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FLAVONOID INDUCTION OF CYTOCHROME P450 (CYP) IN HUMAN ESOPHAGEAL CARCINOMA CELLS: IMPLICATIONS FOR CHEMOTHERAPY

By

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

September 2010

Supervisors: *Dr Collet Dandara & Professor Rob Veale*

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Master of Science degree at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

_____
(Signature of candidate)

_____ Day of _____ 2011

ABSTRACT

Esophageal cancer ranks as the second most common cancer among black males in South Africa and patients often seek medical attention when the cancer has already reached advanced stages. Thus, there is a lot of work investigating factors associated with increased risk, biomarkers for early diagnosis as well as a search for suitable drugs to treat esophageal cancer. In this project, flavonoids were used on esophageal cancer cell lines to identify enzymes that are differentially expressed as well as to evaluate the flavonoids' direct toxic effects on the cancer cells using ß-Naphthoflavone (BNF) on human esophageal cancer cell lines, WHCO1 and WHCO6. Differential expression of drug metabolising enzymes, CYP1A1, CYP1A2 and CYP1B1 was also investigated. BNF showed a moderate antiproliferative activity in WHCO6 cells (IC50~ 10μM) and a weak activity in WHCO1 cells (IC50~25 μM). Thus, suggesting that esophageal cancer cells may be responsive to the treatment with BNF. BNF resulted in the differential expression of CYP1A1, CYP1A2, and CYP1B1. These results, implicate CYP1 enzymes as potential therapeutic targets for esophageal cancer prevention.

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Last, but not least, I thank my family: My parents Merriam and Ramagodi Molepo for their unconditional support and encouragement to pursue my interests. My sister Molebogeng Molepo for listening to my complaints and frustrations, and for believing in me.

LIST OF ABBREVIATIONS

2-OHE2	2-Hydroxyestradiol
4-OHE2	4-Hydroxyestradiol
AFB_1	Aflatoxin B_1
AHR	Aryl Hydrocarbon receptor
AHRR	Aryl hydrocarbon receptor repressor
AIP	Aryl Hydrocarbon Receptor interacting protein
ANF	α-Naphthoflavone
ANOVA	One way analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
BNF	ß-Naphthoflavone
BSA	Bovine serum albumin
BP	Benzo(a)pyrene
BPDE	Benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide
COMT	Catechol O-Methyltransferase
CYP	Cytochrome P450
DMSO	Dimethylsulfoxide
E2	17-ß Estradiol
FBS	Fetal bovine serum
GST	Glutathione-S-transferase
HOSCC	Human esophageal squamous carcinoma cell

HRP	Horseradish peroxidise conjugate
HSP	Heat shock proteins
HSP90	Heat shock protein 90
IC_{50}	Median inhibition concentration
MTT	3-4, 5-Dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide
PAHs	Polyaromatic hydrocarbons
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNPs	Single nucleotide polymorphisms
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween-20
TCA	7.5% tichloro-actic acid
TCDD	2, 3, 7, 8-Tetrachlorodibenzo-P-dioxin
XRE	Xenobiotic response element

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CHAPTER 1: General introduction

1.1. Variation in xenobiotic metabolism

Humans are continuously exposed to harmful xenobiotic compounds which if allowed to accumulate in their bodies could become toxic. Most of these xenobiotics require to be changed or biotransformed in order to be removed from the body. The biotransformation results in mainly detoxification of the xenobiotics but in some cases, metabolic activation occurs. Detoxication or biotranformation of xenobiotics is catalysed by enzymes that belong to gene superfamilies. Detoxification or metabolism of xenobiotics is classified into three phases, I, II and III. The phase I enzymes most of which are the cytochrome P450 (CYP) enzymes, catalyze the functionalization reactions resulting in more polar metabolites (Conney, 2003; Guengerich, 2004; Caro and Cederbaum, 2004; Coon, 2005; Kim and Guengerich, 2005). The phase II enzymes, such as the glutathione transferases, UDP glucuronosyltransferases and N-acetyl transferases, conjugate the phase I products with water-soluble endogenous metabolites such as glutathione, glucuronic acid, sulfate, cysteine or acetate, producing hydrophilic products that can be easily excreted (Burchell, 2003; Kuuranne *et al*; 2003). However, the phase I products are not always conjugated by phase II enzymes since the phase I products are often highly reactive metabolites and can bind to macromolecules such as proteins and nucleic acids (Figure 1.1), thus leading to cancer development

(Guengerich, 2000, Schwarz *et al*, 2001). Phase III metabolism is involved in the transport of the conjugated xenobiotics.

Individuals with an imbalance in phase I, phase II enzyme and phase III activity may have a differential susceptibility to cancer. This project focuses on the CYP enzymes, since these enzymes play a major role in the toxicity and carcinogenesis of several xenobiotic compounds.

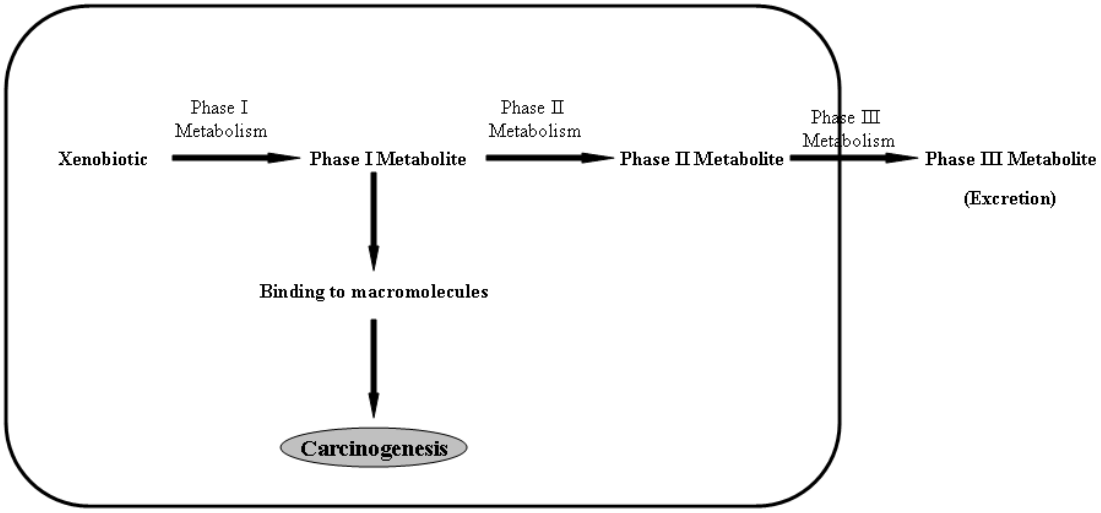


Figure 1.1: A Simplified schematic showing the metabolism of xenobiotics. Xenobiotics entering the cell are metabolized by phase I enzymes and detoxified by phase II enzymes and their transport from the cells is facilitated by phase III enzymes. Phase I metabolites may either bind to macromolecules thus causing cancer or be conjugated with water-soluble endogenous molecules leading to excretion of these metabolites (Oyama *et al*, 2003).

1.2. Cytochrome P450 (CYP) enzymes

Cytochrome P450 (CYP) enzymes are a superfamily of hemoprotein monooxygenases that catalyze the oxidation of a wide variety of both endogenous compounds such as steroids, bile acids and fatty acids, and xenobiotic compounds including drugs, toxins and carcinogens (Caro and Cederbaum, 2004). The CYP enzymes or microsomal carbon monoxide binding pigments as they were initially called, were first discovered in the late 1950's when Klingenberg identified a pigment in the rat liver that produced a unique spectrum ranging between 350 and 500nm upon reduction with carbon monoxide and gave an optical absorption peak at 450nm (Klingenberg, 1958). A few years later, the hemoprotein nature of this pigment was identified and was then given the name cytochrome P450 (Omura and Sato, 1964).

CYP enzymes have been identified in all studied organisms, from bacteria to humans (Nelson *et al*, 2004). Fifty seven active (57) genes encoding cytochrome P450 and fifty eight(58) pseudogenes have been identified in humans to date (Nelson *et al*, 2004, Nelson, 2009).Using sequence homology, the CYP enzymes have been grouped into families and subfamilies and there are eighteen(18) families and fourty three(43) subfamilies currently known in humans (Nelson *et al*, 2004). The majority of CYP enzymes metabolize endogenous substrates in a highly substrate-specific manner (Lang *et al*, 2001;

Jinno *et al*, 2003; Nebert and Dalton, 2006) while others show some overlap in substrate specificity (Yamazaki *et al*, 2001; Shimada and Fujii-Kuriyama, 2004; Mahadevan *et al*, 2007). Of the fiftyseven (57) CYP genes found in humans it is mostly the members of the CYP1, CYP2 and CYP3 families that are responsible for xenobiotic metabolism, while other CYP families only metabolize endogenous substrates (see Table 1.1.).The focus of this project will be on the CYP1 family of enzymes.

Table 1.1.: Human CYP genes and their functions

Family	Members	Main substrates/pathway
CYP1	3 Subfamilies, 3 genes, 1 pseudogene	Polyaromatic hydrocarbon
CYP2	13 subfamilies, 16 genes,16 pseudogenes	Caffeine and testosterone
CYP3	1 subfamily,4 genes, 2 pseudogenes	Xenobiotics, steroids
CYP4	6 subfamilies, 11 genes, 10 pseudogenes	Arachidonic acid
CYP5	1 subfamily, 1 gene	Thromboxane synthesis
CYP7	2 subfamilies, 2 genes	Cholesterol 7α-hydroxylation
CYP8	2 subfamilies, 2 genes	Prostacyclin synthesis
CYP11	2 subfamilies, 3 genes	Steroid biosynthesis
CYP17	1 subfamily, 1 gene	Steroid 17α-hydroxylation
CYP19	1 subfamily, 1 gene	Androgen aromatization
CYP20	1 subfamily, 1 gene	Unknown
CYP21	2 subfamilies, 2 genes,1 pseudogene	Steroid biosynthesis
CYP24	1 subfamily, 1 gene	Vitamin D degradation
CYP26	3 Subfamilies, 3 genes	Retinoic acid hydroxylation
CYP27	3 subfamilies, 3 genes	Steroid 27-hydroxylation
CYP39	1 subfamily, 1 gene	Cholesterol
CYP46	1 subfamily, 1 gene	Cholesterol 24-hydroxylation
CYP51	1 subfamily, 1 gene, 3 pseudogene	Cholesterol biosynthesis

Data extracted from Lund *et al*, 1999 and Nelson, 1999

1.3. Human xenobiotic- metabolizing CYP1 enzymes

The members of the CYP1 family are, CYP1A1, CYP1A2, CYP1B1 and a pseudogene (Nelson, 2009). The CYP1 enzymes have been studied extensively because of the major roles they play in chemical carcinogenesis. They catalyze the bioactivation of mostly polyaromatic hydrocarbons such as benz[a]anthracene, 7,12- dimethylbenz[a]anthracene, arylamines, and benzo(a)pyrene (BP) (Murray *et al*, 2001; Iwanari *et al*, 2002 ; Shimada and Fuji-kuriyama, 2004). The same substrates are also known to act as their inducers (Iwanari *et al* 2002; Guruge *et al*, 2009). The three CYP1 genes are expressed in a tissue specific manner (Jana *et al*, 2000; Murray *et al*, 2001; Coumoul *et al*, 2001; Iwanari *et al*, 2002;) and their transcriptional regulation is via the aryl hydrocarbon receptor complex (Nebert *et al*, 2004). The functions, tissue-specific expression and the regulation of these genes are discussed in the following sections.

1.3.1. *CYP1A1*

1.3.1.1. Gene structure

The human *CYP1A1* gene is located on chromosome 15 on loci 15q22-qter and is composed of seven exons and six introns (Jaiswal *et al*, 1987; Corchero *et al*, 2001). The *CYP1A1* gene codes for a 2.8 kb mRNA and a protein of 512 amino acids. Figure 1.2 shows a schematic of the gene organisation for *CYP1A1*.

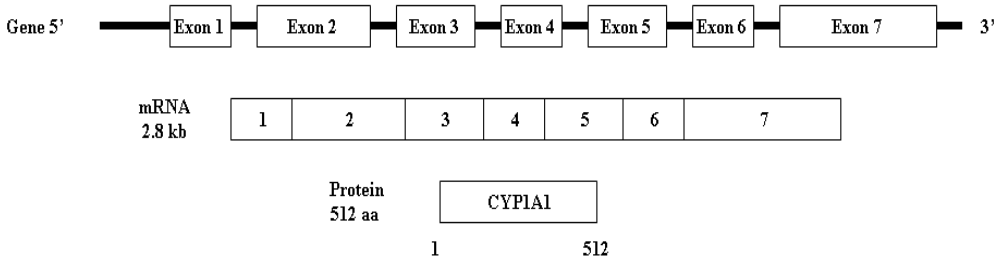


Figure 1.2.: Schematic representation of the human *CYP1A1* gene (adapted from Jaiswal *et al*, 1987 & Corchero *et al*, 2001).

1.3.1.2. Metabolic reactions catalyzed by CYP1A1

Human CYP1A1 is one of the key enzymes in the activation of pro-carcinogenic polycyclic aromatic hydrocarbons (PAHs) (Guengerich, 1992; Shimada *et al*, 1992; Guengerich *et* al, 1999; Minsavage *et al*, 2004; Schwartz *et al*, 2007). PAHs are the major carcinogens found in the environment as pollutants. Prolonged exposure to PAH contaminated air has been associated with increased lung cancer risk (Boffetta *et al*, 1997). A classic example is the activation of benzo (a) pyrene (BP). BP is a PAH generated during incomplete combustion such as in cigarette smoke (Pfeifer *et al*, 2002). BP has been associated with immune suppression, birth defects and carcinogenesis (Miller and Ramos, 2001). CYP1A1 biotransforms BP to produce the ultimate carcinogen BP-7, 8-dihydrodiol-9, 10-epoxide (BPDE). BPDE can covalently bind to DNA thus causing guanine (G) to thymine (T) transversions (Tokiwa *et al*, 1993; Conney *et al*, 1994; Nebert *et al*, 2000; Schwarz *et al*, 2001; Pfeifer *et al*, 2002;). These transversions have been found to activate a number of transcriptional factors such as P53 (Blattner *et al*, 1999; Ueno *et al*, 1999). An

excessive amount of G to T transversions in the P53 gene has been associated with lung cancer. Figure 1.3 is a diagrammatic representation of a reaction catalysed by CYP1A1 and epoxide hydrolase (EH) resulting in the formation of the reactive metabolite, BPDE that covalently binds to DNA resulting in DNA damage and ultimately cancer.

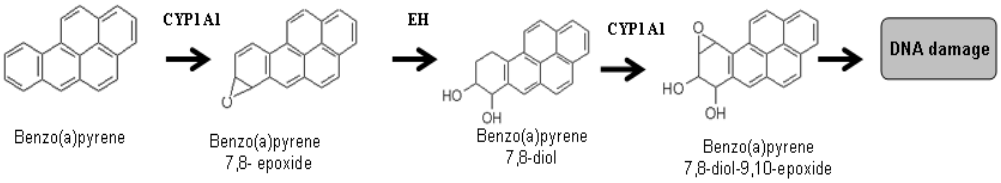


Figure 1.3: Metabolic activation of Benzo[a] pyrene into a carcinogenic Benzo (a) pyrene 7.8-diol-9, 10-epoxide. (Adapted from Kim *et al*, 1998)

1.3.1.3. Tissue specific expression of CYP1A1

CYP1A1 constitutive expression is very low in extrahepatic tissues (Shimada *et al*, 1992). However, high levels of this enzyme have been reported in almost every tissue studied, including vascular endothelium, smooth muscle cells, lung gastrointestinal tract, placenta, brain and others (Celander *et al*, 1997: Zhao *et al*, 1998; Ding and Kaminsky, 2003), only in the presence of an inducer. CYP1A1 is highly inducible by PAHs such as 2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin (TCDD) (Whitlock, 1999; Kim and Sheen, 2000; Chang *et al*, 2009), BP, 3-methylcholanthrene (3MC) and omeprazole (Whitlock, 1999;

Bowen *et al*, 2000; Rodriguez-Antona *et al*, 2000), through the aryl hydrocarbon receptor pathway (Whitlock, 1999; Denison and Nagy, 2003). Previous studies have shown CYP1A1 to be highly expressed in tumors compared with the surrounding normal tissues. For example, CYP1A1 was shown to be over expressed in ovarian cancer cells and esophageal tumors as opposed to normal cells (Murray *et al*, 1994; Nakajima *et al*, 1996; Leung *et al*, 2005). Recently, CYP1A1 was found to be highly expressed in oral squamous cell carcinoma after BP treatment (Hecht, 2003; Nagaraj *et al*, 2006; Chi *et al*, 2009). Therefore, CYP1A1 can be suggested as a target for novel chemotherapeutic agents (Mcfadyen *et al*, 2004; Rooney *et al*, 2004).

1.3.1.4. *CYP1A1* Polymorphisms

Several *CYP1A1* single nucleotide polymorphisms (SNPs) have been reported in the human population (http://www.cypalleles.ki.se/). Four common SNPS in *CYP1A1* have been studied concerning their potential implications in the risk of breast cancer. These SNPs include T3801C, T3205C, A2455G and C2453A (Ambrosone *et al*, 1995; Taioli *et al*, 1995; Huang *et al*, 1999). *CYP1A1* SNPs have also been reported to be associated with other cancer types such as prostate (Acevedo *et al*, 2003; Chang *et al*, 2003, Suzuki *et al*, 2003; Aktas *et al*, 2004; Quinones *et al*, 2006, Shaik *et al*, 2009), colorectal (Jin *et al*, 2011) esophageal (Zhuo *et al*, 2009), oral and pharyngeal (Varela-Lema *et al*, 2008), lung cancer (Shi *et al*, 2008, San Jose *et al*, 2010), ovarian, endometrial and

cervical cancer (Murata *et al*, 1998; Longuemaux *et al*, 1999; Esteller *et al*, 1999; Goodman *et al*, 2001a; 2001b).

1.3.2. *CYP1A2*

1.3.2.1. Gene structure

The human *CYP1A2* loci is found on chromosome 15q22-qter and is composed of seven exons, six introns (Corchero *et al*, 2001), a 3.2 kb mRNA and a protein of 515 amino acids as shown in Figure 1.4 below. The *CYP1A1* and *CYP1A2* genes are separated by 23 kb and are orientated in opposite directions (Corchero *et al*, 2001). There is no open reading frame between these two genes, thus showing that they share a 5'-flanking region and share 80% amino acid sequence homology.

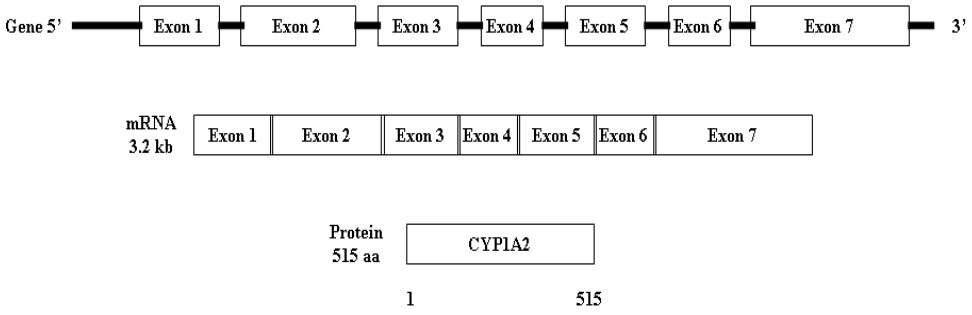


Figure 1.4: A schematic representation of the human *CYP1A2* gene (adapted from Corchero *et al*, 2001).

1.3.2.2. Metabolic reactions catalyzed by CYP1A2

CYP1A2 is responsible for the oxidative metabolism of a wide range of therapeutic drugs such as theophylline, lidocaine, imipramine, verapamil and phenothrazines (Yamazaki *et al*, 2001; Daniel *et al*, 2002; Llibre *et al*, 2002). This enzyme is also involved in the activation of endobiotic compounds such as melatonin, estradiol and uroporphy rinogen (Nichols *et al*, 2003; Tsuchiya *et al*, 2005; Faber *et al*, 2005; Ma and Lu, 2007). In addition, CYP1A2 metabolizes various carcinogenic compounds, such as heterocyclic and aromatic amines to reactive metabolites, leading to toxicity and cancer (Brosen, 1995; Guengerich *et al*, 1999; Van schaik, 2005; Ma and Lu, 2007).

Aflatoxin B_1 (AFB_1) is a potent food carcinogen and has been reported as a causative agent in hepatocellular carcinoma (Kew, 2003; Sudakin, 2003; Williams *et al*, 2004). AFB_1 on its own is not a potent toxin since it requires metabolic activation to exert its genotoxicity. CYP1A2 catalyzes the biotransformation of AFB_1 to the highly reactive AFB_1-8, 9-epoxide (McLean and Dutton, 1995; Guengerich *et al*, 1998) (Figure 1.5.). This epoxide is a mixture of both the exo- and endo-8, 9-epoxide (Ueng *et al*, 1995). Only the exo-8, 9-epoxide has been reported to have genotoxic characteristics (Guengerich *et al*, 1998). Exo-8, 9-epoxide is highly unstable and binds to guanine bases in DNA thus forming aflatoxin-N^7-guanine (Guengerich, 2001; Nayak *et al*, 2001). The aflatoxin-N^7-guanine has been reported to be capable

of forming guanine to thymine transversion mutations in DNA (Bailey *et al*, 1996) (Figure 1.5).

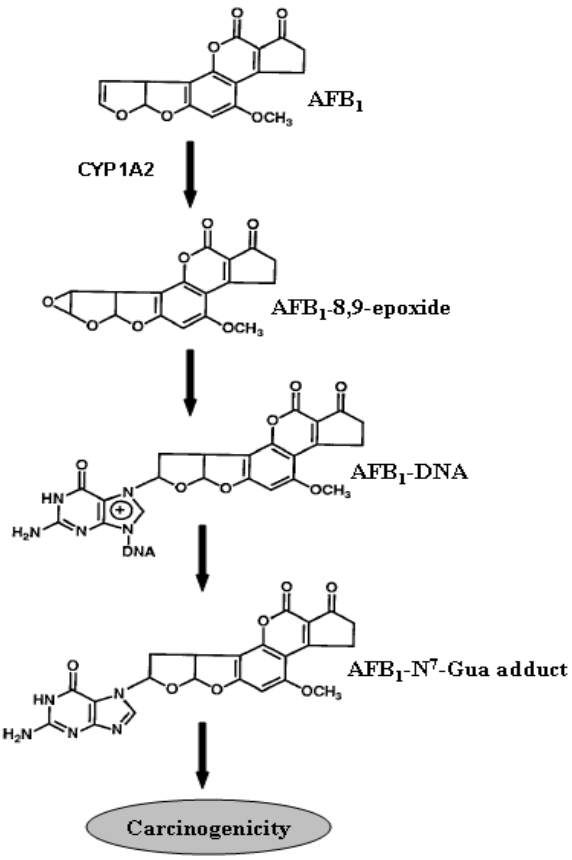


Figure 1.5: Metabolic activation of Aflatoxin B_1 to AFB_1 –8, 9-epoxide leading to the formation of AFB_1 –DNA adduct (AFB_1 –N^7-guanine) (Chou and Chen, 1997).

1.3.2.3. Tissue specific expression of CYP1A2

Human CYP1A2 is constitutively expressed in the liver (Mckinnon *et al*, 1991; Schweikl *et al*, 1993; Landi *et al*, 1999), thus accounting for 13% of the total CYP in this organ. However, a few studies have reported the expression of this gene in other organs such as the lung (Macé *et al*, 1998; Wei *et al*, 2001; Bernauer *et al*, 2006), esophagus (Lechevrel *et al*, 1999), brain (Bhagwat *et al*, 2000), prostate (Williams *et al*, 2000) and gastrointestinal tract (Tatemichi *et al*, 1999). The constitutive level of expression of the *CYP1A2* gene is increased by exposure to PAHs from cigarette smoke and TCDD via the Aryl hydrocarbon receptor/Aryl nuclear translocator (AHR/ARNT) complex (Shen *et al*, 1994; Hankinson *et al*, 1995; Landi *et al*, 1999).

1.3.2.4. CYP1A2 polymorphisms

Several *CYP1A2* polymorphisms have been reported in the past few years (http://www.cypalleles.ki.se). These polymorphisms are associated with individual differences in CYP1A2 activity (Saruwatari *et al*, 2002; Zhang *et al*, 2007). It has been demonstrated that the *CYP1A2* SNP G3860A is associated with decreased enzyme inducibility in Japanese smokers (Nakajima *et al*, 1999). The *CYP1A2* SNP C163A was also reported to be associated with higher enzyme inducibility in white smokers (Sachse *et al*, 1999). In a recent study the *CYP1A2* haplotype (T739G, C729T, and C163A) was reported to be linked with decreased enzyme activity in Ethiopian non-smokers (Aklillu *et al*,

2003). These interindividual differences influence individual susceptibility to cancer risk caused by procarcinogens and the therapeutic efficacy of drugs. Recently, low inducibility of CY1A2 polymorphism was reported to be associated with a high risk of myocardial infarction (Cornelis *et al*, 2004).

1.3.3. *CYP1B1*

1.3.3.1. Gene structure

The human CYP1 gene family was initially thought to consist of *CYP1A1* and *CYP1A2* only. It was only in 1994 that a new member of the CYP1 gene family, *CYP1B1*, was discovered. This gene was induced in human keratinocyte cell line treated with tetrachloro-dibenzo-p-dioxin (Sutter *et al*, 1994). The *CYP1B1* cDNA was cloned to facilitate the characterization of the protein. Nucleic acid and amino acid sequence analysis revealed ~40% homology of *CYP1B1* with *CYP1A1* and *CYP1A2*. There are various distinct properties that separate *CYP1B1* from *CYP1A1* and *CYP1A2*. *CYP1B1* gene is located on position 2p21-22, spanning approximately 12 kilobases (kb) of DNA and contains three exons and two introns (Tang *et al*, 1996). Although it has the simplest gene structure, *CYP1B1* is one of the largest known human CYPs in terms of the mRNA size and number of amino acids. *CYP1B1* has a 5.2kb mRNA with an open reading frame beginning at the 5' end of the second exon (Murray *et al*, 2001). The predicted protein sequence of this CYP is 543 amino acids (Figure 1.6) and this differs with other CYPs which have their

open reading frames beginning in exon 1 (Murray *et al*, 2001). Figure 1.6 below shows the schematic organisation of the *CYP1B1* gene.

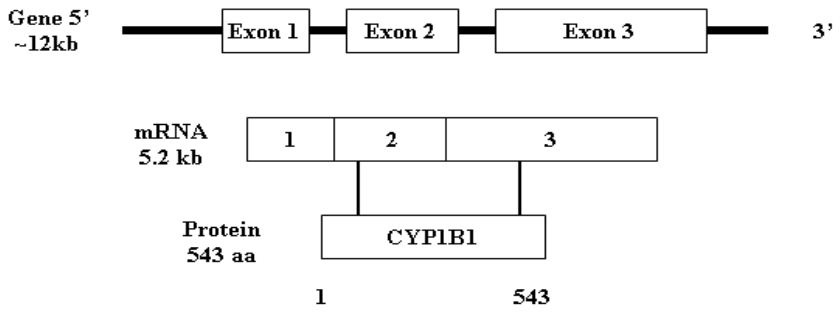


Figure 1.6: A schematic representation of the human *CYP1B1* gene (adapted from Murray *et al*, 2001).

1.3.3.2. Metabolic reactions catalyzed by CYP1B1

The human CYP1B1 activates many structurally diverse environmental procarcinogens, such as PAHs, heterocyclic and aryl amines, as well as the nitroaromatic hydrocarbons (Shimada *et al*, 1996; Kim *et al*, 1998; Guengerich, 2000; Guengerich *et al*, 2003). CYP1B1 also detoxifies cancer drugs such as flutamide and docetaxel, and this reduces their cytotoxic potential and effectiveness (Rochat *et al*, 2001; MacFadyen *et al*, 2001; Bournique and Lemarie, 2002). CYP1B1 has also been found to be effective in activating aflatoxin B_1 to its mutagenic metabolite (Crespi *et al*, 1997). When expressed in *S.cerevisiae*, the human CYP1B1 was found to metabolize a dietary heterocyclic amine, 2-amino-1 methyl-6-phenylimidazo [4, 5-b]

pyridine which has been associated with breast and colon cancer (Crofts *et al*, 1997).

In addition to its role in procarcinogen activation, CYP1B1 is also a key enzyme involved in the production of potentially carcinogenic estrogen metabolites. Estrogens have various biological effects, such as female sexual differentiation and development, and arterial vasodilation, the maintenance of bone density and neuroprotective actions. However, prolonged exposure to estrogens has been associated with breast cancer and endometrial cancer (Pike *et al*, 1993; Jordan, 2000; and Liehr, 2000). Xenoestrogens, such as oral contraceptives and drugs for hormone replacement therapy are also associated with breast cancer risk through their estrogenic properties

17-β estradiol (E2) is the most common steroidal estrogen present in women. The principal source of this estrogen is from pre-menopausal females, ovary and adrenal glands .CYP1B1 encodes for an enzyme that metabolizes E2 to catechol estrogens 4-hydroxyestradiol (4-OHE2) and 2-Hydroxy estradiol (2-OHE2) (Figure 1.7). This enzyme usually produces higher levels of 4-OHE2 than 2-OHE2 (Yager and Liehr, 1996; Hayes *et al*, 1996; Spink *et al*, 1997; Cavalieri *et al*, 1997; Badawi *et al*, 2001; Cavalieri and Rogan, 2004). The 4-OHE2 gets oxidized to E2-3, 4-Quinone which readily reacts with DNA thus

causing damage to the DNA (Nutter *et al*, 1991; Nutter *et al*, 1994; Cavalieri *et al*, 1997) (Figure 1.7). High levels of 4-OHE2 have been detected in human uterine myometrium, benign uterine leiomyoma (Liehr *et al*, 1995), as well as in benign and malignant mammary tumors (Lemon *et al*, 1992; Liehr and Ricci, 1996). Human breast cancer tissues have been shown to have significantly higher levels of 4-OHE2 than normal breast tissues (McKay *et al*, 1995; Liehr and Ricci, 1996; Murray *et al*, 1997).The catechol estrogen 4-OHE2 has also been shown to be carcinogenic in animal models (Kristensen and Borresen-Dale, 2000; Hanna *et al*, 2000). CYP1B1 is therefore one of the risk factors for breast cancer because of the role it plays in steroid metabolism.

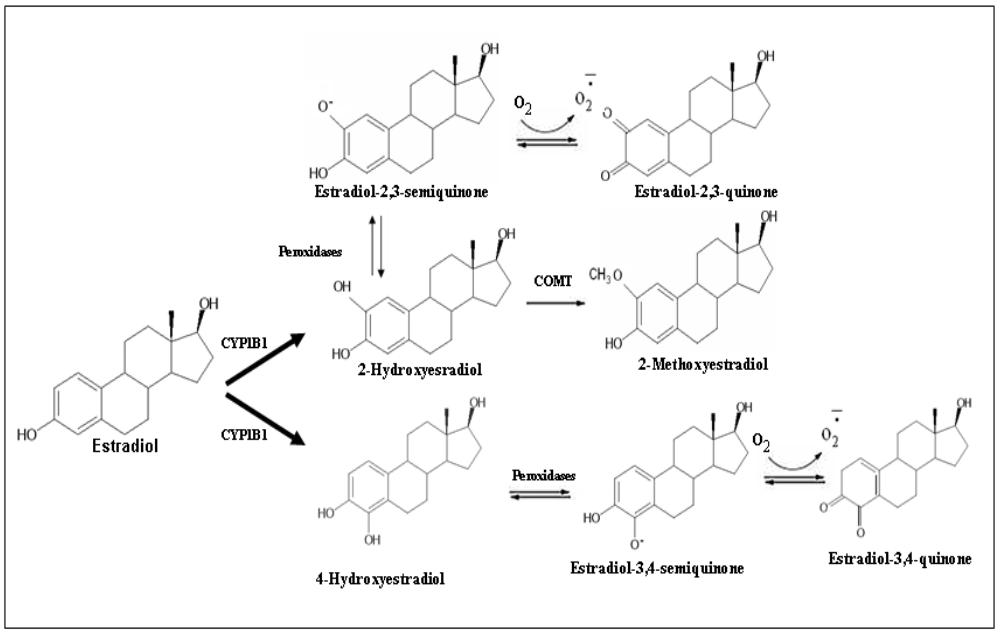


Figure 1.7: Metabolic activation of estradiol and the formation of non-carcinogenic 2-methoxyestradiol and the carcinogenic 4-hydroxyestradiol. Abbreviations used CYP1B1 (Cytochrome P450 1B1), COMT (Catechol O-methyltransferase) (adapted from Chun and Kim, 2003).

1.3.3.3. Tissue specific expression of CYP1B1

The human *CYP1B1* mRNA and protein are constitutively expressed in extrahepatic tissues such as the ovaries, testes, adrenal glands, breast, uterus and prostate, but are poorly expressed in the liver, kidney and lung tissues (Shimada *et al*, 1996; Hakkola *et al*, 1997 and Tang *et al*, 1999). One of the most significant discoveries regarding CYP1B1 expression is its high frequency of expression in various types of tumors compared to the corresponding normal tissues (Murray *et al*, 1997; Cheung *et al*, 1999;

McFadyen *et al*, 1999). For example, the expression of CYP1B1 was detected in breast tumors, but not in normal breast tissues (Huang *et al*, 1996; Kaminsky and Spivack, 1999). In one study, CYP1B1 was found to be over expressed in prostate tumors when compared to the normal prostate tissues (Finnström *et al*, 2001; Chaib *et al*, 2001; Carnell *et al*, 2004). Together these findings implicate CYP1B1 as a potential therapeutic target for reducing the development of a wide variety of cancers and that this CYP may play a major role in anticancer drug resistance (McFadyen *et al*, 2001; Rochat *et al*, 2001).

1.3.3.3. CYP1B1 polymorphisms

Six different polymorphisms of the CYP1B1 gene have been reported and four of these result in amino acid substitutions (Bailey *et al*, 1998; Stoilov *et al*, 1998; Mitrunen and Hirvonen, 2003). Two of these amino acid substitutions are encoded by exon 3, which encodes the heme-binding domain, at codon 453 Asn is substituted with Ser (Asn453Ser) (Bailey *et al*, 1998). The other amino acid substitutions are encoded by exon 2 (Stoilov *et al*, 1998), and they include, Arg48Gly and Ala119Ser.

The CYP1B1 polymorphisms have been reported to have an association with breast or endometrial cancer risk (Bailey *et al*, 1998; Zheng *et al*, 2000; De vivo *et al*, 2002; Kocabas *et al*, 2002; Lee *et al*, 2003 and Sasaki et al, 2003;

Ozbek *et al*, 2010). These polymorphisms have also been found to lead to alterations in estrogen metabolism, thus causing 2 to 3 fold higher catalytic activity than the wild type enzyme (Shimada *et al*, 1999 and Hanna *et al*, 2000). For example, the Leu432Val polymorphism was reported to have the highest effect on the catalytic properties of the enzyme since the Val432 variant displayed three-fold higher 4-hydroxylase activity than the Leu432 variant (Li *et al*, 2000; Hanna *et al*, 2000 and Aklillu *et al*, 2002). Other studies also showed that Val432 variant is associated with higher 4-hydroxyestradiol: 2-hydroxyestradiol and 4-hydroxyestrone: 2-hydroxyestrone ratios than the Leu432 variant (Shimada *et al*, 1999). Higher catalytic activities towards estrogen metabolism have also been reported for the Gly48, Ser119, and Ser453 polymorphisms (Hanna *et al*, 2000).

1.3.4. The Aryl hydrocarbon receptor

1.3.4.1. Gene structure

The Aryl hydrocarbon receptor (AHR) is a ligand activated basic helix-loop-helix (bHLH) protein that belongs to the Per-Arnt-Sim (PAS) family of transcription factors (Schmidt and Bradfield, 1996). It regulates the expression of human CYP genes (Quattrochi *et al*, 1994; Tang *et al*, 1996; Whitlock, 1999; Nebert *et al*, 2000) as well as some Phase II metabolizing enzymes (Hankinson, 1995; Schimidt and Bradfield, 1996; Hahn, 2002). The AHR was first discovered in the livers of the C5BL/6 mice treated with TCDD (Bradfield

et al, 1991). In humans, this receptor encompasses 47,146 nucleotides and is located on chromosome 7 at location 7p15-21 (Micka *et al*, 1997). The human AHR consists of 11 introns and 12 exons**,** which encode an mRNA of 5.483 nucleotides thus giving rise to a protein with 848 amino acids (Harper *et al*, 1991). An illustration on the structure of how the gene is organised is given in Figure 1.8 below.

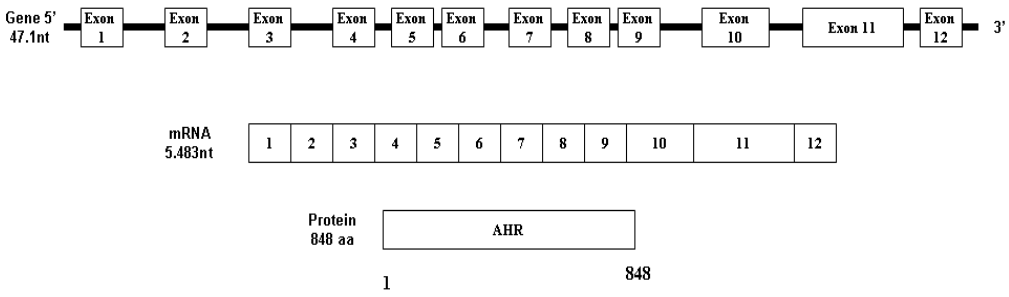


Figure 1.8: A schematic representation of the human AHR gene (adapted from Harper *et al*, 1991).

1.3.4.2. The AHR pathway

In its inactive form, the AHR is localized in the cytoplasm as a complex with a dimmer of heat shock proteins (HSP90), and one molecule of the AHR interacting protein (AIP) (Perdew, 1988; Holmes and Pollenz, 1997; Petrulis and Perdew, 2002; Hollingshead *et al*, 2004; Bock and Kohle, 2006), which are responsible for the AHR folding and stabilization (Gu *et al*, 2000). Halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons such as polychlorinated biphynyls, BP and polychlorinated dibenzo-p-dioxins

are the most common classes of the AHR ligands known (Denison *et al*, 1998; Denison and Heath-pagliuso, 1998). AHR ligands enter the cell by passive diffusion, where they encounter and bind to the AHR. Upon ligand binding, the AHR dissociates from the associated proteins and form a ligand-AHR complex. This complex translocates into the nucleus where it forms a heterodimer with the aryl nuclear translocator (ARNT) (McGuire *et al*, 1994; Goldberg, 1997; Heid *et al*, 2000; Fujii-Kuriyama and Mimura, 2005). The liganded AHR-ARNT heterodimer binds to specific regions of DNA known as xenobiotic response elements (XRE) thus regulating the expression of numerous genes, including *CYP1A1*, *CYP1A2*, and *CYP1B1*, in a positive or negative way as shown in Figure 1.9 (Hankinson, 1995; Nebert *et al*, 2000; Mimura and Fujii-Kuriyama, 2003; Okey *et al*, 2005; Ramadoss *et al*, 2005).

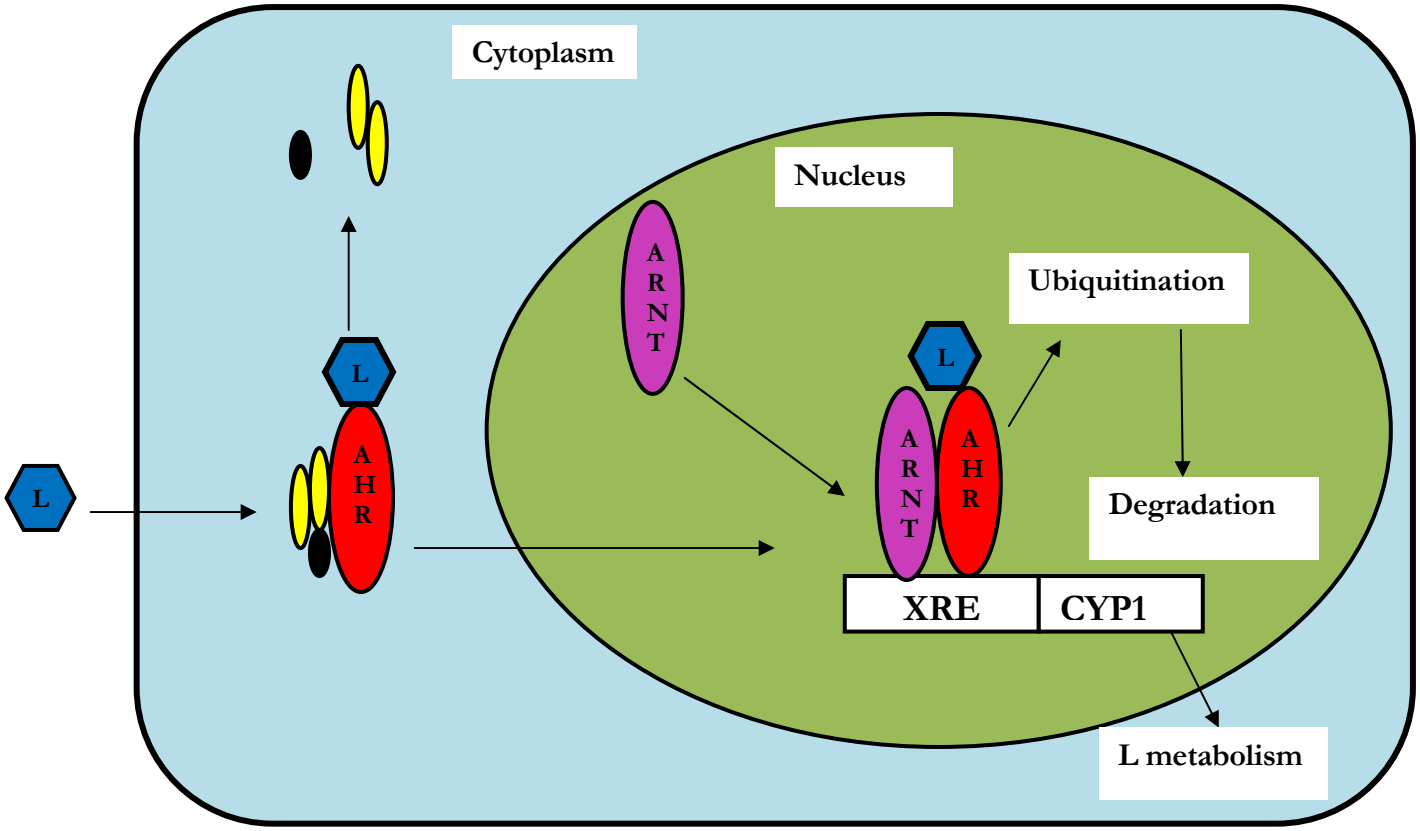


Figure 1.9: Outline of the function of aryl hydrocarbon receptor (AHR) as a ligand-activated transcription factor. AHR, Aryl hydrocarbon receptor; ARNT, AH-receptor-nuclear-translocator; CYP 1, Cytochrome P450 1; HSP90, heat shock protein 90 (shown in yellow), AIP, AHR interacting protein (shown in black); L, Ligand; XRE, Xenobiotic responsive element (adapted from Pollenz, 2002)

1.3.4.3. Regulation of the AHR pathway

The AHR signalling may be down-regulated by several mechanisms; one of which is through the degradation of AHR protein through the ubiquitin-proteasome pathway (Pollenz, 2002). This pathway involves the binding of ubiquitin to the target protein (Pahl and Baeuerle, 1996; Tanaka, 1998,

Ciechanover *et al*, 2000; Kornitzer and Ciechanover, 2000). The ubiquitinated protein is then degraded rapidly by the 26S proteasome (Pahl and Baeuerle, 1996; Tanaka, 1998, Ciechanover *et al*, 2000; Kornitzer and Ciechanover, 2000).

The ubiquitin proteasome degradation pathway plays a role in the regulation of the AHR signal transduction pathway (Pollenz, 1996; Pollenz *et al*, 1998; Giannone *et al*, 1998; Pollenz and Barbadour, 2000; Ma and Baldwin, 2000). The degradation of AHR through the ubiquitin proteasome pathway can occur either in the cytosol or nucleus (Pollenz, 2002). For AHR degradation to occur the AHR/ARNT complex dissociates from the XRE and the ARNT, ubiquinated in the nucleus and gets degraded (Pollenz *et* al, 1994; Pollenz, 1996; Pollez, 2002) (Figure 1.9). Alternatively, after dissociating from the XRE and ARNT, AHR can be exported from the nucleus, ubiquinated in the cytoplasm and gets degraded by the 26S proteasome (Pahl and Baeuerle, 1996; Tanaka, 1998; Davarinos and Pollenz, 1999; Ciechanover *et al*, 2000; Kornitzer and Ciechanover, 2000; Pollenz, 2002).

The other mechanism responsible for AHR downregulation involves the aryl hydrocarbon receptor repressor (AHRR) which acts as a negative regulator of the AHR function by competing with AHR for ARNT (Gradin *et al*, 1993;

Mimura *et al*, 1999; Gradin *et al*, 1999). A liganded AHR in a heterodimer with ARNT activates the expression of the AHRR gene (Mimura *et al*, 1999). The expressed AHRR inhibits the activity and binds to the ARNT, thus forming the AHRR-ARNT heterodimer (Gradin *et al*, 1993; Mimura *et al*, 1999; Haarmann-Stemmann and Abel, 2006). This heterodimer binds to the XREs in DNA.

1.3.4.4. Association of AHR polymorphisms with human diseases

Human genetic variation has been widely studied for phase I and phase II metabolizing enzymes (Nebert *et al*, 1999; Park *et al*, 2000; Kalow, 2001; Ingelman-Sundberg, 2001; Lin and Lu, 2001; Miller and Kumar, 2001; Xie *et al*, 2001). Most of the sequence variants that have been reported in human AHR protein are due to SNPs.

An association of SNPs with complex diseases such as cancer has been reported in most human genes; however, no striking effects of AHR SNPs on human health have been reported. For example, human exposure to dioxin-like compounds such as TCDD has been shown to cause chloracne, which is a severe skin disorder (Guo *et al*, 1999; Geusau *et al*, 2001). However, in one study, no association between the codon 554 polymorphism and chloracne was found in Caucasian population (Wanner *et al*, 1999). To date, no study has

reported a link between cancer and AHR polymorphism. For example, in previous studies there was no link found between the polymorphism associated with position 554 and lung cancer risk in Japanese smokers (Kawajiri *et al*, 1995). Cauchi *et al*, (2001), further confirmed this when he reported no link between the G459A polymorphism and lung cancer risk. Recently, Zhang *et al*, (2002) also found no association of the codon 554 polymorphism with bladder cancer risk in Chinese population.

1.4. Cancer Chemotherapy

Each year an estimated 11 million people are diagnosed with cancer worldwide (Ferlay *et al*, 2004). Current chemotherapeutic options for cancer include drugs most of which have been shown to possess severe side effects (Marsh and McLeod, 2007). Due to this, there is a focus towards new chemotherapeutic agents that show limited toxicity to normal tissue as well as limited side effects.

Flavonoids represent a large class of phenolic compounds present in plants. These compounds have often been associated with cancer prevention (Messina *et al*, 2006; Verheus *et al*, 2007; Arroo *et al*, 2008). In recent studies, their interaction with CYPs has been of particular interest. Flavonoids have been shown as inhibitors of CYP1A and CYP1B1 enzyme activity (Kim *et al*, 2005;

Chang *et al*, 2006) thus blocking the activation of procarcinogens into crcinogens (Shimada and Kuriyama, 2004). In the current study, the effect of β-Naphthoflavone (BNF), a synthetic derivative of a naturally occurring flavonoid, on CYP1 expression was investigated. This is the first study that reports the effect of BNF on the expression of CYP1 enzymes in esophageal cancer cells WHCO1 and WHCO6 cells.

1.5. AIMS

1. To evaluate the antiproliferative activity of a synthetic flavonoid, β-Naphthoflavone, on esophageal cancer cells, WHCO1 and WHCO6 using MTT cell viability assay

2. To investigate the effect of β-Naphthoflavone on the expression of the CYP1 proteins (CYP1A1, CYP1A2 and CYP1B1) using Western blot analysis.

3. To determine whether the regulation of CYP1A1, CYP1A2 and CYP1B1 is associated with the AHR pathway by using Western blot analysis.

CHAPTER 2: The model

2.1. Introduction

Cancer of the esophagus is the ninth most common cancer worldwide and recent evidence shows that its incidence is rising (Munoz, 1993; Parkin *et al*, 2001a; 2001b). Exposure to foreign chemical compounds is one of the main risk factors of this disease. Unfortunately, the hydrophobicity of these compounds is often an obstacle to their elimination. Hence, these compounds may accumulate to toxic levels, unless they are transformed to water soluble molecules that can be easily excreted. That is one of the reasons that Cytochrome P450 (CYP) enzymes were the chosen research topic since these enzymes play a major role in the oxidative metabolism of a wide range of toxic chemical compounds (Conney, 2003; Guengerich, 2004; Caro and Cederbaum, 2004; Coon, 2005; Kim and Guengerich, 2005). A characteristic of CYP enzymes is their inducibility by chemical compounds thus allowing the cell to adapt to changes in its chemical environment. The aim of the current study is to determine the effect of ß-naphthoflavone (BNF), a synthetic derivative of a naturally occurring flavonoid, on the expression of CYP enzymes in moderately differentiated esophageal cancer cell lines, WHCO1 and WHCO6. First, an assessment of the BNF potency against the target cells was done in order to rule out the effect of toxicity. One of the obstacles in this assessment, however, was the poor solubility of BNF in the culture media; therefore, dimethyL sulfoxide (DMSO) was used to dissolve BNF.

DMSO was successfully used to increase the solubility of BNF, however, because DMSO has been reported to interact with cell membranes and affect metabolism in other cells (PenninCKx *et al*, 1983; Brayton, 1986), an experiment was done to determine the cytotoxic effects of DMSO alone on WHCO1 and WHCO6 cell lines. The 3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide (MTT) assay was used to detect cytotoxicity or cell viability following exposure to DMSO according to the method of Mosmann *et al*, (1983). Briefly, the MTT assay measures the ability of viable cells to convert a soluble yellow tetrazolium salt (MTT) into insoluble purple formazan crystals by the mitochondrial dehydrogenase enzymes. The crystals are insoluble in aqueous solutions but can be dissolved in acidified isopropanol. The absorbance of the dissolved formazan solution is then quantified by measuring at a wavelength of between 500 and 600nm using a spectrophotometer. An increase in cell number leads to an increase in the amount of MTT formazan formed and an increase in absorbance (Mosmann *et al*, 1983).

The aim was to determine the DMSO concentration with the least effect on the viability of WHCO1 and WHCO6 cells and subsequently dissolve BNF in that least toxic DMSO concentration. This study will also be of help in future to use these cell lines to study different types of proteins and their interaction in esophageal cancer.

2.2. Materials and Methods

2.2.1. Cell line and Cell Culture

The human esophageal squamous carcinoma cell (HOSCC) lines WHCO1 and WHCO6 were derived from moderately differentiated squamous cell carcinoma tumors (Veale and Thornley, 1989). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, L-alanyl-glutamine, sodium bicarbonate and pyridoxine, HCl (Sigma), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma) at 37ºC, 95% humidity and 5% CO_2.

2.2.2. Cell treatment

WHCO1 and WHCO6 cells at 60-80% confluence were harvested and plated at a density of 5000 cells per well in 96-well flat-bottomed plates in a final volume of 90µl DMEM containing 10% FBS. The cells were left to grow at 37ºC and 5% CO_2 atmosphere for 24 hours. The cells were then treated with DMSO at different concentrations (0%, 0.2%, 1%, 2.5%, and 5% v/v) in triplicate. To make a final volume of 100µl, DMEM (Sigma) containing 10% FBS was added. The cells were incubated at 37ºC and a 5% CO_2 atmosphere for 24 hours.

2.2.3. MTT Cell Viability Assay

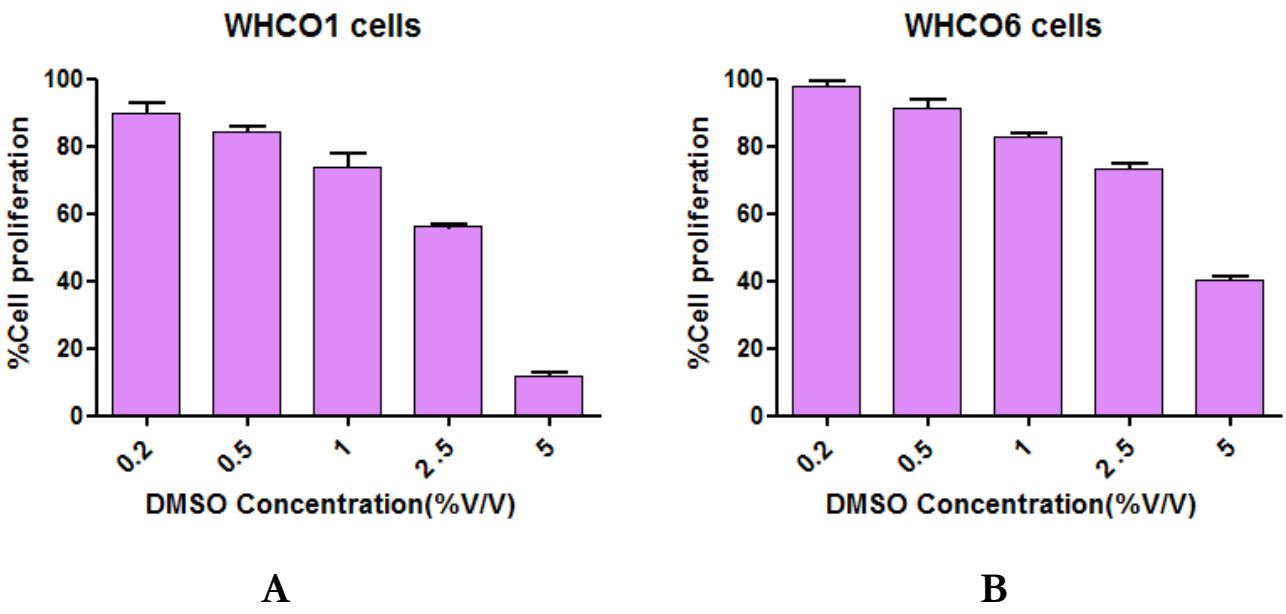
The MTT cell viability and proliferation assay was done by following the method previously used by Mosmann *et al*, (1983). To each well 10µl of MTT solution (5mg/ml) (Appendix 1.1) was added and the cells were incubated at 37ºC for 4 hours. After 4 hours 100µl of the solubilisation reagent (10% SDS in 0.01M HCl) was added to each well and incubated at 37ºC and a 5% CO_2 atmosphere for 16 hours. The absorbance was read with an iMark^{TM} microplate reader (Bio-Rad) at a wavelength of 590nm. The percentage of cell viability/DMSO cytotoxicity was calculated relative to control cells designated as 100% viable cells. This experiment was repeated 3 times.

2.2.4. Statistical Analysis

The results were expressed as means ± standard deviations (SD) for at least three independent experiments. Significant differences were determined using one way analysis of variance (ANOVA) followed by Tukey's post comparison test (Graphpad prism 5) (Appendix 2-3). A probability level of $P<0.05$ was considered significant.

2.3. Results

MTT cell viability test was used to determine the effect of DMSO on the viability of two esophageal cancer cell lines WHCO1 and WHCO6 after 24-hour incubation. Figure 2.1 shows the percentage cell viability in relation to DMSO concentration after 24-hour incubation. The results showed a gradual decrease of cell number with an increase in DMSO concentration. It was also found that there was a significant difference ($P<0.05$) in the toxic effect of DMSO at concentrations from 0-5% (v/v). At higher DMSO concentrations, the number of cells decreased drastically when compared to the lower DMSO concentrations. Figure 2.1 below shows the effects of varying concentrations of DMSO on the two cell lines, WHCO1 and WHCO6.



A **B**

Figure 2.1: The effect of DMSO on cell viability. WHCO1 (A) and WHCO6 (B) cells were incubated with increasing concentrations of DMSO for 24 hours. Cell viability was measured by the MTT assay and the percentage of viable cells relative to the control was calculated for each concentration. Error bars represent the mean ± S.D. from three independent experiments. Statistical differences were determined with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (Graph pad prism 5).

2.4. Discussion and Conclusion

Compounds that are poorly soluble in water or culture media are often encountered during the process of drug discovery. Solvents are thus used to increase the solubility of such compounds. DMSO is an amphipathic molecule consisting of a polar domain and two non-polar methyl groups thus making it to be easily soluble in both aqueous and organic media. DMSO is one of the most common solvents that have been used to dissolve hydrophobic compounds. However previous studies have shown DMSO to penetrate through cells, thus influencing the structure and conformation of proteins and cell membrane, thus affecting cell viability (PenninCKx *et al*, 1983; Brayton, 1986). Therefore, in experiments whereby DMSO is to be used as a solvent, it is always important to determine the effect of only the solvent and use a correction factor when the toxicity of another compound is calculated.

DMSO was used as a solvent to dissolve the compound of interest BNF. In order to determine what concentration of DMSO was suitable to dissolve BNF and had least effect on the viability of WHCO1 and WHCO6 cells, different concentrations of DMSO were added to the two cell lines and the effect was examined using the MTT cell viability test.

The effects of DMSO on cell viability have been studied in a large number of cell types, but variable results have been reported in these studies. For example, most studies have shown insignificantly lower or no effect on cell

viability after 24- hour treatment with 0.1% DMSO (Skupinska *et al*, 2007; Maruyama *et al*, 2007; Tampio *et al*, 2008). Studies with lymphoma cells have shown DMSO concentrations of 1-2% to prevent apoptosis (Lin *et al*, 1995). Other cell types appear to be more resistant to the cytotoxic effects of DMSO, for example, DMSO concentration of 10% had no cytotoxic effects on Caco2/Tc7 cells (Da Violante *et al*, 2002). In this study, 5% (v/v) DMSO which is the highest DMSO concentration which was used was found to have high cytotoxic effects when compared to the other concentrations, causing a reduction of cell viability to 12% in WHCO1 and 40% in WHCO6 cells (Figure 2.1). Only treatment with 0.2% v/v DMSO gave similar percentage viability to that of the control cells in both cell lines. However, DMSO was found to be more toxic in WHCO1 cells than in WHCO6 cells in all the different concentrations , this can be seen on figure 2.1 where the less toxic DMSO concentration, 0.2% (v/v), reduced cell viability to 90% in WHCO1 cells (Figure 2.1A) and 98% in WHCO6 cells (Figure 2.1B). These results are in agreement with previous studies which also showed 0.2% (v/v) DMSO to be a safe concentration for use in tissue culture since DMSO at this concentration seems to cause less or no effect in cell viability (Hukkanen *et al*, 1999; Gelardi *et al*, 2001; Nishi *et al*, 2002).

In summary, the results observed here indicate that 24 hour treatment with DMSO concentrations of 5% (v/v) have higher cytotoxic effects on esophageal cancer cell lines WHCO1 and WHCO6 , with WHCO1 cells being more

sensitive to the cytotoxic effects of DMSO at both higher and lower DMSO concentrations when compared to the WHCO6 cells. The results show that at concentrations up to 0.2% (v/v), DMSO had no cytotoxic effects on WHCO1 and WHCO6 cells; hence, BNF was dissolved in 0.2% DMSO for use in subsequent experiments. Although concentrations lower than 0.2% were shown to be associated with much reduced toxicity, they were not good in dissolving the required drug.

CHAPTER 3: The value of BNF as an antiproliferative agent for esophageal cancer cells

3.1. Introduction

Prognosis of esophageal cancer is poor with an overall 5-year survival rate of less than 10%. There are many factors that have been associated with increased risk of developing esophageal cancer and these include, gender, ethnicity, environmental factors as well as genetic factors. Therefore, early detection and search for potential anticancer compounds is important in the control of this carcinoma. Studies have revealed a large variety of phyto-chemicals that have proven successful against a wide range of cancers. In particular, flavonoids have been shown to influence a variety of biological functions including inhibition of cancer cell growth (Marchand *et al*, 2000).

The chemical structure of flavonoids is derived from a heterocyclic hydrocarbon, chromane, by substituting its ring C with phenyl group (ring B) in either position 2 or 3 (Gary, 2003) (Figure 3.1). Flavonoids are classified as flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavonoids based on the differences in the chemical structure of the heterocyclic C ring, the number and position of the double bonds and hydroxyl(OH) groups (Kozikowski *et al*, 2003). It has been reported that flavonoids with 4-6 OH groups often act as strong antioxidants, while those with more or fewer OH

groups show low or no antioxidant activities (Rice-Evans *et al*, 1995; Rice-Evans *et al*, 1996).

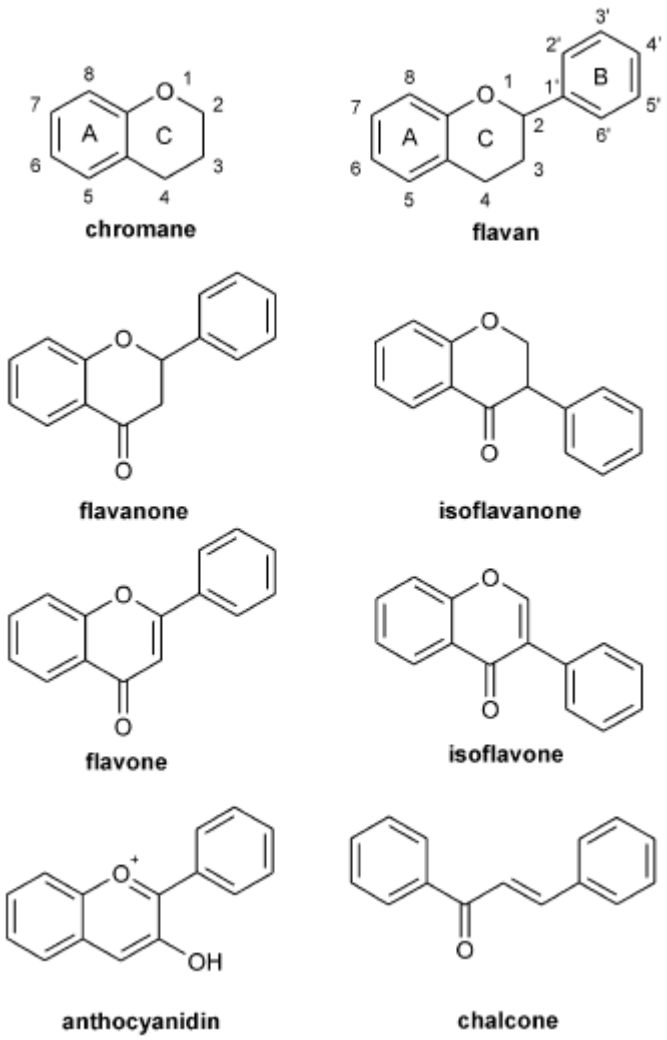


Figure 3.1: Different types of flavonoid structures (adapted from Hodek *et al*, 2002).

Compounds of flavonoid structure have shown their antiproliferative effects by delaying or reversing the process of carcinogenesis. They achieve this by protecting membranes, proteins and DNA from oxidative damage, by

scavenging hydroxyl radicals, superoxide anion radicals and lipid peroxyradicals (Chan *et al*, 1998, Doostdar *et al*, 2000, Henderson *et al*, 2000). They also inhibit enzymes implicated in procarcinogenesis and detoxification of xenobiotics (Bravo, 1998). Considering the antiproliferative activity of some flavonoids on certain tumor cells and due to lack of such knowledge on esophageal cancer cells, in the current study the antiproliferative activity of a synthetic derivative of a naturally occurring flavonoid, β-Naphthoflavone (BNF) on WHCO1 and WHCO6 cells was evaluated. This study may provide some new knowledge about esophageal cancer therapy.

3.2. Materials and Methods

3.2.1. Cell line and Cell Culture

WHCO1 and WHCO6 cells were maintained by following the protocol described in chapter 2 (Section 2.2.1)

3.2.2. Cell treatment

At least three independent studies were conducted in triplicates each time. WHCO1 and WHCO6 cells at 60-80% confluence were harvested and plated at a density of 5000 cells per well in 96-well flat-bottomed plates. α-Naphthoflavone (ANF) and Benzo pyrene (BP) were used as negative controls and 3-Methylcholanthrene(3MC) was used as a positive control in order to assess the validity of the MTT cytotoxicity assay. Stock solutions of BNF, ANF, BP and 3MC (50 Mm and 10 Mm) were dissolved in DMSO and added to the media to give final concentrations of 5, 10, 25, 50 and 100 μM BNF (in each case the final concentration of DMSO in media was 0.2%) in a final volume of 100 μl. Cells treated with the solvent alone (0.2% DMSO) also acted as the negative control.. The cells were treated with BNF, ANF, BP and 3MC at these concentrations for 24, 48 and 72 hours and incubated at 37ºC, 95% humidity and 5%CO_2

3.2.3. MTT Cell Viability Assay

After the incubation period, 10µl of the MTT (final concentration of 0.5 mg/ml) (Appendix 1.1) was added to each well and incubated for 4 hours at 37ºC, 95% humidity and 5% CO_2. 100µl of the solubilization solution (10% SDS in 0.01M HCL) was added to each well. The plate was incubated overnight at 37ºC, 95% humidity and 5% CO_2. The absorbance of the formazan produced by the viable cells was measured for each well at a wavelength of 590nm using an iMARK^{TM} microplate reader (Bio-Rad). The percentage of cell viability was calculated relative to control cells (0.2% DMSO treated cells) designated as 100% viable cells.

3.2.4. Determining the median inhibition concentration (IC_{50})

To assess the effectivity of a compound in inhibiting cell proliferation, an IC_{50}, which is a concentration at which 50% of cell growth is inhibited, is usually calculated. The IC_{50} for BNF was calculated after treatment in WHCO1 and WHCO6 cells (Appendix 6).

3.2.5. Statistical Analysis

The results were expressed as means ±standard deviations (S.D) for at least three independent experiments. Significant differences were determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple

comparison test (Graphpad prism 5) (Appendix 4-5) A probability level of $p<0.05$ was considered significant.

3.3. Results

3.3.1. The antiproliferative activity of BNF against WHCO1 and WHCO6 esophageal cancer cells

To evaluate the effect of BNF, WHCO1 and WHCO6 cells were treated with increasing concentrations of BNF for 24, 48 and 72 hours. Cell viability was measured using the MTT assay. To assess the validity of the MTT cell viability assay BP and ANF were used as negative controls and 3MC was used as a positive control. The assay lasted for 72 hours because other situations may arise at longer incubation periods, such as quiescence, metabolic stopping or induction of apoptosis.

BNF showed the biggest antiproliferative effects against WHCO1 and WHCO6 esophageal cancer cells which was dose and time dependent (see Figure 3.2 and 3.3). However, the effects of BNF seemed to diminish overtime in the WHCO1 cell while in the WHCO6 cell line the effects increased with time.

The 3MC, used as a positive control in this study was associated with a significant ($P< 0.05$) reduction in proliferation of both WHCO1 and WHCO6 and its effects were also time and dose dependent. However, compared to the effect of BNF, 3MC was less potent against the proliferation of both WHCO1 and WHCO6 cells.

BP and ANF were used as negative controls. These two compounds seemed not to have any activity against the proliferation of both WHCO1 and WHCO6 cells. The results demonstrate that with increasing concentrations of BP and ANF from 5µM to 25µM the percentage of cell proliferation increased progressively after 24-48 hour of exposure. It was only after 72h of 100µM BP and ANF exposure that cell proliferation was inhibited to below 80% (Figure 3.3).

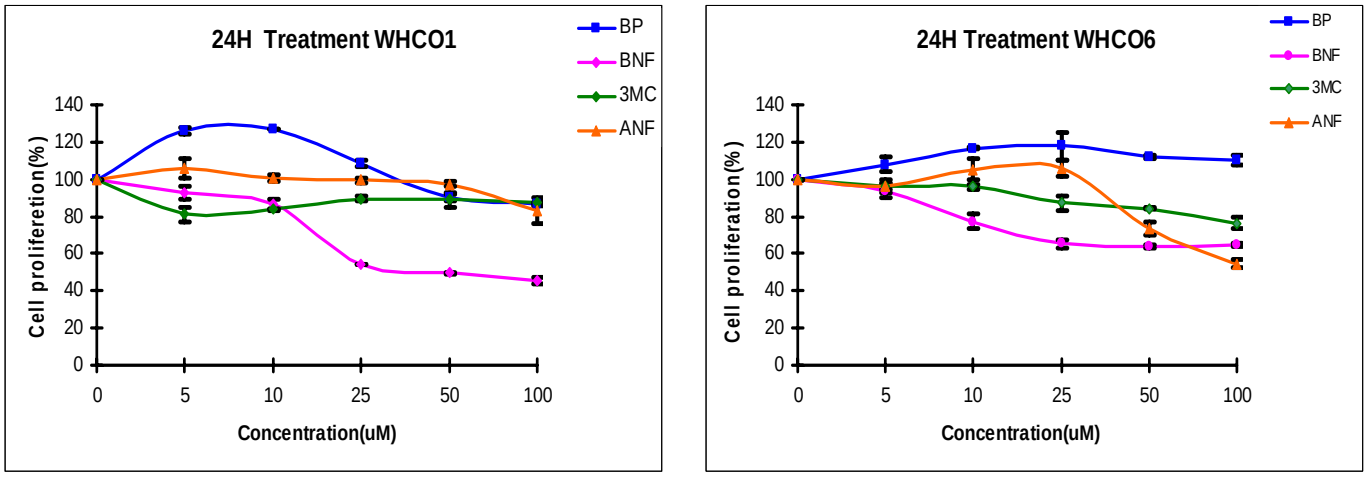


Figure 3.2: The effect of BNF on the proliferation of human esophageal carcinoma cells (WHCO1 and WHCO6) after 24 hours. At least three independent studies were conducted in triplicates each time. Cells were plated at a density of 5000 cells/well in a 96 well plate and incubated with increasing concentrations of BNF for 24 hours. BP and ANF were used as negative controls and 3MC as a positive control. Cell proliferation was measured by the MTT assay and the percentage of viable cells relative to the control (cells treated with DMSO only) was calculated for each concentration. Results are presented as the means ± SD from three independent experiments. Statistical differences were determined with the one-way ANOVA followed by Tukey's post comparison test. $P<0.05$ was considered significant.

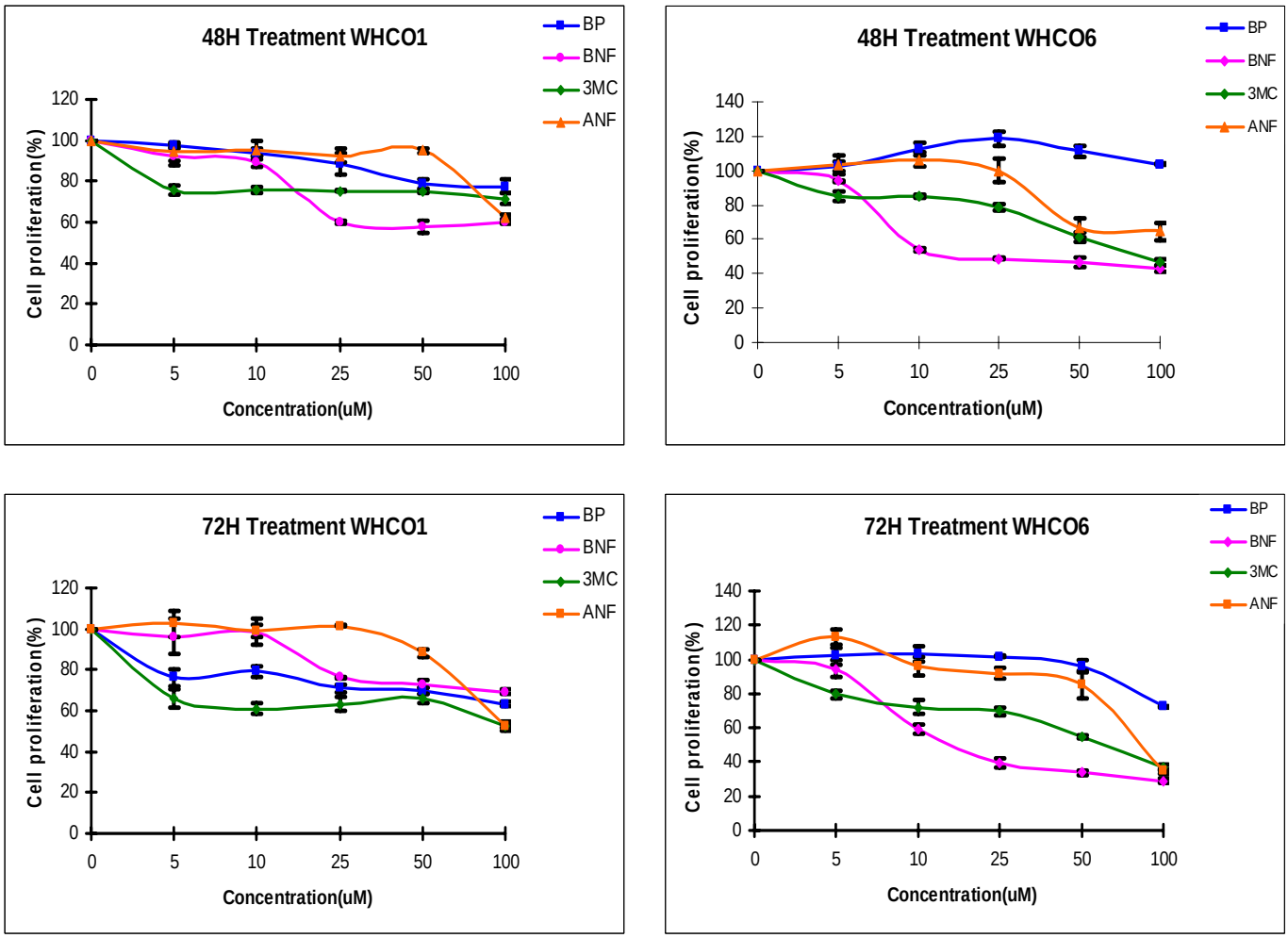


Figure 3.3: The effect of BNF on the proliferation of human esophageal carcinoma cells (WHCO1 and WHCO6) after 48 and 72 hours. At least three independent studies were conducted in triplicates each time. Cells were plated at a density of 5000 cells/well in a 96 well plate and incubated with increasing concentrations of BNF for 48 and 72 hours. BP and ANF were used as negative controls and 3MC as a positive control. Cell proliferation was measured by the MTT assay and the percentage of viable cells relative to the control (cells treated with DMSO only) was calculated for each concentration. Results are presented as the means ± SD from three independent experiments. Statistical differences were determined with the one-way ANOVA followed by Tukey's post comparison test. $P<0.05$ was considered significant.

3.3.2. The median inhibition concentration (IC_{50}) values of BNF in WHCO1 and WHCO6 cells.

For a compound to be considered as anticancerous it must display the desired level of inhibitory activity against the target cell type. Activity is expressed as the median inhibition concentration (IC_{50}) which is the concentration at which a given test compound inhibits cell proliferation by 50%. The 50% proliferation was calculated relative to control cells (0.2% DMSO treated cells) designated as 100% viable cells. Figure 3.4 shows the effects of varying BNF concentrations on the two cell lines. For the WHCO1, to reach 50% toxicity, a concentration of 25 uM was required while for the WHCO6, 50% toxicity was reached with the use of 10 uM BNF.

BNF resulted in IC_{50} after treating WHCO1 cells with 25µM BNF and incubating them for 24 hours while 10µM BNF resulted in IC_{50} in the WHCO6 after incubating them for 48 hours period.

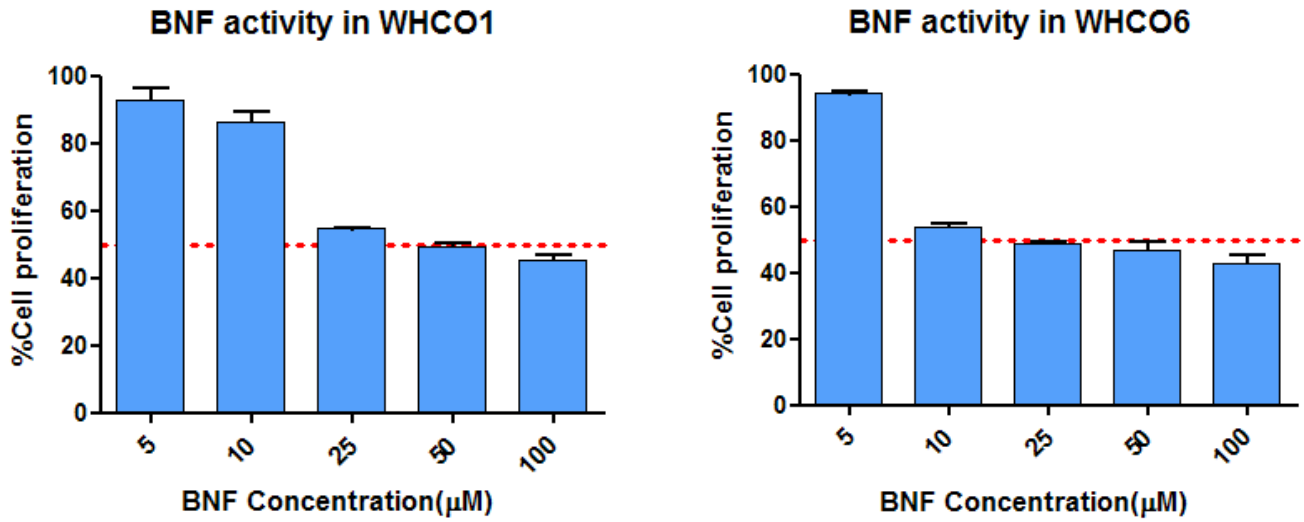


Figure 3.4: The median inhibition concentration (IC_{50}) of BNF in WHCO1 and WHCO6 cells. IC_{50} is a concentration at which 50% of cell growth is inhibited. The red dotted line shows 50% cell growth inhibition. Results represent means ± SD of at least 3 different experiments conducted in triplicates each time. $P<0.05$ is considered significant

3.4. Discussion and Conclusion

Flavonoids are polyphenolic compounds occurring in plants. They have been found in dietary components such as fruits, vegetables, and tea (Barnes *et al*, 2001; Liu, 2003). Besides their role in plants, these compounds have shown to reduce the risk of cancer development and other major chronic diseases (Marchand *et al*, 2000).

ß-Naphthoflavone (BNF) is a synthetic derivative of a naturally occurring flavonoid. The antitumor effects of BNF have been investigated only recently. This flavonoid has been shown to possess antiproliferative effects in breast cancer cells (MCF7) (Cárdenas *et al*, 2006). However, there are few reports documenting the anticancerous activities of BNF in other cancer cell types. Therefore, in the current study the antitumor activities of BNF in human esophageal cancer cells, WHCO1 and WHCO6 cells were further explored.

BNF exerted significant antiproliferative effects against both cancer cell lines. The antiproliferative effects were expressed as the median inhibition concentration (IC_{50}) values. Based on the IC_{50} values, compounds are usually classified as either highly active (IC_{50}< 10μM) or moderately active (IC_{50}~10-20μM) (Cárdenas *et al*, 2006). The magnitude of the antiproliferative effects of BNF were different in the two cell lines, WHCO6 responded very well to low BNF concentrations while WHCO1 cells needed a higher concentration of BNF. Interestingly, BNF effect diminished over time in WHCO1 cells while in

WHCO6 cells the effect increased. BNF caused moderate antiproliferation in WHCO6 (IC_{50} =10μM) but weak effect in WHCO1 cells (IC_{50} =25μM).The reason for the differences in response to BNF in both WHCO1 and WHCO6 could be due to the contribution of metabolizing gene polymorphisms in the two cell lines. These results therefore support the observation that cancerous tumors usually contain cell subpopulations with different biological properties and suggest a differential selective action in different cell types.

The effect of BNF on proliferation was compared to some of the well studied compounds, BP, 3MC and ANF. BP, a polyaromatic hydrocarbon (PAH) which is commonly present in tobacco smoke has been implicated in the induction of cell proliferation (Jeffy *et al*, 1999, Culp *et al*, 2000; Jeffy *et al*, 2002). To exert its adverse health effects, BP requires metabolic activation to a biologically reactive intermediate. The cell proliferation effect of BP has been studied in several cell lines, thus this hydrocarbon together with ANF, one of the most common CYP1 inhibitors (Gasiewicz and Rucci, 1991; Gasiewicz *et al*, 1996) were used as negative controls in this study. The data clearly showed that both BP and ANF did not have detectable antiproliferative effects in both WHCO1 and WHCO6 but instead, treatment of cell lines with these compounds resulted in significantly increasing cell proliferation even at low concentrations. These results are in agreement with previous findings that have shown BP to enhance proliferation of human breast cancer cells and lung cancer cells (Tsai *et al*, 2004; Kometani *et al*, 2009).

3MC which is one of the well known CYP1 classical inducers was shown to have antiproliferative effects in both WHCO1 and WHCO6 cell lines. This type of behavior was also reported in previous studies whereby 3MC displayed antiproliferative effects in human keratinocyte cell line NCTC 2544 (Gelardi *et al*, 2001), rat calvarial osteoblast-like cells and mouse calvarial clonal preosteoblastic cells (MC3T3-E1) (Naruse *et al*, 2002).

The results also showed that BNF was associated with significant antiproliferative effects on esophageal cancer cells and thus it would be suggested that esophageal cancer cells may be responsive to the treatment with BNF. However, further investigations in this direction are required.

CHAPTER 4: The effects of BNF on the expression of CYP1A1, CYP1A2, CYP1B1 and AHR in the two esophageal cancer cell lines, WHCO1 and WHCO6

4.1. Introduction

The human cytochrome P450 (CYP) 1 gene family is one of the main CYP families involved in the metabolism of a wide range of xenobiotics (Gonzalez and Gelboin, 1994; Shimada *et al*, 1996; 1997; Crespi *et al*, 1997) and consists of three known enzymes, CYP1A1, CYP1A2 and CYP1B1. The inducible expression of the CYP1 family is regulated via the aryl hydrocarbon receptor (AHR) complex (Hankinson, 1995; Schmidt and Bradfield, 1996). In its inactive form, the AHR is located in the cytosol where it is bound to a dimmer of heat shock proteins (HSP90), one molecule of p23 and one molecule of the AHR interacting protein (AIP) (Hollingshead *et al*, 2004; Bock and Kohle, 2006). Upon ligand binding the AHR dissociates from the proteins and forms a ligand-AHR complex. This complex is translocated to the nucleus and forms a heterodimer with the aryl nuclear translocator (ARNT) (Fujii-Kuriyama and Mimura, 2005). This heterodimer binds to the xenobiotic response element and activates the transcription of numerous genes, including *CYP1A1*, *CYP1A2*, and *CYP1B1* (Hankinson, 1995; Mimura and Fujii-Kuriyama, 2003).

This chapter describes the experiments that were carried out using Western blot analysis to investigate the effect of BNF on the expression of individual members of the CYP1 gene family in WHCO1 and WHCO6 esophageal cancer derived cell lines. The expression of these forms of CYPs was compared to that of the AHR.

4.2. Materials and Methods

4.2.1. Cell Culture and Treatment

WHCO1 and WHCO6 cells were maintained by following the protocol described in chapter 2 (Section 2.2.1). At 60-80% confluence the cells were rinsed with 1X phosphate buffer saline (PBS), (136.8 mM NaCl, 2.68 mM KCl, 10.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.76 mM KH_2PO_4 pH 7.2), trypsinized with trypsin/ EDTA (0.01% trypsin, 0.004% EDTA) and seeded into10 cm tissue culture dishes (Falcon). After 24 hours the cells were rinsed with 1X PBS and treated with BNF(WHCO1 cells treated with 25µM BNF for 24 hours and WHCO6 cells treated with 10µM BNF for 48 hours) and incubated at 37ºC, 95% humidity and 5% CO_2. Control cells were treated with 0.2% DMSO. Whole cell extractions of the treated and control cells were performed after the incubation period of each cell line.

4.2.2. Whole Cell protein Extraction

Cells were rinsed three times with ice-cold PBS and scraped off the dish in a final volume of 1ml PBS and transferred into a sterile eppendorf tube. The cells were centrifuged at 1145 x g for 2 minutes using the bench top mini centrifuge. The supernatant was discarded and the pellet resuspended in 100µl Laemmli sample buffer (Appendix 7.1). The samples were boiled for 5 minutes followed by centrifugation at 10 000g for 10 minutes at 4ºC. The samples were then stored at -70°C.

4.2.3. Protein Estimation

The protein concentrations of the cell extracts were determined using the method proposed by Bramhall *et al*, 1969. This method is described below. A 15cm filter paper (Whatman) was rinsed in distilled water for 20 minutes. This was followed by rinsing in 95% ethanol for 5 minutes, 99% ethanol for 5 minutes, acetone for 5 minutes, consecutively, in order to dehydrate the filter paper. The filter paper was then left under a fume hood to dry. Bovine serum albumin (BSA) was dissolved in Laemmli sample buffer (Appendix 7.1) to make a final concentration of 2µg/µl. A range of protein standards with a final concentration of between 1 and 20µg BSA were prepared and loaded onto the dry filter paper. Five (5) µl of each protein sample was also loaded onto the filter paper. The filter paper was left under the fume hood to dry.

The BSA standards and the protein samples were fixed within the filter paper by rinsing the filter paper with 7.5% tichloro-acetic acid (TCA) (Appendix 7.2) for 40 minutes and stained for 45 minutes with 0.25% coomassie blue stain solution (Appendix 7.2). The filter paper was destained in destaining solution (Appendix 7.2) until only the stained protein spots and BSA standards sports remained. The filter paper was left to dry and the stained spots were individually cut and placed in 5ml elution solution (Appendix 7.2) overnight in the dark. The absorbance of each sample was measured at 596nm. The BSA standards absorbance values were used to construct a standard curve. The concentration of each protein sample was calculated from the standard curve.

4.2.4. SDS-PAGE

The SDS-PAGE method used was adapted from Laemmli, 1970. A 10% separating gel (Appendix 7.3) and 5% stacking gel (Appendix 7.3) were prepared. Each sample was loaded to the gel at the required volume to obtain 35µg of protein for the detection of CYP1A1, 25µg for the detection CYP1A2, 15µg for the detection of CYP1B1 and 10µg for the detection of AHR. Protein molecular weight marker (Fermentas) was also loaded. The gel was electrophoresed at a constant current of 25 milli-Amps (mA) in running buffer (Appendix 7.3).

4.2.5. Western Blotting

For the detection of 56kDa CYP1A1, CYP1A2, and CYP1B1 as well as 122kDa AHR proteins, the proteins were electrically transferred from the gel onto the nitrocellulose membranes, in transfer buffer (Appendix 7.4) for 1 hour at a constant current of 400mA using the Biorad mini trans-blot system. The membranes were rinsed 3 times with PBS. To block non-specific proteins, the membranes were incubated for 1 hour in 5% non-fat milk powder in Tris buffered saline (TBS) or 5% BSA in Tris buffered saline containing 0.1% Tween-20(TBST). The membranes were rinsed 6 times with PBS or TBST and incubated overnight at 4ºC with rabbit anti-CYP1A1 antibody (1:1000, SANTA CRUZ), rabbit anti-CYP1A2 (1:1500, SANTA CRUZ), rabbit anti-CYP1B1 (1:1000, SANTA CRUZ) or rabbit anti AHR antibody (1:800,

SANTA CRUZ) in 1% BSA. After 6 X 5 minutes rinsing in PBS or TBST the membranes were incubated with 1:5000 horseradish peroxidase conjugate (HRP) goat anti-rabbit antibody (Sigma) for an hour in 1% BSA or 5% non-fat milk powder at room temperature in the dark. The membranes were then rinsed for a further 30 minutes in PBS or TBST at 5-minute intervals. The super signal west pico chemiluminescent substrate kit (Pierce) (Appendix 7.4) was used for the detection of of the proteins. The membranes were then exposed to the hyperfilm for 10 minutes. The film was developed for 5 minutes in the developer (Appendix 7.4), rinsed with water and placed in fixer (Appendix 7.4) for 5 minutes and finally rinsed with water.

4.2.6. Densitometry

Densitometric methods are used to measure the relative quantities of proteins on the blot compared to the control proteins. LabWorks^{TM} Image Acquisition and analysis Software (LabWorks version 4.5) was used to quantitatively determine the concentration level of CYP1A1, CYP1A2, CYP1B1 and AHR.

4.2.7. Statistical Analysis

The results were expressed as means ±standard deviations (S.D) for at least three independent experiments. Significant differences between the control cells and BNF treated cells were determined using the T-test (Excel 2003) (Appendix 8). A probability level of $p<0.05$ was considered significant.

4.3. Results

4.3.1. SDS-PAGE for protein estimation

To compare a change in expression between treated and non- treated samples using Western blotting the same concentration of both samples was used. Each sample was loaded to the gel at the required volume to obtain 15µg of protein. Figure 4.1 shows SDS-PAGE of samples extracted from WHCO1 and WHCO6 cell lines. The uniform band intensities obtained for background protein for all samples analyzed by SDS-PAGE (Figure 4.1), is confirmation that similar concentrations of cell lysate were loaded in each well.

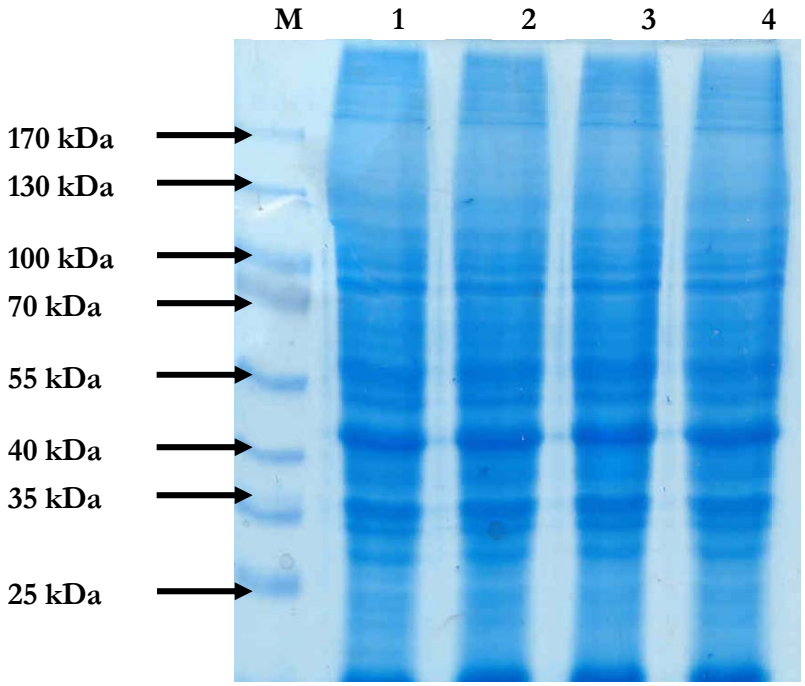


Figure 4.1 SDS-PAGE of samples extracted from WHCO1 and WHCO6 cell lines. Whole cell protein extraction was used and the total protein was estimated as explained in materials and methods. 15µg of each sample was loaded in each lane. Lanes- **M:** Marker, **Lane 1:** WHCO1 control, Lane 2: WHCO1 BNF treated, Lane 3: WHCO6 control, Lane 4: WHCO6 BNF treated.

4.3.2. The effect of BNF on CYP1A1, CYP1A2, CYP1B1 and AHR protein expression

To examine the effect of BNF on the expression of CYP1A1, CYP1A2, CYP1B1 and AHR, both WHCO1 and WHCO6 cells were treated with their respective BNF IC_{50} concentrations whereby the WHCO1 cells were treated with 25µM BNF for 24 hours and WHCO6 cells were treated with 10µM BNF for 48hours. The expression of these proteins was measured using Western blot analysis. Fold increases or decreases in protein expression was determined by

comparing protein levels of CYP1A1, CYP1A2, CYP1B1 and AHR in BNF treated cells to protein levels of each respective gene in control cells by using the densitometric band intensities of Western blots.

4.3.2.1. The effect of BNF on CYP1A1 protein expression

The CYP1A1 protein was constitutively expressed in both WHCO1 and WHCO6 cell lines. Treatment of these cells with BNF resulted in an increase in CYP1A1 protein expression that was significantly different (Appendix 8) from the controls. WHCO1 cells treated with BNF displayed a 1.3 fold increase in CYP1A1 protein expression compared to control cells (P=0.02) (Figure 4.2) while WHCO6 cells treated with BNF displayed a 1.1 fold increase in CYP1A1 protein expression compared to control cells (P=0.01).

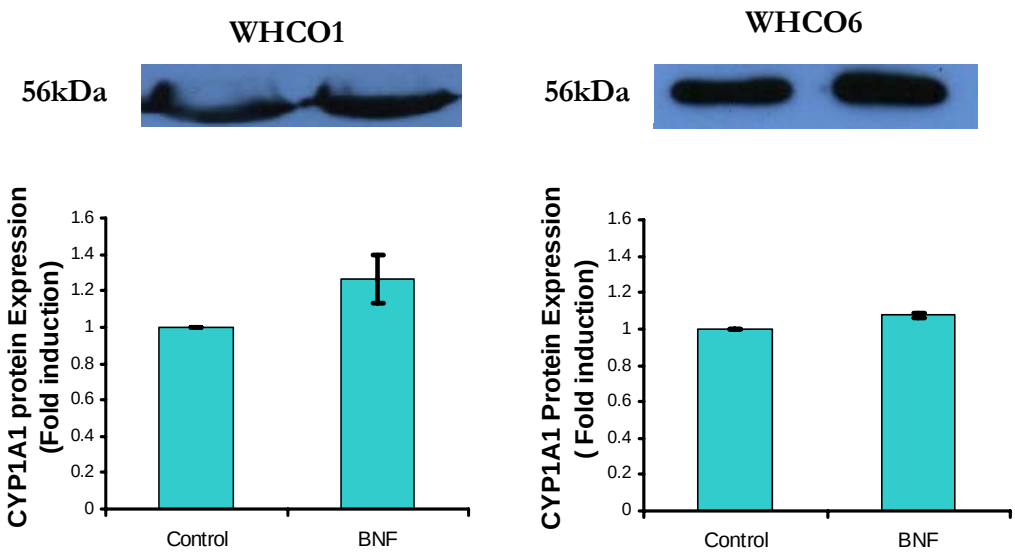


Figure 4.2 The effect of BNF on the expression of CYP1A1 protein in WHCO1 and WHCO6 cells. Representative Western blots are shown in this figure. WHCO1 and WHCO6 cells were exposed to 25μM BNF for 24 hours and 10μM BNF for 48 hours, respectively, while control cells were treated with 0.2% DMSO. 35μg of the whole cell protein extract of each sample was loaded in each lane. The transferred proteins were probed with rabbit anti-CYP1A1 antibody followed by (HRP) goat anti-rabbit antibody. The densitometric band intensities of Western blots are expressed as fold change relative to the control. Statistical differences were determined by using the T-test whereby $P < 0.05$ was considered significant.

4.3.2.2. The effect of BNF on CYP1A2 protein expression

Similar to the CYP1A1, CYP1A2 protein was also discovered to be constitutively expressed in WHCO1 and WHCO6 cells. The treatment of these cells with BNF displayed an increase in CYP1A2 protein expression as shown on figure 4.3. The change in CYP1A2 protein expression was

significantly increased to approximately 1.4 fold over control cells in both cell lines (P=0.01 in WHCO1, P=0.05 in WHCO6).

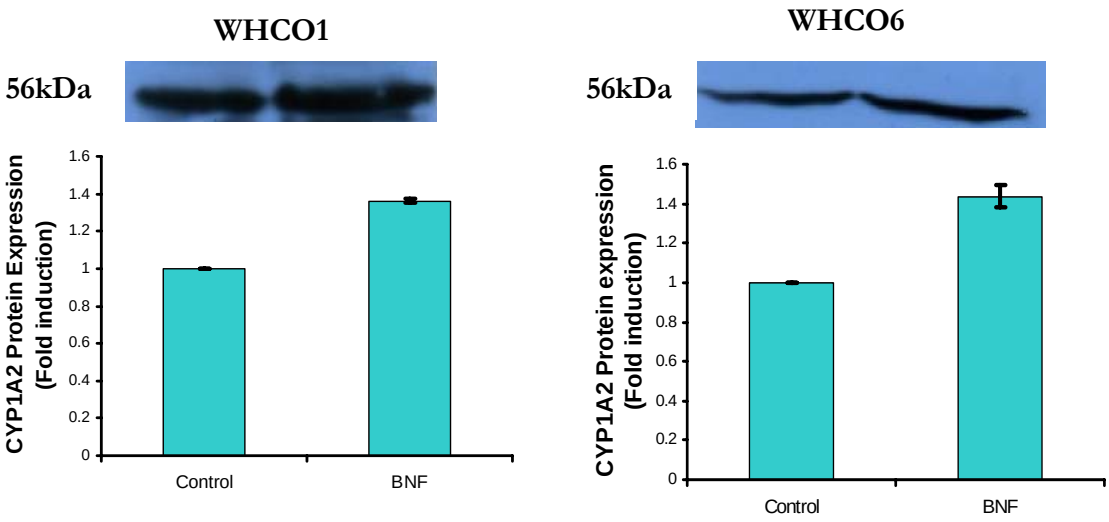


Figure 4.3 The effect of BNF on the expression of CYP1A2 protein in WHCO1 and WHCO6 cells. Representative Western blots are shown in this figure. WHCO1 and WHCO6 cells were exposed to 25µM BNF for 24 hours and 10µM BNF for 48 hours, respectively, while control cells were treated with 0.2% DMSO. 25µg of the whole cell protein extract of each sample was loaded in each lane. The transferred proteins were probed with rabbit anti-CYP1A2 antibody followed by (HRP) goat anti-rabbit antibody. The densitometric band intensities of Western blots are expressed as fold change relative to the control. Statistical differences were determined by using the T-test whereby $P < 0.05$ was considered significant.

4.3.2.3. The effect of BNF on CYP1B1 protein expression

Figure 4.4 shows the effect of BNF on the expression of CYP1B1 protein. The CYP1B1 constitutive expression was very low in both cell lines, however, significant inductions in CYP1B1 protein expression were observed after BNF

treatment. WHCO1 cells treated with BNF showed a 2.4 fold increase in CYP1B1 protein expression compared to control cells (P=0.003) (Figure 4.4) while WHCO6 cells treated with BNF displayed a 1.7 fold increase in CYP1B1 protein expression compared to control cells (P=0.05) (Figure 4.4).

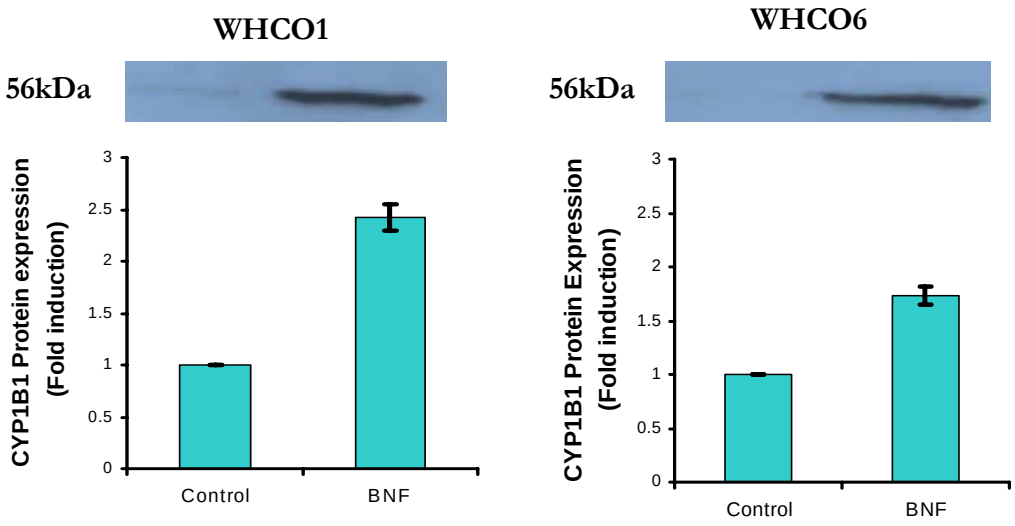


Figure 4.4 The effect of BNF on the expression of CYP1B1 protein in WHCO1 and WHCO6 cells. Representative Western blots are shown in this figure. WHCO1 and WHCO6 cells were exposed to 25µM BNF for 24 hours and 10µM BNF for 48 hours, respectively, while control cells were treated with 0.2% DMSO. 15µg of the whole cell protein extract of each sample was loaded in each lane. The transferred proteins were probed with rabbit anti-CYP1B1 antibody followed by (HRP) goat anti-rabbit antibody. The densitometric band intensities of Western blots are expressed as fold change relative to the control. Statistical differences were determined by using the T-test whereby $P < 0.05$ was considered significant.

4.3.2.4. The effect of BNF on AHR protein expression

Treatment of WHCO1 and WHCO6 cells with BNF caused a down regulation in AHR protein expression that was significantly different (Appendix 8) from the controls. WHCO1 cells treated with BNF caused a 1.3 fold decrease in AHR protein expression compared to the control cells (P=0.003) (Figure 4.5). With regard to the WHCO6 cells, BNF caused a 4.5 fold decrease in AHR protein expression compared to the control cells (P=0.004) (Figure 4.5).

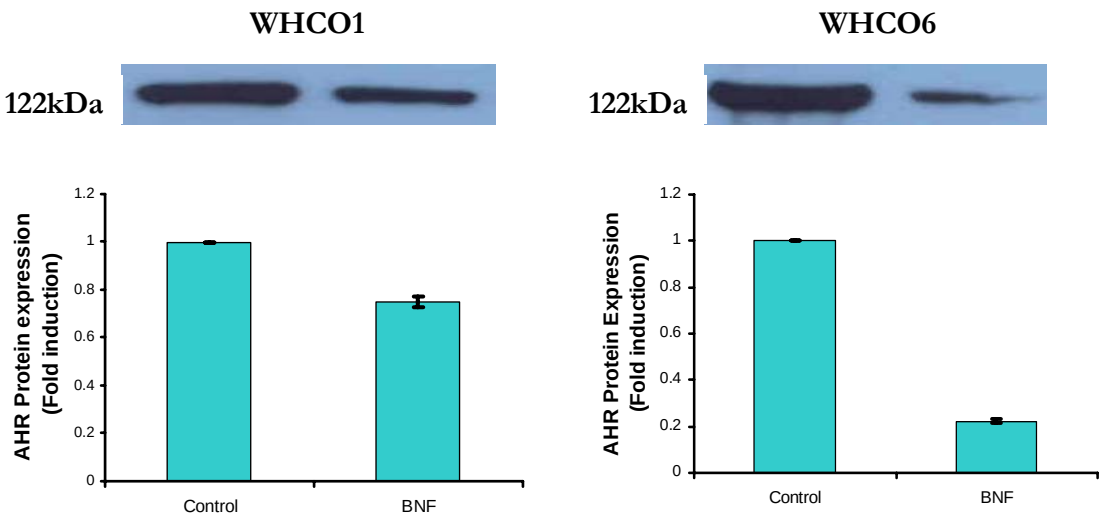


Figure 4.5: The effect of BNF on the expression of AHR protein in WHCO1 and WHCO6 cells. Representative Western blots are shown in this figure. WHCO1 and WHCO6 cells were exposed to 25µM BNF for 24 hours and 10µM BNF for 48 hours, respectively, while control cells were treated with 0.2% DMSO. 10µg of the whole cell protein extract of each sample was loaded in each lane. The transferred proteins were probed with rabbit anti-AHR antibody followed by (HRP) goat anti-rabbit antibody. The densitometric band intensities of Western blots are expressed as fold change relative to the control. Statistical differences were determined by using the T-test whereby $P < 0.05$ was considered significant.

4.3.3. BNF induces the expression of CYP1B1 protein to significantly higher levels than CYP1A1 and CYP1A2 proteins in WHCO1 and WHCO6 cells

To compare the relationship amongst CYP1A1, CYP1A2 and CYP1B1 protein expression in response to BNF treatment in WHCO1 and WHCO6 cells, the ratios of CYP1B1 to CYP1A1 fold changes (CYP1B1 fold change/CYP1A1 fold change), CYP1B1 to CYP1A2 fold changes (CYP1B1 fold change/CYP1A2 fold change), and CYP1A2 to CYP1A1 fold changes (CYP1A2 fold change/CYP1A1 fold change) were calculated for each cell line (Table 4.1). Ratios that are greater than one, for example in terms of the CYP1B1 to CYP1A1 fold change, would mean that the expression of CYP1B1 was induced to a higher level than the expression of CYP1A1 protein (Table 4.1). Similar conclusions were made for the other protein ratio changes.

Table 4.1: Comparison of CYP1A1, CYP1A2 and CYP1B1 expression in BNF treated cells.

Cell line	Dose (μM))	Time (h)	CYP1B1/1A1 ratio	CYP1B1/1A2 ratio	CYP1A2/1A1 ratio
WHCO1	25	24	2.2	1.7	1.1
WHCO6	10	48	1.6	1.2	1.3

Ratios that are greater than one (1) show a higher level of induction

4.4. Discussion

4.4.1. BNF induces the expression of CYP1 enzymes

Flavonoids are a large class of phenolic compounds present in fruits, vegetables and other plant foods and have been shown to reduce the risk of cancer and other major chronic diseases. They show their effects by either delaying or reversing the process of carcinogenesis. At the first entry site, procarcinogens are metabolized in phase I reactions catalyzed by CYP enzymes (Conney, 2003; Guengerich, 2004; Caro and Cederbaum, 2004; Coon, 2005; Kim and Guengerich, 2005) and are then conjugated with water soluble endogenous metabolites by phase II enzymes, thus producing hydrophilic products that can be easily excreted (Burchell, 2003; Kuuranne *et al*, 2003). However, phase I products are not always conjugated by phase II enzymes since the phase I products are often highly reactive metabolites capable of causing toxicity (Guengerich, 2000; Schwarz *et al*, 2001).

Flavonoids have been shown to interact with CYP enzymes in at least three ways: they induce the biosynthesis of several CYPs, they modulate (inhibit or stimulate) the activities of CYP enzymes and flavonoids are also metabolized by several CYPs (Hodek *et al*, 2002). Some of the beneficial properties of flavonoids include inhibition of CYPs involved in carcinogen activation and scavenging of reactive radicals formed from carcinogens by CYP-mediated reactions (Chan *et al*, 1998; Zhai *et al*, 1998; Doostdar *et al*, 2000; Henderson

et al, 2000). Flavonoids can also prevent the process of carcinogenesis by induction of phase II enzymes as well.

In the current study, the inducible expression of the enzymes (CYP1A1, CYP1A2 and CYP1B1) in response to treatment of esophageal cancer cells, WHCO1 and WHCO6 by the synthetic flavonoid BNF was investigated. An induction of CYP1A1, CYP1A2 and CYP1B1 by BNF is reported in this study. This flavonoid was shown to induce CYP1A1 by 1, 3 and 1, 1 fold in WHCO1 and WHCO6 cells, respectively. It was also shown to induce CYP1A2 by 1, 4 fold in both cell lines. In addition, this flavonoid also induced CYP1B1 by 2.4 and 1, 7-fold induction in WHCO1 and WHCO6 cells, respectively.

Although CYP enzymes generally convert xenobiotics to less toxic compounds, the reactions frequently involve the formation of reactive intermediates capable of causing toxicity. Elevation of activities of CYP1 family of enzymes (CYP1A1, CYP1A2 and CYP1B1) is highly unwanted since these enzymes are responsible for activation of carcinogens such as BP, 7, 12-dimethylbenz (a) anthracene (DMBA), Aflatoxin B_1 and meat derived heterocyclic aromatic amines (Omiecinski *et al*, 1999). CYP1 induction can also cause an imbalance between phase I and phase II reactions, for example when CYP1 enzymes are more active than phase II enzymes, this can cause an accumulation of phase I products capable of causing toxicity (Guengerich, 2000; Schwarz *et al*, 2001). CYP1 enzymes have broad substrate specificities

therefore induction of these enzymes may result in alteration of pharmaceutical drugs thus causing an overdose or loss of their therapeutic effect (Tang and Stearns, 2001; Rochat *et al*, 2001; Mcfadyen *et al*, 2001; Bournique and Lemarie, 2002).

The induction of CYP1 enzymes is recognized as a factor in determining the risk of the development of cancer. Increased expression of CYP1A1 in the lungs increases the risk of lung cancer (McLemore *et al*, 1990; Guengerich, 1988) as well as colorectal cancer (Sivaraman *et al*, 1994). CYP1A2 also has a role in the development of cancers associated with tobacco smoking. Differential expression of the CYP1 enzymes was observed in WHCO1 and WHCO6 cells, with CYP1B1 induction being the highest compared to the other enzymes after BNF exposure. CYP1A1 was the second highly induced enzyme while CYP1A2 was induced the least after BNF exposure. These results are in agreement with previous results that showed CYP1B1 enzyme to be more active than CYP1A enzymes in metabolizing several compounds to reactive intermediates (Shimada *et al*, 1996; Li *et al*, 1998; Yengi *et al*, 2003; Wen and Walle, 2005; Walle *et al*, 2006). CYP1B1 induction can thus be used as a target in cancer therapy and prevention, especially since this enzyme has been shown to be up-regulated in a wide variety of cancers (Murray *et al*, 1997; Ko *et al*, 2001; Murray *et al*, 2001; McFadyen *et al*, 2001; Tanaka *et al*, 2002; Chang *et al*, 2003; Carnell *et al*, 2004; Wen and Walle, 2005; Walle *et al*, 2006).

When comparing the ratio of protein level in both WHCO1 and WHCO6 cells, the induction of the CYP1 enzymes was higher in WHCO1 than in WHCO6 cells. These results therefore suggest that the ability of BNF in increasing the CYP1 protein level differs with different cell lines. One reason for the variation in the ratio of protein level in both cell lines could be due to CYP1 polymorphisms, especially since these cell lines originate from different individuals. Genetic polymorphisms of CYP1A1 enzymes have been linked with a higher risk for the development of a wide range of cancers (San Jose *et al*, 2010; Jin *et al*, 2011). Several studies have also reported the association of CYP1A2 with an increased risk of a wide range of cancers (Landi *et al*, 1999; Williams *et al*, 2000). In addition to the above studies, the up-regulation of CYP1B1 has also been shown to be associated with various cancer types (Watanabe, 2000;Ko *et al*, 2001; Goodman *et al*, 2001; Tanaka *et al*, 2002 ; Gattás *et al*, 2006; Varela-Lema, 2008; Ozbek *et al*, 2010). The results presented in this study provide more evidence about the potential of the CYP1 enzymes as targets in cancer therapy and prevention.

4.4.2. BNF down-regulates the expression of AHR

The AHR is a ligand activated basic helix-loop-helix (bHLH) protein that belongs to the Per-Arnt-Sim (PAS) family of transcription factors (Schimidt and Bradfied, 1996). In this study, BNF, which is one of the most potent AHR ligands, was used to investigate the effect it had on the AHR protein expression in esophageal cancer cells, WHCO1 and WHCO6 cells. The results showed a

1.3 and 4.5 fold down-regulation of AHR in WHCO1 and WHCO6, respectively, in the presence of the ligand BNF. These results are in agreement with previous studies that showed a down-regulation of AHR protein upon ligand exposure both *in vivo* and *in vitro* (Prokipcak and Okey, 1991; Swanson and Perdew, 1993; Giannone *et al*, 1995; Pollenz, 1996; Giannone *et al*, 1998; Pollenz *et al*, 1998; Roman *et al*, 1998; Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Wormke *et al*, 2000; Santiago-Josefat *et al*, 2001).

Previous studies have also shown that the mechanism of AHR downregulation upon ligand binding involves nuclear export of the AHR, ubiquination followed by protein degradation by the 26S proteasome (Pollenz, 1996; Pollenz *et al*, 1998; Giannone *et al*, 1998; Pollenz and Barbadour, 2000; Ma and Baldwin, 2000; Pollenz, 2002), which thus cause the reduction of the protein available. This does not occur in control cells, since in control cells, the AHR protein remains inactive and localized in the cytosol where it is not at risk of being ubiquinated and degraded via the 26S proteasome.

Another reason for the AHR downregulation in BNF treated cells compared to control cells could involve the AHRR, where the liganded AHR in a heterodimer with ARNT leads to the activation of the AHRR protein, while the up-regulated AHRR, in turn, reduces the expression of AHR (Mimura *et al*, 1999).

4.5. Conclusion

In general, the induction of CYP1 enzymes is highly unwanted, since these enzymes have been shown to convert xenobiotics to reactive and carcinogenic metabolites. According to the results obtained, BNF was shown to be a CYP1 inducing compound. BNF may therefore be considered as harmful since exposure to it could lead to an increase in the rate of metabolism of other carcinogenic compounds. The results also showed the differential induction of the CYP1 protein levels after BNF exposure, which suggests the presence of polymorphisms of these enzymes. Together, these results, implicate CYP1 enzymes, especially the CYP1B1, as potential therapeutic targets for cancer prevention.

CHAPTER 5: General discussion

In South Africa, esophageal cancer is the most prevalent cause of cancer related deaths in black males (Sitas, 1992). Although different factors can play a role in the development of this disease, reports have shown that alcohol consumption and tobacco smoking are the main risk factors for esophageal cancer (Montesano *et al*, 1996, Castellsaqũe *et al*, 1999). The development of esophageal cancer is asymptomatic, thus resulting in late diagnosis and poor prognosis. Success in treatment has been limited in terms of esophageal cancer management; therefore, surgery is still the major intervention when the disease is detected relatively early. Therefore, early detection and search for potential anticancer compounds are important in the control of esophageal cancer.

Flavonoids, a large class of phenolic compounds present in plants, have been shown to influence a wide range of biological functions, including the inhibition of tumor growth and the prevention of cancer (Harborne *et al*, 1999). Various natural flavonoids were also shown previously to have antiproliferative activities against several human cancer cell lines (HeLa, MCF7, SK-Mel-28 and KB) (Cárdenas *et al*, 2006). Considering the antiproliferative activities of some flavonoids on certain cancer cells and due to a lack of such information on esophageal cancer cells, in the current study the antiproliferative activity of a synthetic flavonoid, ß-

Naphthofglavone (BNF), on esophageal cancer cells WHCO1 and WHCO6 was evaluated. BNF showed a moderate activity in WHCO6 cells (IC_{50} ~ 10μM) and a weak activity in WHCO1 cells (IC_{50} ~ 25μM).Viewed generally, the results show that the growth inhibitory activity of BNF against the two cell lines is not always the same, thus indicating differences in the sensitivity of cancer cells to BNF. The number and position of hydroxyl groups and double bonds are the determining factors for flavonoid antiproliferative activities (Kozikowsk *et al*, 2003). Flavonoids with 4-6 hydroxyl groups have been shown to have strong antiproliferative activities, whereas those with more or fewer hydroxyl groups show low or no antiproliferative activities (Rice-Evans *et al*, 1995; Rice-Evans *et al*, 1996; Rice-Evans, 2001). One of the reasons that BNF did not show the greatest effect on the proliferation of the WHCO1 and WHCO6 cells could be due to its lack of hydroxyl groups. However, the current results still demonstrate that even though the antiproliferative activity of BNF was not outstanding, esophageal cancer cells may still be responsive to the treatment with this flavonoid.

Flavonoids contribute to the prevention of cancer through several mechanisms. They may act in different stages of the development of cancer by scavenging free radicals, thus protecting the DNA from oxidative damage (Ferguson, 2001). Flavonoids have been shown to inactivate carcinogens by inhibiting the expression of mutagenic genes.

They have also been shown to inactivate enzymes responsible for the activation of procarcinogens and activate the ones responsible for the detoxification of xenobiotics (Bravo *et al*, 1998).

Among the proteins that interact with flavonoids, CYP monooxygenase metabolizing xenobiotics and endogenous substrates play a major role (Hodek *et al*, 2002). The CYP inhibitory capacity of flavonoids has been widely studied due to their potential use as agents blocking carcinogenesis (Chan *et al*, 1998; Zhai *et al*, 1998; Doostdar *et al*, 2000; Henderson *et al*, 2000). Certain flavonoids alter the expression of CYP1 enzymes thus inhibiting the activation of procarcinogens to carcinogens and ultimately to a reduction of DNA adduct formation. Reports have shown that flavonoids possessing hydroxyl groups are usually associated with an inhibition of CYP enzyme activity, whereas those lacking hydroxyl groups often stimulate CYP enzyme activity. BNF, which does not possess any hydroxyl groups, and was found to significantly induce the expression of CYP1A1, CYP1A2 and CYP1B1 in both WHCO1 and WHCO6 cells.

CYP1A1 is one of the key enzymes in the activation of procarcinogens to carcinogens (Guengerich, 1992; Shimada *et al*, 1992; Guengerich, 1999; Minsavage *et al*, 2004; Schwartz *et al*, 2007). This enzyme was induced by 1.3 and 1.1 fold in WHCO1 and WHCO6 cells, respectively, upon exposure to BNF. The induction of CYP1A1 by BNF may result in an

increase in the capacity of CYP1A1 mediated carcinogen activation thus leading to a development of neoplastic diseases. On the other hand, the induction of this enzyme may mean that when exposed to potential carcinogens these carcinogens will be activated and thus be detoxified by phase II enzymes and readily eliminated from the body (Burchell, 2003; Kuuranne *et al*; 2003).

CYP1A2 enzymes are known for their oxidative metabolism of a wide range of therapeutic drugs (Yamazaki *et al*, 2001; Daniel *et al*, 2002; Llibre *et al*, 2002). BNF induced this enzyme by 1.4 fold in both cell lines. Since CYP1A2 metabolizes therapeutic drugs, the simultaneous administration of BNF and therapeutic drugs may cause an altered pharmacokinetics of the drugs thus resulting in either overdose or loss of their therapeutic effect (Tang and Stearns, 2001). Some people are slow drug metabolizers, therefore, the simultaneous administration of their therapeutic drugs and BNF may be beneficial in the activation and improvement of drug transportation in such people.

CYP1B1 is well known for the steroid metabolism to the carcinogenic metabolite 4-hydroxyesstradiol (4-OHE_2) (Badawi *et al*, 2001; Cavalieri and Rogan, 2004). This metabolite has been implicated in causing several types of cancer, including breast cancer (Mckay *et al*, 1995; Liehr and Ricci, 1996; Murray *et al*, 1997). BNF induced CYP1B1 by 2.4 and 1.7 in

WHCO1 and WHCO6 cells, respectively. Induction of CYP1B1 is a risk factor for breast cancer because it is a major enzyme for the carcinogenic estrogen metabolism.

A differential expression of the CYP1 proteins was observed in both WHCO1 and WHCO6 cell lines. One reason for the variation in the ratio of protein level in both cell lines could be due to CYP1 polymorphisms that may contribute to interindividual susceptibility to environmental carcinogens. Genetic polymorphisms of CYP1A1 enzymes have been linked with a higher risk for the development of a wide range of cancers. Several studies have also reported the association of CYP1A2 with an increased risk of a wide range of cancers (Landi *et al,* 1999; Williams *et al*, 2000). CYP1B1 was highly expressed compared to the CYP1A proteins. This could be because CYP1B1 has been shown to be expressed at high frequencies in various human cancers, including breast, colon, lung, esophagus, skin, lymph node, brain, and testicles but not in normal tissues (Murray *et al*, 1997; Watanabe, 2000; Ko *et al*, 2001; Tanaka *et al*, 2002; Gattás *et al*, 2006; Varela-Lema, 2008). Together, these findings implicate CYP1 enzymes, especially the CYP1B1, as potential therapeutic targets for esophageal cancer prevention.

Aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor which regulates CYP1 enzymes. Inhibiting the CYP1 family of enzymes

through blocking the AHR plays a critical role in the cancer chemopreventive properties of flavonoids. For instance, quercetin, one of the well known naturally occurring flavonoids, binds as an antagonist to AHR and consequently inhibits benzo(a)pyrene (BP) induced CYP1A1 mRNA transcription and protein expression and thus resulting in decreased BP-DNA adduct formation (Kang *et al*, 1999). In the present study, BNF did not show an induction but a down-regulation of the AHR in both cell lines. BNF down-regulated AHR by 1.3 and 4.5 fold in WHCO1 and WHCO6, respectively. The mechanism of AHR down-regulation upon ligand binding has been reported to involve nuclear export of the AHR, ubiquination, followed by protein degradation by the 26S proteasome (Pahl and Baeuerle, 1996; Tanaka, 1998; Davarinos and Pollenz, 1999; Ciechanover *et al*, 2000; Kornitzer and Ciechanover, 2000; Pollenz, 2002).

Conclusion and Future studies

Summarizing the above results, the agonist function of BNF seems not to be in line with its anticancer properties. However, BNF induction of the CYP1 enzymes may facilitate the removal of other compounds that are already active in the body. This flavonoid may prevent the process of carcinogenesis by other mechanisms such as the induction of both the phase I and phase II enzymes, thus the reactive intermediates that are formed as a result of metabolism of other compounds are removed from the body in a coordinated way. In future, it will be important to evaluate the effects of BNF on phase II enzymes such as the glutathione transferases, UDP glucuronosyltrallsferases and Nacetyl transferases, in order to find out how this flavonoid affects the detoxification pathway

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APPENDICES

Appendix 1

1.1. MTT cell viability assay

1.1.1. MTT labeling reagent

MTT (3-[4, 5-dimethyl thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide)

Dissolved in phosphate buffred saline to make a final concentration of 5mg/ml

1.1.2. MTT Solubilization solution

10g SDS

Dissolved in 100ML 0.01M HCL

Appendix 2

2.1. The effect of DMSO on WHCO1 cell viability (One-Way anova and Tukey posttest)

Table 1.1.B: One-Way ANOVA column statistics for DMSO treated WHCO1 cells

	Control	0.2 %V/V	0.5%V/V	1%V/V	2.5%V/V	5%V/V
Number of values	3	3	3	3	3	3
Minimum	0.612	0.528	0.512	0.424	0.337	0.067
25% Percentile	0.612	0.528	0.512	0.424	0.337	0.067
Median	0.612	0.548	0.513	0.465	0.346	0.067
75% Percentile	0.612	0.569	0.526	0.466	0.346	0.08
Maximum	0.612	0.569	0.526	0.466	0.346	0.08
Mean	0.612	0.5483	0.517	0.4517	0.343	0.07133
Std. Deviation	0	0.0205	0.00781	0.02397	0.0052	0.00751
Std. Error	0	0.01184	0.00451	0.01384	0.003	0.00433
Lower 95% CI	0.612	0.4974	0.4976	0.3921	0.3301	0.05269
Upper 95% CI	0.612	0.5993	0.5364	0.5112	0.3559	0.08998

Table 1.2: Repeated measures ANOVA for DMSO treated WHCO1 cells

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	503.6
R squared	0.996
Was the pairing significantly effective?	
R squared	0.000000772
F	0.0009757
P value	0.999
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.3: General ANOVA for DMSO treated WHCO1 cells

	SS	**df**	**MS**
Treatment (between columns)	0.5735	5	0.1147
Individual (between rows)	4.44E-07	2	2.20E-07
Residual (random)	0.002278	10	0.00023
Total	0.5757	17	

Table 1.4: Tukey's multiple comparison test showing the significant difference between the different DMSO concentrations used to treat WHCO1 cells

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs 0.2 %V/V	0.06367	7.307	Yes	**	0.02087 to 0.1065
Control vs 0.5%V/V	0.095	10.9	Yes	***	0.05220 to 0.1378
Control vs 1%V/V	0.1603	18.4	Yes	***	0.1175 to 0.2031
Control vs 2.5%V/V	0.269	30.87	Yes	***	0.2262 to 0.3118
Control vs 5%V/V	0.5407	62.05	Yes	***	0.4979 to 0.5835
0.2 %V/V vs 0.5%V/V	0.03133	3.596	No	ns	-0.01147 to 0.07413
0.2 %V/V vs 1%V/V	0.09667	11.09	Yes	***	0.05387 to 0.1395
0.2 %V/V vs 2.5%V/V	0.2053	23.57	Yes	***	0.1625 to 0.2481
0.2 %V/V vs 5%V/V	0.477	54.75	Yes	***	0.4342 to 0.5198
0.5%V/V vs 1%V/V	0.06533	7.498	Yes	**	0.02253 to 0.1081
0.5%V/V vs 2.5%V/V	0.174	19.97	Yes	***	0.1312 to 0.2168
0.5%V/V vs 5%V/V	0.4457	51.15	Yes	***	0.4029 to 0.4885
1%V/V vs 2.5%V/V	0.1087	12.47	Yes	***	0.06587 to 0.1515
1%V/V vs 5%V/V	0.3803	43.65	Yes	***	0.3375 to 0.4231
2.5%V/V vs 5%V/V	0.2717	31.18	Yes	***	0.2289 to 0.3145

Appendix 3

3.1 The effect of DMSO on WHCO6 cell viability (One-Way anova and Tukey posttest).

Table 1.5: One-way Anova column statistics for DMSO treated WHCO6 cells

	Control	0.2 %V/V	0.5%V/V	1%V/V	2.5%V/V	5%V/V
Number of values	3	3	3	3	3	3
Minimum	0.4007	0.387	0.355	0.326	0.287	0.158
25% Percentile	0.4007	0.387	0.355	0.326	0.287	0.158
Median	0.4007	0.391	0.365	0.334	0.296	0.159
75% Percentile	0.4007	0.398	0.377	0.335	0.297	0.166
Maximum	0.4007	0.398	0.377	0.335	0.297	0.166
Mean	0.4007	0.392	0.3657	0.3317	0.2933	0.161
Std. Deviation	0	0.005568	0.01102	0.00493	0.005508	0.00436
Std. Error	0	0.003215	0.00636	0.00285	0.00318	0.00252
Lower 95% CI	0.4007	0.3782	0.3383	0.3194	0.2797	0.1502
Upper 95% CI	0.4007	0.4058	0.393	0.3439	0.307	0.1718

Table 1.6: Repeated measures ANOVA for DMSO treated WHCO6 cells

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	689.2
R squared	0.9971
Was the pairing significantly effective?	
R squared	0.0008797
F	1.521
P value	0.265
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.7: General ANOVA for DMSO treated WHCO6 cells

	SS	**df**	**MS**
Treatment (between columns)	0.1194	5	0.02388
Individual (between rows)	0.0001054	2	0.00005272
Residual (random)	0.0003466	10	0.00003466
Total	0.1199	17	

Table 1.8: Tukey's multiple comparison test showing the significant difference between the different DMSO concentrations used to treat WHCO6 cells

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs 0.2 %V/V	0.008667	2.55	No	ns	-0.008028 to 0.02536
Control vs 0.5%V/V	0.035	10.3	Yes	***	0.01831 to 0.05170
Control vs 1%V/V	0.069	20.3	Yes	***	0.05231 to 0.08570
Control vs 2.5%V/V	0.1073	31.58	Yes	***	0.09064 to 0.1240
Control vs 5%V/V	0.2397	70.51	Yes	***	0.2230 to 0.2564
0.2 %V/V vs 0.5%V/V	0.02633	7.748	Yes	**	0.009638 to 0.04303
0.2 %V/V vs 1%V/V	0.06033	17.75	Yes	***	0.04364 to 0.07703
0.2 %V/V vs 2.5%V/V	0.09867	29.03	Yes	***	0.08197 to 0.1154
0.2 %V/V vs 5%V/V	0.231	67.96	Yes	***	0.2143 to 0.2477
0.5%V/V vs 1%V/V	0.034	10	Yes	***	0.01730 to 0.05069
0.5%V/V vs 2.5%V/V	0.07233	21.28	Yes	***	0.05564 to 0.08903
0.5%V/V vs 5%V/V	0.2047	60.22	Yes	***	0.1880 to 0.2214
1%V/V vs 2.5%V/V	0.03833	11.28	Yes	***	0.02164 to 0.05503
1%V/V vs 5%V/V	0.1707	50.21	Yes	***	0.1540 to 0.1874
2.5%V/V vs 5%V/V	0.1323	38.94	Yes	***	0.1156 to 0.1490

Appendix 4

4.1 The effect of BNF on WHCO1 cell proliferation (One-way anova and Tukey posttest)

Table 1.9: One-way ANOVA column statistics for WHCO1 cells treated with BNF for 24 hours

	Control	5μM	10μM	25μM	50μM	100μM
Number of values	3	3	3	3	3	3
Minimum	1.04	0.9387	0.8637	0.5657	0.5057	0.4557
25% Percentile	1.04	0.9387	0.8637	0.5657	0.5057	0.4557
Median	1.04	0.9917	0.9087	0.5667	0.5177	0.4617
75% Percentile	1.04	0.9917	0.9217	0.5687	0.5207	0.4837
Maximum	1.04	0.9917	0.9217	0.5687	0.5207	0.4837
Mean	1.04	0.974	0.898	0.567	0.5147	0.467
Std. Deviation	0	0.0306	0.03044	0.00153	0.00794	0.01474
Std. Error	0	0.01767	0.01757	0.00088	0.00458	0.00851
Lower 95% CI	1.04	0.898	0.8224	0.5632	0.4949	0.4304
Upper 95% CI	1.04	1.05	0.9736	0.5708	0.5344	0.5036

Table 1.10: Repeated measures ANOVA for WHCO1 cells treated with BNF for 24 hours

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	474.9
R squared	0.9958
Was the pairing significantly effective?	
R squared	0.0001873
F	0.2233
P value	0.8037
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.11: General ANOVA for WHCO1 cells treated with BNF for 24 hours

	SS	**df**	**MS**
Treatment (between columns)	0.9752	5	0.195
Individual (between rows)	0.00018	2	9.17E-05
Residual (random)	0.00411	10	0.000411
Total	0.9795	17	

Table 1.12: Tukey's multiple comparison test showing the significant difference between the different BNF concentrations used to treat WHCO1 cells after 24 hours

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs 5M	0.06633	5.669	Yes	*	0.008860 to 0.1238
Control vs 10M	0.1423	12.16	Yes	***	0.08486 to 0.1998
Control vs 25M	0.4733	40.45	Yes	***	0.4159 to 0.5308
Control vs 50M	0.5257	44.93	Yes	***	0.4682 to 0.5831
Control vs 100M	0.5733	49	Yes	***	0.5159 to 0.6308
5M vs 10M	0.076	6.495	Yes	**	0.01853 to 0.1335
5M vs 25M	0.407	34.78	Yes	***	0.3495 to 0.4645
5M vs 50M	0.4593	39.26	Yes	***	0.4019 to 0.5168
5M vs 100M	0.507	43.33	Yes	***	0.4495 to 0.5645
10M vs 25M	0.331	28.29	Yes	***	0.2735 to 0.3885
10M vs 50M	0.3833	32.76	Yes	***	0.3259 to 0.4408
10M vs 100M	0.431	36.84	Yes	***	0.3735 to 0.4885
25M vs 50M	0.05233	4.473	No	ns	-0.005140 to 0.1098
25M vs 100M	0.1	8.547	Yes	**	0.04253 to 0.1575
50M vs 100M	0.04767	4.074	No	ns	-0.009807 to 0.1051

Table 1.13: One-way ANOVA column statistics for WHCO1 cells treated with BNF for 48 hours

48H BNF	Control	5µM	10µM	25µM	50µM	100µM
Number of values	3	3	3	3	3	3
Minimum	1.608	1.469	1.392	0.97	0.9028	0.9503
25% Percentile	1.608	1.469	1.392	0.97	0.9028	0.9503
Median	1.608	1.543	1.414	0.971	0.9847	0.974
75% Percentile	1.608	1.578	1.46	0.9752	0.9954	0.98
Maximum	1.608	1.578	1.46	0.9752	0.9954	0.98
Mean	1.608	1.53	1.422	0.9721	0.961	0.9681
Std. Deviation	0	0.05592	0.03508	0.00276	0.05063	0.01572
Std. Error	0	0.03228	0.02025	0.00159	0.02923	0.00908
Lower 95% CI	1.608	1.391	1.335	0.9652	0.8352	0.929
Upper 95% CI	1.608	1.669	1.509	0.9789	1.087	1.007

Table 1.14: Repeated measures ANOVA statistics for WHCO1 cells treated with BNF for 48 hours

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	288.9
R squared	0.9931
Was the pairing significantly effective?	
R squared	0.003087
F	2.252
P value	0.1558
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.15: General ANOVA statistics for WHCO1 cells treated with BNF for 48 hours

	SS	df	MS
Treatment (between columns)	1.429	5	0.2858
Individual (between rows)	0.004456	2	0.002228
Residual (random)	0.009895	10	0.0009895
Total	1.443	17	

Table 1.16: Tukey's multiple comparison test showing the significant difference between the different BNF concentrations used to treat WHCO1 cells after 48 hours

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs 5M	0.07845	4.319	No	ns	-0.01076 to 0.1677
Control vs 10M	0.1863	10.26	Yes	***	0.09705 to 0.2755
Control vs 25M	0.6363	35.03	Yes	***	0.5471 to 0.7255
Control vs 50M	0.6474	35.65	Yes	***	0.5582 to 0.7366
Control vs 100M	0.6402	35.25	Yes	***	0.5510 to 0.7294
5M vs 10M	0.1078	5.937	Yes	*	0.01861 to 0.1970
5M vs 25M	0.5578	30.72	Yes	***	0.4686 to 0.6470
5M vs 50M	0.5689	31.33	Yes	***	0.4797 to 0.6581
5M vs 100M	0.5618	30.93	Yes	***	0.4726 to 0.6510
10M vs 25M	0.45	24.78	Yes	***	0.3608 to 0.5392
10M vs 50M	0.4611	25.39	Yes	***	0.3719 to 0.5503
10M vs 100M	0.454	25	Yes	***	0.3648 to 0.5432
25M vs 50M	0.0111	0.611	No	ns	-0.07811 to 0.1003
25M vs 100M	0.003957	0.218	No	ns	-0.08525 to 0.09316
50M vs 100M	-0.007144	0.393	No	ns	-0.09635 to 0.08206

Table 1.17: One-way ANOVA column statistics for WHCO1 cells treated with BNF for 72 hours

72H BNF	Control	5µM	10µM	25µM	50µM	100µM
Number of values	3	3	3	3	3	3
Minimum	1.668	1.664	1.676	1.264	1.181	1.135
25% Percentile	1.668	1.664	1.676	1.264	1.181	1.135
Median	1.668	1.715	1.676	1.264	1.231	1.161
75% Percentile	1.668	1.715	1.726	1.278	1.246	1.173
Maximum	1.668	1.715	1.726	1.278	1.246	1.173
Mean	1.668	1.698	1.692	1.268	1.219	1.156
Std. Deviation	0	0.02944	0.02887	0.00808	0.03403	0.01943
Std. Error	0	0.017	0.01667	0.00467	0.01965	0.01122
Lower 95% CI	1.668	1.625	1.621	1.248	1.134	1.108
Upper 95% CI	1.668	1.771	1.764	1.288	1.304	1.204

Table 1.18: Repeated measures ANOVA for WHCO1 cells treated with BNF for 72 hours

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	327.6
R squared	0.9939
Was the pairing significantly effective?	
R squared	0.0003594
F	0.2963
P value	0.7499
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.19: General ANOVA for WHCO1 cells treated with BNF for 72 hours

	SS	df	MS
Treatment (between columns)	1.021	5	0.2042
Individual (between rows)	0.0003693	2	0.0001847
Residual (random)	0.006233	10	0.0006233
Total	1.028	17	

Table 1.20: Tukey's multiple comparison test showing the significant difference between the different BNF concentrations used treat WHCO1 cells for 72 hours

	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs 5M	-0.02967	2.058	No	ns	-0.1005 to 0.04114
Control vs 10M	-0.02433	1.688	No	ns	-0.09514 to 0.04647
Control vs 25M	0.3997	27.73	Yes	***	0.3289 to 0.4705
Control vs 50M	0.449	31.15	Yes	***	0.3782 to 0.5198
Control vs 100M	0.512	35.52	Yes	***	0.4412 to 0.5828
5M vs 10M	0.005333	0.37	No	ns	-0.06547 to 0.07614
5M vs 25M	0.4293	29.78	Yes	***	0.3585 to 0.5001
5M vs 50M	0.4787	33.21	Yes	***	0.4079 to 0.5495
5M vs 100M	0.5417	37.58	Yes	***	0.4709 to 0.6125
10M vs 25M	0.424	29.41	Yes	***	0.3532 to 0.4948
10M vs 50M	0.4733	32.84	Yes	***	0.4025 to 0.5441
10M vs 100M	0.5363	37.21	Yes	***	0.4655 to 0.6071
25M vs 50M	0.04933	3.422	No	ns	-0.02147 to 0.1201
25M vs 100M	0.1123	7.793	Yes	**	0.04153 to 0.1831
50M vs 100M	0.063	4.371	No	ns	-0.007804 to 0.1338

Appendix 5

5.1 The effect of BNF on WHCO6 cell proliferation (One-way anova and Tukey posttest)

Table 1.21: One-way ANOVA column statistics for WHCO6 cells treated with BNF for 24 hours

	Control	5µM	10µM	25µM	50µM	100µM
Number of values	3	3	3	3	3	3
Minimum	0.7955	0.7305	0.6435	0.5275	0.5015	0.5155
25% Percentile	0.7955	0.7305	0.6435	0.5275	0.5015	0.5155
Median	0.7955	0.7375	0.6475	0.5305	0.5045	0.5175
75% Percentile	0.7955	0.7425	0.648	0.5415	0.5175	0.5215
Maximum	0.7955	0.7425	0.648	0.5415	0.5175	0.5215
Mean	0.7955	0.7368	0.6463	0.5332	0.5078	0.5182
Std. Deviation	0	0.00603	0.00247	0.00737	0.00851	0.00306
Std. Error	0	0.00348	0.00143	0.00426	0.00491	0.00176
Lower 95% CI	0.7955	0.7219	0.6402	0.5149	0.4867	0.5106
Upper 95% CI	0.7955	0.7518	0.6525	0.5515	0.529	0.5258

Table 1.22: Repeated measures ANOVA for WHCO6 cells treated with BNF for 24 hours

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	1441
R squared	0.9986
Was the pairing significantly effective?	
R squared	0.00019
F	0.6711
P value	0.5327
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.23: General ANOVA for WHCO6 cells treated with BNF for 24 hours

ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	0.2267	5	0.04535
Individual (between rows)	4.2E-05	2	2.1E-05
Residual (random)	0.00031	10	3.1E-05
Total	0.2271	17	

Table 1.24: Tukey's multiple comparison test showing the significant difference between the different BNF concentrations used to treat WHCO6 cells for 24 hours

	Mean Diff.	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs 5M	0.05867	18.12	Yes	***	0.04276 to 0.07457
Control vs 10M	0.1492	46.06	Yes	***	0.1333 to 0.1651
Control vs 25M	0.2623	81.01	Yes	***	0.2464 to 0.2782
Control vs 50M	0.2877	88.83	Yes	***	0.2718 to 0.3036
Control vs 100M	0.2773	85.64	Yes	***	0.2614 to 0.2932
5M vs 10M	0.0905	27.95	Yes	***	0.07459 to 0.1064
5M vs 25M	0.2037	62.89	Yes	***	0.1878 to 0.2196
5M vs 50M	0.229	70.71	Yes	***	0.2131 to 0.2449
5M vs 100M	0.2187	67.52	Yes	***	0.2028 to 0.2346
10M vs 25M	0.1132	34.95	Yes	***	0.09726 to 0.1291
10M vs 50M	0.1385	42.77	Yes	***	0.1226 to 0.1544
10M vs 100M	0.1282	39.58	Yes	***	0.1123 to 0.1441
25M vs 50M	0.02533	7.823	Yes	**	0.009426 to 0.04124
25M vs 100M	0.015	4.623	No	ns	-0.0009070 to 0.03091
50M vs 100M	-0.01033	3.191	No	ns	-0.02624 to 0.005574

Table 1.25: One-way ANOVA column statistics for WHCO6 cells treated with BNF for 48 hours

	Control	**5µM**	**10µM**	**25µM**	**50µM**	**100µM**
Number of values	3	3	3	3	3	3
Minimum	0.9363	0.8732	0.5039	0.4511	0.4068	0.3836
25% Percentile	0.9363	0.8732	0.5039	0.4511	0.4068	0.3836
Median	0.9363	0.8832	0.5086	0.4562	0.4349	0.3963
75% Percentile	0.9363	0.8845	0.509	0.4613	0.4559	0.3998
Maximum	0.9363	0.8845	0.509	0.4613	0.4559	0.3998
Mean	0.9363	0.8803	0.5072	0.4562	0.4325	0.3932
Std. Deviation	0	0.00617	0.00285	0.00511	0.02463	0.0085
Std. Error	0	0.00357	0.00165	0.00295	0.01422	0.00491
Lower 95% CI	0.9363	0.865	0.5001	0.4435	0.3713	0.3721
Upper 95% CI	0.9363	0.8956	0.5143	0.4689	0.4937	0.4143

Table 1.26: Repeated measures ANOVA for WHCO6 cells treated with BNF for 48 hours

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	1617
R squared	0.9988
Was the pairing significantly effective?	
R squared	0.000479
F	1.938
P value	0.1945
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.27: General ANOVA for WHCO6 cells treated with BNF for 48 hours

	SS	df	MS
Treatment (between columns)	0.8754	5	0.1751
Individual (between rows)	0.00042	2	0.00021
Residual (random)	0.00108	10	0.00011
Total	0.8769	17	

Table 1.28: Tukey's multiple comparison test showing the significant difference between the different BNF concentrations used to treat WHCO6 cells for 48 hours

	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs 5M	0.05603	9.326	Yes	***	0.02652 to 0.08554
Control vs 10M	0.4292	71.43	Yes	***	0.3996 to 0.4587
Control vs 25M	0.4802	79.92	Yes	***	0.4506 to 0.5097
Control vs 50M	0.5038	83.86	Yes	***	0.4743 to 0.5333
Control vs 100M	0.5431	90.39	Yes	***	0.5136 to 0.5726
5M vs 10M	0.3731	62.1	Yes	***	0.3436 to 0.4026
5M vs 25M	0.4241	70.59	Yes	***	0.3946 to 0.4536
5M vs 50M	0.4478	74.53	Yes	***	0.4183 to 0.4773
5M vs 100M	0.4871	81.07	Yes	***	0.4576 to 0.5166
10M vs 25M	0.05099	8.487	Yes	**	0.02148 to 0.08050
10M vs 50M	0.07466	12.43	Yes	***	0.04515 to 0.1042
10M vs 100M	0.1139	18.96	Yes	***	0.08443 to 0.1435
25M vs 50M	0.02367	3.94	No	ns	-0.005842 to 0.05318
25M vs 100M	0.06295	10.48	Yes	***	0.03344 to 0.09247
50M vs 100M	0.03928	6.538	Yes	**	0.009772 to 0.06880

Table 1.29: One-way ANOVA column statistics for WHCO6 cells treated with BNF for 72 hours

72h BNF	**Control**	**5µM**	**10µM**	**25µM**	**50µM**	**100µM**
Number of values	3	3	3	3	3	3
Minimum	1.135	1	0.637	0.423	0.375	0.314
25% Percentile	1.135	1	0.637	0.423	0.375	0.314
Median	1.135	1.025	0.688	0.446	0.377	0.332
75% Percentile	1.135	1.035	0.688	0.478	0.394	0.34
Maximum	1.135	1.035	0.688	0.478	0.394	0.34
Mean	1.135	1.02	0.671	0.449	0.382	0.3287
Std. Deviation	0	0.01803	0.02944	0.02762	0.01044	0.01332
Std. Error	0	0.01041	0.017	0.01595	0.006028	0.007688
Lower 95% CI	1.135	0.9752	0.5979	0.3804	0.3561	0.2956
Upper 95% CI	1.135	1.065	0.7441	0.5176	0.4079	0.3617

Table 1.30: Repeated measures ANOVA for WHCO6 cells treated with BNF for 72 hours

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	6
F	945.8
R squared	0.9979
Was the pairing significantly effective?	
R squared	0.000432
F	1.024
P value	0.3939
P value summary	ns
Is there significant matching? (P < 0.05)	No

Table 1.31: General ANOVA for WHCO6 cells treated with BNF for 72 hours

	SS	**df**	**MS**
Treatment (between columns)	1.76	5	0.3519
Individual (between rows)	0.000762	2	0.000381
Residual (random)	0.003721	10	0.000372
Total	1.764	17	

Table 1.32: Tukey's multiple comparison test showing the significant difference between the different BNF concentrations used to treat WHCO6 cells for 72 hours

Tukey's Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Control vs 5M	0.1147	10.3	Yes	***	0.05997 to 0.1694
Control vs 10M	0.4637	41.64	Yes	***	0.4090 to 0.5184
Control vs 25M	0.6857	61.57	Yes	***	0.6310 to 0.7404
Control vs 50M	0.7527	67.59	Yes	***	0.6980 to 0.8074
Control vs 100M	0.806	72.38	Yes	***	0.7513 to 0.8607
5M vs 10M	0.349	31.34	Yes	***	0.2943 to 0.4037
5M vs 25M	0.571	51.27	Yes	***	0.5163 to 0.6257
5M vs 50M	0.638	57.29	Yes	***	0.5833 to 0.6927
5M vs 100M	0.6913	62.08	Yes	***	0.6366 to 0.7460
10M vs 25M	0.222	19.93	Yes	***	0.1673 to 0.2767
10M vs 50M	0.289	25.95	Yes	***	0.2343 to 0.3437
10M vs 100M	0.3423	30.74	Yes	***	0.2876 to 0.3970
25M vs 50M	0.067	6.016	Yes	*	0.01230 to 0.1217
25M vs 100M	0.1203	10.81	Yes	***	0.06563 to 0.1750
50M vs 100M	0.05333	4.789	No	ns	-0.001368 to 0.1080

Appendix 6

6.1 The median inhibition concentration (IC_{50}) values

Table 1.33: The median inhibition concentration (IC_{50}) values of BNF in WHCO1 cells

BNF CONC	Absorbance values			Percentage			Average	IC_{50}	STDEV
0µM	1.040334	1.040334	1.040334	100	100	100	100		0
5µM	0.938667	0.991667	0.991667	90.22747	95.32198	95.32198	93.62381		2.941321
10µM	0.921667	0.863667	0.908667	88.59337	83.01824	87.34378	86.31846		2.925572
25µM	0.566667	0.568667	0.565667	54.46972	54.66196	54.3736	54.50176	**~ 25µM**	0.14683
50µM	0.505667	0.520667	0.517667	48.60622	50.04806	49.75969	49.47132		0.762952
100µM	0.461667	0.483667	0.455667	44.37681	46.49151	43.80007	45.43416		1.417067

Table 1.34: The median inhibition concentration (IC_{50}) values of BNF in WHCO6 cells

BNF CONC	Absorbance values				Percentage				Average	IC_{50}	STDEV
0µM	0.936333	0.936333	0.936333	0.93633	100	100	100	100	100		0
5µM	0.884473	0.873209	0.883224		94.46137	93.2584	94.328		94.01591		0.659422
10µM	0.508988	0.503887	0.489733	0.50864	54.35972	53.8149	52.3033	54.3231	53.70025	**~10µM**	0.9639
25µM	0.451072	0.456183	0.461294		48.17431	48.7202	49.266		48.72017		0.545853
50µM	0.45609	0.406796	0.434851	0.45589	48.71023	43.4457	46.4419	48.6891	46.82172		2.489687
100µM	0.430515	0.383627	0.396276	0.39978	45.97883	40.9712	42.3221	42.6967	42.99221		2.124516

Note: The one-way anova results for the other compounds are not shown.

Appendix 7

7.1. Protein extraction

Laemmli sample buffer

1.5% Tris.HCL (pH 6.8)
2% SDS
10% Glycerol
5% β-mercaptoethanol
Make up in dH_2O
Store at 4ºC

7.2 Protein estimation

Trichloroacetic acid (TCA)

7.5% TCA
Make up to the required volume with dH_2O

Coomassie Blue solution (0.25%)

0.25% Coomassie brilliant blue powder
50% Methanol
10% Glacial acetic acid
Make up to final volume with dH2O

Destaining solution

12% Glacial acetic acid
10% Methanol
Make up to final volume with dH2O

Elution solution

66% Methanol
33% dH2O
1% Ammonia

Estimation of the total protein content of cells

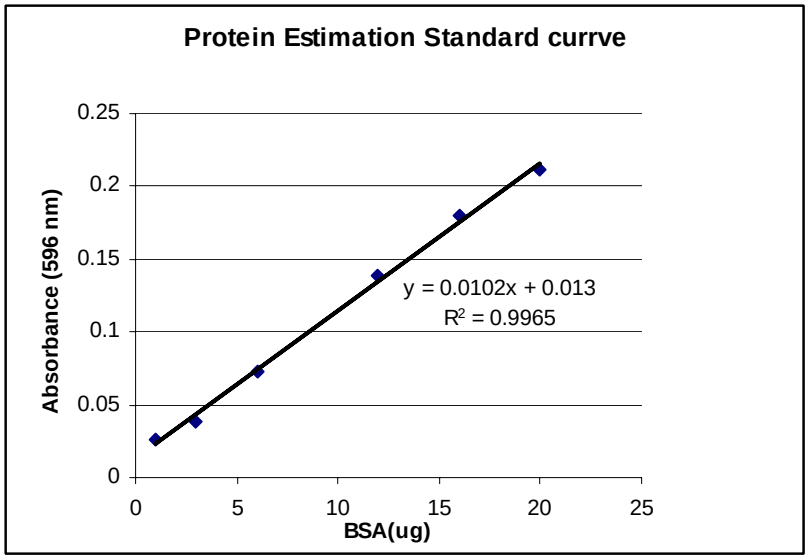


Figure 7.1: BSA standard curve for protein estimation. Shown is a plot of the absorbance values at 596nm versus BSA concentrations (1-20µg). The equation of the standard curve(y=0.0102x + 0.013) was used to calculate the unknown concentrations of the whole cell extracts. R^2 (Linear regression) =0.9965.

7.3 SDS-PAGE

Separating gel

10% Acrylamide
0.1% NN'-methylenebisacrylamide
375 mM Tris-HCl, pH 8.8
0.2% SDS
Make up to final volume with dH2O
Just before use add:
1mM Ammonium persulphate
0.25% N, N, N'N'-tetramethylenediamine (TEMED)

Stacking gel

10% Acrylamide
0.1% NN'-methylenebisacrylamide
125 mM Tris-HCl, pH 6.8
0.2% SDS
Make up to final volume with dH2O
Just before use add:
1mM Ammonium persulphate
0.25% TEMED

Running buffer

3.74 mM SDS
25 mM Tris-HCl, pH 8.3
192.5 mM Glycine
Make up to final volume with dH2O

Destain solution

10% Acetic acid
10% Methanol
Make up to final volume with dH2O

7.4 Western Blot

Transfer buffer

25 mM Tris-HCl, pH 8.3
1.41% Glycine
20% Methanol

Make up to final volume with dH2O (Store at4ºc)
Blocking solution

50 mM Tris-HCl, pH 7.8
2 mM Calcium chloride dihydrate
5% non-fat milk powder
0.01% anti-foam
0.05% Triton-X 100
Make up to final volume with dH2O
Store at 4ºC

Super signal west pico chemiluminescent substrate kit

Before use mix**:**
50% Luminol/Enhancer solution
50% Stable peroxide buffer
Store in the dark

Developer

6.4 M Metol
0.6 M Sodium sulphite (anhydrous)
80 mM Hydroquinine
0.45 mM Sodium carbonate (anhydrous)
34 mM Potassium bromide
Make up to final volume with dH2O
Store in the dark

Fixer

0.8 M Sodium trisulphate
0.2 M Sodium metasulphite
Make up to final volume with dH2O
Store in the dark

Appendix 8

8.1 T-test results for comparing protein expression level

Table 1.35: CYP1A1 fold induction

	WHCO1 BNF treatment			WHCO6 BNF treatment		
Fold induction	1.348312	1.327196		1.098469	1.075601	1.075601
Fold induction average	1.337754			1.083224		
STDEV	0.014931			0.013203		
P	0.019894			0.008285		

Table 1.36:CYP1A2 fold induction

	WHCO1 BNF treatment			WHCO6 BNF treatment		
Fold induction	1.352497	1.36784		1.476955	1.399223	
Fold induction average	1.360169			1.438089		
STDEV	0.010849			0.054965		
P	0.013558			0.056332		

Table 1.37:CYP1B1 fold induction

	WHCO1 BNF treatment			WHCO6 BNF treatment		
Fold induction	2.467483	2.276516	2.517369	1.676021	1.794708	
Fold induction average	2.420456			1.735364		
STDEV	0.127127			0.083925		
P	0.002659			0.051264		

Table 1.38: AHR fold induction

	WHCO1 BNF treatment			WHCO6 BNF treatment		
Fold induction	0.751155	0.722407	0.768886	0.22846	0.218438	
Fold induction average	0.747483			0.223449		
STDEV	0.023456			0.007087		
P	0.002864			0.004108		