstable domain due to a natural amino-terminal truncation and this compromises the molecules ability to function as a caspase inhibitor (Shin et al., 2005). In this study FB_1 was able to significantly increase both the mRNA (5.7 fold) and protein expression (2.3 fold) of BIRC-8 in HepG2 cells (Figures 5.3A and 5.3B). FB_1 initiated apoptosis via the intrinsic pathway as indicated by caspase-9 activity, but the lack of executioner caspase -3/-7 activity can be attributed to increased expression of both ILP-2 protein BIRC-8 mRNA. Furthermore, Smac/DIABLO, a mitochondrial pro-apoptotic protein, is able to antagonise the inhibitory effects of XIAP due to the presence of IAP-binding protein motifs that fit into the BIR domains of XIAP (Chai et al., 2000; Fischer and Schulze-Osthoff, 2005).

We showed that FB_1 caused a decrease in protein expression of Smac/DIABLO (Figure 5.3C). A recently published finding by our group illustrated the effect of FB_1

on microRNA (miRNA) expression in HepG2 cells and found a significant down-regulation of both miR-27b and miR-27a (Chuturgoon et al., 2014b). Interestingly, both miR-27a and miR-27b also target and specifically bind to *XIAP* (NM_001167) in the 3' UTR (position 6748-6754) with an exact match to positions 2-8 of the mature miRNA seed sequence. Thus elevated IAP components observed in FB_1 treatment may also be attributed to compromised post-transcriptional regulation by miR-27a/b.

Therefore, functionally compromised cells that persist as a result of incomplete apoptosis may have a high propensity to become oncogenic. The over expression of DAPK1 suggests a cytoprotective effect that is mediated through both the intrinsic and extrinsic apoptotic signaling pathways and results in inhibition of cytochrome c release from the mitochondria as well as inhibition of caspase-3 and caspase-9 activity. This suggests that DAPK1 is a negative regulator of TNF-induced apoptosis (Jin et al., 2002). Overexpression of CARD8 was noted in some cancers that suppress caspase mediated apoptosis (Bagnall et al., 2008).

Our data clearly shows that FB_1 modulates apoptosis of HepG2 cells in a complex manner – and it is not a matter of simply switching on or off. The HepG2 cells do perceive toxic stress, as indicated by membrane changes and oxidation (Reactive oxygen species/GSH – Addendum C), but more importantly these cells can reverse apoptosis by the induction of the IAP machinery in response to FB_1.

5. Conclusion:

In conclusion, our results show that FB_1 inhibits apoptosis in HepG2 cells by inducing increased expression of Birc-8/ILP-2 that may then lead to liver tumourigenesis.

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CHAPTER 6

CONCLUSION

FB_1, a type 2B carcinogen, is known to promote tumourigenesis but its cancer initiating properties have not been conclusively proved. Many studies on laboratory animals showed that FB_1 possesses liver cancer initiating effects.

FB_1 exhibited cancer initiating properties in male BD IX, Fischer 344 and Sprague Dawley rats (Gelderblom et al., 1994; Gelderblom et al., 2001; Gelderblom et al., 2008; Mehta et al., 1998). FB_1 can initiate cancer in rat liver similar to other genotoxic carcinogens. However, rat liver cancer initiation was only effected after prolonged feeding and depended on the dose of FB_1. An important observation was that the levels of DNA mutations occurred at a significantly lower rate when compared to other genotoxic agents (Gelderblom et al., 1992; Norred et al., 1992). In comparison to other genotoxins, cell proliferation associated with hyperplasia was minimal, while stimulation of regenerative cell proliferation enhanced the cancer initiating potency of FB_1 (Gelderblom et al., 2001).

Currently, there is minimal evidence about the nature of the initiating step of either genotoxic or non-genotoxic carcinogens such as FB_1, although parameters such as protein and RNA can be altered independently from changes to the DNA (Gelderblom and Marasas, 2012). Apoptosis plays a critical role in the outcome of the cancer initiating event, as initiated cells are more prone to undergo apoptosis (Bursch et al., 1992). FB_1 induces necrosis as well apoptosis in the liver that could determine the outcome of cancer initiation (Gelderblom et al., 2008).

This study showed that FB_1 has epigenetic and apoptotic properties in a human liver cell line (HepG2). Firstly, FB_1 (with an IC_{50} value of 200 μM) disrupted DNA methylation and induced chromatin modifications in the HepG2 cells. The data shows, for the first time, that FB_1 induced global DNA hypomethylation in human hepatoma cells by altering the balance of DNA methylases/demethylases, thereby disrupting the structural integrity of DNA. FB_1 significantly decreased the methyltransferase activities of DNMT1, DNMT3A and DNMT 3B, and significantly up-regulated the demethylases (MBD2 expression and enzyme activity). In addition, FB_1 also significantly increased the expression of two histone demethylase genes *KDM5B* and *KDM5C* (members of the Jumonji family of proteins that catalyse the demethylation of tri- and dimethylated lysines). They specifically act on tri- and dimethylated lysine 4 on histone H3 (H3K4) (only found at transcribed genes) and are associated to euchromatin and active transcription. Trimethylated H3K4 is highly enriched around transcriptional start sites whilst dimethylated H3K4 is present throughout the coding region of transcribed genes. *KDM5B* is regulated by the oncogene *c-ErbB2* and is highly expressed in ductal breast carcinomas and is associated with the malignant phenotype in breast. In addition to its role in promoting cancer, *KDM5B* is needed for the proliferation of MCF-7 breast cancer cells and tumour growth of mammary carcinoma cells in nude mice; its target genes in breast cancer proliferation have been identified as *BRCA 1, CAV 1* and *HOXA5*. FB_1 increased *KDM5B* and *KDM5C* expression in liver HepG2 cells that will result in increased H3K4Me3/Me2 demethylation. A consequence of this increased H3K4 demethylation will disrupt its role as a transcriptional repressor.

FACS data (using anti- 5'-MeCyt) showed a significant increase in global DNA hypomethylation, resulting in gross changes in structural DNA, an observation

supported by the significantly long DNA comet tails. This is the first evidence that shows FB_1 induced epigenetic DNA hypomethylation in human liver cells leading to chromatin instability and aberrant transcriptional activation.

FB_1 results in aberrant gene expression in liver cells; the cells may alter their normal miRNA profiles and create a micro-environment which facilitates carcinogenesis. It is likely that the cells may change their miRNA milieu in response to FB_1 toxicity. Five miRNAs were down regulated (miR-27b, miR-27a, miR-30c, miR-181d and miR-135b) with miR-27b significantly down regulated by 11 fold. MiR-27b modulates *CYP1B1* expression, suggesting an additional mode of hepatic neoplastic transformation by FB_1. CYP1B1 catalyses the metabolic activation of many pro-carcinogens including PAHs and aryl amines, and the metabolism of oestradiol to form 4-hydroxyoestradiol, which causes DNA damage and contributes to oestrogen-dependent cancers. Liver cells transfected with the mimic to miR-27b showed that FB_1 significantly down-regulated expression of miR-27b; whilst *CYP1B1* mRNA and protein expression was significantly upregulated by 1.8- fold and 2.6- fold respectively. *CYP1B1* is post-transcriptionally regulated by miR-27b in HepG2 by FB_1. FB_1- induced epigenetic modulation of miR-27b in hepatic cells may be an additional mode of hepatic neoplastic transformation.

The ability of cells to escape apoptosis is critical in the development of cancer and FB_1 may selectively manipulate apoptotic pathways leading to resistance to apoptosis, thus allowing altered growth capabilities. Interestingly, the cancer promoting effects of FB_1 are closely correlated with increased apoptosis and subsequent regenerative cellular proliferation. Using a panel of 84 apoptosis associated genes FB_1 significantly increased only one gene, from the IAP protein family – BIRC 8 (8 fold). The AIP's inhibit apoptosis by either inhibiting caspases, or via ubiquitin-targeted

proteasome degradation. Birc-8 or ILP-2 contains the surface elements required for caspase-9 inhibition. FB_1 significantly increased both the mRNA and protein expression of BIRC-8/ILP-2 in HepG2 cells. FB_1 initiated apoptosis via the intrinsic pathway as indicated by increased caspase-9 activity, but the lack of executioner caspase-3/-7 activity can be attributed to increased expression of both ILP-2 protein and *BIRC-8* mRNA. Further, Smac/DIABLO protein expression, a mitochondrial pro-apoptotic protein, was decreased by FB_1. FB_1 influences microRNA expression in HepG2 cells. Thus elevated AIP components observed in FB_1 treatment may also be attributed to compromised post-transcriptional regulation by miR-27a/b.

The data clearly shows that FB_1 modulates liver cell apoptosis in a complex dose-dependent regulation of pro- and anti-apoptotic molecules. The HepG2 cells do perceive toxic stress, but more importantly these cells can reverse apoptosis by the induction of the IAP machinery in response to FB_1.

Finally, taken together, this study shows that FB_1 possess epigenetic properties by inducing global DNA hypomethylation, modulating miRNA expression and increasing expression of the IAP protein (BIRC8/ILP-2); FB_1 is a genotoxic agent that can lead to liver tumourigenesis.

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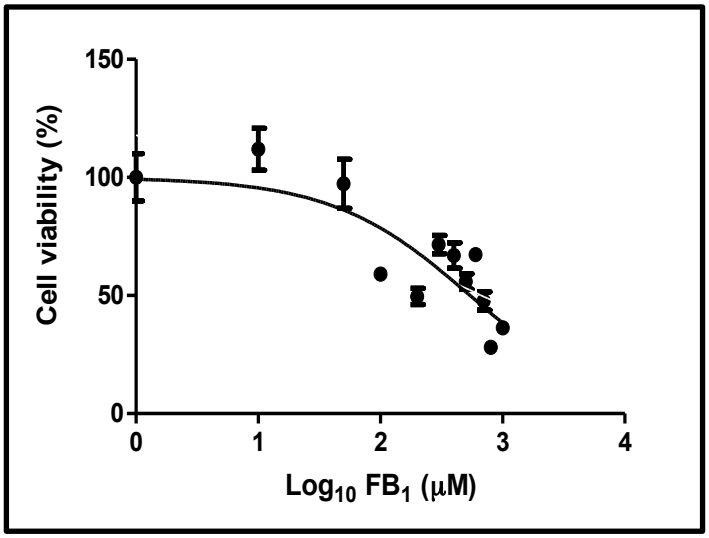
Addendum A

HepG2 cell viability treated with a range of FB_1 concentrations

HepG2 cells were treated with a range (0- 1000µM) of concentrations of FB_1 over 24hours. A dose dependent decline in HepG2 cell viability was observed and an IC_{50} value of 200µM was determined (Addendum Table 1 and Figure 1).

Addendum A-Table 1: HepG2 cell viability treated with a range of FB_1 concentrations (0-1000µM) for 24 hours

FB_1 [µM]	Mean Viability ± SEM	95% CI of Difference
0	100.0 ± 10.0	-38.86 to 15.02
10	111.9 ± 8.8	-24.25 to 29.63
50	97.3 ± 10.4 **	14.02 to 67.90
100	59.0 ± 2.5 ***	23.44 to 77.32
200	49.6 ± 3.5 *	1.522 to 55.40
300	71.5 ± 3.8 *	6.137 to 60.02
400	66.9 ± 5.4 **	17.10 to 70.98
500	56.0 ± 3.3 ***	25.37 to 79.25
800	28.1 ± 2.7 ***	44.98 to 98.86
1000	36.4 ± 1.3 ***	36.71 to 90.59

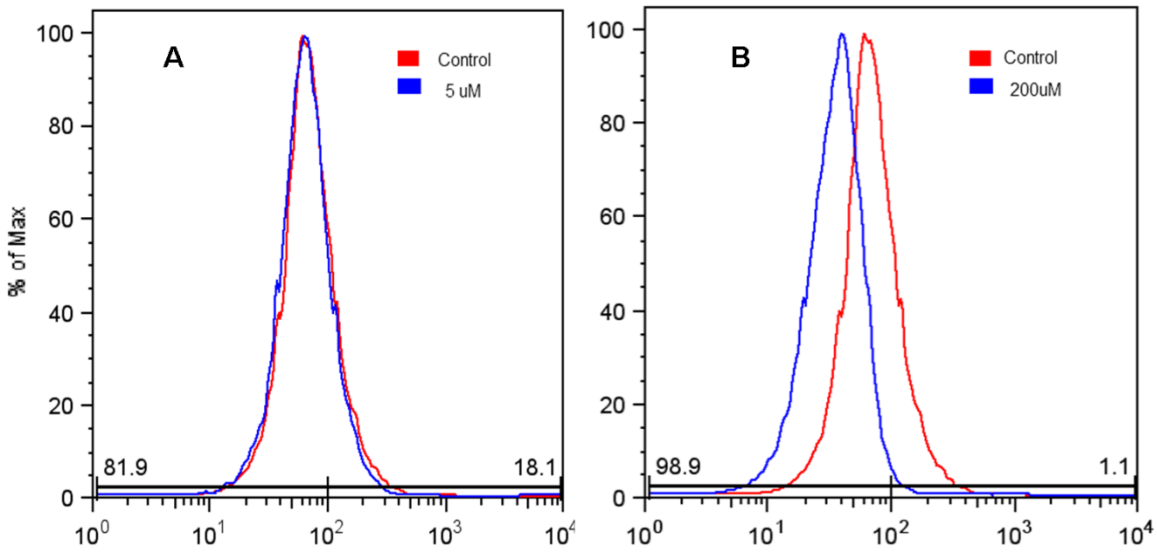


Addendum A- Figure 1: A dose dependent decline in HepG2 cell viability following treatment for 24hr with a range (0-1000μM) of concentrations of FB_1. Data represented as a percentage of the means from 3 separate experiments. Where *p<0.05, **p<0.01, ***p<0.001 compared to 0 μM.

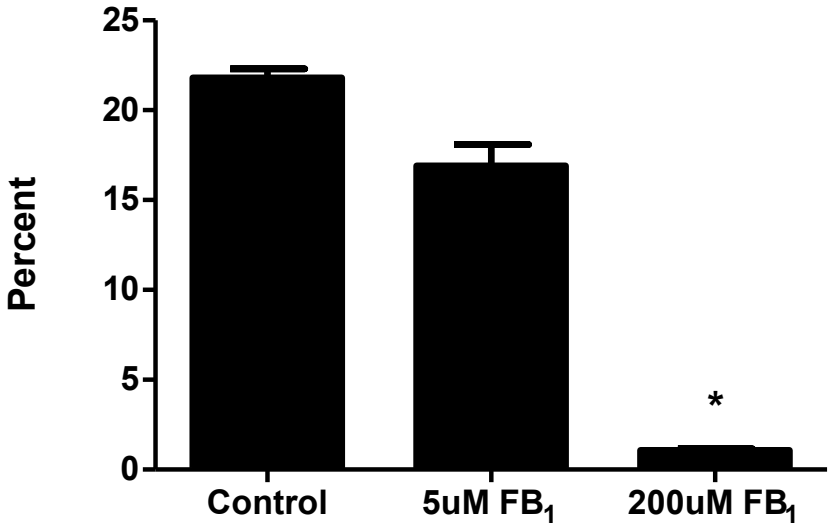
Addendum B

DNA hypomethylation by 5µM FB_1

Using FACs, methylation assays (anti-5'-MeCyt) on HepG2 cells were performed with 5µM FB_1 (mitogenic concentration) after 24 hours incubation. At 5µM FB_1 induced hypomethylation (Addendum B -Figures 2A and 3), but the hypomethylation was not significant as that observed at 200µM FB_1 (Addendum B -Figures 2B and 3).



Addendum B- Figure 2: Histograms showing global DNA hypomethylation in HepG2 cells after 24hr culture with FB_1. **A**: 5-methylcytosine detection in 5µM FB_1 treatment. **B**: 5-methylcytosine detection in 200µM FB_1 treatment.



Addendum B- Figure 3: Flow cytometric detection of 5-methylcytosine in HepG2 cells (* p = 0.0006; One way ANOVA).

Thus using both a low concentration of FB_1 (5µM; mitogenic) and a cytotoxic concentration (200µM; IC_{50}) – hypomethylation was induced in HepG2 cells, with significant hypomethylation at the 200µM FB_1 concentration.

ADDENDUM C

Oxidative stress induced by FB_1

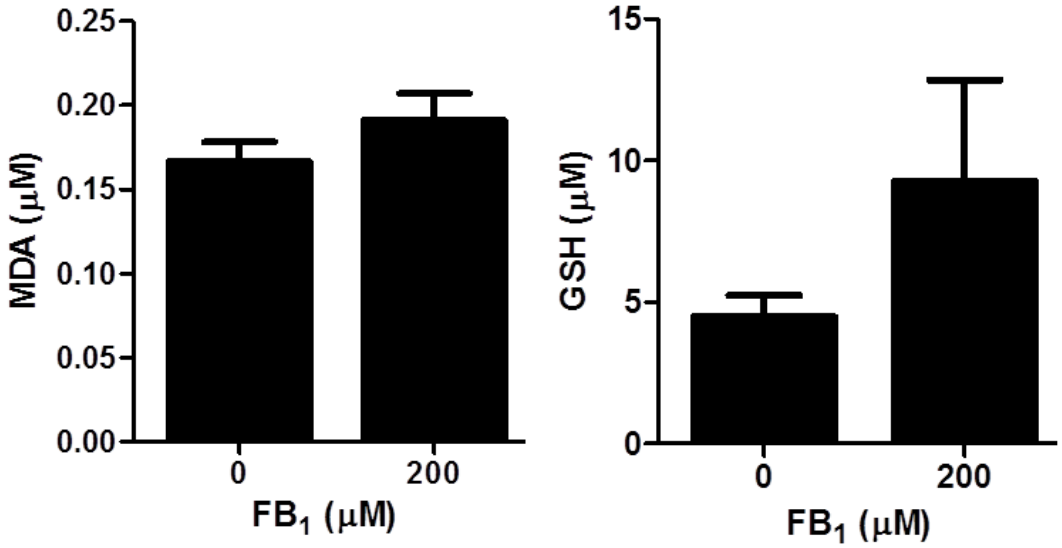
Lipid peroxidation assay

The by-product of lipid peroxidation, malondialdehyde (MDA), was assessed using the thiobarbituric acid reactive substances (TBARS) as an indicator of oxidative damage in the cell. Briefly, 200µl of 2% H_3PO_4, 400µl of 7% H_3PO_4, 400µl of TBA/BHT solution and 200µl of 1M HCL were added to thoroughly cleaned test tubes. The supernatants from each sample (100µL) was added to the relevant test tube. A positive control was prepared by adding 1µL MDA to a test tube. All tubes were incubated in a water bath (100°C, 15min) and after cooling; butanol (1.5ml) was added to each tube, vortexed for 10 seconds and allowed to separate into two distinct phases. 100µL of the butanol phase (upper phase) was transferred in triplicate into a 96-well microtitre plate and read using a spectrophotometer (Bio-tek µQuant) at 532/600nm. Results are expressed as mean optical density which correlates directly to MDA levels.

Quantification of Glutathione

Glutathione-Glo™ Assay (Promega) was used according to manufacturer's guidelines to quantify glutathione (GSH) levels. 50µl from each sample (MOE treated and untreated control) was added in six replicates to the wells of an opaque polystyrene 96-well microtitre plate. GSH standards (0-50µM) were prepared from a 5mM stock diluted in de-ionised water. 50µl of each GSH standards and 50µl of the GSH-Glo™ Reagent 2X was added per well and incubated in the dark (30min, RT). Reconstituted Luciferin Detection Reagent (50µl) was added per well and incubated (15min, RT).

The luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). The data was analysed and expressed as relative light units (RLU).



Addendum C- Figure 4: FB_1 increased both MDA levels (lipid peroxidation) and intracellular GSH levels inHepG2 cells