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SCREENING HUMAN CANCER PATHWAYS FOR A ROLE OF AFLATOXIN B1

BY

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DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Aflatoxin B1 (AFB1) has been shown to negatively affect the functions of major body organs such as the liver, kidney, spleen and heart, as well as decreasing cellular immunity and causing cancers, primarily hepatocellular carcinoma. Some studies have suggested that mutation in the codon 249 of p53 tumour suppressor gene is a key event in AFB1 induced carcinogenesis but the effects of AFB1 on other cancer pathways are not known. The aim of the study was to screen 45 cancer pathways for a role of AFB1 using human embryonic kidney cells (HEK 293) as a model system. Cytotoxicity was determined by treating HEK 293 cells with increasing concentrations of AFB1 for 24, 48 and 72 hours and the viability of cells was evaluated by an MTS based assay. It was established that increasing concentration of AFB1 killed cells in a dose dependent manner with 32 μ M being established as the working concentration. The cells were then reversed transfected with pre-coated pathway reporters in Cignal Finder 45-pathway reporter array for 24 hours and treated with or without 32 μ M of AFB1 for 24 hours and harvested. The influence of AFB1 on the pathways was then determined by measuring the luciferase activity of the pathway reporters using dual luciferase reporter gene assay. Validation of data from the Cignal Finder 45-pathway reporter array was achieved by measuring the influence of AFB1 on interferon (IFN) induced signal transduction pathway in an independent assay. The results of Cignal Finder 45-pathway reporter array showed that AFB1 differentially modulates multiple pathways. Key pathway reporters upregulated included AARE, ARE, ATF-6, GRE, MTF-1, ISRE, NF κ B and PAX-6 whereas p53, FoxO, NFAT, PPAR, SP-1 and STAT3 were down-regulated. Results from the validation experiment showed that AFB1 up-regulated IFN induced signal transduction which was consistent with that of Cignal Finder 45-pathway reporter arrays. These results show that AFB1 could cause cancer by deregulating multiple pathways aside the p53 pathway suggesting that further studies should be done to establish the exact point of influence of AFB1 on these pathways.

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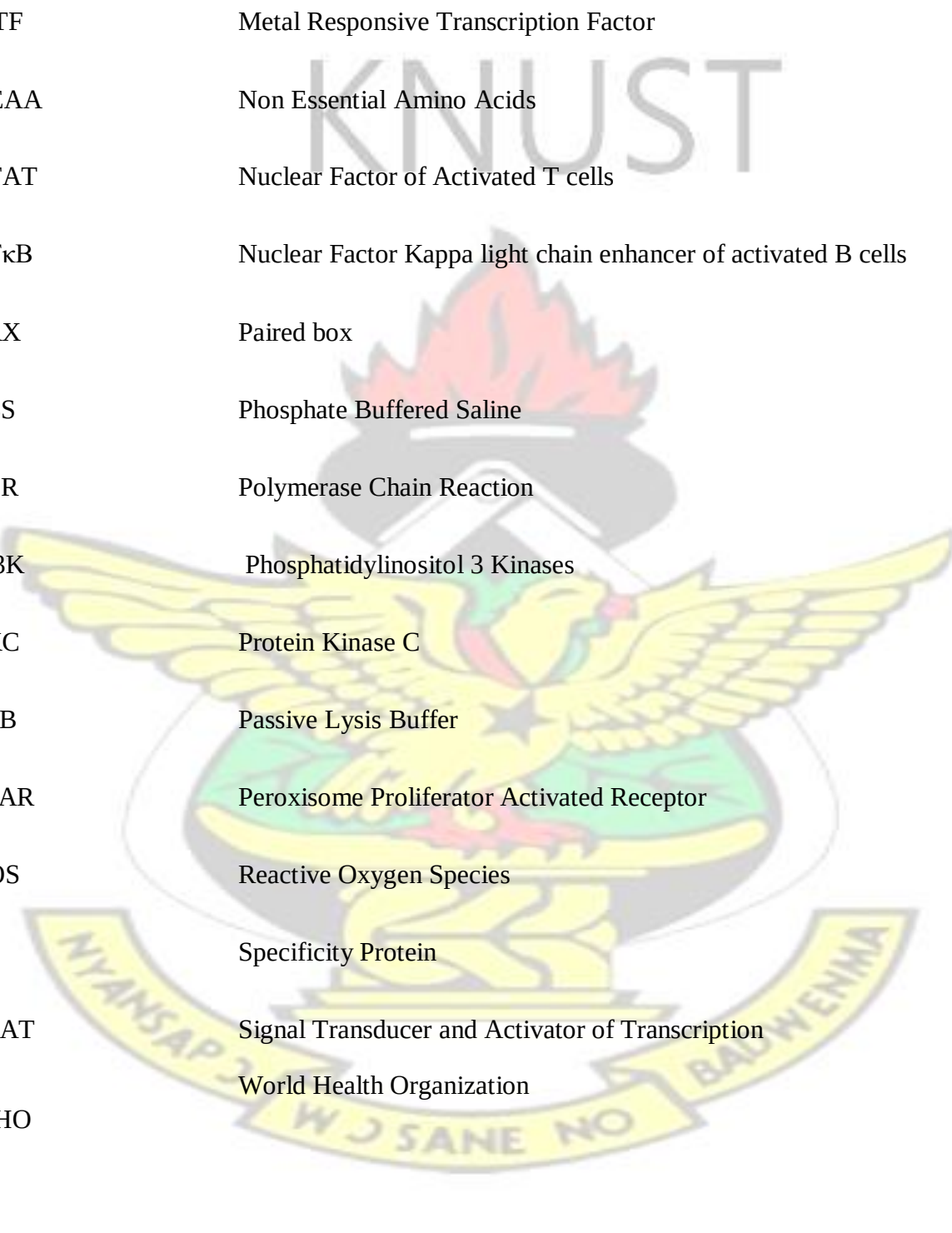
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List of Acronyms



AADR	Amino Acid Deprivation Response
AR	Antioxidant Responsive
ARE	Antioxidant Responsive Elements
ATF	Activation Transcription Factor
CNS	Central Nervous System
CYP	Cytochrome
DMEM	Dulbecco's Minimum Essential Medium
DNA	Deoxyribonucleic Acid
ER	Endoplasmic Reticulum
GFP	Green Fluorescence Protein
GLOBOCAN	Global Burden of Cancer
GR	Glucocorticoid Receptor
HCC	Hepatocellular Carcinoma
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
IARC	International Agency for Research on Cancer
IFN	Interferon
JAK	Janus activated kinase



LAR	Luciferase Assay Reagent
MTF	Metal Responsive Transcription Factor
NEAA	Non Essential Amino Acids
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor Kappa light chain enhancer of activated B cells
PAX	Paired box
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3 Kinases
PKC	Protein Kinase C
PLB	Passive Lysis Buffer
PPAR	Peroxisome Proliferator Activated Receptor
ROS	Reactive Oxygen Species
Sp	Specificity Protein
STAT	Signal Transducer and Activator of Transcription
WHO	World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background

The incidence of cancer has increased in most countries. In 2013 alone, a study on global burden of diseases across 188 countries, recorded 14.9 million new cancer cases and 8.2 million deaths due to cancer (Fitzmaurice *et al.*, 2015). In the United States of America, up to 60% of all cancer deaths have been linked to 8 risk factors. The risk factors were tobacco, alcohol, ionizing and solar radiations, infectious agents, obesity, occupations and physical inactivity (Schottenfeld *et al.*, 2013). The relationship between cancers, lifestyle and environmental risk factors presents the scientific community with questions. Questions such as how significant is aflatoxin's contribution towards the high burden of cancers and what are the molecular mechanisms underlying its contribution? Over the years, enough evidence has been gathered to confirm that aflatoxins cause cancers, primarily hepatocellular carcinoma (HCC) (Bressac *et al.*, 1991). The World Health Organization (WHO) reports HCC to be the third leading cause of death due to cancer worldwide (WHO, 2008). Of the total HCC new cases recorded each year, HCC resulting from exposure to aflatoxin accounts for about 25,200 – 155,000 (4.6–28.2%) out of 550,000 – 600,000 globally (Yan & Wu, 2010). Although the geographical distribution of HCC varies significantly, studies have shown that about 80% of cases occur in developing countries (Lodato *et al.*, 2006). In Ghana, HCC records the highest cancer mortality in males and third highest in females (Wiredu & Armah, 2006). Other works have shown that an individual's risk of liver cancer is about 30 times greater when exposed to HBV (hepatitis B virus) infection and aflatoxin as compared to when exposed to aflatoxin only (Groopman *et al.*, 2008). A study on the 2004 aflatoxicosis outbreak in eastern

Kenya, which resulted in 317 illnesses and 125 deaths, revealed a strong synergy between aflatoxin and HBV surface antigens in causing liver cancers and death (Azziz-Baumgartner *et al.*, 2005).

In addition to causing HCC, other works have shown that aflatoxins negatively influence the cellular immunity and thus makes the host susceptible to a wide range of infections (Jiang *et al.*, 2005). Aflatoxins have also been found to negatively affect the functions of some major organs in the body including liver, kidney, lungs, uterus, testes, heart and brain (Bbosa *et al.*, 2013). Others have also estimated that about 40% of disease burden in developing countries where short lifespan is prevalent has a form of relationship with aflatoxin exposure (Williams *et al.*, 2004).

Aflatoxins are a group of structurally related compounds produced as secondary metabolites by some species of *Aspergillus* (Jiang *et al.*, 2005). Although there are different types of naturally occurring aflatoxins, aflatoxin B1 is the most potent and has been classified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen (IARC, 2002). These ubiquitous moulds can successfully establish themselves during both pre-harvest period and post-harvest period. Their growth is further enhanced by tropical conditions as well as poor post-harvest practices. Thus, countries that lie within latitudes 40°N and 40°S of the equator are at a greater risk, especially developing countries within sub-Sahara Africa (Williams *et al.*, 2004). These moulds are however often found growing in maize, rice, peanuts, cassava, chilies, spices, oilseeds, cocoa beans and even smoked fish (Dorostkar & Mabodian, 2011; Williams *et al.*, 2004). The aflatoxins produced by these moulds are not only highly toxic, mutagenic, carcinogenic, teratogenic but also thermally stable. These toxins require a minimum of 160 °C for 30 minutes to achieve 100% degradation (Raters & Matissek, 2008). This suggests that the presence of aflatoxins in food is hard to manage since the normal cooking temperatures can't degrade it completely. Ingestion of foods containing aflatoxin therefore remains the primary non-occupational means by

which humans are exposed to these toxins (IARC, 2002). Aflatoxin exposure through food thus remains a significant risk factor for HCC (Wild & Gong, 2009). However, workers who handle infected food either in the farm or the processing unit may be exposed to airborne aflatoxin (IARC, 2002; Kauppinen *et al.*, 2000). Studies have shown that about 5.5 billion people are exposed to uncontrolled amounts of aflatoxins (Strosnider *et al.*, 2006). This therefore suggests that more than half of the world's population stand at some form of risks of developing aflatoxin related diseases.

Aflatoxin is readily assimilated into the blood upon ingestion or inhalation. Through blood circulation, aflatoxins reach various body organs, especially the primary target organ which is the liver. Within the body, aflatoxin undergoes both metabolic and genotoxic pathways and also interacts with other signalling pathways to bring about their physiological effects on the body (Bbosa *et al.*, 2013).

1.2 Problem statement

Studies have suggested strong correlations between aflatoxin exposure and HCC (AzzizBaumgartner *et al.*, 2005). Other works speculate that mutation in codon 249 of p53 tumour suppressor gene is the key event in HCC (Bressac *et al.*, 1991). However, the details of its interactions in the p53 pathway and other pathways as well as other organs are not known. As a result of this limited information, prognosis of HCC is often poor as most cases are detected at an advanced stage. This limits the potential curative treatment, which is surgery, to a few cases of HCC with small malignancies (Stefaniuk *et al.*, 2010). In order to determine an individual's risk and possibly manage the effects of aflatoxin on the body, the details of its interactions in the body systems should be known. It therefore befalls on the scientific community to investigate into the intricate nature of these interactions, develop specific biomarkers, enhance prognosis and ultimately save more lives.

1.3 Justification

This work was to identify which of the 45 cancer pathways are modulated by aflatoxin. This will open up several avenues for further studies into these pathways to know the exact points of influence of aflatoxin. The knowledge derived could further be explored to identify specific biomarkers of HCC and probably make early detection of HCC possible. In a nut shell, this study would thus contribute to the existing knowledge on the subject area and go a long way to influence future therapeutic interventions.

1.4 Aim/Objectives

The aim of the study was:

- To determine the modulation of aflatoxin B1 on the activities of 45 human cancer pathways.

The specific objectives were:

- To determine the cytotoxic effects of aflatoxin B1 on human embryonic cell line (HEK 293).
- To determine the human cancer pathways which are modulated by aflatoxin B1.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aflatoxins

Aflatoxins are a group of mycotoxins chemically regarded as difuranocyclopentanocumarines or difuranopentanolidocumarines compounds which consists of a dihydrofuran or a tetrahydrofuran ring (Talebi *et al.*, 2011). They were discovered in 1960 after causing a disease outbreak among turkeys in England (Richard, 2008). Subsequent research revealed that these mycotoxins are produced by closely related species within the *Aspergillus* genus. They were first found to be produced by *Aspergillus flavus* and thus named aflatoxin (A-fla-toxin). Other species later found to produce aflatoxins include *A. parasiticus*, *A. nomius*, *A. bombycis*, *A. ochraceoroseus*, *A. pseudotamarii*, and *A. australis* (IARC, 2002). Currently, there are about 20 known aflatoxins. Only 4 out of the 20 occur naturally and thus are regarded as major aflatoxins whereas the rest are metabolites. The 4 major aflatoxins are; aflatoxin B1, B2, G1, and G2. Of the metabolites, aflatoxin M1 and M2 are considered the most important because they are direct contaminants of food, especially dairy product (Talebi *et al.*, 2011). Aflatoxin B1 and B2 (AFB1 and AFB2 respectively) exhibit a strong blue fluorescence under ultraviolet lights and are referred to as the B group. Aflatoxins G1 and G2 (AFG1 and AFG2 respectively) are also referred to as the G group because they give off greenish yellow fluorescence under UV light. Chemically, the B group differs from the G group by the fusion of a cyclopentenone ring to its lactone ring (Raney *et al.*, 1990). AFB1 is considered the most common and potent aflatoxin, followed by AFG1. This is due to the presence of unsaturated bond at the 8, 9 position on the terminal furan ring. The unsaturated bond allows AFB1 and AFG1 to easily form epoxides which play a role in their carcinogenicity. AFB2 and

AFG2 are less potent, in that they first need to be metabolically oxidized to AFB1 and AFG1 (Groopman & Kensler, 2005). On the other hand, AFM1 and AFM2 are hydroxylated metabolites of AFB1 and AFB2 respectively. They are found in milk and other dairy products from animals fed with AFB1 and AFB2 contaminated feed (Iha *et al.*, 2011). Recently, a study on lactating mothers in Nigeria revealed the presence of AFM1 in their breast milk (Adejumo *et al.*, 2013). The IARC classifies AFM1 as a class 2B carcinogen and thus suggests considerable risks to babies and infants when fed with contaminated milks.

2.2 Toxic effects of aflatoxins

Aflatoxicosis can be defined as poisoning due to aflatoxin exposure either through ingestion of contaminant food or feed and in some case through inhalation (Bbosa *et al.*, 2013; Larsson & Tjälve, 2000). Based on the length and dosage of exposure, aflatoxicosis can broadly be classified into acute aflatoxicosis and chronic aflatoxicosis. Large doses of aflatoxin cause acute aflatoxicosis and result in direct damage to the liver, which is the primary target organ, followed by illness or death. Experimentally, most laboratory animals readily die upon administration of large doses. On the other hand, chronic aflatoxicosis is due to low dose exposure to aflatoxins over a long period. These sub-lethal doses have been shown to have detrimental effects on the nutritional and immune system of both humans and animals. Nonetheless, all doses have an accumulated effect on the liver and thus increase the risk of HCC. Although the liver is the primary target organ, aflatoxins can cause tumours in kidney and colon as well. (Jiang *et al.*, 2008; Kensler *et al.*, 2011; Talebi *et al.*, 2011; Williams *et al.*, 2004). However, in cell cultures such as immortalized human bronchial epithelial BEAS-2B cells, AFB1 causes cell death in both time and dose dependent manners (Yang *et al.*, 2012). It is reported that AFB1 causes chromatin breakage during cell cycle thus disrupting key physiological processes leading to cell death by apoptosis (Ribeiro *et al.*, 2010)

2.3 Aflatoxin contamination of foods in Ghana

Staple foods in Ghana include; maize, rice, millet, sorghum, yam, cassava and plantain. Unfortunately, the high humidity and warm temperature in Ghana make most of these staples susceptible to the aflatoxin producing fungi *Aspergillus*. Furthermore, poor pre and post-harvest practices as well as poor food handling and preparation have been flagged as significant factors for aflatoxin contamination in Ghana (Jolly *et al.*, 2006; Perrone *et al.*, 2014). Although Ghana Standards Boards permits a maximum of 15µg/kg of aflatoxin in food, a study in 2005 revealed as high as 24,873µg/kg in groundnut kernels, 1,260µg/kg in groundnut products, and 1,156µg/kg in maize products (Kpodo *et al.*, 2005). Again, a recent study revealed that “weanimix”, which is a locally prepared blend of maize and groundnut for newly weaned babies, had as much as 145.2µg/kg (Kumi *et al.*, 2014). Unfortunately, food crops grown in Ghana do not go through any rigorous scrutiny for these aflatoxins before going to the markets. Likewise, foods imported from neighbouring countries do not undergo scrutiny except for some processed foods. Although, there are guidelines to minimize aflatoxin contamination in Ghana, they remain un-enforced. This poses a considerable risk to Ghanaian consumers.

2.4 State of HCC in Ghana

Ghana lies within the Sub-Saharan African region of the continent. This region has been flagged as the most affected region in the world next to Eastern Asia (Nordenstedt *et al.*, 2010). Factors attributed to this high incidence are dietary exposure to aflatoxin and chronic HBV infection (Kew, 2012). A 10-year retrospective review of cancer cases in Korle-Bu Teaching Hospital, Accra, was done. The results showed that liver cancer was the second most common cancer. The study further revealed that HCC was the highest mortality in males with cancers and third highest in females with cancers (Wiredu & Armah, 2006). Although Ghana is challenged by incomplete cancer registry, the global cancer registry database (GLOBOCAN) estimates that, the incidence of HCC

in males is 17.6 per 100,000 people with a mortality of 17.1. In females however, the incidence is 4.9 per 100,000 people and with a mortality of 4.6 (WHO, 2013). In Ghana, diagnosis of HCC is mainly by magnetic resonance imaging (MRI) scans and histopathological analysis. However, these facilities are limited to a few selected hospitals. Diagnosis is often delayed due to the limited infrastructure, limited trained hepatologist and late presentation of the diseases. Furthermore, most people only seek health care when the cancer is in the advanced stage thus making curative measures almost impossible (Ladep, 2014).



Figure 1: Patient with Liver Cancer

2.5 Kinetics of aflatoxins

Dietary exposure of aflatoxins poses a significant risk for HCC (Wild & Gong, 2009). AFB1 is readily assimilated into the blood after ingestion or inhalation of contaminated food (Bbosa *et al.*, 2013). As the blood circulates the body, the aflatoxins enter the various body organs including the liver which is the main site of metabolism of xenobiotics. Within the liver microsomes, the CYP3A4 and CYP1A2 of the cytochrome P450 (CYP) oxidize AFB1 into a reactive AFB1-8,9-epoxide and to hydroxylated AFB1 (Forrester *et al.*, 1990; Gallagher *et al.*, 1996). The AFB1-8,9-epoxide upon reaching the mammary gland via the blood is secreted through breast milk (Battacone *et al.*, 2003). The AFB1-8,9-epoxide is integrated into the DNA by forming covalent bonds between the C8 of the AFB1 and N7 of the guanine bases of the DNA. An AFB1-N7-guanine adduct forms as a result (Figure 1). (Shen & Ong, 1996; Wang & Groopman, 1999).

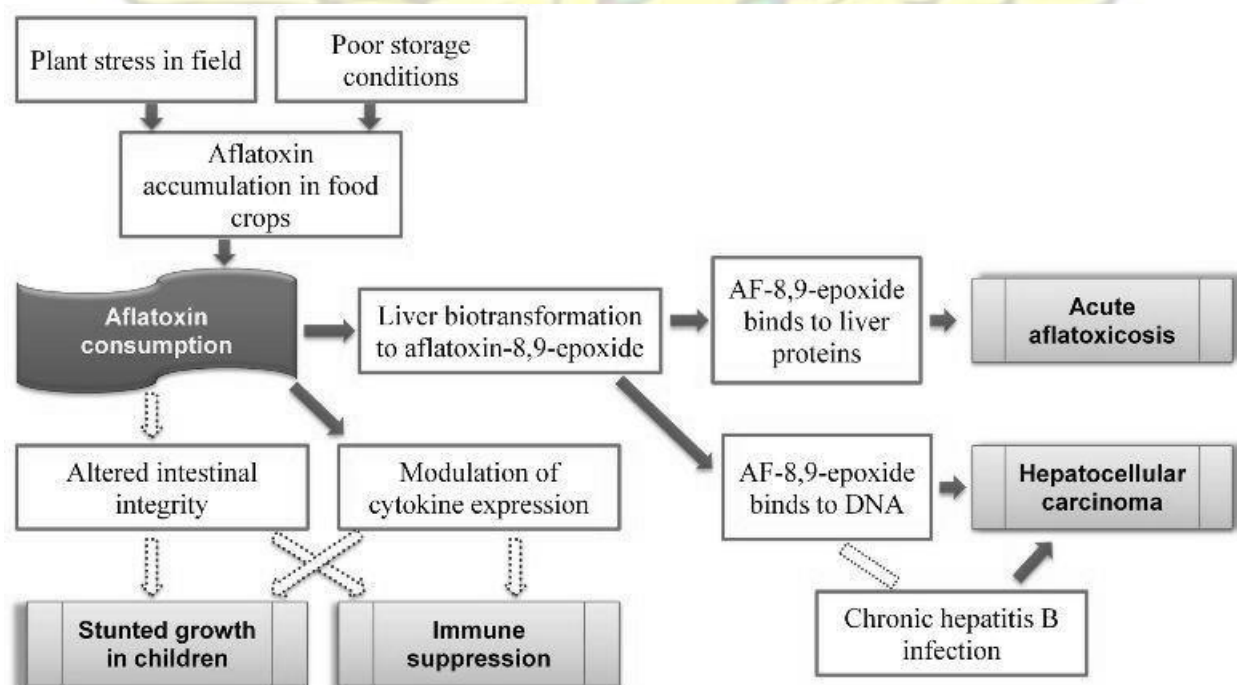


Figure 2: Aflatoxin and disease pathways in humans.

Source: (Wu *et al.*, 2011)

2.6 Main mechanism of carcinogenesis of aflatoxin

Although aflatoxins are known to cause cancers, primarily HCC (Liu *et al.*, 2012), the tendency of aflatoxin in causing this cancer, to a large extent, is dependent on the expression levels of specific cytochrome P450 enzymes within the liver (Zhang *et al.*, 2000). This suggests different levels of susceptibility to genotoxic actions of aflatoxins due to individual variation within human population (Murray, 2000). The cytochrome P450 enzymes are oxidase enzymes belonging to a family of hemoproteins (Hrycay & Bandiera, 2012). They play major role in drug response, cell signalling as well as carcinogenesis (Rooney *et al.*, 2004). By the use of human liver epithelial cell lines, research has shown that expression levels of CYP 1A2 and CYP 3A4 significantly modulates formation of AFB1 – DNA adduct (Macé *et al.*, 1997). Again, using oltipraz to inhibit expression of CYP 1A2 and CYP 3A4 in human hepatocytes, AFB1 activation was significantly reduced (Langouët *et al.*, 1995). This suggests that activation of AFB1 into AFB1-8,9-epoxide which forms AFB1 – DNA adduct is dependent on the expression of CYP 1A2 and CYP 3A4.

The replication of AFB1 – DNA adduct leads to a mutation in the p53 tumor suppressor gene. This mutation arises from a guanine to thymidine transversion within the codon 249. This leads to the substitution of arginine by serine and ultimately alters the protein functions (Aguilar *et al.*, 1993). The p53 tumour suppressor gene, also known as the “guardian of the genome”, thus loses its ability to prevent the formation of cancers. Its normal role includes; inducing apoptosis, growth arrest, senescence and also inhibiting angiogenesis (Wang & Sun, 2010). This allows cells to eliminate any damage due to the classical DNA transcription pathways and also develop an adaptive response to stresses such as metabolic stress, ribosomal stress, oxidative stress as well as viral infection. This suggests that the loss of functions of p53 tumour suppressor gene would make the cell prone to several cancer forming agents. This accounts for the elevated risk of HCC among HIV, hepatitis B

and C patients (Jiang *et al.*, 2008). However, the exact mechanisms of aflatoxins in cancer development is poorly understood.

2.7 Aflatoxins and cancers pathways

Cancer, like many other diseases, exploits the physiological processes in the body. In a healthy body, a good balance between cell proliferation and programmed cell death is maintained (Denekamp, 1993). On the cellular level, the processes that maintain this balance are regulated by genes. These genes can broadly be grouped into proto-oncogenes and tumor suppressor genes. Proto-oncogenes promote normal cell growth and division whereas tumour suppressor genes inhibit cell growth and division. These genes are activated by transcription factors and through signal transduction pathways regulates cellular processes such as cell cycle, cell proliferation, cell survival, apoptosis and among others. Cancer often arises as a result of the overexpression or loss of functions of these genes (Grizzi *et al.*, 2006). In addition, cancer development is a cascade of cellular events involving several factors and signalling pathways which ultimately results in metastasis. This suggests that the inhibition or deletion of a single event could have detrimental repercussion on all subsequent dependent processes (Wang & Sun, 2010). Although several mechanisms within the eukaryotic cells seek to repair damaged DNA (Gursoy-Yuzugullu *et al.*, 2011), cancer cells have activated pathways that lie outside these repair mechanisms. A review on hormone related cancers revealed that cancer cells exhibited multiple deregulation of signalling pathways (Li *et al.*, 2011) Research has shown that although environmental factors can influence the expression of genes, hormonal, neurological and nutritional factors also do play similar role though on a lesser scale (Heck *et al.*, 2004; Liss & Roeper, 2004; Ing, 2005). The increase in the expression of genes or the up-regulation of a signalling pathway results in an increase of the gene product such RNA and proteins (Spitz & Furlong, 2012). Likewise, down regulation of a signalling

pathway also results in the decrease of the gene products. However, studies are yet to establish how aflatoxins modulates the activities of these genes or pathways.

2.7.1 Amino Acid Deprivation Response (AADR) pathway

Cellular concentrations of amino acids have been found to regulate the expression of mammalian genes. Its abundance or deprivation triggers some responses. (Bruhat *et al.*, 2000). In response to amino acids deprivation, the AADR pathway is triggered (Kilberg *et al.*, 2012). Upon depletion of amino acid, General Control Non-derepressible-2 (GCN2) kinase activates and phosphorylates Eukaryotic Initiation Factor 2a (eIF2a). This results in the increase in translation of Activation Transcription 4 (ATF-4) as well as ATF-3 and ATF-2 (Wek *et al.*, 2006). Activation Transcription Factors share a common basic zipper domain which is made of amino acids and a leucine zipper region. By this feature, they are able to form dimers and bind to specific DNA region and regulate their expression (Yamasaki *et al.*, 2009). ATFs have been shown by research to play central role in Amino Acid Pathway (Cherasse *et al.*, 2009; Kilberg *et al.*, 2012). They are involved in regulating expression of genes in different type of cancers (Vlahopoulos *et al.*, 2008). Example, ATF -1 has been shown to be a survivor factor for human melanoma cells and also acts as a promoter of tumour invasion of thyroid papillary carcinoma (Leslie & Bar-Eli, 2005). Also, the over-expression of ATF-2 has been shown to play a vital role in cell proliferation of human and mouse cancer cell lines (Ronai *et al.*, 1998; Papassava *et al.*, 2004; Ricote *et al.*, 2006). Again, ATF-2 has been implicated in several cancers including prostate, breast, hepatic and lung cancers as well as leukemia, melanoma and tumours of the nervous system (Vlahopoulos *et al.*, 2008). The AFT-3 has also been shown to play key roles in prostate and ovary cancers (Syed *et al.*, 2005; Bandyopadhyay *et al.*, 2006). ATF-4 plays a role in drug resistivity in human cancer cell lines (Igarashi *et al.*, 2007). However, studies are yet to show the role of AFB1 in AADR pathway.

2.7.2 Activation Transcription Factor (ATF) -6 pathway

AFT-6 is also a member of the leucine zipper protein family. However, AFT-6 is bounded to the membrane of the Endoplasmic Reticulum (ER) (Adachi *et al.*, 2008). The ER serves as the site for the synthesis of both secretory and transmembrane proteins. The ER has an oxidative environment with high calcium ion concentration which is necessary for the formation of disulfide bonds and proper folding of proteins into their functional conformation (Orrenius *et al.*, 2003). Under certain pathological and physiological conditions such as calcium depletion, ER homeostasis can be disrupted. This results in accumulation of misfolded and unfolded proteins. This phenomenon is referred to as ER stress (Kim *et al.*, 2008). ER stress thus signals unfolded protein response (UPR) which aims at removing the misfolded and unfolded proteins and ultimately restoring ER homeostasis. Studies have shown that ATF-6 is key in this process. It does so by specific and direct interactions with ER stress response element which leads to the induction of ER chaperones and other transcription factors (Li *et al.*, 2000; Yoshida *et al.*, 2000). Down-regulation of ATF-6 would thus be detrimental to the cells, in that, defective proteins will accumulate. On the other hand, among the induced ER chaperones is glucose-regulated protein (grp) 78. This grp78 is a transformation-associated gene in HCC (Shuda *et al.*, 2003). Studies have implicated the overexpression of AFT-6alpha in hepatocarcinogenesis (Arai *et al.*, 2006). That is to say that the more AFT-6 is expressed, the more the transformation-associated gene is induced, and this alters the integrity of the liver.

2.7.3 Antioxidant Responsive (AR) pathway

In order for eukaryotic cells to survive in their aerobic environment without damage to their DNA, they have mechanisms which counteract oxidative damage, environmental stress and restore cellular redox homeostasis (Halliwell, 2007). This is achieved by induction of several cytoprotective enzymes which metabolize carcinogens into less reactive forms and also detoxify

other reactive agents. The transcription of these enzymes are regulated by cis-acting enhancer sequence called Antioxidant Responsive Elements (ARE) (Hur *et al.*, 2010). The AREs have unique structural and biological features which allow them to bind to chemical compounds that undergo redox cycling or have a potential to be metabolically transformed into a reactive or electrophilic intermediate (Rushmore *et al.*, 1990; Rushmore *et al.*, 1991). The AREs are responsible for encoding phase II detoxification enzymes and antioxidant proteins, such as reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H):glutathione S-transferases, quinone oxidoreductase 1 and glutamate-cysteine ligase (Lee & Johnson, 2004). Thus the up-regulation or otherwise of ARE is crucial, in that, protection of the cells is dependent on the abundance of these protective enzymes (Wasserman & Fahl, 1997). Studies have shown that Nuclear Factor-Erythroid 2-related factor 2 (Nrf2) is a promoter of ARE. The binding of this basic leucine transcription factor to ARE leads to up-regulation of ARE and subsequent protection of cells in many different cell types. This is achieved through coordinated up-regulation of the ARE-driven detoxification and antioxidant genes together with cell type-specific target genes required for the defense system of each cell type in its unique environment (Lee *et al.*, 2005). Using Nrf2 deficient mice, research has established that Nrf2 play two roles in carcinogenesis. Firstly, it functions to prevent tumour initiation especially in the case of chemical carcinogens (Osburn & Kensler, 2008). Secondly, in late stages of carcinogenesis, the activities of Nrf2 have been found to promote malignant transformation of benign tumours (Satoh *et al.*, 2013). Again, using Nrf2 knockout mice, hepatotoxicity was associated with decreased expression of ARE regulated antioxidant genes and enzymes (Enomoto *et al.*, 2001). However, there is no information to suggest reduction of aflatoxins into less reactive forms by ARE pathway.

2.7.4 Metal-Responsive Transcription Factor-1 (MTF-1) pathway

The metal-responsive transcription factor-1 (MTF-1) also plays a role in cellular adaptation to various stress conditions, chiefly exposure to heavy metals and oxidative stress (Günther *et al.*, 2012). Although MTF-1 has been shown to be essential for the development and differentiation of embryonic hepatocytes, recent evidence suggests their role in promoting malignancy of cancer cells (Günes *et al.*, 1998; Murphy, 2004). As tumour cells develop, a microenvironmental hypoxia and in some cases anoxia is formed. This is due to the malformation during angiogenesis and its resulting malfunction of the blood vessels and also due to the high energy demands of the proliferative cells (Vaupel, 2004). Thus the suspected role of MTF-1 is to help the tumour cells adapt to the oxidative stress formed. This suspicion has been strengthened as researchers have shown that the loss of MTF-1 suppresses the growth of tumour cells (Haroon *et al.*, 2004). That is to say, up-regulation of the MTF-1 by a carcinogen has the potential of promoting tumour development. However, relationship between MTF-1 and aflatoxins is however yet to be determined.

2.7.5 Glucocorticoid Receptor (GR) pathway

Glucocorticoids collectively are a group of steroid hormones involved in the metabolism of carbohydrates, proteins, fats and also in anti-inflammatory and immunosuppressive activities (Spies *et al.*, 2011). These hormones are produced by the adrenal gland cortex and function primarily in maintaining body homeostasis and in the body's response to external stressors (Dickmeis, 2009; Whirledge & Cidlowski, 2010). They modulate the transcription of some key genes and thus influence a myriad of cellular functions by binding to glucocorticoid receptors (GR) (Evans, 2005). The binding of these hormones which acts as ligands to GR has been shown to have two outcomes. One, the binding of GR to specific DNA could act directly as a transcription factor. On the other hand, the binding of GR to other transcription factors suppresses inflammatory transcription factors activator protein-1 and NFκB (Nuclear Factor Kappa-light-chain-enhancer of

activated B cells) and also induces anti-inflammatory genes which codes for the protein inhibitor of NF κ B (Saklatvala, 2002). Again, glucocorticoids exert anti-proliferative and anti-angiogenic effects on their target cells (Vilasco *et al.*, 2011). In ovarian cancers, GR has been shown to play a role in its pathogenesis through the signalling apoptosis and aberrant cell migration (Fang *et al.*, 2014). Others have also suggested a cross-talk with Estrogen receptors and GR in breast cancers (Miranda *et al.*, 2013). In HepG2 hepatocarcinoma cells however, GR specifically binds to D-loop region of the mitochondrial genome and ultimately results in the induction of mitochondrial transcription factors, ribosomal RNA and several oxidative phosphorylation genes. This binding of GR results in increased RNA synthesis, cytochrome oxidase subunit I protein expression and ATP production (Psarra & Sekeris, 2011). In AFB1-induced rat hepatoma Kagura-2 (K2) cell line, glucocorticoids act as tumour promoters by significantly suppressing apoptosis induced by arachidonic acid (Iida *et al.*, 1998; Sugiyama & Tashiro, 2000). Furthermore, a mutation in the GR results in functional defects on the glucocorticoid signal transduction pathway (Charmandari *et al.*, 2004).

2.7.6 Nuclear Factor Kappa-B (NF κ B) pathway

The NF κ B (Nuclear Factor Kappa-light-chain-enhancer of activated B cells) collectively refer to dimeric transcription factors consisting of members of Rel family of DNA binding proteins (Karin & Ben-Neriah, 2000). They are essential in diverse physiological processes but chiefly regulate cell proliferation, inflammatory and immune response (Gilmore, 2006). Evidence mounting up suggests a major role of NF κ B in oncogenesis (Dolcet *et al.*, 2005). They have been found to cooperate or crosstalk with a multiple of other signalling molecules and pathways which results in different expression of NF κ B target genes (Hoesel & Schmid, 2013). Innate immune response of neutrophil induced by NF κ B, releases reactive oxygen species (ROS) which is targeted at killing invading pathogens. However, the ROS released has a tendency of causing damage to the host DNA thereby

triggering tumor initiation (Liou & Storz, 2010). Again studies have shown that NF κ B partially modulates transcription of angiogenic and tumorigenic chemokine genes that enhances the growth of tumours (Richmond, 2002). Mutations in NF κ B signalling genes have also been found in some cancers including cancer of the liver and breast. These mutations were found to impart their protein functions (Arsura *et al.*, 2000; Jiao *et al.*, 2012). However, AFB1 has been shown to up-regulate NF κ B pathway in hepatocarcinogenesis (Castelino, 2013).

2.7.7 Interferon (IFN)-Induced Signal Transduction pathway

Interferons (IFN) are naturally occurring cytokine mediators released by host cells in response to the presence of pathogens, their products and tumour cells (De Andrea *et al.*, 2002). They play roles in immune modulation and anti-proliferative activities. IFN-alpha and IFN-beta are classified as type I IFN and they are secreted by virus infected cells. IFN-gamma is regarded as type II IFN and it is secreted by T cells, natural killer (NK) cells and macrophages (Le Page *et al.*, 2000). IFN binds to IFN receptors and regulate the transcription of genes downstream. IFN signalling can occur through both signal transducer and activator of transcription (STAT) dependent and independent mechanisms (Nguyen *et al.*, 2000). STAT is a family of cytoplasmic transcription factors responsible for mediating intercellular signalling generated by receptors on the cell surface to the nucleus (Siveen *et al.*, 2014). In the STAT dependent mechanisms, Janus activated kinase (JAK) binds with IFN and IFN receptors leading to the phosphorylation of STAT1 and STAT2.

This initiates the JAK-STAT signalling pathway (Platanias, 2005). An IFN-stimulated gene factor 3 (ISGF3) complex is formed as a result (Icardi *et al.*, 2012). The complex is made up of STAT1, STAT2 and IFN regulatory transcription factor 9 (IRF9). The complex moves into the cell nucleus where it binds to IFN-stimulated response elements (ISREs) of the IFN stimulated genes (ISGs) and thus promotes their transcription (McComb *et al.*, 2014). ISRE has been shown to be mainly responsible for the up-regulation of IFN induced programmed death of macrophages (Cho *et al.*,

2008). Again, dysfunctioning of the ISRE has been shown to lead to decrease resistance to human immunodeficiency virus (HIV) and has also been implicated in some cancers (Cremer *et al.*, 2002; Sen & Sarkar, 2007). In human lymphoblastoid Jurkat T-cell model, AFB1 has been shown to stimulate the IFN pathway in a dose dependent manner (Luongo *et al.*, 2014). The study further suggested an existence of a concentration threshold beyond which AFB1 will induce biological activity. Using the rat model, IFN-alpha gene were significantly expressed and this exerted significant protective effects against aflatoxin initiated carcinogenesis in the liver (Aziz *et al.*, 2005). On the other hand, a study using rhesus monkey kidney (LLC-MK2) cell monolayers demonstrated that aflatoxins have the ability to inhibit viral induction of IFN-alpha and IFN-beta (Hahon & Chen, 1992).

2.7.8 Signal Transducer and Activator of Transcription 3 (STAT3) pathway

STAT3, which is another member of the STAT family of cytoplasmic transcription factors, has been shown to play critical roles in a wide variety of human tumours including hepatocellular, colorectal, breast and prostate cancers (Bowman *et al.*, 2000; Bromberg, 2002; Siveen *et al.*, 2014). Its normal functions include signalling cell proliferation, differentiation, survival, development as well as inflammation (Yue & Turkson, 2009). Activation of STATs by phosphorylation is mediated by growth factor receptor tyrosine kinases, cytokine receptor-associated Janus kinases (JAK) and Src family kinases (Buettner *et al.*, 2002). Upon phosphorylation of STAT monomers, they dimerize via reciprocal phosphotyrosine-SH2 domain interactions and move from the cytoplasm and accumulate within the nucleus. In the nucleus, the dimers bind to specific STAT DNA-response elements to regulate the expression of those genes (Yue & Turkson, 2009). Evidence mounting up suggests that JAK-STAT3 signalling pathway promotes cancer through various means including; tumour cell proliferation, anti-apoptosis, immunosuppression, obesity

and pre-metastatic niche (Yu *et al.*, 2014). However, AFB1 was shown to up-regulate STAT3 pathway in hepatocarcinogenesis (Castelino, 2013).

2.7.9 Peroxisome Proliferator-Activated Receptor (PPAR) pathway

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear hormone receptors which modulate gene expression. They play essential roles in diverse cellular activities such as metabolism of carbohydrate, lipid and protein, and in cellular differentiation and development (Feige *et al.*, 2006). There are 3 isotypes of PPARs. However, these 3 are encoded by different genes and also show different tissue distribution (Michalik & Wahli, 1999). PPAR α controls various aspects of fatty acid catabolism thus expressed in liver, kidney, heart, muscle and adipose tissues (Willson *et al.*, 2000). PPAR δ on the other hand is involved in embryo implantation, myelination of corpus callosum, lipid metabolism, development and epidermal cell proliferation and thus expressed predominantly in brain, adipose tissues, and skin (Lim & Dey, 2000). Lastly, PPAR γ controls adipocyte differentiation, systemic glucose levels, and lipid homeostasis and thus found virtually in all tissues (Willson *et al.*, 2000). All 3 PPARs heterodimerize with Retinoid X receptor (RXR) and bind to the peroxisome proliferator hormone response elements (PPREs) within the promoter regions to induce anti-tumour effects (Berger & Moller, 2002; Ditsch *et al.*, 2012). Consequently, the binding of PPAR to its ligand results in either increase or decrease in transcription depending on the gene. The exact molecular mechanisms of PPAR in carcinogenesis remains unclear. However, the deregulation of PPAR has been found to support tumour progression of human colon, breast, prostate and liver cell line (Matsuda & Kitagishi, 2013; Park *et al.*, 2001; Stephen *et al.*, 2004; Toyoda *et al.*, 2002; Wang *et al.*, 2006). Nevertheless, a relationship between aflatoxin and PPAR is yet to be established.

2.7.10 Paired Box (PAX-6) pathway

Paired box 6 (PAX-6) is a protein-coding gene. It plays key roles in the development of the eye, pancreas and central nervous system (CNS) (Shaham *et al.*, 2012; Zhang *et al.*, 2010). The encoded protein has two DNA binding sites namely; paired box domain and homeo box domain. The domains may function separately in regulating specific functions of Pax6 either separately or in a cooperative manner (van Heyningen & Williamson, 2002). The exact molecular mechanisms underline its role in proliferation and neurogenesis are however yet to be fully understood. Nonetheless, works have established Pax6 as a vital regulator of fate and patterning decisions as well as cell proliferation in achieving appropriate CNS and eye development (Walcher *et al.*, 2013). Again, the works have implicated Pax6 in some cancers. In pancreatic cancer, Pax6 plays a role by activating the Met tyrosine kinase receptor gene (Mascarenhas *et al.*, 2009). In breast cancer, Pax6 is suspected to facilitate regulatory roles in proliferation of the cancer cells as well as tumour progression (Zong *et al.*, 2011). However, the effect of AFB1 on Pax6 pathway is yet to be studied.

2.7.11 Phosphatidylinositol-3 Kinases - Akt (PI3K-AKT) pathway

The PI3K-Akt pathway promotes cell proliferation and survival in response to extracellular signals (Osaki *et al.*, 2004). Phosphatidylinositol 3-kinase (PI3K) and Akt (or Protein Kinase B) are the key proteins in this pathway. PI3Ks are a family of lipid kinases with the ability to phosphorylate inositol ring 3'-OH group in inositol phospholipids to generate phosphatidylinositol-3,4,5trisphosphate (PIP3) (Fresno-Vara *et al.*, 2004). The PI3K-Akt pathway starts with the activation of receptor tyrosine kinases which results in the formation of in PIP3 and PIP2 by PI3K at the inner side of the plasma membrane. The interaction between Akt and these phospholipids causes translocation of Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase (PDK) 1 and PDK2 (Osaki *et al.*, 2004). The activation of Akt thus modulates fundamental cellular functions including cell proliferation and phosphorylation of

numerous substrates involved in the regulation of cell survival, cell cycle progression and cellular growth (Mosca *et al.*, 2012). Upon activation, Akt regulates the activities of the forkhead box O (FoxO) protein encoded by the FoxO (Tzivion *et al.*, 2011). FoxO proteins function in myriad of cellular and physiological processes such as reactive oxygen species (ROS) response, apoptosis, longevity, as well as in the regulation of cell cycle and metabolism (van der Vos & Coffey, 2008). The binding of Akt to FoxO triggers a cascade of reactions which prevents the tumour suppressor FoxO from inhibiting cell proliferation (Zhang *et al.*, 2011). PI3K-Akt pathway has been found to be activated in almost all cancers, thus creating the belief that it is the most commonly activated signalling pathway in human cancers (Liu *et al.*, 2009). However, in AFB1 induced liver lesion, PI3K/Akt pathway was found to contribute to the neutralization of apoptosis induced by AFB1 (Yang *et al.*, 2014).

2.7.12 Protein Kinase C – Calcium ions (PKC/Ca²⁺) pathway

Protein kinase C (PKC) consists of a family of serine/threonine kinases. They are activated by a rise in diacylglycerol (DAG) or calcium ions (Ca²⁺) concentration (Spitaler & Cantrell, 2004). The fifteen isozymes found in humans have been divided into subfamilies based on their second messenger requirements. The subfamilies are conventional (or classical), novel, and atypical PKCs. However, all members of the PKC family have a similar architecture. They are made up of a carboxyl-terminal kinase domain which acts as the catalytic domain hinged by a flexible segment to an amino-terminal region containing regulatory modules (Newton, 2003; Parker & MurrayRust, 2004). The regulatory modules allow sensitivity to diacylglycerol (C1 domain) or Ca²⁺ (C2 domain) although variants of the modules in some of the isozymes do not bind ligand (Blumberg *et al.*, 2008; Guerrero-Valero *et al.*, 2007). Upon activation, receptor for activated C kinases (RACK proteins) translocate PKC to the plasma membrane. The plasma membrane serves as a platform for PKC function. It also supports maturation of PKC through phosphorylation and its

allosteric activation by binding specific lipids (Newton, 2010). The PKCs bind to several substrates involved in cell proliferation, differentiation, apoptosis and angiogenesis (Mackay & Twelves, 2007). They have been found to be involved in the activation of nuclear factor of activated T-cells (NFAT) (Pfeifhofer *et al.*, 2003). NFAT is a family of transcription factors that induces primarily important genes in immune response and development of cardiac muscle cells (Hogan *et al.*, 2003). NFAT proteins are weak in their binding to DNA and thus cooperate with PKC and other proteins to form complexes on DNA (Crabtree & Olson, 2002). The overexpression of various NFAT isoforms have been found in human solid tumors and multiple compartments in the tumour microenvironment (Jauliac *et al.*, 2002). They contribute to tumourigenesis by promoting cell growth, survival, invasion and angiogenesis (Mancini & Toker, 2009). In Jurkat E6-1 human Tcell leukemia, AFB1 treatment inhibits the binding of NFAT to DNA thus down-regulating the activities of NFAT (Han *et al.*, 1999).

2.7.13 Specificity Protein 1(Sp1) pathway

The Specificity protein 1 (Sp1) is a zinc-finger transcription factor and plays multiple roles in cellular functions and tumour progression (Choi *et al.*, 2014). They have been found to influence the expression of vascular endothelial growth factor and genes involved in apoptosis, cell cycle as well as DNA damage response (Olofsson *et al.*, 2007; Safe & Abdelrahim, 2005). They bind to guanine-cytosine (GC) boxes of promoters and cooperate with the transcription machinery in regulating the genes they influence (Safe & Abdelrahim, 2005). Post-translational modification of Sp1 through phosphorylation and methylation significantly affects the binding affinity to DNA thus making it act as an activator or a repressor (Waby *et al.*, 2008). Mounting evidence suggests that the over expression of Sp1 is a key contributor in various cancers (Kanai *et al.*, 2006; Safe & Abdelrahim, 2005; Shen *et al.*, 2009). In AFB1 induced mouse lung tumours, AFB1 was found to methylate Sp1 binding site thus causing aberrant expression of Sp1(Tam *et al.*, 2003).

2.7.14 DNA Damage pathway

DNA damage can occur by both endogenous and exogenous factors such as replication errors and mutagenic agents respectively (Chagin *et al.*, 2010). Upon DNA damage, the cell elicit a response to either arrest cell cycle, repair the damage DNA, induce senescence or apoptosis (Huen & Chen, 2008). The tumour suppressor p53 (TP53) is one of the major transcription factors recruited in DNA damage response (Surget *et al.*, 2013). The TP53 encodes about twelve p53 protein isoforms through usage of alternative promoters, alternative splicing and alternative initiation of translation (Khoury & Bourdon, 2011). All 12 isoforms share a C-terminal domain, tetramerisation domain, DNA binding domain and N-terminal domain and thus are able to differentially regulate gene expression (Khoury & Bourdon, 2011). P53 remains inactivated by its association with mouse double minute 2 (MDM2) until it receives signals from upstream mediators such as Chk2 (Brooks & Gu, 2006). P53 can also bind directly as a tetramer to its response element in inducing or repressing the expression of a gene (Pan & Nussinov, 2007). Studies have shown that p53 directly regulates at least 3600 target genes (Li *et al.*, 2012). The p53 has thus assumed the status as “guardian of the genome” due to its varied role in gene expression (Ananiev *et al.*, 2011). Upon activation of p53 due to cellular injury, p53 either induces cell cycle arrest or initiates DNA repair or triggers programme cell death depending on the extent of injury (Surget *et al.*, 2013). The deregulation of p53 pathway therefore exerts detrimental consequences on the cell and has been flagged as a major factor in most cancers (Vogelstein *et al.*, 2000). Studies have shown that the deregulation of this pathway is often due to point mutation in the TP53 gene (Zilfou & Lowe, 2009). The mutant proteins formed as a result has acquire oncogenic characteristics and thus are able to promote invasion, metastasis, proliferation and survival of transformed cells (Muller & Vousden, 2013). AFB1 has been shown to cause a point mutation in the p53 gene and this could account for its role in p53 related cancers (Aguilar *et al.*, 1993).

2.8 Measuring the activity in a pathway

The strength of activity in a pathway can be measured either by quantifying the gene products of the pathway under study or by measuring the activity of its reporter enzymes. However, because gene products such as RNA can spontaneously disintegrate, measuring the activity of a reporter enzyme is a better tool in estimating the activity in a pathway. Using reporter enzyme as a tool in determining the strength of activity of a pathway in a cell cultures would require transfecting the cells with pathway specific regulatory elements (transcription factors) coupled to reporter enzymes. The activity of the reporter enzyme is then measured after treating or stimulating the cells with molecules of interest, example AFB1. Pathway specific regulatory elements coupled to reporter enzymes are available commercially.

2.8.1 QIAGENTM SIGNAL FINDER 45-PATHWAY REPORTER ARRAYS

The Signal Finder 45-Pathway Reporter Arrays is a multi-pathway reporter which enables researchers to pinpoint on pathways regulated by gene product and chemical compounds under study (Devgan et al., 2008). They provide rapid, sensitive and quantitative assays for measuring the activity of specific signal transduction pathways. It functions on the dual luciferase principle. They are ready-to-transfect cell culture plates with each duplicate well consisting of a unique pathway reporter assay; an inducible transcription factor-responsive construct and a constitutively expressing *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferase construct in a ratio of 20:1 respectively. The inducible transcription factor-responsive construct encodes firefly (*Photinus pyralis*) luciferase reporter gene and monitors both the increase and decrease in the activity of the coupled regulatory element in a said signalling pathway. On the other hand, the constitutively expressing *Renilla* construct encodes the *Renilla* luciferase reporter gene which serves as an internal control. The plate also has three negative control wells consisting of noninducible reporter construct which encodes firefly luciferase only. The three positive control wells however consisted

of constitutively expressing green fluorescence protein (GFP), firefly luciferase and *Renilla* luciferase constructs. However, each experiment is conducted using 2 plates, of which one plate serve as the treatment condition while the other serves as a control

Figure 3: Overview of Cignal Finder 45-Pathway Reporter Array Protocol

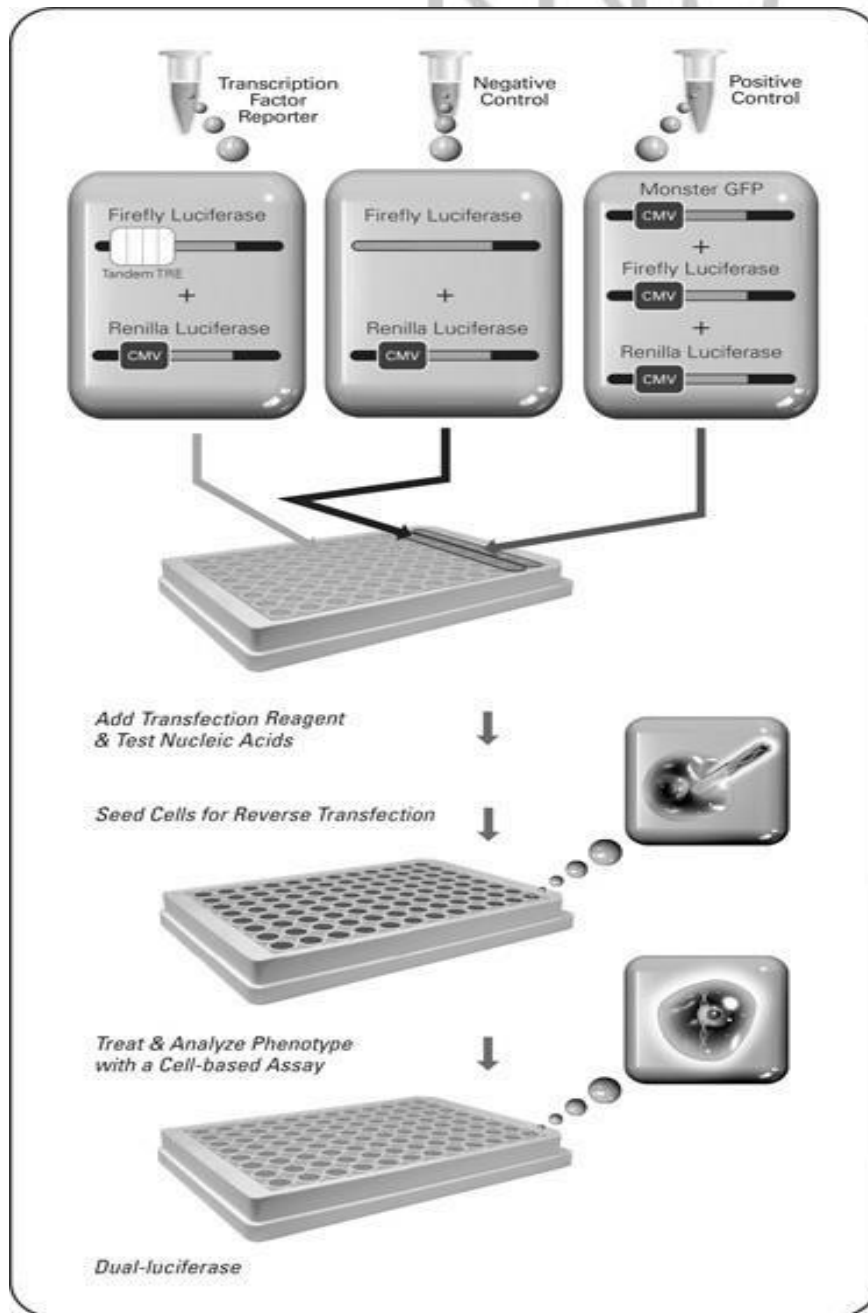


Table 1: Summary of pathways measured using Qiagen™ Signal Finder 45-Pathway Reporter Arrays

Reporters	Pathways	Transcription Factors	Position (wells)
AARE	Amino Acid Deprivation Response	ATF4/ATF3/ATF2	A01, A02
AR	Androgen Receptor	Androgen Receptor	A03, A04
ARE	Antioxidant Response	Nrf2 & Nrf1	A05, A06
ATF6	ATF6	ATF6	A07, A08
C/EBP	C/EBP	C/EBP	A09, A10
CRE	cAMP/PKA	CREB	A11, A12
E2F	Cell Cycle	E2F/DP1	B01, B02
p53	p53/DNA Damage	p53	B03, B04
EGR1	EGR1	EGR1	B05, B06
ERSE	Endoplasmic Reticulum Stress	CBF/NF-Y/YY1	B07, B08
ERE	Estrogen Receptor	Estrogen Receptor	B09, B10
GATA	GATA	GATA	B11, B12
GRE	Glucocorticoid Receptor	Glucocorticoid Receptor	C01, C02
HSR	Heat Shock Response	HSF	C03, C04
MTF1	Heavy Metal Stress	MTF1	C05, C06
GLI	Hedgehog	GLI	C07, C08
HNF4	Hepatocyte Nuclear Factor 4	HNF4	C09, C10
HIF	Hypoxia	HIF-1	C11, C12
IRF1	Interferon Regulation	IRF1	D01, D02

ISRE	Type I Interferon	STAT1/STAT2	D03, D04
GAS	Interferon Gamma	STAT1/STAT1	D05, D06
KLF4	KLF4	KLF4	D07, D08
LXR	Liver X Receptor	LXR α	D09, D10
SRE	MAPK/ERK	Elk-1/SRF	D11, D12
AP1	MAPK/JNK	AP-1	E01, E02
MEF2	MEF2	MEF2	E03, E04
Myc	c-myc	Myc/Max	E05, E06
Nanog	Nanog	Nanog	E07, E08
RBP-Jk	Notch	RBP-Jk	E09, E10
NFkB	NFkB	NFkB	E11, E12
4-Oct	Oct4	Oct4	F01, F02
Pax6	Pax6	Pax6	F03, F04
FOXO	PI3K/AKT	FOXO	F05, F06
NFAT	PKC/Ca ⁺⁺	NFAT	F07, F08
PPAR	PPAR	PPAR	F09, F10
PR	Progesterone Receptor	Progesterone Receptor	F11, F12
RARE	Retinoic Acid Receptor	Retinoic Acid Receptor	G01, G02
RXR	Retinoid X Receptor	Retinoid X Receptor	G03, G04
Sox2	Sox2	Sox2	G05, G06
SP1	SP1	SP1	G07, G08
STAT3	STAT3	STAT3	G09, G10
SMAD	TGF β	SMAD2/SMAD3/SMAD4	G11, G12

VDR	Vitamin D Receptor	Vitamin D Receptor	H01, H02
TCF/LEF	Wnt	TCF/LEF	H03, H04
XRE	Xenobiotic	AhR	H05, H06
Negative Control			H07, H08, H09
Positive Control			H10, H11, H12

2.8.2 DUAL-LUCIFERASE® REPORTER ASSAY SYSTEM

The Dual-Luciferase® Reporter Assay System is a genetic reporter system which allows simultaneous expression and measurements of two individual reporter enzymes within a single system. In this system, the “experimental” reporter which is firefly luciferase is correlated with the effect of specific experimental conditions such as AFB1 treatment. The co-transfected “control” reporter which is *Renilla* luciferases provides an internal control thus serving as the baseline response. The two luciferases are measured sequentially from a single sample and yield linear assays with subattomole sensitivities and no endogenous activity of either reporter in the experimental host cells. Therefore, the normalization of the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell viability or transfection efficiency. It also effectively eliminates other sources of variability such as differences in pipetting volumes, cell lysis efficiency as well as assay efficiency. It therefore gives a more reliable interpretation of the experimental by reducing extraneous influences. (Promega, 2003).

CHAPTER 3

MATERIALS AND METHODOLOGY

This chapter describes the procedures used in this study. All reagents and materials used in the experiments have been listed in appendix A. References will be made to appendices when necessary.

3.1 Cell cultures

Human embryonic kidney (HEK 293) cell line was used in this study. The cells were kindly donated by Prof. David J. Blackburn of University of Surrey, UK. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% v/v foetal bovine serum (FBS), 1% v/v sodium pyruvate, 1% v/v L-glutamine, 1% v/v non-essential amino acids (NEAA) and 0.1% v/v gentamicin. Henceforth this medium shall be referred to as culture medium. The cells were incubated in 5% CO₂ under humidified atmosphere at 37°C until the desired confluence was obtained. The cells were mostly cultured in T-25 tissue culture flask. However, depending on the nature and the type of experiment being conducted, they were cultured in T-75, 6-well plates and 96-well plates.

3.2 Sub-culturing and counting of cells

To sub-culture the cells, the used culture medium was removed and discarded. Appropriate volume of phosphate buffered saline (PBS) pre-warmed to 37°C was gently added to the side of the flask opposite the cell monolayer. The flask was gently rocked back and forth to remove any left-over culture medium. The wash solution was removed and discarded. The cells were washed to remove calcium and magnesium ions which could inhibit the activity of trypsin during cell dissociation in the next step. The appropriate volume of trypsin pre-warmed to 37°C was added over the cell

monolayer and incubated for 5 minutes at 37°C. When about 90% of the cells had detached, the cells were suspended in appropriate volume of culture medium. The suspended cells were then transferred to 15ml falcon tube and centrifuged (250 x g, 5 minutes). After centrifugation, the supernatant was discarded and the cells were re-suspended in 1 ml of the culture medium. Twenty (20) microliters of the re-suspended cells were transferred into a PCR tube and 80 µl of trypan blue exclusion dye was added providing a ratio of 1:5 dilutions. Depending on the cell density, 1:2 or 1: 3 dilutions were also used. After carefully mixing the cells with the trypan blue dye, 10 µl of the cell-trypan blue suspension was loaded into each chamber of the haemocytometer. The cells in the two chambers of the haemocytometer were counted and the total number of cells per ml was determined using the formula;

$$\text{Cells/ml} = \frac{\text{number of cells (chamber 1 + chamber 2)}}{2} \times \text{dilution factor} \times 10^4$$

× volume of cell suspension

3.3 Cytotoxicity of AFB1 on HEK 293 cells.

Cytotoxicity of AFB1 was performed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay which is an MTS based assay. This assay uses colorimetric analysis which makes determination of the number of viable cells within a sample or well possible. Its reagent contains a MTS tetrazolium compound which is biologically reduced by the metabolically active cells to form a soluble coloured product called formazan within the culture medium. Thus measuring the absorbance of the formed formazan product at 490nm provides a direct proportion to the number of viable cells. AFB1 stock solution was obtained from Sigma-Aldrich, USA. Briefly, cells were seeded at 2×10^4 cells per well in 96 well plates and incubated in 5% CO₂ under humidified atmosphere at 37°C until they were at about 80% confluence. The cells were treated with or without increasing concentrations of AFB1 (0, 3.2, 32, 320, 800, 1600µM) in duplicate wells for 24, 48

and 72 hours. After each treatment condition, 20µl of the reagent was added to the wells and incubated again in 5% CO₂ under humidified atmosphere at 37°C for 2 hours. At the end of the 2 hours, 100µl of reagent-culture medium was transferred into an ELISA plate and absorbance was recorded at 490nm using iMark™ Microplate Absorbance Reader (see Appendix A for photograph). Percentage cell viability was calculated as a ratio between AFB1 treated cells and non-treated cells. This assay was conducted two times and each independent experiment was performed in duplicate.

3.4 Effect of AFB1 on cell signalling pathways in HEK 293 using Cignal Finder 45pathway reporter array

The influence of AFB1 on the 45 signalling pathways was assessed using the Cignal Finder 45pathway reporter array (see section 2.8.1) and the expression of the regulatory genes in the signalling pathways was measured using dual-luciferase reporter assay (see section 2.8.2)

3.4.1 Reverse transfection and AFB1 treatment of HEK 293 cells

Briefly, fifty (50) microliters of DMEM only was added to each well of the Cignal Finder-45 pathway array plate to re-suspend the dried down DNA construct. The plate was gently tapped on every side and slightly rocked back and forth, left to right, five times each and incubated for 5 minutes at room temperature to further enhance re-suspension of the DNA constructs. A dilution of Trans IT 2020 transfection reagent was prepared (see appendix C) and 50µl was added to the re-suspended nucleic acids and gently mixed by tapping the sides of the plate for at least 30 seconds, making a ratio of 1:1. The plate was incubated at room temperature for 20 minutes to allow DNA-Trans IT 2020 complex to form. Fifty (50) microliters of cells suspended in the culture media was added to each well and mixed gently by rocking the plate back and forth, then left to

right and incubated in 5% CO₂ under humidified atmosphere at 37°C. An AFB1 dilution of 32µM was prepared by adding appropriate volume of the stock AFB1 to the culture media. After 24 hours of incubation, the cells were treated with 100µl of AFB1 (32µM) dilution and incubated in 5% CO₂ under humidified atmosphere at 37°C for 24 hours.

3.4.2 Measuring the expression of regulatory genes using dual luciferase reporter assay.

Briefly, after 24 hours of AFB1 treatment, the cells were gently washed with adequate volume of cold phosphate buffered saline (PBS). Passive lysis buffer (PLB, 1X) was prepared by adding 1ml of the stock 5X PLB to 4ml of distilled water. Twenty (20) microliters of 1X PLB was added to each well and the 96 well plate was gently rocked by placing on a rocking platform for 15 minutes to lyse the cells at room temperature. Luciferase Assay Reagent II (LAR II) was prepared by resuspending the lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II and aliquoted and stored at -80°C until used. However, Stop & Glo reagent was always freshly prepared before use. This was done by adding appropriate volume of Stop & Glo substrate to its buffer and storing in the dark briefly till use (see appendix C). Both reagents were then loaded into a pre-programmed Berthold Orion Microplate luminometer (see Appendix A for photograph). The luminometer automatically dispensed appropriate volume of LAR II to the well to generate a stabilized luminescent signal which is measured as the firefly luciferase reporter. Appropriate volume of Stop & Glo was added to quench the LAR II reaction while simultaneously initiating *Renilla* luciferase reaction to create a stabilized signal from which *Renilla* luciferase reporter was measured.

3.5 Validating results from Cignal Finder 45-pathway reporter array

The effects of AFB1 on IFN-Induced Signal Transduction pathway was tested in an individual reporter assay as a validation test of the Cignal Finder 45-pathway reporter array. This individual

reporter assay involved measuring the expression of IFN-stimulated response elements (ISREs) which is the promoter of IFN stimulated genes (ISGs). Thus if results yielding from this individual assay was found to be consistent with the results from the Cignal Finder 45-Pathway reporter array, then the results of all 45 signalling pathways from the Cignal Finder 45-Pathway Reporter Array were considered valid.

3.5.1 Establishing the minimum concentration of IFN- α that can induce a maximal activity in IFN-Induced Signal Transduction pathway

Briefly, cells were seeded at 2×10^4 cells per well in a 96 well plate and then incubated in 5% CO₂ under humidified atmosphere at 37°C for 24 hours until they were about 80% confluence.

Appropriate volume of DNA dilution containing pISRE (encodes firefly luciferase) and pRLSV40 (encodes *Renilla* luciferase) in DMEM only was prepared. A corresponding appropriate volume of Trans IT 2020 transfection reagent was added to the prepared DNA dilution and incubated at room temperature for 20 minutes to enhance the formation of DNA-Trans IT 2020 complex (see appendix C). The cells were transiently co-transfected by the addition of 100 μ l of the complex formed gently to each well and incubated in 5% CO₂ under humidified atmosphere at 37°C. After 24 hours of incubation, the cells were treated with or without increasing concentrations of IFN- α (0, 100, 200, 300 and 400 IU/ml) for 24 hours. The cells were harvested and expression levels of the genes measured using dual luciferase reporter assay (see section 2.8.2).

3.5.2 Effects of AFB1 treatment on IFN-Induced Signal Transduction pathway

After establishing the minimum concentration of IFN- α which could induce a maximal activity in IFN-Induced Signal Transduction pathway, the effect of AFB1 on the said pathway was then determined. Briefly, cells were seeded at 2×10^4 cells per well in 96 well plate and incubated in 5% CO₂ under humidified atmosphere at 37°C until they were about 80% confluence. Appropriate volume of DNA-Trans IT 2020 complex was prepared and 100 μ l was gently added to each well

(see section 3.5.1) and incubated in 5% CO₂ under humidified atmosphere at 37°C. After 24 hours, the cells were treated with or without IFN- α (400IU/ml) and with or without increasing concentrations of AFB1 (32, 64 μ M) for 24 hours. The cells were harvested and expression levels of the genes measured using dual luciferase reporter assay (see section 2.8.2). This experiment was conducted three times and each independent experiment was done in duplicate wells.

3.6 Statistical analysis

All data were entered using Microsoft Excel 2016. Data from Cignal Finder 45 pathway reporter array were analyzed using spreadsheet provided by the manufacturer online. (Date accessed; 14th January 2015, http://www.sabiosciences.com/reporter_assay_product/HTML/CCA-901L.html). One-way analysis of variance (ANOVA) and unpaired t-test were calculated using GraphPad prism (version 5.01) and significance level was determined at $p < 0.05$.



CHAPTER FOUR

RESULTS

4.1 AFB1 differentially regulates 45 cancer pathways in HEK 293

AFB1 is a known toxin to human cell lines and so before the effects of AFB1 on the 45 cancer pathways was determined, the cytotoxicity of AFB1 on HEK 293 cells was first determined by treating HEK 293 cells with or without increasing of AFB1 (0, 3.2, 32, 320, 800, 1600 μ M) for 24, 48 and 72 hours after which the percentage viability of the cells was evaluated by an MTS based assay. One-way analysis of variance (ANOVA) was calculated for the set of results for 24, 48 and 72 hours using GraphPad Prism version 5. The p value was found to be 0.983 and thus the difference between 24, 48 and 72 hours was considered to be not statistically significant. At 32 μ M of AFB1 for 24 hours, more than 80% of the cells survived (Figure 4). This experiment was done to establish the working concentration of AFB1.

Having established the working concentration of AFB1, the effect of AFB1 on the 45 different pathways was assessed using the Signal Finder 45-pathway reporter array. This was achieved by determining the fold change in each pathway by comparing the normalized luciferase activity of each pathway reporter in the AFB1 treated plate to the untreated plate. The results indicated that AFB1 differentially modulated or affected the 45 pathways studied or assayed (Figure 4). Of the 45 pathways studied, 22 pathways were up-regulated, 16 were down-regulated and 7 pathways had no measurable influence. Key pathway reporters up-regulated included AARE, ARE, ATF-6,

GRE, MTF-1, ISRE, NF κ B and PAX-6. On the other hand, key pathway reporters down-regulated by AFB1 included p53, FoxO, NFAT, PPAR, SP-1 and STAT3.

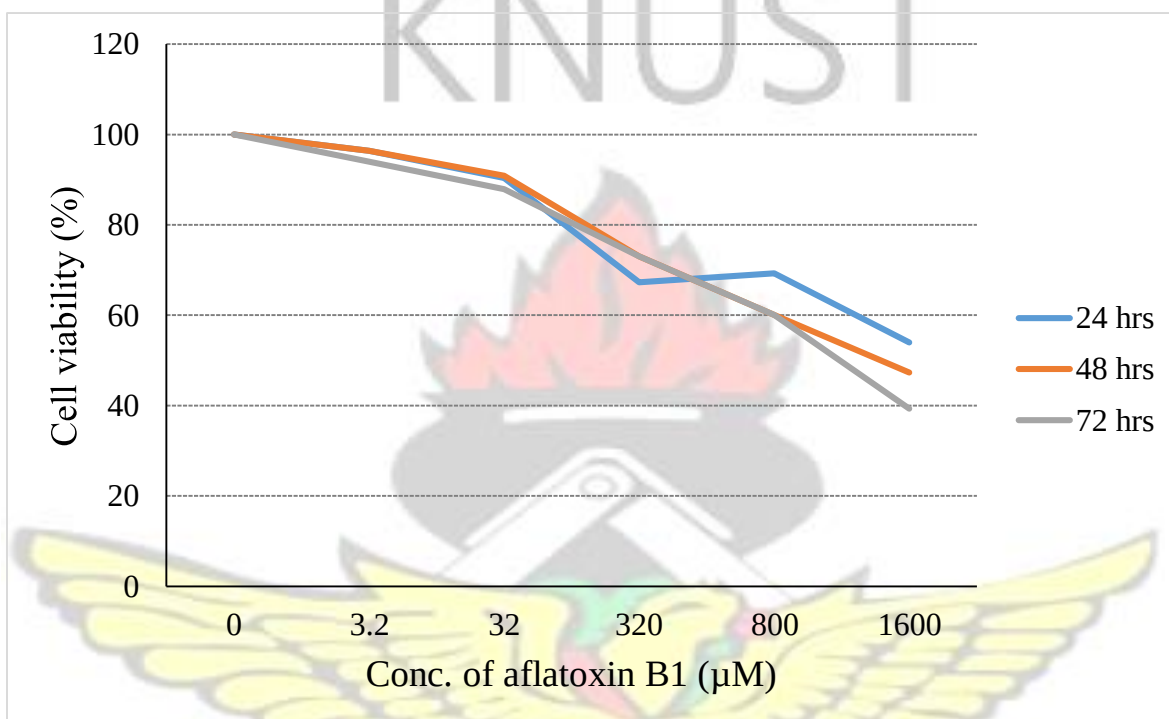


Figure 4: Cytotoxicity of AFB1 on HEK 293. HEK 293 cells were seeded at 2×10^4 cells per well in 96 well plates until they were at about 80% confluence and treated with or without increasing amounts of AFB1 (0, 3.2, 32, 320, 800, 1600 μ M) in duplicate wells for 24, 48 and 72 hours and cytotoxicity was evaluated by an MTS based assay. The viability of cells was calculated as the ratio between AFB1 treated cells and non-treated cells. The results are presented as mean percentage cell viability of two independent experiments each performed in duplicate. There was no significant difference between AFB1 treatment for 24, 48 and 72 hours (p value = 0.983)

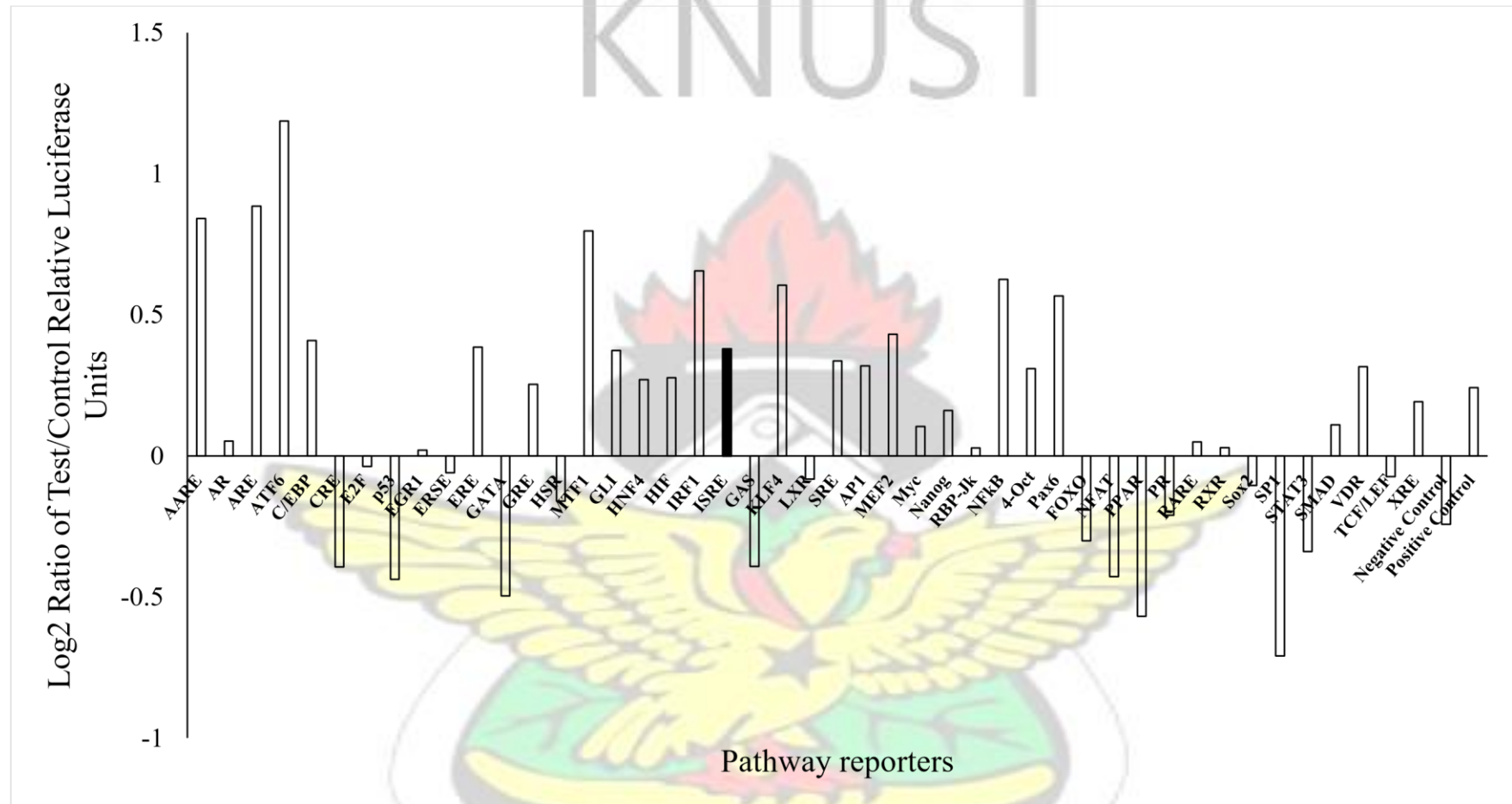


Figure 5: AFB1 differentially modulates 45 human cancer pathways in HEK 293 cells. The cells were reversed transfected at a density of 8×10^4 per well with transcription factors for 24 hours. At 24 hours post transfection, the cells were treated with $32 \mu\text{M}$ of AFB1 for 24 hours. Expression of the transcription factors were measured using dual luciferase reporter gene assay. The results are expressed as log2 of fold change of the expression of transcription factors between AFB1 treated cells and non-treated cells.

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4.2 Confirming the influence of AFB1 on IFN-Induced Signal Transduction pathway

The results of the Cignal Finder 45-pathways reporter array was confirmed or validated by determining the effects of AFB1 on IFN-induced signal transduction pathway in HEK 293 cells. This was achieved by transfecting the cells with the pathway specific firefly luciferase plasmid (pISRE-luc) together with *Renilla* luciferase plasmid (pRLSV40) which serves as a baseline response. Firstly, the minimum concentration of IFN- α which could induced maximal activity in the said pathway was determined by transiently co-transfecting the cells with pISRE-luc and pRLSV40 for 24 hours followed by treatment with or without increasing concentration of IFN- α (0, 100, 200, 300 and 400 IU/ml) for 24 hours after which the activated pathway (see appendix B) was measured using dual luciferase reporter gene assay. One-way analysis of variance (ANOVA) was calculated for 100, 200, 300 and 400 IU/ml using GraphPad Prism version 5. The p value was found to be 0.947 and thus the difference was considered to be not statistically significant. However, 400IU/ml of IFN- α was selected as the minimum IFN- α concentration that could induce maximal activity in the pathway (Figure 5) taking the error bars into consideration and thus was establish as the working concentration for subsequent experiment. Next, the effect of AFB1 on IFN- induced signal transduction pathway was assessed by activating the pathway, that is treating cells transfected with pISRE-luc with or without IFN (400) and with or without AFB1 (32, 64 μ M). The results suggested that AFB1 up-regulates the IFN- induced signal transduction pathway in a dose dependent manner (Figure 6) and this was consistent with that of Cignal Finder 45-pathways reporter array. Using unpaired t test, the difference between pISRE-luc activity of cells treated with IFN- α only and cells treated with IFN- α and 64 μ M of AFB1 was considered to be statistically significant (p-value $\leq$ 0.037).

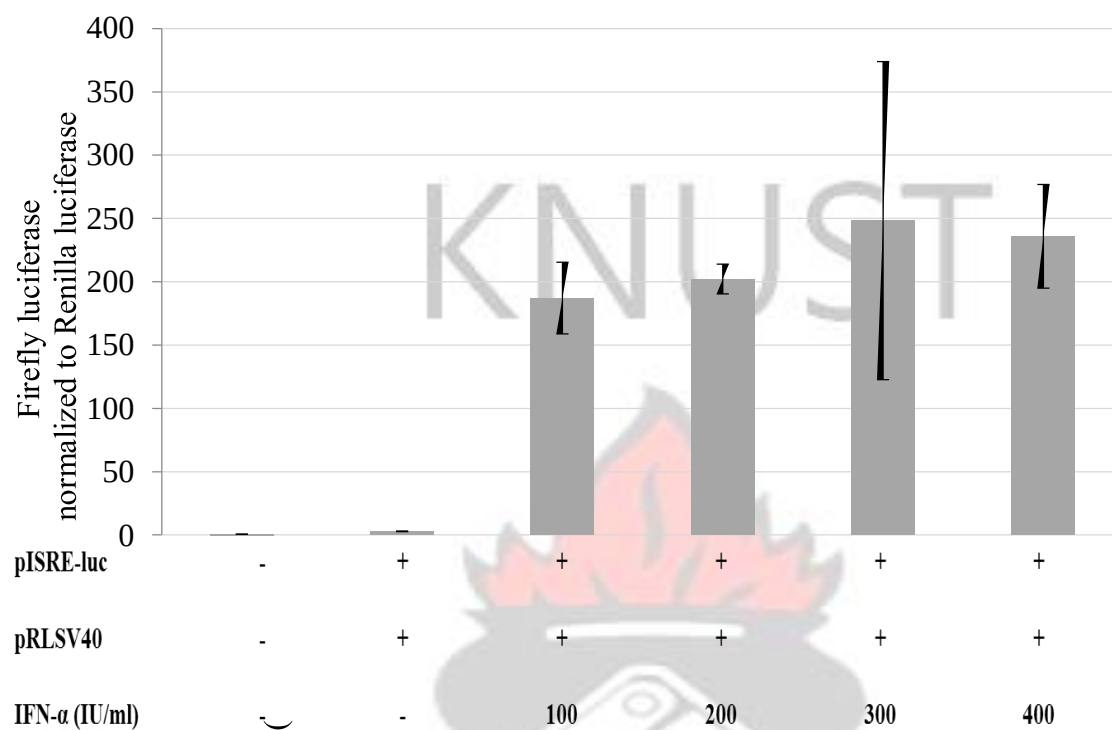


Figure 6: IFN- α (400UI/ml) induces maximal activity in IFN-induced signal transduction pathway in HEK 293 cells. The cells were transiently co-transfected with pISRE-luc (250ng) and pRLSV40 (1ng) for 24 hours and treated with increasing concentrations of IFN- α (0, 100, 200, 300 and 400 IU/ml) for 24 hours. Luciferase activity was measured and results presented as mean and standard deviation of three independent experiments each performed in duplicate.

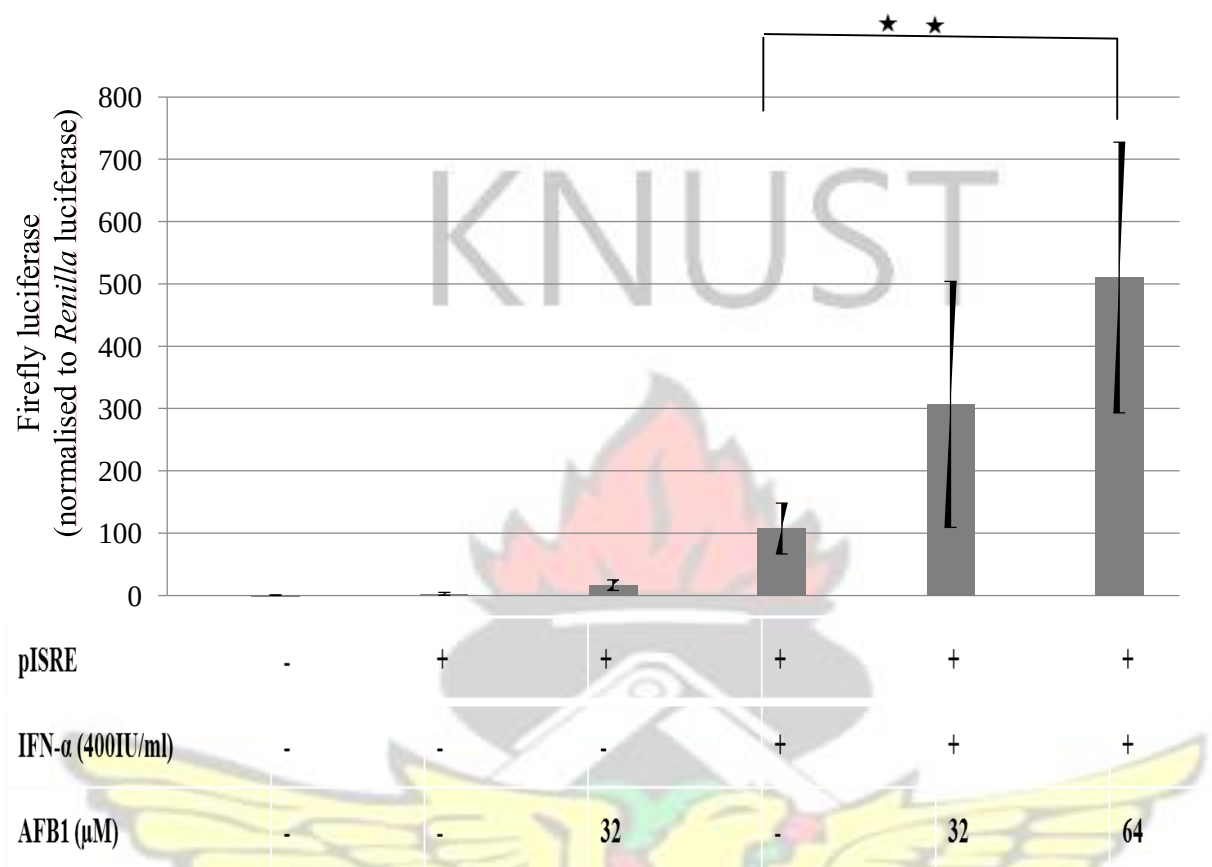


Figure 7: AFB1 up-regulates IFN-induced signal transduction pathway in HEK 293 cells.

The cells were transiently co-transfected with pISRE-luc and pRLSV40 as described in Figure 5 and treated with or without IFN- α (400IU/ml) and with or without increasing amount of AFB1 (32, 64 μ M) for 24 hours. Transfected cells which were treated with IFN- α but not AFB1 were calculated to have 100% pISRE-luc activity. Results are presented as mean and the standard deviation of three independent experiments each conducted in duplicate. There was a significant difference in pISRE-luc activity of cells treated with IFN- α alone compared to cells treated with IFN- α and 64 μ M of AFB1 (**p-value ≤ 0.037).

CHAPTER FIVE

DISCUSSION

AFB1 treatment of cells results in growth arrest, necrosis or apoptosis in both dose and time dependent manner (Yang *et al.*, 2012). Expectedly, the results from the cytotoxicity assay in these experiments indicated that the higher the dosage of AFB1, the less number of cells survived. As reported by Ribeiro *et al* (2010), AFB1 was shown to cause cell death by causing the breakage of chromatin during cell cycle in rat primary hepatocytes. The chromatin breakage was suspected to disrupt physiological processes leading to cell membrane damage and cell death. This could possibly account for the death of HEK 293 cells when treated with higher doses of AFB1 as shown in this study.

Low dosage of AFB1 was however found to differentially modulate the activities of the signalling pathways. An up-regulation in a signalling pathway consequentially leads to an increase in the gene product such as RNA and proteins and likewise down regulation leads to a decrease in the gene products (Spitz & Furlong, 2012). Of the 45 pathways assayed, key pathway reporters upregulated included AARE, ARE, ATF-6, GRE, MTF-1, ISRE, NF κ B and PAX-6 pathways. The up regulation of ISRE for example is consistent with study done by Luongo *et al* (2014) who reported that AFB1 up-regulates the activities of ISRE of the IFN induced signal transduction pathway in human lymphoblastoid Jurkat T-cell model in a dose dependent manner. The IFN induced signal transduction pathway functions chiefly in anti-cancer, anti-viral and antiinflammatory mechanisms within the cell (De Andrea *et al.*, 2002). Therefore, the up regulation of ISRE could confer more cellular protection and can be exploited for therapeutic interventions. In addition, the up regulation of NF κ B pathway established in this study was consistent with study

conducted by Castelino (2013) who also reported that AFB1 up-regulates the activities of NF κ B Chapter 5; Discussion in hepatocarcinogenesis. NF κ B plays essential roles in regulating cell proliferation, inflammatory and immune responses (Gilmore, 2006). Likewise, the up-regulation of NF κ B could also confer cellular protection and could be exploited for therapeutic purposes. Furthermore, the antioxidant responsive pathway encodes phase II detoxification enzymes and antioxidant proteins enzymes which metabolize carcinogens into less reactive forms and also detoxify other reactive agents (Lee *et al.*, 2005). Therefore, the up-regulation of ARE could imply induction of more detoxification enzymes and antioxidant proteins to counter the activities of AFB1

Conversely p53, FoxO, NFAT, PPAR, SP-1 and STAT3 pathway reporters were down regulated in this study. The down regulation of p53 observed in this study was consistent with already established key event in AFB1 induced carcinogenesis (Zilfou & Lowe, 2009). The p53 pathway directly regulates more than 3600 target genes and crosstalks with several other pathways (Li *et al.*, 2012). Its activities in DNA damage response are key to the survival of the cell. This is because, upon cellular injury, p53 initiates DNA repair or cell death and prevents mutations from accumulating within cell (Surget *et al.*, 2013). Thus, a down regulation of the p53 could lead to the accumulation of mutant genes and their products and ultimately loss of functions of key genes and their products due to mutations. Again, the down regulation of NFAT in this study was consistent with Han *et al.* (1999). Their work indicated that AFB1 inhibited the binding of NFAT to DNA thus down-regulating the activities of NFAT in Jurkat E -1 human T-cell leukemia. A possible scenario could have occurred in HEK 293 cells thus accounting for the down-regulation observed. However further studies are needed to confirm this. The NFAT transcription factors regulate important genes in immune response of cell mediated immunity (Hogan *et al.*, 2003). Therefore, a down regulation in the activities of NFAT would possibly result in decreased

immunity. STAT3 is a proto-oncogene which functions mainly in cell proliferation, differentiation Chapter 5; Discussion and cell survival (Yue & Turkson, 2009). Its up-regulation therefore promotes cancer via tumour cell proliferation, anti-apoptosis and immunosuppression (Yu *et al.*, 2014). However, the STAT3 pathway was down regulated in this study as opposed to its up-regulation in liver cells reported by Castelino (2013). Thus the down regulation observed in this study could imply an anti-cancer mechanism and could be exploited in therapeutic interventions. However, more studies are needed to establish the relationship between AFB1 and STAT3. Also, FoxO was up-regulated in liver lesions induced by AFB1 (Yang *et al.*, 2014) as opposed to the down regulation observed in this study. FoxO plays key roles in inducing apoptosis, reactive oxygen species (ROS) response as well as cell proliferation (van der Vos & Coffey, 2008). Therefore, a down regulation of this pathway could inhibit apoptosis and ROS response and cause deregulated cell cycle. This suggest that the effect of AFB1 on this pathway could lead to the accumulation of mutant genes and their products and promote cell longevity. Furthermore, the deregulation of cell cycle resulting from the down regulation of FoxO could contribute to the transformation of cells into cancer cells. However, more studies are needed to determine the relationship between AFB1 and FoxO. The down regulation of Sp1 by AFB1 in mouse lung tumours observed by Tam *et al.*, (2003) is consistent with this study Sp1 regulates genes involved in apoptosis, cell cycle as well as DNA damage response (Olofsson *et al.*, 2007; Safe & Abdelrahim, 2005). Therefore, a down regulation of this pathway could inhibit apoptosis and DNA damage response and deregulate cell cycle. This could lead to the accumulation of mutant genes and their products which may ultimately result in cancers.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Altogether, this study showed that higher concentration of AFB1 was found to be toxic to HEK 293 cells. AFB1 was also found to differentially modulate the 45 cancer pathways assayed. Key pathway reporters including AARE, AR, ATF-6, GRE, MTF-1, ISRE, NF κ B and PAX-6 were upregulated while FoxO, NFAT, PPAR, SP-1 and STAT3 were down-regulated. These results show that AFB1 could cause cancer by deregulating multiple pathways aside the p53 pathway

6.2 Recommendation

Based on the results of this study, the following recommendations were made:

1. Similar studies should be conducted in human liver cells since the liver is the primary target organ of AFB1.
2. Further studies should be done on each key pathway to elucidate the exact molecular mechanisms utilized by AFB1 in deregulating the pathways.

CHAPTER SEVEN

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KNUST



Appendix A

Table 2: List of solutions and reagents

Item	Source
CellTiter 96 AQueous One Solution Reagent	Promega, USA
Dual Luciferase Gene Assay	Promega, USA
Cignal Finder 45-Pathway Report Array	Qiagen, USA
Cignal ISRE Reporter (luc) Kit	Qiagen, USA
Aflatoxin B1 (AFB1) Solution	Sigma-Aldrich, USA
Interferon- α A human Solution	Sigma-Aldrich, USA
TransIT®-2020 Transfection Reagent	Mirus Bio, USA
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific, USA
Fetal Bovine Serum	Thermo Fisher Scientific, USA
MEM Non-essential Amino Acid Solution	Sigma-Aldrich, USA
Gentamicin solution	Sigma-Aldrich, USA
Phosphate Buffered Saline	Sigma-Aldrich, USA
Trypsin-EDTA Solution	Sigma-Aldrich, USA

Photographs of equipment used.

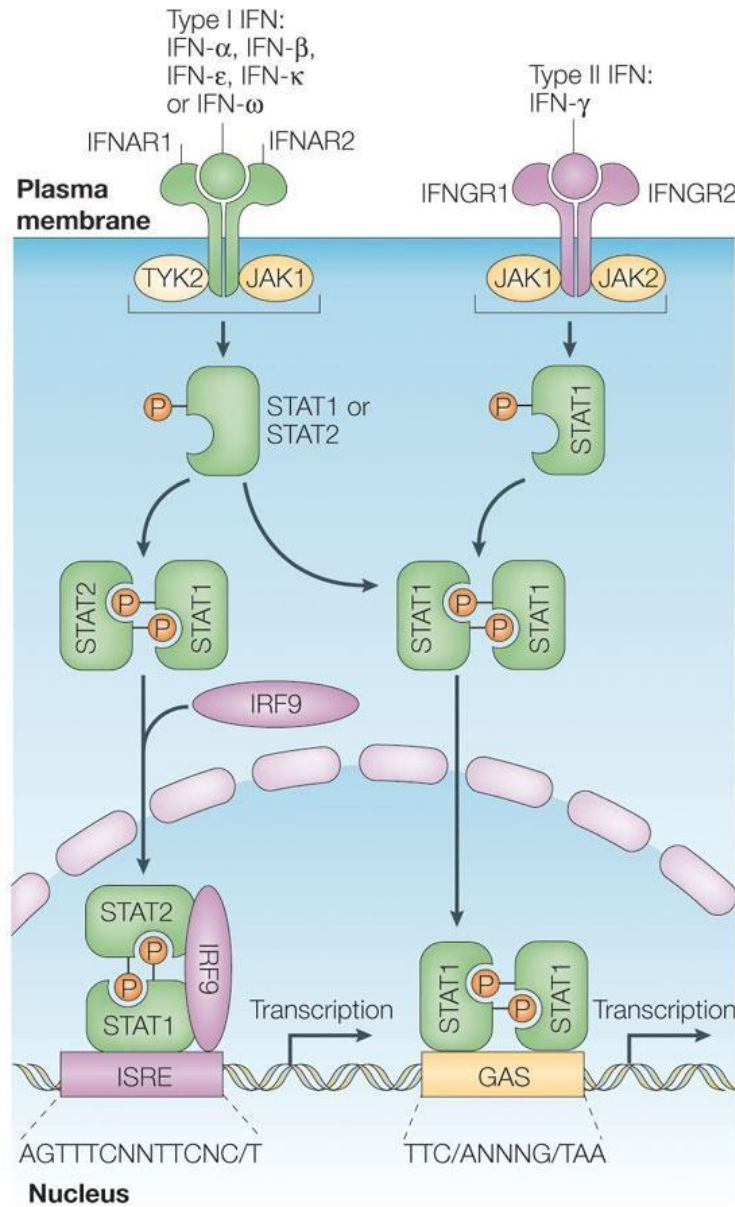


iMark™ Microplate Absorbance Reader



Berthold Orion Microplate Luminometer and Injection Unit

Appendix B



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IFN induced signal transduction pathway. Source; (Platanias, 2005) IFN- α binds to type I IFN receptor which consists of IFNAR1 and IFNAR2 which are associated with Janus activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1, respectively. This leads to the phosphorylation of signal transducer and activator of transcription I and 2 (STAT1 and STAT2) and the recruitment of IFN-regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3) complex. The complex translocates to the nucleus where it binds to IFN-stimulated response elements (ISREs) of the IFN stimulated genes (ISGs) to initiate transcription.

Appendix C

Table 3: Reagents preparation

Dilution of Trans IT 2020 transfection reagent used in Signal Finder 45-pathway reporter assay

	Volume needed per well	Total volume per 96 plate
Trans IT 2020 reagent	0.6 μ l	0.6 $\times$ 108=64.8 μ l
Diluent (DMEM only)	49.4 μ l	49.4 $\times$ 108=5335.2 μ l
		Total =5400 μ l

Preparation of Stop & Glo reagent.

	Volume needed
Stop & Glo substrate	0.2ml
Stop & Glo buffer	10ml

Preparation of DNA:Trans IT 2020

Plasmid stock	Final concentration	Volume per well (μ l)	Total volume (μ l)
pISRE (1000ng/ μ l)	250ng/ μ l	$\frac{250}{1000} = 0.25$	0.25 $\times$ 12=3
pRLSV40 (1ng/ μ l)	1ng/ μ l	1	1 $\times$ 12=14
Diluent (culture medium)	-	7.9	7.9 $\times$ 12=94.8
Trans IT 2020	-	0.3	0.3 $\times$ 12=3.6

Total=115.4