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Modulation of gene expression and DNA adduct formation by chlorophyllin in human mammary cells exposed to benzopyrenes

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MODULATION OF GENE EXPRESSION AND DNA ADDUCT FORMATION BY CHLOROPHYLLIN IN HUMAN MAMMARY CELLS EXPOSED TO BENZOPYRENES

Kaarthik John

Dissertation submitted to the
Davis College of Agriculture and Forestry and Consumer Sciences at
West Virginia University in partial fulfillment of the requirements for
the degree of

**Doctor of Philosophy in Genetics and Developmental
Biology**

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ABSTRACT

Modulation of gene expression and DNA adduct formation by chlorophyllin in human mammary cells exposed to benzopyrenes

Kaarthik John

This study investigated metabolic activation by benzopyrenes (BP and B[e]P) in human mammary cells and modulation by chlorophyllin (CHL, a chemopreventive agent). Among 6 NHMEC strains monitored using microarrays, 54 genes were up-regulated and 11 down-regulated by signal log ratio (SLR) ≥ 1.5 on treatment with BP alone. Pre CHL + post BP+CHL treatment up-regulated the expression of 129 genes and down-regulated those of 35 genes by SLR ≥ 1.5 . Studies on *CYP1* gene induction and BP-DNA adduct formation among 20 NHMECs revealed wide inter-individual variations both in the induction (3-96-fold for *CYP1A1* and 4-43-fold for *CYP1B1*, respectively) and modulation (2-54-fold and 1-39-fold for *CYP1A1* and *CYP1B1*, respectively) of *CYP1* gene expression and reduction of BP-DNA adduct formation (0% to 86%) on treatment with BP $\pm$ CHL. B[e]P was a very poor inducer of *CYP1* gene expression and also exhibited no detectable adduct formation among the 2 NHMEC strains used, when compared to BP. There was a reduction of BP induced *CYP1A1* (0-40% among the 2 cell strains) and *CYP1B1* expression (5-50% among the 2 cell strains) across all CHL treatments except for pre CHL+ post BP treatment and pre CHL + post BP+CHL treatment in one of the cell strains. CHL enhanced B[e]P induced *CYP1* gene expression on treatment of cells with B[e]P + CHL and pre CHL + post B[e]P + CHL. When MCF-7 cells were compared to NHMECs, though basal *CYP1B1* expression was nearly 348 times that of *CYP1A1*, MCF-7 cells exhibited highly inducible *CYP1A1* expression (114 fold) compared to *CYP1B1* expression (5 fold). None of the different CHL treatments modulated *CYP1* gene expression or BP-DNA adduct formation in MCF-7 cells as opposed to NHMECs. When MCF-7 cells were compared to M00012, a NHMEC, across a range of CHL concentrations, only 3 μ M, 4 μ M and 16 μ M CHL mitigated *CYP1* expression to different extents. BP-DNA adduct levels were unaltered in MCF-7 cells but reduced in concentration dependent manner in M00012. These studies show the wide inter-individual variability in response to carcinogens and chemopreventive agents which are to be accounted for while designing intervention strategies.

Dedicated to my Parents and Sisters'

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LIST OF ABBREVIATIONS

4-OHE ₂	4 hydroxy estradiol
AhR	Aryl hydrocarbon receptor
ARNT	AhR nuclear translocator
ATCC	American Type Culture Collection
B[e]P	Benzo(e)pyrene
BP	Benzo(a)pyrene
BPDE	(±)-7β,8α, dihydroxy 9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene
BPdG	(7R)-N ² -(10[7b,8a, 9a-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]yl)-deoxyguanosine
BSA	Bovine serum albumin
cDNA	Copy DNA
CHL	Chlorophyllin
cRNA	Complementary RNA
CYP1A1	Cytochrome P4501A1
CYP1B1	Cytochrome P4501B1
CYP3A4	Cytochrome P4503A4
DMSO	Dimethyl sulfoxide
DMT	Data Mining Tool
DNA	Deoxyribose nucleic acid
FC	Fold change
GC	Gene chip
HCC	Hepatocarcinoma
HH	Halogenated hydrocarbon
IARC	International Agency for Research on Cancer
MAS	Microarray Suite
NHMEC	Normal human mammary epithelial cell

PAH	Polycyclic aromatic hydrocarbon
PBST	phosphate buffered saline containing 0.05% Tween-20
PKA	protein kinase A
RNA	Ribose nucleic acid
RT-PCR	Real time polymerase chain reaction
SAPE	streptavidin phycoerythroprotein
SC	Solvent control/control vehicle
SFS	synchronous fluorescence spectrometry
SLR	Signal log ratio

1.0 INTRODUCTION

Human exposures to various compounds such as polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons (HHs), aromatic amines (AAs), microbial toxins, drugs and other xenobiotic compounds occur through diet, occupation, medicines and/or environment. Benzo(a)pyrene (BP) is a PAH found in tobacco smoke, vehicle exhaust, indoor heating and various industrial processes, and it is a ubiquitous environmental pollutant and potent procarcinogen (IARC 1987; Osborne et al. 1987). On entering the body it can follow a number of routes some of which lead to its bioactivation or detoxication.

Metabolic activation of PAHs (e.g., BP) is necessary for them to exert their adverse biological and carcinogenic effects, and cytochrome P450 metabolism is a key factor in this process. Cytochrome P450s (CYPs) constitute a superfamily of heme thiolate proteins, that are likely the most important classes of enzymes involved in the metabolism of all xenobiotics (Guengerich 2001). Through their action, together with other enzymes (epoxide hydrolases) the bioactivation of BP proceeds, leading to the generation of highly reactive electrophiles capable of damaging cellular macromolecules such as DNA, RNA and protein (Weston et al. 2005). In addition, CYP450s have a normal role in hormone metabolism, and since BP acts to generally induce the activity of a range of CYP450s through activation of the aryl hydrocarbon receptor, BP and other PAHs may be a significant factor in hormonal carcinogenesis

BP, by acting as a ligand for the AhR, enters the cell and induces CYP genes (Oesch-Bartlomowicz et al. 2005). CYP1A1, CYP1B1 and CYP3A4 are the predominant CYP isoforms documented to be involved in the metabolism of BP. The predominant pathway of

metabolism involves the initial oxidation of BP by CYP1A1/CYP1B1 at the 7, 8 position followed by hydrolysis by epoxide hydrolase to dihydrodiols and then further oxidation at the 9, 10 olefinic bond to yield dihydrodiol epoxides, which being highly electrophilic bind covalently to DNA leading to genetic damage (Weston et al. 2005). CYP1A1 and CYP1B1 are also potent steroid hydroxylases with CYP1B1 being a potent estradiol 4 hydroxylase. 4-OHE₂ has been shown to possess a strong cell proliferative effect in various estrogen responsive tissues such as those of the breast and the endometrium and is thereby implicated in their carcinogenesis (Cavalieri et al. 2002).

Given the ubiquitous nature of BP, a complete avoidance of exposure may be difficult. Chemopreventive agents that block or even reverse the carcinogenic process may therefore be a useful alternative consideration. CHL, a water soluble sodium-copper or potassium-copper metalloporphyrin has been shown to exhibit anti-carcinogenic, anti-mutagenic, anti-tumorigenic and anti-oxidant properties in various studies and model systems (Sarkar et al. 1994; Tachino et al. 1994; Dashwood 1997a; Reddy et al. 1999; Kamat et al. 2000). It has also been used as a safe and effective intervention strategy in various developing parts of Africa and China with populations at high risk for developing hepatocarcinoma (HCC) due to the regular consumption of aflatoxin contaminated foods (Kensler et al. 2004).

Due to its carcinogenic properties, BP has been a compound of intense research interest since its isolation in the 1930s. However, very few studies have been conducted on the effect of BP on genome wide gene expression patterns, effect of intervention agents on modulating these expression patterns on a genomic scale and in turn the effects of this modulation to the individual in question. In addition, malignantly transformed cells are often

used as alternatives to normal cells in *in vitro* studies due to their ease in maintenance and the assumption of their similarity to normal cells in response to carcinogenic stress. However, whether or not malignantly transformed cells are appropriate models to use in studies of metabolic activation and chemical carcinogenesis is an important question to consider.

This dissertation considers the metabolism and activation of an important, carcinogenic polycyclic aromatic hydrocarbon to which everyone is exposed. It further considers inter-individual variation in certain metabolic factors that impact an individual's risk of cancer, and it examines the extent to which these factors may be mitigated by the introduction of a chemical intervention.

Objectives

The goals and objectives of these studies were to:

- Assess genome-wide changes in gene expression patterns among a panel of 6 normal human mammary epithelial cells (NHMECs) on exposure to BP in the absence or presence of CHL in order to seek biomarkers for chemopreventive intervention.
- Determine the extent of inter-individual variation in *CYP1A1* and *CYP1B1* gene expression among a larger panel of 20 NHMECs on exposure to BP in the absence or presence of CHL.
- Determine the extent of inter-individual variation in BP-DNA adduct formation among a larger panel of 20 NHMECs on exposure to BP in the absence or presence of CHL.
- Examine BP and B[e]P in parallel for their effect on *CYP1A1* and *CYP1B1* gene expression and DNA adduct formation in 2 NHMECs in the absence or presence of CHL.
- Examine NHMEC(s) in parallel with a cancerous cell line, MCF-7 for their effect on *CYP1A1* and *CYP1B1* gene expression and DNA adduct formation on exposure to BP in the absence or presence of CHL.

2.0 REVIEW OF LITERATURE

2.1 Introduction

Cancer is the second largest cause of mortality next only to cardiovascular diseases. Nearly 1.4 million new cancer cases (excluding noninvasive cancer) are expected to be diagnosed in the US in 2006 and more than 500,000 will die (<http://www.cancer.org>). A large proportion of these cancer deaths are attributable to nutrition, lifestyles and occupational exposures to many carcinogens.

Cancers arise in epithelial tissues (carcinomas), mesenchymal tissues (sarcomas), haemato-lymphoid system (leukemias and lymphomas) and other tissues of the body (Knowles et al. 2005). These cancers are both benign and malignant.

Human exposures to a range of chemicals and toxins many of which may be potentially carcinogenic occur through air, water, diet, lifestyle and/or occupation. Biotransformation and metabolism of many of these chemicals are the primary cause of toxic manifestations. Cytochrome P450s (CYPs) form the major superfamily of enzymes involved in many of these Phase I metabolic reactions in addition to their roles in steroid genesis and drug metabolism. Given the widespread distribution and potentially multiple routes of exposure of many of these chemicals alternative strategies involving the use of chemopreventive measures to block or even reverse some of the genetic damage induced by these agents may be a viable approach.

2.2 Chemical carcinogenesis

Chemical carcinogenesis typically involves a complex series of events starting with the bioactivation of the chemicals to DNA damaging metabolites followed by damage of cellular macromolecules and mutation fixation. Reports on increased incidences of nasal polyps among dippers and enhanced rates of scrotal cancer in English chimney sweeps were some of the early documented cases of chemical carcinogenesis (Poirier 2004).

Early chemical carcinogenesis studies in the 1930s with attempts to isolate some of the carcinogenic constituents of coal tar has progressed rapidly ever since (Phillips 1983). An important landmark was the understanding that binding of reactive electrophiles (in case of genotoxic agents) to cellular macromolecules being associated with enhanced risk of carcinogenesis. However, these early studies believed the interaction to occur with proteins rather than DNA (Miller 1951). The work by Brookes and Lawley served to be some of the seminal work which demonstrated the binding of PAHs to DNA and its correlation to carcinogenicity (Brookes et al. 1964). Subsequently, it has also become known that free radicals that may be generated by CYP mediated catalytic cycling of xenobiotics could also potentially cause free radical damage of cellular macromolecules. Non-genotoxic agents may cause damage by oxyradical formation, depurination, toxic cell death or even by modifying the effects of genotoxic agents (Yuspa et al. 1997). All these can be involved in the multistage process of chemical carcinogenesis that can be conceptually divided into 4 component process: tumor initiation, tumor promotion, malignant conversion and tumor progression.

Tumor initiation is a rare event the frequency of which is dependent on the carcinogen dosage. It may involve DNA adduct formation (as in case of genotoxic agents) leading to the inactivation of tumor suppressor genes and/or activation of protooncogenes or events such as methylation of the promoters of tumor suppressor genes (as in case of non-genotoxic agents) leading to their silencing. This requires the changes to occur in a population of dividing cells so as to 'fix' the mutations. In general a strong correlation has been observed between the extent of DNA adduct formation and tumor formation.

Tumor promotion involves a clonal expansion of the initiated cells. Tumor promoters are agents that are generally non-mutagenic and non-carcinogenic by themselves but which enhance the carcinogenicity of tumor initiators. These usually possess multiple mechanisms of action and generally do not require any metabolic activation for the activity. They mainly serve to reduce the latency period for tumor formation when dosed in conjunction with the initiator. Tumor promoters generally bring about their effects through activation of cell surface receptors, stimulation of cell proliferation, perturbation of signal transduction pathways, inhibition of apoptosis or by modulation of cytosolic and nuclear factors. Agents capable of functioning both as a tumor initiator and promoter are regarded as complete carcinogens e.g.: 4-amino biphenyl. Tumor promotion is considered the chief rate limiting step in experimental carcinogenesis.

Malignant conversion involves accumulation of further genetic changes leading to the transformation of preneoplastic cells to express a malignant phenotype. This often involves multi-focal changes in premalignant lesions such as up-regulation of AP-1, gene

amplification, exposure of cells to DNA damaging agents and over-expression of cell cycle regulatory proteases. The changes occurring may be reversible where regression of lesions occur when the promoting agent is removed prior to malignant conversion.

Tumor progression involves acquisition of more aggressive characteristics by the malignant cells facilitating their spread and invasion beyond the primary site and often involves events such as complex genetic alterations (translocations, deletions, gene amplifications, duplications), ectopic changes in hormone production and activation of protooncogenes (through point mutations or gene amplifications) and inactivation of tumor suppressor genes.

2.3 Chemical classes

2.3.1 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are a class of more than 100 known compounds that are composed of variable numbers of fused benzene rings. They are ubiquitous environmental pollutants formed from the incomplete combustion of organic matter and are also generated as by-products from various industrial processes. Pure PAHs are usually colorless, white or pale yellow solids, generally odorless, but in some cases have a mild faint odor. These compounds almost most occur as complex chemical mixtures in nature.

Considerable interest in PAHs stems from the ability of many of them to cause toxicity and cancers of various organs (Ramesh et al. 2004). PAHs are chemically inert, and they require metabolic activation to exert their biological effects (Weston et al. 2005). Benzopyrenes, are members of the PAH family of compounds. Two of these benzo(a)pyrene (BP) and benzo(e)pyrene (B[e]P) were used in various experiments in this study.

Benzopyrenes

Benzopyrenes, prototypical carcinogens found generally with various other PAHs in tobacco smoke, charbroiled foods and in the combustion products of organic matter and various organic fuels are ubiquitous environmental pollutants (Hooven et al. 2005). BP, a highly carcinogenic PAH, has been a compound of extensive research interest since its first isolation by Kennaway *et. al.*, in 1930s from 2 tons of coal tar pitch (Kennaway 1955). According to the International Agency for Research on Cancer (IARC) it has been placed under Group 2A (probably carcinogenic to humans) (IARC 1987). The structure of BP is shown in Figure 1(A).

Figure 1: (A) Benzo[a]pyrene (BP)

(B) Benzo[e]pyrene (B[e]P)

B[e]P is a structural isomer of BP. The low carcinogenicity of B[e]P in contrast to BP has caused very few studies to be carried out on B[e]P compared to BP. B[e]P is only weakly mutagenic and carcinogenic compared to BP and has been assigned IARC rank of Group 3 (not classifiable as to the carcinogenicity to humans) (IARC 1987). The structure of B[e]P is presented in Figure 1(B).

However, because it is found with BP and other highly carcinogenic hydrocarbons in the environment, its effect on modulating the carcinogenicity of these hydrocarbons is to be addressed.

2.3.2 Aromatic amines and heterocyclic amines

Aromatic amines are carcinogens present in tobacco smoke, diesel exhaust and some cooked foods. They consist of one or more rings of unsaturated or cyclic hydrocarbons along with nitrate (NH₂) groups. They are implicated in cancers of the bladder, colon and ureter (Weston et al. 2005).

Heterocyclic amines are formed from high temperature cooking of foods (>150°C) mainly from pyrolysis of the amino acids, creatine and creatinine and sugars such as glucose. These substances have been found to be potent mutagens in *Ames Salmonella* assay and are carcinogens in laboratory animals (Layton et al. 1995). These have also been found to form liver tumors in primates (Adamson 1989).

2.3.3 Aflatoxins

These are chemicals which represent a family of structurally related difuranocoumarin derivatives produced by certain fungi (*Aspergillus flavus* and *A. parasiticus*) that contaminate peanuts, soybean and certain cereals. The production of these toxins are affected by environmental and strain specific factors (Sudakin 2003). Aflatoxin B1 and G1 are more mutagenic and carcinogenic than aflatoxin B2 and G2 (Weston et al. 2005). Exposure to aflatoxin B1 has been shown to be linked to enhanced risk of liver cancer in

human populations exposed to dietary aflatoxins in certain parts of China and Africa (Kensler et al. 2004).

2.3.4 N-nitrosamines

These are ubiquitous environmental pollutants found in certain foods such as cured meats, alcoholic beverages, certain cosmetics, oils and tobacco. N-nitrosamines can also be formed endogenously through nitrosation where an amine reacts with nitrate alone or in the presence of acid (Weston et al. 2005). Exposures to dietary nitrosamines have been associated with risk of esophageal and gastrointestinal cancers while exposure to tobacco specific nitrosamines such as 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone have been associated with enhanced risk of lung cancer (Goldman et al. 2003).

2.3.5 Non-genotoxic agents

These are mainly agents which tend to bioaccumulate in biological systems (e.g.: pesticides, herbicides) and cause disease upon prolonged exposure. There is no conclusive evidence of their role in causing cancers but they may modify the effects of some of genotoxic carcinogens. In other cases they may manifest their effects by causing toxic cell death, oxyradical formation, disruption of hormonal balance, dysregulation of growth factors and by disruption of certain signaling pathways (Yuspa et al. 1997; Weston et al. 2005).

2.4 Metabolism of carcinogens

Once internalized most chemical carcinogens are subject to competing processes of metabolic activation (Phase I reactions) versus detoxication (Phase II reactions). The metabolic route of some of the chemical classes listed above is discussed below.

2.4.1 PAHs

Benzopyrenes

BP is one of the best characterized PAHs whose metabolism has been studied extensively. It acts as a ligand for Ahr, a basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) homology domain family of transcription factors. Various other planar aromatic compounds and many of their halogenated derivatives also act as Ahr ligands. BP has been found to be a strong Ahr ligand while B[e]P a weak Ahr ligand (Sterling et al. 1994). In vertebrates, Ahr exists in the cytosol complexed with a dimer of Hsp90 and several Hsp90 accessory proteins (Puga et al. 2005). Ahr ligands following entry of the cell by diffusion, bind Hsp90 associated Ahr and subsequently cause a conformational change in Ahr leading to its increased affinity for DNA. The conformation change causes liganded Ahr to translocate to the nucleus, exchange Hsp90 for Arnt (aryl hydrocarbon nuclear translocator), another bHLH/PAS transcription factor ultimately leading to the formation of an Ahr, Arnt and ligand complex. This complex in turn binds to xenobiotic response elements (XRE) in the upstream regions of CYP genes leading to their enhanced transcription (Schmidt et al. 1996; Vrzal et al. 2004). In some rare cases unliganded Ahr has been found to undergo nuclear translocation and it is suggested that a universal secondary mediator like cAMP may play a role in this mechanism (Oesch-Bartlomowicz et al. 2005).

Many studies on the mechanism of BP metabolism occurred during the period between 1950 -1990. The metabolism of BP generally proceeds in 2 phases; Phase I involves the conversion of BP to oxidized derivatives (epoxides, phenols, diols and quinines) and Phase II reactions involve the conversion of these derivatives to polar metabolites by conjugation with sulfate, glucuronic acid or glutathione (Osborne et al. 1987).

The primary metabolites are three epoxides: 4,5-epoxide, 7,8-epoxide, and 9,10-epoxide while 1,2-epoxide and 2,3-epoxide are theoretical intermediates that are non-enzymatically converted to phenols. 4, 5-epoxide is the most stable of the epoxides that can be isolated. These products of initial oxidation are generated by the action of various CYPs principally CYP1A1, CYP1B1 and CYP3A4. The epoxides can subsequently be hydrolyzed to the corresponding trans-diols/dihydrodiols (t-4,5 diol, t-7,8 diol or t-9,10 diol) by the action of epoxide hydratase (EH) or be converted non-enzymatically to phenols or undergo conjugation by glutathione S-transferases to form the corresponding glutathione conjugates (Gelboin 1980). Diols formed can exist in either the *cis* or *trans* configuration. All diols formed from PAHs other than BP in the presence of mammalian microsomal enzymes exist in the *trans* configuration. However, all diols formed enzymatically from BP are trans and (-) enantiomers of high optical purity (Gelboin 1980).

1-OH, 3-OH, 6-OH, 7-OH and 9-OH are the 5 major phenol metabolites (with the 3-OH, 7-OH and 9-OH metabolites being the most common) which can subsequently be converted to quinones/diones (1,3 quinone, 3,6 quinone and 6,12 quinone) or be conjugated to glucuronic acid by UDP glucuronosyl transferases (encoded by 2 gene families, UGT1 and UGT2 with several sub-families and members) to yield glucuronide conjugates. The glucuronide conjugates may be acted upon by β -glucuronidase present in the intestinal

microflora to release the parent compound, which, being electrophilic may attack DNA and other cellular macromolecules (Parkinson 2003). The quinones formed from the phenols may be conjugated to sulfates by sulfotransferases (encoded by 5 gene families, SULT1-SULT5 with several subfamilies) and subsequently be converted to the corresponding sulfate metabolites or be conjugated to glutathione to produce glutathione conjugates (Guillen et al. 2003). The phenols may also be directly converted to sulfate metabolites by the action of sulfotransferases. The formation of phenols, sulfates, glutathione and glucuronide conjugates are all considered to be more of detoxification pathways enhancing the hydrophilicity of the compounds and therefore aiding in their excretion from the system. Dihydrodiol metabolites can undergo further oxidation by CYPs (mainly CYP1B1 and CYP3A4) to produce dihydrodiol epoxides (Gelboin 1980) or may undergo conversion to more polar phenol diols (6-OH-7,8 diol, 1-OH-9,10 diol, 3-OH-9,10 diol, 9-OH-4,5 diol).

The two common stereoisomeric dihydrodiol epoxides (of 4 possible forms) are r-7,t-8 diol c-9,10 hydroxy BP/ ($\pm$)7 α ,8 β -dihydroxy-9 α ,10 α -epoxy-7,8,9,10 tetrahydroBP (BPDE1/*syn* isomer /*cis* isomer) and r-7,t-8 diol t-9,10 dihydroxy BP/ ($\pm$)7 α ,8 β -dihydroxy-9 β ,10 β -epoxy-7,8,9,10 tetrahydroBP (BPDE2/*anti* isomer/*trans* isomer) (Kim et al. 1998; Rubin 2001). The preferred oxygenation site and subsequently the preferred dihydrodiol epoxide formed appears to depend on the CYP isoform catalyzing the process and hence is a function of the tissue or enzyme preparation used (Gelboin 1980). Both BPDE1 and BPDE2 are reactive capable of binding to DNA, RNA and proteins forming bulky covalent addition compounds called adducts. The first experimental evidence that the anti isomer of BPDE may be the ultimate DNA binding metabolite was from work of Sims *et. al.* (Sims et al. 1974). Both, BPDE1 and BPDE2 form a C-10 carbonium ion intermediate which is

responsible for their reactivity towards cellular nucleophiles such as DNA and proteins. Among the two stereoisomers BPDE2 has been found to be even more reactive than BPDE1 and hence has been studied extensively (Kim et al. 1998). Both diol epoxides however are unstable in aqueous media and are hydrolyzed spontaneously to tetrols. BPDE1 can be hydrolyzed to 2 tetrols, ($\pm$)BP r-7,t-8 c-9,c-10 tetrahydrotetrol (RTCC), the major tetrol and ($\pm$)BP r-7,t-8 c-9,t-10 tetrahydrotetrol (RTCT), the minor tetrol while BPDE2 can also be hydrolyzed to 2 tetrols, ($\pm$)BP r-7,t-8 t-9,c-10 tetrahydrotetrol (RTTC), the major tetrol and ($\pm$)BP r-7,t-8 t-9,t-10 tetrahydrotetrol (RTTT), the minor tetrol. [The R, T, C system of nomenclature was suggested by the American Chemical Society where r = relative, the position to which all other positions relate, t = trans and c = cis]. Both BPDE1 and BPDE2 can be reduced in the presence of NADH or NADPH to the corresponding triols. This reduction occurs non-enzymatically and is dependent on the tissue concentration of NADH or NADPH. The triols and tetrols are polar and undergo elimination through excretion. A summary of these various routes during the metabolism of BP is presented in Figure 2. A more detailed representation of the pathway leading to metabolic activation and the formation of adducts is presented in Figure 3.

Metabolically activated BP (BPDE) binds DNA, RNA and proteins. The binding to proteins may be 10 times greater than to nucleic acids. Apart from BPDE, the oxides as well as diols are also known bind to proteins and nucleic acids (Osborne et al. 1987). Electron rich amino acids such as tyrosine, histidine and tryptophan may play a vital role in protein binding. When binding to DNA, deoxyguanosine appears to be the preferred base though adducts with all other bases of DNA are also known (Dipple et al. 1984). Some studies have also found modification of the phosphate backbone of nucleic acids. In case of binding to

RNA there seems to be some interaction of the diol epoxides with uracil residues (Gelboin 1980). In case of guanine the major site of modification is the exocyclic amino group at position 2 though a minor product may also be formed at N-7. Adducts are also possible with the exocyclic amino group at position 6 of deoxyadenosine and the amino group of deoxycytidine (Dipple et al. 1984). The diol epoxide seems to be the predominant adduct involved in DNA binding.

Given the lower carcinogenicity of B[e]P compared to BP relatively fewer studies have been carried out on this compound. Metabolism of B[e]P has been found to yield B[e]P-4,5-dihydrodiol, 1-hydroxy-BeP and 3-hydroxy-BeP along with small amounts of B[e]P-9,10-dihydrodiol and a few other secondary metabolites. Of these the 4,5 dihydrodiol appears to be the major metabolite. Other studies have also found an unidentified phenol (probably 1-hydroxy-BeP), 3-hydroxy-BeP and a quinone as metabolites but their proportions are much lower than B[e]P-4, 5-dihydrodiol. However, B[e]P-9,10-dihydrodiol is the only derivative whose metabolism has been studied. On incubation of this derivative with liver microsomal about 13% was converted to 9,10-epoxy-11,12-dihydroxy-9,10,11,12-tetrahydro-BeP while the rest was recovered as B[e]P-4,5,9,10-tetrahydrodiol and 2 triols. 9,10-epoxy-11,12-dihydroxy-9,10,11,12-tetrahydro-B[e]P reacts directly with DNA, the principal adducts having C9 of guanine attached to exocyclic N2 of guanine. A few other minor adducts including ones with deoxyadenosine have also been found (Osborne et al. 1987).

Figure2: Metabolic pathways of BP. The different routes of metabolism listed here are however not exhaustive.

Figure 3: The metabolic activation of BP has been widely studied. (1) CYP1A1 and CYP1B1 catalyze the conversion of the parent compound to arene oxides. (2) Arene oxides are converted to dihydrodiols by EH (3) The resulting dihydrodiols are further oxidized at the site of the olefinic bond (4) Vicinal diol epoxides are highly unstable and undergo spontaneous arene ring opening to yield carbocation. (5) The electrophilic carbocation can form a covalent bond with exocyclic amino group of deoxyguanosine. The resulting adducts resides in the minor groove of DNA.

2.4.2 Aromatic amines and heterocyclic amines

The metabolism of aromatic amines is rather complex. They may be converted to an aromatic amide catalyzed by acetyl co-enzyme A dependent acetylation or may undergo N-oxidation to give N-hydroxylated products which may subsequently be protonated by acidic conditions in the urinary bladder giving rise electrophiles capable of binding DNA. The initial activation step of both aromatic amines and amides is through CYP1A2 mediated N-oxidation (Weston et al. 2005).

Heterocyclic amines are bioactivated by CYP1A2 mediated N-hydroxylation followed by enzymatic O-esterification. The nitrenium ion is likely to be the ultimate carcinogen capable of adduct formation (Goldman et al. 2003).

2.4.3 Aflatoxins

Aflatoxins may be activated by various CYPs (CYP2A3, CYP1A2, CYP2A6, CYP3A4, and CYP3A5). In case of aflatoxin B1, the process involves a CYP3A4 or CYP1A2 mediated oxidation at the exo 8, 9 position to form an electrophilic exo 8, 9 epoxide which can subsequently bind to DNA and proteins and form adducts. Formation of these adducts has been shown to cause G:C to T:A transversion at codon 249 of the p53 tumor suppressor gene (Kensler et al. 2004). Formation of CYP1A2 mediated aflatoxin M1 is considered a detoxification pathway (Sudakin 2003).

2.4.4 N-Nitrosamines

Endogenous nitrosamines form during the reaction of an amine with a nitrate alone or a nitrite in the presence of an acid. Dietary and tobacco specific nitrosamines constitute some

of the exogenous sources of nitrosamines. N-nitrosodimethylamine formed from exposure to nitrosamines undergo enzymatic α -hydroxylation (CYP2E1/ CYP2A6 mediated) to form an unstable α -hydroxynitrosamine which subsequently hydrolyzes to an aldehyde and a monoalkylnitrosamine. The monoalkylnitrosamine can subsequently release a carbocation which can alkylate DNA bases.

2.5 DNA damage

Exposures to carcinogens through various routes lead to DNA damage. The damage may involve formation of addition compounds by covalent attachment to DNA bases (adduct formation), alterations in DNA structure (single and double strand breaks, DNA strand cross-links, chromatid exchanges), oxyradical damage of bases, transitions, transversions, dimerization, deamination as well as epigenetic changes (Yuspa et al. 1997; Weston et al. 2005).

Most carcinogens are mutagens causing heritable changes in the structure of DNA. Different nucleophilic sites on DNA form targets for attack by different adduct forming species. These include N1, N3 and N7 of adenine, N3 of cytosine, O2, N3 and O4 of thiamine and N2, O6 and N7 of guanine. DNA adduct formation may involve the addition of just a portion of the chemical carcinogen such as a small alkyl group (as in the case of carcinogens like N-nitrosamines, N-alkylureas, mustards and haloalkanes) or the entire carcinogen leading to the formation of large, bulky DNA adducts (as in case of many PAHs) (Weston et al. 2005). In many cases these changes lead to the formation of 'signature' mutations in certain key oncogenes and /or tumor suppressor genes. For example, in many cases of tobacco smoke induced lung cancer the common targets for adduct formation appear

to be the *ras* oncogene as well as the p53 tumor suppressor gene. Most cases of aflatoxin induced hepatocarcinomas (from either China or South Africa) were found to harbor G → T transversions in the 3rd position of codon 249 of p53 (Yuspa et al. 1997; Weston et al. 2005).

Activated benzopyrenes bind preferentially to the exocyclic amino group of deoxyguanosine (N2) to form (7R)-N2-(10[7b, 8a, 9a-trihydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene]yl)-deoxyguanosine (BPdG) adducts which appears to be the most common adduct of BP though adducts with other bases in DNA have also been reported. With regard to aromatic amines the C8 of deoxyguanosine appears to be the predominant site for adduct formation though adducts with C8, N2 and O6 are also possible (Weston et al. 2005). Heterocyclic aromatic amines also have a preference for the C8 and amino groups of purines, especially deoxyguanosine for forming adducts (Dipple 1995). Aflatoxin B1 and G1 undergo oxidation at the 8, 9-position to form N7 deoxyguanosine adducts. This in turn could undergo ring opening to form two pyrimidine adducts N-nitrosamines upon metabolic activation bring about alkylation of DNA at some of the similar sites on DNA bases as mentioned above though the N7 of guanine appears to be the preferred site.

Apart from these, oxidative damage of DNA from various endogenous and/or exogenous sources of oxygen radicals can lead to the formation of thymine glycol or 8-hydroxydeoxyguanine adducts. The catalytic cycling of BP, endogenous lipid peroxidation and catalytic cycling of some enzymes include some of these (Guengerich 2001). Etheno adducts can result from reaction of the bases in DNA with xenobiotics such as urethane and chemicals such as carbon tetrachloride and ethanol.

2.5.1 DNA repair

Maintenance of the genome integrity may be achieved at various levels such as by prevention of carcinogen activation, prevention of interaction of the activated carcinogen with the target site, abrogation of the damage caused, or various combinations of these. Impaired capacity for DNA repair is associated with various cancers and syndromes. More than 140 gene products are implicated to play roles either directly or indirectly in different mechanisms of DNA repair mechanisms (Knowles et al. 2005). There are six main mechanisms of DNA repair: direct DNA repair, nucleotide excision repair (NER), base excision repair (BER), mismatch repair, non-homologous end joining and homologous recombination (Yuspa et al. 1997; Weston et al. 2005).

Generically DNA repair involves damage recognition, excision of the damaged strand, resynthesis and ligation. All these repair mechanisms involve multiprotein complexes with many of their components being involved in common in more than one repair process (Weston et al. 2002).

Direct DNA repair involves alkyltransferases which remove alkyl moieties from alkylated bases followed by transfer of the moiety to a cysteine in the active site of the repair enzyme. The best known example of this mechanism is the removal of O⁶-methyl guanine.

NER and BER are similar with both involving multi-protein complexes which recognize the damage, remove the damage, fill in the gaps created and finally seal the nick. NER however, is employed in the repair of large bulky adducts such as those of benzopyrenes while BER is typically involved in the removal of smaller alkyl groups.

DNA mismatches involving single base mismatches due to errors in DNA repair or replication can be corrected by mismatch DNA repair mechanism which involves removal of

large pieces of mismatched DNA using protein complexes that bind to the mismatch and the nearest unmethylated adenine in the sequence GATC, followed by resynthesis of excised part by DNA polymerase and finally ligation by a ligase complexed to the polymerase.

Large bulky adducts such as those of BP can also distort DNA causing it to break. In addition, other mechanisms such as exposures to ionizing radiations and a damage tolerance mechanism known as post replicative repair also causes DNA double strand breaks. These double strand breaks may be repaired by homologous or non-homologous recombination mechanisms. Homologous recombination involves the simultaneous resection of both strands followed by invasion of the homologous duplex by 3' tails of the disrupted DNA, resynthesis followed by polymerization and ligation.

Non-homologous end joining is a repair mechanism that is homology independent. The breaks may be symmetric or asymmetric; in case it is asymmetric resection occurs to create blunt ends before they are joined. This method of repair is therefore prone to introduction of small deletions in the sequence of the repaired DNA.

2.6 Potential impact on hormonal carcinogenesis

BP is an inducer of steroid hydroxylases, CYP1A1 and CYP1B1, which in turn bring about the metabolism of BP to reactive intermediates. These enzymes along with CYP3A4 also play a vital role in the oxidative metabolism of two of major natural estrogens, estrone (E1) and estradiol (E2) (produced by the ovaries) as well as other synthetic estrogens such as 17 α ethinyloestradiol (Liehr et al. 1990). The major pathway of estrogen oxidation involves their P450 mediated hydroxylation to form catechol estrogens (CEs), their 2-OH and 4-OH derivatives, and 16 α -OH metabolite (Cavalieri et al. 1997). Although these CEs can be

inactivated through conjugation, their incomplete or ineffective conjugation may lead to the production of reactive semiquinones and/or quinones radicals. These quinones can subsequently be inactivated by conjugative mechanisms. However, incomplete removal may allow them to bind to DNA and form stable DNA or depurinating DNA adducts. The formation of N7 guanine adducts by CE-3,4-quinone is thought to be a tumor-initiating event (Cavalieri et al. 1997). Apart from this, free radicals generated from the quinone-semiquinone redox cycling of estrogens can lead to free radical damage (Kirkman 1959; Liehr et al. 1990). Further, the 4-OH and 16 α -OH metabolites of estrogen are found to have a potent cell proliferative effect in estrogen responsive tissues

2.7 Chemoprevention

Given the ubiquitous nature of benzopyrenes a complete avoidance of their contact is impossible. An alternative more practical approach may therefore involve reducing their uptake, preventing their metabolism or preventing the activated metabolites from reaching/interacting with the target site. The use of agents (natural, synthetic, biological or chemical) which may reverse, suppress or prevent the carcinogenic process at various phases of the multistep carcinogenic process may form a viable strategy. This constitutes the concept of chemoprevention. Though known for about four decades or so a greater insight into the idea was brought by two pioneers in the field, Lee W. Wattenberg and Michael B. Sporn. Initially called ‘chemoprophylaxis of carcinogenesis’ by Wattenberg (Wattenberg 1966), the term ‘chemoprevention’ was coined only later by Sporn *et al.*, (1976). The original concept of chemoprevention was to inhibit or delay the occurrence of cancers by agents that would block one or more pathways in the induction of cancer. However, the concept has expanded

over the years to include agents which would even reverse the carcinogenic process by transforming malignancy to pre-malignancy.

2.7.1 Chemopreventive agents

Currently there are approximately 400 compounds being studied as potential chemopreventive agents of which around 10% are in clinical trials (<http://www.cancer.gov/cancertopics/factsheet/Prevention/chemoprevention>).

Chemopreventive agents can be classified into 4 main categories: blocking agents, agents reducing tissue vulnerability to carcinogenesis and suppressing agents (Wattenberg 1997).

Blocking agents act through prevention of carcinogen activation, enhancement of carcinogenic detoxification or prevention of activated metabolites from reaching critical target sites. Agents reducing tissue vulnerability to carcinogenesis act through effecting cellular maturation, reducing the activity of the target cells, decreasing cell proliferation or modulating signal transduction and hormone/growth factor activity.

Suppressing agents prevent the evolution of the neoplastic process in cells that may be on the verge of becoming malignant. Some suppressing agents may act by producing differentiation and inhibiting cellular proliferation. Another large group of compounds not fitting into any of these categories have the capacity to inhibit components of the arachidonic acid pathway and induce antioxidant enzymes.

Ideal chemopreventive agents are ones which combine multiple mechanisms of action, have low toxicity, high efficacy, are easily acceptable in intervention strategies and are economically viable (Egner et al. 2001).

Chlorophyllin (CHL)

Porphyrins are compounds with four pyrrole nuclei (a five-membered heterocyclic aromatic ring compound with the formula C_4H_5N) joined by -CH- bridges.

Porphyrins, both natural as well as modified versions are generally, effective antimutagens and anticarcinogens against heterocyclic amines and various other mutagens possessing polycyclic structures. Hemin (the natural porphyrin found in hemoglobin and myoglobin), copper phthalocyanin (a blue pigment similar in structure to hemin) and chlorophyll (the green pigment in photosynthesizing plants) have all been found to be effective antimutagens (Hayatsu 1995). The structures of these compounds are presented in Figure 4.

Chlorophylls and even more so CHL have been gaining prominence over the last decade as effective chemopreventive agents (Dashwood 1997b). Commercially available CHL consists mainly of a trisodium copper salt apart from small amounts of mono- and di-carboxylic acid chlorins (Dashwood 1997b).

Though several medical and clinical uses of CHL exist (Kephart 1955) its increasing use in cancer biology stems from its ability to exhibit various activities in *in vitro* and *in vivo* studies making it an attractive chemopreventive agent. It has been found to be antimutagenic being effective against various chemicals such as aflatoxin B₁, BP, heterocyclic amines, coal dust and airborne particles (Terwel et al. 1985; Ong et al. 1986; Dashwood et al. 1991; Warner et al. 1991). Anticlastogenic activities of CHL have been demonstrated in a few studies (Sarkar et al. 1996; Negraes et al. 2004). CHL has also been shown to exhibit antigenotoxic (Bez et al. 2001; Torres-Bezauri et al. 2002), anticarcinogenic (Park et al. 1996; Reddy et al. 1999), antioxidant (Kamat et al. 2000; Kumar et al. 2004), apoptosis inducing (Diaz et al. 2003; Chiu et al. 2005) and immunomodulatory properties (Yun et al. 2005; Sharma et al. 2006).

Hemin

Cu-phthalocyanine

Chlorophyll

Chlorophyllin (CHL)

Figure 4: Structures of some of the biologically important metalloporphyrins. The groups that differ in CHL compared to chlorophyll are circled. The positions occupied by sodium (Na) can also be occupied by potassium (K) depending on reaction conditions during the conversion of chlorophyll to CHL. Commercial CHL is mainly the trisodium salt shown here along with small amounts of mono-sodium and di-sodium salts.

The most conclusive evidence of CHL as an anticarcinogenic agent probably comes from its role in the chemoprevention of hepatocarcinoma (liver cancer) in aflatoxin endemic areas of the world such as China (Kensler et al. 2004). Chronic hepatitis B virus (HBV) infection along with exposure to aflatoxins through diet contributes to extremely high risk of liver cancer in Quidong. Exposure to aflatoxins is also widespread in Beijing, Taiwan, certain parts of Africa and other developing areas of the world leading to high risk of liver cancer of exposed populations. While primary intervention through HBV vaccination has helped to some extent, secondary intervention strategies through the use of CHL or other drugs such as oltipraz have greatly helped reduce the risk of disease through various mechanisms discussed below. Administration of CHL helped reduce AFB1- N7-guanine adducts by as much as 55%. In this aspect CHL has an advantage over other agents such as oltipraz, given its low cost and high efficacy leading to its easy adoptability by a larger section of at risk human populations in developing areas (Sudakin 2003; Kensler et al. 2004).

However, there are conflicting reports with regard to the use of chlorophyll or CHL as chemopreventive agents of choice with some studies pointing to chlorophyll being a better choice (de Vogel et al. 2005) others CHL (Terwel et al. 1985) and yet others showing both to be equally effective (Bez et al. 2001). The nature of the carcinogen, system under study and end points observed may contribute to some of the differences. Again, with respect to CHL, there are some studies which point not only to the lack of a positive modulatory effect (Torres-Bezauri et al. 2002) but also to an enhancement of the tumor promotion (Nelson 1992) or increase in mutation frequency (Negraes et al. 2004). In another study, CHL when administered after or during and after carcinogen exposure (1, 2 dimethyl hydrazine)

enhanced the number of colon tumors produced in rats. Additionally, in the same study, when a range of CHL concentrations were administered through drinking water, following exposure to 1,2 dimethyl hydrazine (DMH) or 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), the lowest concentration of CHL (0.001%) enhanced the multiplicity of DMH induced tumors while having no promotion effect on tumors induced by IQ (Dashwood et al. 2001). Thus, here too the test species involved, carcinogen used, the concentration of CHL employed, the route of administration and temporal aspects of the exposure protocol may all be key factors playing an important role in deciding the final role of CHL as an inhibitor or a potentiator.

Mechanisms of action

Several mechanisms of CHL action have been proposed. By acting as a suicidal inhibitor, CHL has been shown to bring about non-specific inhibition of CYPs probably involving destruction of the heme prosthetic group of CYPs (Yun et al. 2005). However, other investigations involving spectral studies have proposed that the inhibition may be rather indirect by interacting with NADPH-cytochrome P450 reductase, a rate limiting enzyme in mixed-function oxygenase system rather than involving any direct interaction with P450. This interaction may involve a physical interaction of CHL with NADPH-cytochrome P450 reductase preventing docking P450 with or its role as an alternative electron acceptor interfering with the electron flow from NADPH to P450s (Tachino et al. 1994). In addition to inhibition of Phase I enzymes, CHL has also been reported to enhance the expression of Phase II detoxification enzymes. One study revealed that following oral administration, CHL enhanced the amounts of hepatic glutathione S-transferase in lactating mice (Singh et al. 1996). Studies on induction of NADPH quinone reductase (NQO1), a

quintessential Phase II enzymes showed CHL and chlorophylls to be significant inducers of NQO1 (Fahey et al. 2005).

The most widely accepted mechanism is the role of CHL as an ‘interceptor molecule’ or ‘desmutagen’ wherein CHL in addition to acting as a free radical scavenger also forms tight molecular complexes with the activated carcinogen preventing it from interacting with DNA or other cellular macromolecules (molecular trapping). Studies indicate that the mutagen should possess at least 3 fused rings with some degree of planarity in order to complex with CHL (Arimoto et al. 1993). Two forces play a key role in complex formation; van der Waals forces between overlapping ring systems of the mutagen and CHL and ionic or H bonds between substituents on the mutagen and carboxyl groups of the inhibitor (Dashwood et al. 1996). In case of BP, hypothetical complexes between CHL and BPDE (benzo[a]pyrene –trans-7, 8-dihydrodiol-9, 10-epoxide) are thought to be stabilized by multiple π - π interactions between CHL and BPDE. Following sequestration of the activated carcinogen (BPDE) by CHL they may be degraded to more hydrophilic tetrols and excreted (Tachino et al. 1994).

A relatively recent study has also shown the role of CHL as a modulator of carcinogen uptake and transport. The study examining the effects of a range of CHL concentrations on the transport of 3 different carcinogens, dibenzo(a,l)pyrene (DBP), 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) and AFB1 across Caco-2 cell monolayers from the apical to the basolateral side found that all concentrations of CHL reduced the transport of DBP from the apical to the basolateral side though not much effect was observed on the transport of the other two carcinogens (Mata et al. 2004). Though there have hardly been any further studies on this aspect of CHL, it may be possible that CHL may

have a modulatory effect on the intake and transport of BP. CHL has been reported to be a membrane stabilizer and hence it may be reasonable to make the above assumption (Dhir 1989).

As further mechanistic studies and intervention trials are conducted with CHL a greater number of mechanisms of action and properties of CHL may be unveiled.

2.8 Cytochromes P450 (CYPs)

Most carcinogens (including benzopyrenes) requiring metabolic activation to render them carcinogenic and are often bioactivated by various members of the cytochrome P450s. These enzymes possess some of the broadest range of substrate specificities with some being highly specific (such as those involved in steroid anabolism, mitochondrial and bacterial P450s) and yet others (such as those involved in oxidation of various substrates) exhibiting a broad range of catalytic activities (Guengerich 1991; Ioannides 1996; Lewis 2001). These proteins have been reported to be present in most tissues though mainly concentrated in the liver (1-2% of an individual hepatocyte by weight) and localized to the endoplasmic reticulum (12-15%) and to some extent the mitochondria. Some P450s have also been reported to be translocated outside the plasma membrane where they may have role in development of immune responses. As of 2005 there were reportedly >4500 named P450 genes (<http://drnelson.utmem.edu/CytochromeP450.html>).

Structurally, all P450s seem to contain an 8-10-residue signature motif (F G/S x H/R x C x G/A) with a highly conserved cysteine residue serving as a ligand for a central heme (Lewis 2001). The central heme is bound to the four pyrrole nitrogens leaving room for two additional ligands in the axial positions. One of these ligands above the porphyrin plane is

satisfied by the invariant cysteine and the other ligand below the plane generally being a hydroxyl group related to a tyrosinyl radical or an exchangeable water molecule (Janig et al. 1984; Porter et al. 1991).

P450s are mainly mixed function oxygenases which require a source of electrons to function. The electrons which are generally derived from NADPH are delivered to the iron in the CYP by a flavoprotein, NADPH cytochrome P450 reductase in case of P450s bound to the endoplasmic reticulum or by another protein, ferredoxin reductase (adrenodoxin reductase in case of the adrenals) via an iron-sulfur cluster called ferredoxin to mitochondrial P450s (<http://drnelson.utmem.edu/CytochromeP450.html>). In some less common cases, the routing of electrons from NADPH is may be carried out through cytochrome b5 and NADPH cytochrome b5 reductase or microsomal heme oxygenase (Shen et al. 1989).

The CYP superfamily is divided into families, subfamilies and individual genes based on primary amino acid sequence similarities. P450 protein sequences with $\leq 40\%$ amino acid sequence identity are grouped into different families. Members within a family are divided into sub-families and individual genes (<http://drnelson.utmem.edu/CytochromeP450.html>).

P450s catalyze the metabolism of various endogenous and exogenous substrates. In general, P450s in most non-animal species seem to be involved chiefly in the metabolism of endogenous substrates. Different P450 isoforms have been found to be involved in the biosynthesis and metabolism of steroids, fatty acids, prostaglandins, prostacyclins, thromboxanes, Vitamin D, bile acids and also in the metabolism of a large number of drugs (<http://medicine.iupui.edu/flockhart/>).

2.8.1 Regulation of CYPs

The amount of functional P450s is subject to considerable inter-individual variation. Several factors such as the presence of polymorphisms in P450 genes, use of different nuclear receptors, post-transcriptional (mRNA stabilization) and post-translational (enzyme stabilization) mechanisms appear to play a role to regulate the abundance and activity of P450s (Figure 5). Most CYPs however appear to be regulated at the transcriptional level and many seem to be subject to tissue specific regulation.

Figure 5: Multiple levels in the regulation of P450s. The different levels at which CYPs are regulated are indicated in red. The specific CYP isoform(s) regulated by the respective mechanism(s) is indicated below each mechanism.

The most well characterized P450 regulatory mechanisms are with respect to members of the CYP1 family, regulated by the Ahr-Arnt mechanism (discussed in detail in the section on BP in 2.4.1).

Transcriptional regulation of the CYP2 family involves various receptors related to the nuclear receptor family such as the constitutive androstane receptor (CAR) in the case of CYP2B and the retinoic acid receptor (RAR) in the case of CYP2C (Lewis 2001). In

addition, members of the CYP2B family are also regulated by protein kinase A (PKA). mediated phosphorylation (Oesch-Bartlomowicz et al. 2003). The regulation of CYP2A and CYP2D however, are, mediated by HNF-4 (hepatic nuclear factor-4). The regulation of alcohol inducible CYP2E1 appears to take place at multiple levels including post-transcriptional and post-translational. Diabetes and fasting appear to have a stabilizing effect on CYP2E1 mRNA. Presence of chemical inducers also stabilize the protein by preventing its phosphorylation by PKA and subsequent degradation (Oesch-Bartlomowicz et al. 2003).

CYP3 family members are mainly regulated by members of the orphan nuclear receptor family, glucocorticoid receptor (GR) and pregnane-X-receptor (PXR).

The CYP 4 family is regulated via another nuclear receptor, peroxisome proliferator activated receptor (PPAR).

Various other receptors involved in the modulation of different P450s associated with the metabolism of different endogenous substrates are present in Table 1.

Other mechanisms of regulation include regulation by cyclic AMP (cAMP) in the case of trophic hormones (Porter et al. 1991), use of cis and trans acting elements along with co-activator and repressor proteins, cross-talk between specific nuclear receptors (Lewis 2001) and in some rare cases by neonatal imprinting (Porter et al. 1991). In addition, certain chemicals can inhibit P450s through several mechanisms such as heme ligation, heme adduct formation, competitive inhibition, formation of reactive intermediates or various combinations of these and can therefore modulate CYP expression (Lewis 2001).

Polymorphisms in P450s have mostly been addressed in the context of 'drug metabolizer' phenotypes and susceptibility to diseases. However, the underlying cause of these is the differential expression of the respective P450s caused by the respective

polymorphisms. For example, *2A and *2B variant alleles of *CYP1A1* gene have a T3801C base change introducing a new *MspI* restriction site in intron 6. These variant alleles are associated with enhanced enzyme inducibility and subsequently an elevated risk of smoking associated lung cancer in Japanese and Caucasian cancer patients (Chowbay et al. 2005). The P450s form one of the most polymorphic groups of enzymes. A comprehensive list of different polymorphisms in P450 enzymes can be found at (<http://www.imm.ki.se/CYPalleles/>).

In summary, the overall amounts of P450s are governed by the concentration of inducers and repressors, polymorphisms, and cross-talk with various other receptors, ligands, proteins, and signaling molecules with other factors within the cell.

Table 1: List of receptors and factors associated with the induction of some of the P450s involved with endogenous substrate metabolism

CYP	Receptor/Factor	Trophic hormone as mediating factor
7	FXR, LXR	-
11	SF-1	ACTH (adrenals), LH (testis)
17	SF-1	ACTH (adrenals), LH (ovary)
19	SF-1, ER	FSH (ovary)
21	SF-1	ACTH (adrenals),
26	RAR, RXR	-
27	VDR	PTH (kidney)

FXR = farnesoid X receptor (down-regulator of bile acid synthesis), LXR = liver X receptor (up-regulator of bile acid synthesis), SF = steroidogenic factor 1, ER = estrogen receptor, VDR = vitamin D receptor, ACTH = adrenocorticotrophic hormone, LH = lutenizing hormone, FSH = follicle stimulating hormone, PTH = parathyroid hormone

2.9 Biomarker Concept

Traditional epidemiology has investigated the relationship between exposure and disease. The extent that identification of preclinical changes was possible assisted the better understanding of that relationship. Before the introduction of biomarkers the intermediary steps in the exposure-disease progression were considered to be unknown (a “Black Box”). The introduction of biomarkers has assisted greatly in the effort to see into the “Black Box.”

(Hirschhorn et al. 2002; Wogan et al. 2004). The biomarker concept in molecular epidemiology has been refined significantly since it was articulated by Perera and Weinstein in 1982 (Perera et al. 1982; Perera et al. 2000) and promises to elucidate a large number of intermediary steps that have the potential to impact upon risk of disease as depicted in the schematic representation below.

Schematic representation of the components of the exposure-disease continuum emphasizing the need for development of biomarkers of human cancer risk at every pathologic stage

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacological responses to therapeutic intervention (Srivastava et al. 2002). In order for biomarkers to be valuable they need to be sensitive, specific and predictable in the manner they change between the normal and the diseased states.

Human chemical carcinogenesis generally has a long latency. For decades, a pathology approach has used microscopy of biopsied slides to understand structural changes underlying the transformation process. Unfortunately, when these discernable or measurable changes have occurred many molecular changes have also occurred and it is usually too late for successful intervention (Negm et al. 2002).

Emphasis needs to be on the development of early biological markers of the disease process prior to the transformation of normal cells. Detecting and understanding these changes quickly and accurately will be greatly aid in the early detection of cancers. In addition it will also help shape preventive and therapeutic measures and also predict prognostic outcomes.

Conventional epidemiology, which draws on casual associations between certain exposures/lifestyles and risk of certain cancers, requires studies of population risks and may at best help identify and monitor other individuals or populations at risk; however, it cannot help early detection of cancer. Recent technological advances have helped in a greater understanding of the cancer process at the molecular level and in turn has helped develop biomarkers for early detection and risk assessment of different exposure scenarios (Srivastava et al. 2002).

Molecular epidemiology (the use of molecular and biochemical tools to detect early molecular changes associated with cancer risk) can now be used in conjunction with conventional epidemiological studies to detect some cancers in their early stages (Weston et al. 2005). Thus, they are intended to supercede clinical endpoints.

2.9.1 Types of cancer biomarkers

According to schematic representation above there is a hierarchy of biomarkers: general exposure biomarkers include metabolites in urine, saliva and other body fluids (e.g., cotinine and cortisol signaling exposure to nicotine and stress, respectively); biomarkers of biologically effective dose include carcinogen-protein or carcinogen-DNA adducts derived from blood (these are surrogates for potential genetic damage in the DNA of target tissues). BP-DNA adducts have been valuable in the integration of dose and biological propensity of DNA damage from PAHs (Perera et al. 1982). Exfoliated aflatoxin-guanine adducts in urine (AFB1-N7-dG) have been identified as a risk factor for hepatocellular cancer in populations potentially exposed to AFB1 in china and S. Africa (Kensler et al. 2004).

In conjunction with these studies, there is the possibility to assess cancer susceptibility based on genetic variation. When the continuum of events from exposure to disease is considered, it should be noted that the contribution from the Human Genome Project (HGP) has been highly significant in the arena of genetic polymorphisms. Recent studies have shown that molecular epidemiologic association studies offer a powerful approach to the identification of genetic variants that influence disease susceptibility (Hirschhorn et al. 2002; Consortium 2003). Some of these pertain to potential markers of

susceptibility in the context of a specific exposure scenario, for example: bladder cancer and the NAT2 genotype in dye workers and in tobacco smokers (Green et al. 2000).

Gene expression patterns have been used to compare different types of tumor, different tumor grades and tumor response to treatment (Lonning et al. 2005). However, from the perspective of carcinogenesis, recent studies have identified gene expression patterns within the continuum of the carcinogenic process. One such study described gene expression patterns in peripheral white blood cells in response to exposure in tobacco smokers (Lampe et al. 2004).

In addition to gene expression studies, gene mutation studies have been important biomarkers that have been used for both understanding etiology (Olivier et al. 2004) and predicting outcome (Jackson et al. 2003). A prime example is that of p53 mutations. There is good evidence that exposure to specific agents results in specific or limited types of p53 mutations in certain types of cancer; for example AFB1 and liver cancer, polycyclic aromatic hydrocarbons and lung cancer, and UV irradiation and skin cancer (Olivier et al. 2004). From prospective studies of AFB1 related liver cancer, p53 mutations have been identified in fragments of tumor DNA exfoliated into blood serum before clinical manifestation of disease. There by providing a biomarker for early liver cancer detection in high AFB1 exposure regions of the world.

Many genes including oncogenes and tumor suppressor genes are regulated by epigenetic mechanisms. Dysregulation of epigenetic control of various genes such as p15, p16, APC and VHL have been observed to occur early on in several cancers (Negm et al. 2002).

Other penitential biomarkers include measurement of chromosomal aberrations, point mutations and loss of heterozygosity in DNA from various body fluids of cancerous patients. Presence of anti-apoptotic proteins in the urine of patients with recurrent bladder cancer has been reported and has been found to be a useful biomarker (Smith et al. 2001).

2.9.2 Challenges in using biomarkers

In order for biomarkers to be used efficiently and reliably they will need to be highly sensitive and specific. Though there has been significant progress in the field of cancer biomarker research over the last two decades it has still not reached a level of confidence for application in the clinical setting, and validation studies are required. Some of the biomarkers currently in use such as prostate specific antigen (PSA) for prostate cancer, CA125 for ovarian cancer are not very specific for their respective cancers. In addition, in this time of rapidly advancing technological developments, there is a need for more effective as well as novel cancer biomarkers.

2.10 Statement of the problem

Benzopyrenes, a class ubiquitous environmental contaminants are procarcinogens. Due to their widespread distribution, alternative strategies such as the use of chemopreventive measures to mitigate some of the genetic damage caused by these chemicals may form a practical approach. This study was designed to seek biomarkers of BP exposure (using microarrays) which could subsequently form targets for chemopreventive intervention. CHL, a water soluble metalloporphyrin was used as a chemopreventive agent in all these studies. Also, since benzopyrenes are procarcinogens requiring metabolic activation by CYP1 enzymes for their conversion to DNA damaging metabolites, the effect of CHL on mitigating the expression of *CYP1* enzymes, in addition to their efficacy in preventing the interaction of the activated BP metabolites with cellular macromolecules such as DNA as measured by RT-PCR and BP-DNA adduct analysis, respectively was determined. B[e]P, a structural isomer of BP which has been reported to be weakly carcinogenic was also examined in parallel with BP (both in the absence and presence of CHL) to induce *CYP1* gene expression as well as cause DNA adduct formation. In addition, MCF-7, a malignantly transformed breast cell line was observed in parallel with NHMECs across different CHL treatments and concentrations to examine if their responses parallel those of normal cells and in turn whether they can be used as a surrogate to normal cells in studying carcinogenic stress.

3.0 MATERIALS AND METHODS

Most experiments in this study were carried out on a panel of primary NHMECs developed from breast tissue discarded at reduction mammoplasty. In order to compare the responses of cancerous cells, a breast cancer cell line, MCF-7 was used in parallel with NHMEC(s) for some of the studies. Genome-wide changes in gene expression patterns were initially observed in a panel of 6 NHMECs. Subsequently, gene expression patterns of *CYP1A1* and *CYP1B1*, two key BP metabolizing enzymes as well as the level of BP-DNA adducts was observed in a panel of 20 NHMECs. For studies on the comparison *CYP1A1*, *CYP1B1* gene expression and the extent of DNA adducts on treatment of cells with BP or B[e]P, 2NHMECs (M98025 and M98015) were used. Studies for comparison of normal cells with malignantly transformed cells were carried out on a NHMEC strain (M00012) and the cancerous cell line MCF-7 over a range of CHL concentrations and treatments. An overview of the different experiments carried out in this study is shown in Table 2.

3.1 Breast tissue

Breast tissues from otherwise healthy women undergoing reduction mammoplasty were obtained from the Cooperative Human Tissue Network (CHTN) sponsored by the National Cancer Institute (NCI) and National Disease Registry Interchange (NDRI). Information on age, ethnicity of donors and a corresponding pathology report were obtained without personal identifiers. Since tissue samples were provided without personal identifiers the study was granted a waiver by the Human Studies Review Board at NIOSH.

3.2 Development of NHMEC strains

NHMECs were isolated from the breast tissues by a process involving a combination of mechanical disruption, enzymatic digestion and filtration as described (Stampfer et al. 1980) with slight modifications. Briefly, stromal and epithelial tissue masses (following removal of fat deposits (by dissection) were minced to 1-2 cm pieces, filled into 50 ml conical tubes up to 1/3rd the capacity and incubated overnight at 37⁰C, on a wave mixer with constant shaking with Tissue Digestion mix (Appendix). Following partial digestion the following day, the tubes were spun (250g, 10min in a swinging bucket centrifuge), the supernatant removed and the tissue pellet resuspended in fresh Tissue Digestion mix and incubated for another 24-48h to ensure complete tissue digestion. Complete tissue digestion was ascertained by observing for organoids (cell clumps) under 40X and 60X objectives of an Olympus IX70 Inverted Fluorescent light microscope. Completely digested pellets were pooled together (typically 4 tubes into 1) and resuspended in 5 ml freezing media (Appendix). The resuspended pellets were then filtered through a 150 µm nylon filter and the flow through refiltered through a 90 µm nylon filter. The organoids retained on the 150 µm nylon filter were resuspended in a small volume of freezing media (Appendix) to constitute the 150 µm ductal pool consisting mainly of a population of epithelial cells. Similarly, the organoids on the 90 µm filter were resuspended in a small volume of freezing media to constitute the 90 µm fraction consisting of some small epithelial clumps. The 150 µm fraction, 90 µm fraction and the filtrate obtained after retention of the 90 µm fraction were all separately aliquoted to cryogenic vials frozen in isopropanol containers at -80⁰C overnight and then archived in liquid nitrogen until needed.

3.3 Cell culture

NHMECs were grown and sub-cultured up to passage 6. The 150 μm organoids were seeded with a sterile Pasteur pipette to collagen treated flasks, allowed to stand for about 5 minutes and then supplemented with reconstituted serum-free mammary epithelial medium (MEBM; Clonetics™, Wakersville, MD) supplemented with manufacturer supplied Singlequots™ (gentamycin sulfate-amphotericin B, recombinant human epidermal growth factor, bovine insulin, bovine pituitary extract and hydrocortisone). Cell migration from the organoids was generally observed 24-48h after seeding. Cells were allowed to reach 60-70% confluency before they were sub-cultured. Cells were maintained in MEBM mammary epithelial medium at 37°C, 5% CO₂ and 95% relative humidity. NHMECs are adherent cells and were sub-cultured by trypsinization. Cells at 60-70% confluency were washed once with 1X PBS following removal of media, incubated with 0.025% Trypsin-EDTA (Clonetics™, Wakersville, MD) at 2 ml Trypsin-EDTA/25cm² growth area of the flask and then incubated at 37°C for 2-3 min till $\geq 90\%$ of the cells detached from the flask and appeared rounded when viewed under the microscope. At this stage the flask was gently rapped and the Trypsin-EDTA neutralized with Trypsin Neutralizing Solution (TNS) at 4 ml TNS/25cm² growth area of the flask and the detached cells were transferred to a 50 ml conical tube. The residual cells in the flask were collected by rinsing the flask with 1X PBS. The cells were centrifuged at 150g for 6min in a fixed angle centrifuge. The supernatant was aspirated, the cells washed with 1X PBS and cell pellet finally resuspended in MEBM mammary epithelial media. The suspended cells were then seeded into new flasks at 10,000 cells/cm² and maintained in MEBM mammary epithelial medium. In this manner, cells were passaged up to passage 6. MCF-7 cells were from ATCC and were grown, passaged and maintained in a

manner similar to the NHMECs. MCF-7 cells were grown and maintained in MCF-7 media (Appendix).

3.4 Cell treatments

All treatments were carried out on a uniform population of ~ 70% confluent cells at passage 6. The concentration of BP in all experiments was 4 μ M. The concentration of CHL used was 5 μ M for all experiments except for experiments involving a comparison of MCF-7 cells with a NHMEC across a range of CHL concentrations. For these CHL dose response studies, CHL was used at a concentration of 2 μ M, 3 μ M, 4 μ M, 8 μ M and 16 μ M. In all these experiments the concentration of BP and CHL used were decided based on preliminary cytotoxicity assays. For studies on comparison of BP and B[e]P the concentration of B[e]P used was also 4 μ M in order to have a direct comparison with the effects of BP and was also dissolved in the same solvent as BP. BP was initially dissolved in acetone to obtain a 10 mM solution and subsequently diluted to 4 mM with absolute ethanol. The 4 mM solution was stored in amber vials shielded from light at -20⁰C until needed. Nuclease free water served as the solvent for CHL. For treatment of MCF-7 and NHMECs with 5 μ M CHL an initial stock solution of 5 mM was prepared. For the CHL dose response study, 2 mM, 3 mM, 4 mM, 8 mM and 16 mM solutions were prepared. All CHL solutions were prepared fresh just before use and shielded from light. These stock solutions of BP and CHL respectively were later diluted 1000 fold in MEBM mammary epithelial medium just prior to treatment of cells to obtain the desired micromolar concentrations. Acetone: ethanol (2:3) at 1:1000 dilution used as a solvent for BP served as the vehicle control. All treatments were carried out in duplicate (n = 2).

The treatment protocol of 20 NHMECs and MCF-7 is detailed in Table 3. The treatment protocol for comparison of NHMECs on treatment with BP or B[e]P in the absence or presence of CHL is detailed in Table 4. The treatment protocol for the CHL dose response study comparing MCF-7 and a NHMEC (M00012) across a range of CHL concentrations is presented in Table 5.

3.5 Expression analysis

Analysis of genome-wide changes in gene expression patterns following exposure of NHMECs to BP in the absence or presence of CHL was carried out using microarrays. Subsequently, confirmation of gene expression patterns for a selected subset of genes which appeared altered on microarrays was carried out through real time polymerase chain reaction (RT-PCR). The magnitude and patterns of expression of *CYP1A1* and *CYP1B1*, the main CYP isoforms known to be involved in the metabolism of BP/B[e]P was also carried out through RT-PCR.

3.5.1 Microarray analysis

Arrays

Human genome U133A (HG-U133A) arrays containing 22,283 probe sets representing ~ 14,500 genes were purchased from Affymetrix (Affymetrix, Santa Clara, CA). The sequences used in the design of these arrays were derived from GenBank, dbEST, and RefSeq. These arrays form a part of the HG-U133 set which consists of 2 arrays (HG-U133A and HG-U133B) and altogether consists of almost 45,000 probe sets representing

approximately 33,000 well-substantiated human genes. HG-U133B mostly consists of ESTs and was therefore not used in this study.

Target generation

For microarray analysis due to considerations of cost and time involved only 3 treatment groups (solvent control (SC), BP, and pre CHL + post co-treatment) were considered across 6 NHMEC strains (M98035, M99005, M98015, M98025, M99025 and M99016).

Total RNA isolation

Total RNA following treatment of cells was isolated using RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Cells following treatment were washed with 1X PBS, lysed in a chaotropic buffer containing guanidine isothiocyanate and homogenized in a spin column (Qias shredder) to shear high molecular weight DNA. The lysate containing total RNA was bound to a silica gel based membrane (RNeasy column) and the contaminants washed away using the manufacturer supplied wash buffers (RWE and RPE). Finally the bound total RNA was eluted out using RNase free water.

cDNA synthesis

12 µg of total RNA (260:280 ratio >1.9 and clear, non-smearing rRNA bands on agarose gel) were used for synthesis of double stranded cDNA using T7-oligo(dT) promoter primer kit (Affymetrix, Santa Clara, CA) and SuperScript Double-Stranded cDNA Synthesis kit, (Invitrogen Life Technologies, Carlsbad, CA) as per manufacturer's instructions. The generated double stranded cDNA was cleaned by phenol-chloroform extraction using Phase Lock Gels (PLG) and finally ethanol precipitated. The precipitated cDNA was dissolved in 12.0 µl nuclease free water.

cRNA synthesis

A 'one cycle' target labeling assay was used for generation of labeled target. 6.0 µl of cleaned cDNA was used for the generation of biotin labeled complementary RNA (cRNA) target for hybridization on to HG-U133A arrays using the Gene Chip IVT Labeling Kit (Affymetrix, Santa Clara, CA). The generated cRNA was purified using RNeasy Kit (Qiagen, Valencia, CA) as per manufacturer's protocol. The cleaned cRNA was subsequently quantified using a Beckman DU 7500 UV/VIS spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) to determine the yield and quality of the generated cRNA. An adjusted cRNA yield was calculated using the formula below for further down-stream processing.

$$\text{Adjusted cRNA yield} = \text{RNA}_{\text{ivt}} - (\text{Total RNA}) (y)$$

RNA_{ivt} = amount of cRNA obtained following spectrophotometric analysis

Total RNA = starting amount of total RNA used for cDNA synthesis

y = fraction of the purified cDNA used for cRNA synthesis

The cRNA was subsequently fragmented to 35-200nt bases (94°C, 35 min) prior to hybridization on to the arrays by metal induced hydrolysis using a 5X RNA fragmentation buffer (Appendix).

Hybridization

15 µg of fragmented cRNA were used for hybridization on to 'standard' Hu-Gene 133A arrays. The efficiency of target hybridization was assessed by incorporating pre-mixed, staggered concentrations of biotin labeled cRNA transcripts for three bacterial genes of the biotin synthesis pathway (*bioB*, *bioC*, *bioD*) and one bacteriophage gene for recombinase (*cre*) at staggered concentrations of 1.5 pM, 5 pM, 25 pM and 100 pM, respectively in a hybridization cocktail. A B2 oligo used as a positive hybridization control by the analysis

software to place a grid over the scanned image and demarcate the probe area was also spiked into the hybridization cocktail. The remaining components spiked into the hybridization cocktail included herring sperm DNA, acetylated BSA, hybridization buffer and DMSO (Affymetrix 2004). The cocktail was heated to 99°C for 5 min prior to hybridization. The arrays with the hybridization cocktail were then incubated in a rotisserie oven at 45°C for 16h with constant rotation (60 rpm).

Washing, staining and scanning

The hybridized arrays were subsequently washed and stained on the GeneChip Fluidics Station 400 (Affymetrix, Santa Clara, CA) using Eu-GE-W2v4 protocol of Microarray Suite (MAS) 5.0 (Affymetrix 2004). A signal amplification protocol consisting of first using streptavidin phycoerythroprotein (SAPE) followed by a biotinylated antibody to SAPE and then SAPE again was used as opposed to a single stain protocol. Following washing and staining the arrays were scanned with an argon-ion laser on the HP GeneArray 2500 Scanner (Hewlett Packard, Palo Alto, CA).

Data analysis

The scanning of arrays generated image files (.dat files) (Figure 6).

Figure 6: Representative scanned image obtained following scanning of arrays.

These .dat files were analyzed using MAS 5.0 (Affymetrix, Santa Clara, CA) to generate .cel files (with individual cell intensities), .chp files (with the analysis of cell intensities) and .rpt files (report files with an overview of various quality control parameters for each array/hybridization). Initially an ‘absolute analysis’ was conducted to compute absolute signal intensities of the SC as well as treated groups (BP and pre CHL + post co-treatment). ‘Absolute analysis’ involves analysis of each of the individual arrays to determine the presence or absence of a particular transcript (Detection call), measure their relative abundance (Signal) and also provide a ‘Detection p-value’ evaluated against a user defined cut-off to determine the Detection call. Two algorithms, a detection algorithm and a signal algorithm are employed to achieve this.

Subsequently, a ‘comparison analysis’ comparing the fold change (FC) of the treated groups over the SC (BP versus SC and pre CHL + co-treatment versus SC) was also conducted. A ‘change algorithm’ which defines the nature of change (increase, decrease or no change) in expression trends of the experimental array as compared to its counterpart baseline array and another algorithm which provides a quantitative measure of the magnitude of change in terms of signal log ratio (SLR) are used for comparison analysis.

In this study, only probe sets having a Present call (P) and a FC ~ 3.0 or greater (SLR ≥ 1.5) for up-regulated genes and SLR ≥ -1.5 for down-regulated genes in both replicates in at least one of the 6 NHMECs were considered altered. Various quality control parameters such as background, noise, 3'/5' GAPDH and β -Actin ratios, and % Present calls (between replicates) were examined in the .rpt files.

Graphical representation of all the altered genes were generated using Cluster and Tree View software (<http://rana.lbl.gov/EisenSoftware.htm>) using the average-linkage method (Sokal et al. 1958).

To identify common patterns of gene expression patterns across cell strains following different treatments ‘coefficient of correlation clustering’ was conducted using Micro DB 3.0 and Data Mining Tool (DMT) 3.0 (Affymetrix, Santa Clara, CA). In the ‘correlation of coefficient’ clustering, ‘seeding’, a pre-clustering process involving initial grouping of a small subset of genes with similar expression patterns to generate a ‘seed’ is first carried out with a user specified correlation coefficient threshold. Subsequently, a correlation coefficient threshold to assign probe sets to a particular ‘seed’ in order to create a ‘cluster’ is also defined (Affymetrix 2001). In this study, the correlation threshold of forming ‘seeds’ and assignment of genes to respective clusters were both set at 0.90. Clustering was carried out to

first find the genes common across all 6 cell strains on comparison of treated groups versus SC (BP versus SC and pre CHL + co versus SC). Only genes with a Present call (P) in both the replicates were used for the preliminary clustering. Subsequently, an unpaired, one-sided T test was carried out on above set of genes common to all 6 cell strains to find genes significantly altered due to respective treatment ($p < 0.05$) and also determine the direction of change ('up' or 'down'). Gene expression 'cluster maps' were finally generated from the above subset of statistically significantly altered genes exhibiting particular expression pattern.

Due to the importance of host immune responses in better tackling genotoxic insults, cross-talks between immune response genes altered by $SLR \geq 1.5$ in at least one of the 6 NHMECs by the respective comparisons (BP versus SC and pre CHL + co versus SC) as well as any possible statistically significant associations of the altered genes with any of the presently well known and characterized biological pathways in GenMAPP, PharmGKB, KEGG and BioCarta was carried out using Pathway Studio v1.1 software (Ariadne Genomics, Rockville, MD) and ArrayXPath software (<http://www.snubi.org/software/ArrayXPath>), respectively.

Pathway Studio relies on a proprietary ResNet database to query nearly 500,000+ functional relationships distilled from the entire PubMed and 43 full text journals to automatically extract information from scientific literature, and works with leading public and commercial databases of signaling and biochemical pathways, to construct cross-talks between the input genes/ proteins (<http://www.ariadnegenomics.com>). Initially, using Pathway Studio v1.1 all possible interactions of the altered immune response genes with all other processes, entities and proteins in the cell was deciphered. Later, 'shortest path'

interactions were sought. Finally, only direct interactions, if any, between genes altered by the respective treatments were deciphered.

ArrayXPath is web-based software for mapping and visualization of microarray gene-expression data in the context of integrated biological pathway resources. A total of 45 pathways in GenMAPP pathways, 9 pathways PharmGKB pathways, 70 pathways in KEGG pathways and 346 pathways in Biocarta are queried by ArrayXPath to calculate associations of input genes with the respective pathways. A statistically significant association of the input gene(s) with alteration of the respective pathway is defined by a 'p' value (<http://www.snubi.org/software/ArrayXPath>). For genes involved in more than one pathway, a second p value (in this case designated as q value) accounts for the multiple-comparison. In this analysis, both 'p' and 'q' values were set at ≤ 0.05 .

3.5.2 RT-PCR

Confirmation of gene expression on microarray was carried out through RT-PCR using 'SYBR green chemistry' for a selected sub-set of probes altered by $SLR \geq 1.5$ in at least one of the 6 NHMEC strains. 1 μ g of total RNA isolated from 3 treatment groups (SC, BP and Pre CHL + post co-treatment) using RNeasy kit (Qiagen, Valencia, CA) was reverse transcribed to single stranded cDNA using Advantage RT PCR kit (BD Biosciences, Palo Alto, CA) as per manufacturer's instructions

Primers for these genes were designed using Primer Express v1.5 (PE Applied Biosystems, Foster City, CA), got synthesized from Sigma-Genosys (Wodlands, TX) and used at 50 pmol concentration of each primer in a 25 μ l reaction mix with SYBR Green Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Quantitation was carried

out using a standard 96-well block on the 7900HT Fast Real-Time PCR System preloaded SDS software v2.2.2 (Applied Biosystems, Foster City, CA). Each sample was assayed in duplicate and their expression normalized to that of GAPDH using the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001). The specificity of the generated product was confirmed by looking for a single specific product on ‘Amplify’ software v1.2 β (University of Wisconsin, Genetics, Madison) prior to RT-PCR as well as going in for dissociation curves at the end of the run.

CYP1A1 and *CYP1B1* quantitation across the panel of 20 NHMECs and MCF-7 cells for all treatment groups was carried out using ‘Taqman chemistry’. The expression analysis across the 20 NHMECs (on treatment with BP in the absence or presence of CHL as detailed in Table 3) and 2 NHMECs (on treatment with BP/B[e]P in the absence or presence of CHL as detailed in Table 4) were carried out on the ABI7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). *CYP1A1* and *CYP1B1* quantitation in MCF-7 cells and M00012 across different CHL concentrations (as detailed in Table 5) and in MCF-7 cells across different CHL treatments (as detailed in Table 3) was carried out on the ABI 7900HT Fast Real-Time PCR System (PE Applied Biosystems, Foster City, CA) using ‘Taqman chemistry’. 1.0 μ g of total RNA (RNeasy kit; Qiagen, Valencia, CA) from each of the treated groups was used for cDNA synthesis. Single stranded cDNA (Advantage RT PCR kit; BD Biosciences, Palo Alto, CA) was used as template in a 50 μ l reaction mix consisting of 2X Taqman Universal PCR Master Mix and Taqman® Gene Expression Assay primers and probes for *CYP1A1* (Hs00153120_ml) or *CYP1B1* (Hs00164383_ml) (Applied Biosystems, Foster City, CA). Each sample was assayed in duplicate and their expression normalized to that of GAPDH (Hs99999905_ml; Applied Biosystems, Foster City, CA) using the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001).

3.6 Adduct analysis

BP-DNA adducts formed on treatment of 20 NHMECs as well as MCF-7 cells with BP in the absence or presence of CHL was determined through a chemiluminiscence immunoassay (CIA). B[e]P-DNA adducts formed on treatment of 2 NHMEC strains with B[e]P in the absence or presence of CHL was analyzed through synchronous fluorescence spectrometry (SFS).

3.6.1 DNA extraction

DNA was extracted using a non-organic extraction protocol (Laird et al. 1991). Following treatment, cells were washed with 1X PBS, incubated in a lysis buffer (100 mM Tris pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 µg/ml Proteinase K) at 37°C for 3h and the DNA precipitated with an equal volume of isopropanol. The precipitated DNA was washed twice with 70% ethanol and then dissolved in nuclease free water.

3.6.2 BP-DNA (BPdG) adduct analysis

BPdG adducts were measured by a chemiluminiscence immunoassay (CIA) using antiserum elicited against DNA modified with (±)-7β,8α, dihydroxy 9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[α]pyrene (BPDE) (8). Briefly, sonicated (20s, 20% amplitude, Ultrasonic Processor, Sonics & Materials, Inc, Newton, CT) and heat denatured (95°C, 4min and then cooled on ice) sample DNA or serially diluted standard BPDE-DNA (1.0 BPdG adduct/10⁶ nucleotides) was mixed with an equal volume of rabbit anti-BPDE antiserum (1:3000000 in 0.25% casein prepared in phosphate buffered saline containing 0.05% Tween-20 (PBST)), incubated at 37°C for 15 min and added to wells of 96-well high binding LIA microtiter

plates (Greiner Labortechnik, FRG) coated with highly modified BPDE-DNA or calf thymus DNA. These plates were prepared by coating with 100 pg of highly modified, sonicated BPDE-DNA (0.33% modified) or non-adducted calf thymus DNA in 0.1ml Reactibind solution (Pierce Biotechnology, Inc., Rockford, IL) for 48h following which they were washed with PBST containing 0.05% sodium azide and stored at -20⁰C until used. The plates along with the sample or standard DNA were incubated at 37⁰C for 90 min, washed with PBST, incubated with streptavidin alkaline phosphatase (Avidx-AP: 1:5000, 60 min; Applied Biosystems, Foster City, CA) prepared in 0.25% casein, washed again with PBST and Tris buffer (20 mM Tris, 1mM MgCl₂, pH 9.5) before finally incubating with CDP-Star solution containing Emerald II enhancer (Applied Biosystems, Foster City, CA). The plates were incubated overnight at 4⁰C and the luminescence measured at 542 nm on TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA) on the following day after equilibration of the plates to room temperature. All samples were assayed in triplicate experimental wells and two separate CIAs.

3.6.3 B[e]P-DNA adduct analysis

DNA extracted using a non-organic protocol as detailed above were assayed for B[e]P-DNA adducts using synchronous fluorescence spectrometry (SFS). 100 µg of DNA was acid hydrolyzed (90⁰C, 3h), extracted three times with equal volumes of water-saturated isoamyl alcohol and then resuspended in 600 µl HPLC grade water. Benzo(a)pyrene-r-7, t-8, c-9, t-10-tetrahydrotetrol (+/1) was used to generate a standard curve. Synchronous spectra were obtained by driving the excitation and emission monochromators simultaneously with a wavelength difference of 34 nm. Fluorescent emission was obtained at emission wavelength

of 380 nm (345 nm excitation wavelength). All measurements were carried out on a Perkin Elmer LS50B spectrophotometer (Perkin Elmer Corp., Rockville, MD) loaded with WinLab FL software.

3.7 Statistical correlations

Pearson correlation coefficients between the extent of expression of *CYP1A1* and *CYP1B1* on gene-chip (GC) versus RT-PCR as well as correlation coefficients between *CYP1A1* and *CYP1B1* expression and levels of carcinogen-DNA adducts on treatment of NHMECs or MCF-7 cells with BP or B[e]P in the absence or presence of CHL were determined using Excel (Saunders et al. 1994). Positive values indicate an increase in the value of the dependent variable with a corresponding increase in the independent variable and vice versa.

Table 2: Details of different experiments carried out in the present study

Experiment	Number and type of cell strains used	Cell strains used	Treatments used
1. Microarray analysis	6 NHMECs	M98035, M99005, M98015, M98025, M99025, M99016	1, 2 and 5 as detailed in Table 3
2. RT-PCR and DNA adduct analysis			
a. On treatment with BP $\pm$ CHL	20 NHMECs	M98026, M98019, M98014, M98011, M99021, M99006, M99025, M0004, M98025, M98035, M99005, M99003, M00012, M99004, M98016, M99015, M98030, M98015, M98013, M99016	1, 2, 3, 4 and 5 as detailed in Table 3
b. On treatment with BP or B[e]P $\pm$ CHL	2 NHMECs	M98015 and M98025	1, 2, 3, 4 and 5 as detailed in Table 4
c. On comparison of a NHMEC versus a cancerous cell line on treatment with BP $\pm$ CHL	20 NHMECs and MCF-7	The same 20 NHMECs mentioned above and MCF-7 (cancerous cell line)	1, 2, 3, 4 and 5 as detailed in Table 3
d. On treatment with BP $\pm$ a range of CHL concentrations	1 NHMEC strain and MCF-7	M00012 (NHMEC strain) and MCF-7 (cancerous cell line)	1, 2, 3, 4, 5, 6 and 7 as detailed in Table 5

Table 3: NHMECs and MCF-7 cells treatment protocol

Treatment		Treatment protocol	
1. Control vehicle (SC)	-	Acetone: ethanol (1:1000)-24h	
2. BP alone (BP)	-	BP (4 μ M)-24h	
3. CHL + BP (co-treatment)	-	[CHL (5 μ M) + BP (4 μ M)]-24h	
4. Pre CHL + post BP treatment	CHL (5 μ M)-24h	+	BP (4 μ M)-24h
5. Pre CHL + post co-treatment	CHL (5 μ M)-24h	+	[CHL (5 μ M) + BP (4 μ M)]-24h

Table 4: Treatment protocol for the comparison of BP and B[e]P across 2 NHMECs (M98015 and M98025)

Treatment		Treatment protocol	
1. Control vehicle (SC)	-	Acetone: ethanol (1:1000)-24h	
2. BP or B[e]P alone	-	BP or B[e]P (4 μ M)-24h	
3. CHL + BP or B[e]P (co-treatment)	-	[CHL (5 μ M) + BP or B[e]P(4 μ M)]-24h	
4. Pre CHL + post BP or B[e]P treatment	CHL (5 μ M)-24h	+	BP or B[e]P (4 μ M)-24h
5. Pre CHL + post BP co-treatment or post B[e]P co-treatment	CHL (5 μ M)-24h	+	[CHL (5 μ M) + BP or B[e]P (4 μ M)]-24h

Table 5: Treatment protocol for the CHL dose response study comparing MCF-7 and an NHMEC (M00012) across a range of CHL concentrations

Treatment		Treatment protocol	
1. Control vehicle (SC)	-	Acetone: ethanol (1:1000)-24h	
2. BP alone (BP)	-	BP (4 μ M)-24h	
3. Pre 2 μ M CHL + post BP + 2 μ M CHL co-treatment)	CHL (2 μ M)-24h	+	[CHL (2 μ M) + BP (4 μ M)]-24h
4. Pre 3 μ M CHL + post BP + 3 μ M CHL (co-treatment)	CHL (3 μ M)-24h	+	[CHL (3 μ M) + BP (4 μ M)]-24h
5. Pre 4 μ M CHL + post BP + 4 μ M CHL (co-treatment)	CHL (4 μ M)-24h	+	[CHL (4 μ M) + BP (4 μ M)]-24h
6. 8 μ M CHL + post BP + 8 μ M CHL (co-treatment)	CHL (8 μ M)-24h	+	[CHL (8 μ M) + BP (4 μ M)]-24h
7. Pre 16 μ M CHL + post BP + 16 μ M CHL (co-treatment)	CHL (16 μ M)-24h	+	[CHL (16 μ M) + BP (4 μ M)]-24h

4.0 RESULTS

4.1 Gene expression by microarray

The expression of a multitude of genes involved in diverse cellular functions was altered by BP. The expression patterns of all the 22,283 probe sets (14,500 genes) on HuGene 133A array in each of the 6 NHMEC strains on treatment with BP alone or in the presence of CHL (pre CHL + post co-treatment) are presented in Figure 7. The figure depicts a plot of the signal from each of the 22,283 probe sets for each of the respective treatment replicates versus the corresponding FC for those probe sets. It provides an overview of the scatter of expression patterns across cell strains and treatments. Each cell strain exhibited similar expression patterns for some of the probe sets yet unique expression patterns for others. The scatter plot also shows that for some probe sets while the signal may be low the overall FC compared to SC could be high and vice versa. While the expression of majority of the genes across most cell strains exhibited variation within 2-3 fold, there clearly appeared to be outliers which were treatment as well as cell strain specific. Of these probe sets, only those altered by a FC of ~ 3.0 ($SLR \geq 1.5$) in both the replicates upon comparison analysis of a respective treatment versus SC were considered altered. A complete list of all genes altered by $SLR \geq 1.5$ in at least one of the 6 NHMECs on comparison of BP treated as well as pre CHL + post co-treated cells versus SC can be found in Tables A, B, C, D and E at (<http://www.cdc.gov/niosh/ext-suppmat/MTP/index.htm>). Genes of the biotin synthesis pathway, *bioB*, *bioC*, *bioD* and the bacteriophage gene, *cre* spiked in the hybridized cocktail as hybridization controls were all detected on the arrays with signals in increasing order, as expected. Human GAPDH and Actin, house keeping genes were also detected. The average background, noise, percentage

BP versus control vehicle

M98035

M99005

M98015

M98025

M99025

M99016

Pre CHL + co-treated versus control vehicle

Figure 7: 'Fold change' scatter plots showing expression patterns of all the 22,283 probe sets on Hu-Genel33A arrays on comparison of treated groups versus control vehicle. More details in the text.

of genes present, absent and average signal intensities were also examined for each of the arrays.

A total of 49 genes (0.38%) were altered in at least one of the 6 NHMEC strains on comparison of BP treated cells with SC when analyzed by MAS 5.0. Of these 43 were up-regulated and only 6 were down-regulated. Only *CYP1B1* exhibited consistent up-regulation by $SLR \geq 1.5$ in all 6 cell strains. *ALDH1A3* (probe ID 222168_at) showed up-regulation in 4 cell strains. All other genes were up-regulated in less than 4 cell strains. All down-regulated genes were from only one cell strain, M99016 and no gene was consistently down-regulated by $SLR \geq 1.5$ in all the cell strains. *IL1B* and *SECTM1* were immune response genes consistently up-regulated by BP in most individuals with M99016 exhibiting the greatest expression with $SLR \geq 1.5$. Both the above genes also exhibited the greatest expression in M99016 on pre-treatment with CHL followed by co-treatment and were, therefore, represented in the list of genes up-regulated by BP as well as pre CHL + post co-treatment. Overall, genes altered included those involved with xenobiotic metabolism, cell signaling, cell motility, cell proliferation, cellular transcription, metabolism, immune responses and other processes.

A total of 125 genes (0.86%) were altered in at least one of the 6 NHMEC strains on comparison of pre CHL + post co-treated cells with SC when analyzed by MAS 5.0. Of these 103 were up-regulated and 22 down-regulated. Again, only *CYP1B1* was consistently up-regulated by $SLR \geq 1.5$ in all but one cell strain. No gene was consistently down-regulated by $SLR \geq 1.5$ in all cell strains. Gene categories modulated included mainly those altered by BP treatment in addition to genes involved in cell cycle control, apoptosis, DNA repair and various other cellular processes such as neurogenesis, gene regulation, and

hormonal metabolism. A graphical display of all the altered genes generated using Cluster and Tree View (<http://rana.lbl.gov/EisenSoftware.htm>) according to the average linkage method (Sokal et al. 1958) is presented in Figure 8.

As per DMT 3.0 analysis a total of 5 different expression clusters were obtained on clustering of genes altered by comparison of treated groups versus SC (Figure 9). One cluster each was generated on clustering of genes significantly up-regulated and down-regulated by BP as well as genes significantly down-regulated by pre treatment with CHL followed by co-treatment. However, two different expression clusters were generated for genes significantly up-regulated by pre CHL + post co-treatment. In all cases, the correlation of the altered genes belonging to the respective cluster pattern was 0.99. A complete list of all the genes following the respective cluster patterns can be found in Tables G, H, I and J at (<http://www.cdc.gov/niosh/ext-supp-mat/MTP/index.htm>). More details of the characteristics of the genes used for the ‘correlation coefficient’ clustering are displayed in Table 6.

Given the importance of immune responses in parallel with other cellular mechanisms such as detoxication and repair mechanisms to combat toxic stress, immune response genes altered by $SLR \geq 1.5$ in at least one of the 6 NHMECs in both the replicates upon comparison analysis of a respective treatment versus SC by MAS 5.0 were analyzed separately. Any interactions and cross-talks between the significantly altered immune response genes as well as other cellular proteins were analyzed. A total of only 5 immune response genes were altered by $SLR \geq 1.5$ on comparison of BP treated cells versus SC of which 4 were up-regulated and only 1 was down-regulated by $SLR \geq 1.5$ in at least one of the 6 NHMEC strains. The expression of 24 genes was altered by the synergistic

Figure 8: Graphical display (generated using Cluster and Tree View software) of all genes altered by $SLR \geq 1.5$ on Hu-Gene 133A arrays in at least one of the 6 NHMECs on treatment of cells with BP or pre CHL + co-treatment. A total of 49 genes were altered (up or down regulated) on treatment with BP alone and 125 on pre treatment with CHL followed by co-treatment. Though the gene names are not decipherable in the figure above due to the number of genes involved, a complete listing of all the above genes along with their corresponding FCs can be found at (<http://www.cdc.gov/niosh/ext-suppmat/MTP/index.htm>) [Tables A, B, C, D].

Genes altered by BP treatment

Cluster 1: BP versus SC - up-regulated genes

Cluster 2: BP versus SC - down-regulated genes

Genes altered by Pre CHL + co-treatment

Clusters 3(A) and 3(B): Pre CHL + co-treatment versus SC - up-regulated genes

Cluster 4: Pre CHL + co-treatment versus SC - down-regulated genes

Figure 9: Correlation coefficient gene-expression clusters created from genes altered significantly in all 6 donors on comparison of the respective treated groups versus solvent control (SC). Each point on each cluster was calculated using the average signal generated by the respective treatment: **1= SC, 2 = BP alone and 3 = pre CHL + co-treatment**. The number of genes/probes displaying the respective cluster pattern are shown in parenthesis besides each cluster. The correlation coefficient (CC) of the genes following the respective cluster pattern is shown below each cluster. Details about the exact genes in each cluster following the respective cluster pattern can be found at (<http://www.cdc.gov/niosh/ext-suppmat/MTP/index.htm>) [Tables G, H, I and J].

Table 6: Table representing the number and characteristics of the probe sets used to generate the clusters displayed in Figure 9

Comparison	Number of probes common to all 6 NHMECs altered by respective comparison	Number of probes common to all 6 NHMECs altered significantly ($p < 0.05$) by the respective comparison	Number of significantly altered probes ($p < 0.05$) exhibiting common clustering pattern	Correlation coefficient of probes belonging to respective cluster
BP versus control vehicle (up-regulated)	391	106	71	0.99
BP versus control vehicle (down-regulated)	383	134	96	0.93
Pre CHL + co- treatment versus control vehicle (up-regulated)	228	46	39, 3	0.99, 0.99
Pre CHL + co- treatment versus control vehicle (down-regulated)	232	41	40	0.99

interaction of BP and CHL coming together in a pre CHL + co-treatment of which 15 genes were up-regulated and 9 were down-regulated by $SLR \geq 1.5$. The expression of *IL1B* and *SECTMI* were consistently up-regulated by BP in most individuals with M99016 exhibiting the greatest expression with $SLR \geq 1.5$. Both genes also exhibited the greatest expression in M99016 on pre-treatment with CHL followed by co-treatment. A graphical display of all altered immune genes was also generated using Cluster and Tree View according to the average linkage method (Sokal et al. 1958) and is presented in Figure 10.

Altered immune response genes were analyzed for cross-talks/signal transduction between themselves and with other cellular entities as well as other cellular pathways. Though the altered genes are fed as gene names to Pathway Studio, the software takes them as corresponding proteins while constructing interactions between the input entities as well as all other entities within the cell. When genes altered by BP treatment versus SC were queried for all possible interactions with all other molecules and processes within the cell using Pathway Studio, a total of 2248 relationships/interactions involving 1485 entities including proteins, small molecules, and various functional classes of molecules, complexes and cellular processes were found to be involved. When only ‘shortest cross-talk’ interactions were considered for the above genes only 60 relationships were found between 249 entities. However, there were no direct interactions between the 5 altered genes (Figure 11). More details about the various interactions among the immune response genes altered by comparison of BP versus SC can be found at (http://www.cdc.gov/niosh/ext-suppl-mat/IR_Genes/index.htm) (IR-BP genes).

Figure 10: Graphical representation (generated using Cluster and Tree View software) of all immune response genes altered by $SLR \geq 1.5$ on Hu-Gene 133A arrays in at least one of the 6 NHMECs on treatment with BP or pre CHL + co-treatment. A complete listing of all the above immune response genes along with their corresponding FCs can be found at (http://www.cdc.gov/niosh/ext-supp-mat/IR_Genes/index.htm) [IR genes with FC table]

Figure 11: Five immune response genes were altered by $SLR \geq 1.5$ on Hu-Gene 133A arrays in at least one of the 6 NHMECs on exposure to BP alone. These five immune response genes were queried for any direct interactions between themselves using Pathway Studio software. The software takes the input genes as the corresponding proteins while trying to construct any interactions between the respective members. In this case, no direct interactions were observed between any of the input entities. The different immune response proteins are shown according to their respective cellular location.

Genes altered by pre CHL + post co-treatment were found to exhibit 5782 interactions among and between 2299 entities within the cell. Of these 379 entities including the altered genes were found to be involved in 704 ‘shortest cross-talk’ interactions. However, there were only 51 direct interactions between the altered genes (Figure 12). More details about the various interactions among the immune response genes altered by comparison of pre CHL + post co-treatment can be found at (http://www.cdc.gov/niosh/ext-supp-mat/IR_Genes/index.htm) (IR-CHL genes). It must be mentioned that since Pathway Studio relies largely on PubMed to construct interactions and since the content of PubMed is highly ‘dynamic’, the nature and number of interactions between the same input members and all other cellular entities are likely to vary with time.

Using ArrayXPath software, 2 (*IL1B* and *MAL*) out of the 5 genes altered by BP were found to have a statistically significant association ($p < 0.05$) with 5 Biocarta pathways (Table 7). A total of 45 pathways in GenMAPP, 9 pathways in PharmGKB, 70 pathways in KEGG pathways and 346 Biocarta pathways were queried by Array X to obtain the above associations. A total of 2 (*CD80* and *CD86*) out of 23 genes altered by pre CHL + post co-treatment were involved in one GenMAPP pathway and 6 genes (*SLPI*, *IL8*, *IL1B*, *IL1RN*, *CD80* and *CD86*) were found to be involved in 13 Biocarta pathways (Table 8).

Figure 12: Twenty four immune response genes were altered by $SLR \geq 1.5$ on Hu-Gene 133A arrays in at least one of the 6 NHMECs on treatment with pre CHL + co-treatment. IL8 was up-regulated by $SLR \geq 1.5$ in some of the NHMECs but down-regulated by $SLR \geq 1.5$ in the others and therefore represented in common in list of up-regulated and down-regulated genes. These twenty four immune response genes were queried for any direct interactions between themselves using Pathway Studio software. The software takes the input genes as the corresponding proteins while trying to construct any interactions between the respective members. Fifty one direct interactions were observed between the input entities. The different immune response proteins are shown according to their respective cellular location. Genes with no direct interactions between themselves are shown as separate entities.

Table 7: Pathways in which immune response (IR) genes altered by $SLR \geq 1.5$ on Hu-Gene133A arrays on comparison of **BP treated cells versus control vehicle** are involved. A total of 5 IR genes were altered by $SLR \geq 1.5$ of which 2 (40%) (*IL1B*, *MAL*) were found to possess a statistically significant association ($p \leq 0.05$) with the above pathways. For genes involved in more than one pathway, a q value ($q \leq 0.05$, equivalent to p value) accounts for the multiple-comparison.

Pathway	Identified node	p-value	q-value
BioCarta//Hs_IL 5 Signaling Pathway	1/8 (19)	0.0101	0.0154
BioCarta//Hs_NFkB activation by Nontypeable Hemophilus influenzae	1/24 (43)	0.0300	0.0230
BioCarta//Hs_Signal transduction through IL1R	1/30 (41)	0.0374	0.0230
BioCarta//Hs_Role of MAL in Rho-Mediated Activation of SRF	1/19 (26)	0.0238	0.0230
BioCarta//Hs_Msp/Ron Receptor Signaling Pathway	1/6 (27)	0.0075	0.0232

Table 8: Pathways in which immune response (IR) genes altered by SLR ≥ 1.5 on Hu-
Gene133A arrays on comparison of **CHL treated cells (pre CHL + co-treated) versus control vehicle** are involved. A total of 23 IR genes were altered by SLR ≥ 1.5 of which 6 (26%) (*SLP1*, *IL8*, *IL1B*, *IL1RN*, *CD80* and *CD86*) were found to possess a statistically significant association ($p \leq 0.05$) with the above pathways. For genes involved in more than one pathway, a q value ($q \leq 0.05$, equivalent to p value) accounts for the multiple-comparison.

Pathway	Identified node	p-value	q-value
BioCarta//Hs_Proepithelin Conversion to Epithelin and Wound Repair Control	2/6 (40)	0.0002	0.0018
BioCarta//Hs_The Co-Stimulatory Signal During T-cell Activation	2/16 (40)	0.0014	0.0072
BioCarta//Hs_NFkB activation by Nontypeable Hemophilus influenzae	2/24 (43)	0.0032	0.0110
BioCarta//Hs_Signal transduction through IL1R	2/30 (41)	0.0049	0.0128
BioCarta//Hs_Free Radical Induced Apoptosis	1/10 (11)	0.0373	0.0430
BioCarta//Hs_IL 5 Signaling Pathway	1/8 (19)	0.0299	0.0430
BioCarta//Hs_Msp/Ron Receptor Signaling Pathway	1/6 (27)	0.0225	0.0430
BioCarta//Hs_B Lymphocyte Cell Surface Molecules	1/9 (10)	0.0336	0.0430
BioCarta//Hs_Antigen Dependent B Cell Activation	1/10 (24)	0.0373	0.0430
BioCarta//Hs_IL 17 Signaling Pathway	1/15 (16)	0.0554	0.0443
BioCarta//Hs_Regulation of hematopoiesis by cytokines	1/15 (41)	0.0554	0.0443
BioCarta//Hs_Adhesion and Diapedesis of Lymphocytes	1/14 (47)	0.0518	0.0443
BioCarta//Hs_Adhesion and Diapedesis of Granulocytes	1/15 (49)	0.0554	0.0443
BioCarta//Hs_Cells and Molecules involved in local acute inflammatory response	1/17 (19)	0.0626	0.0459
BioCarta//Hs_Th1/Th2 Differentiation	1/18 (88)	0.0662	0.0459
BioCarta//Hs_Cytokine Network	1/22 (103)	0.0804	0.0522
BioCarta//Hs_Cytokines and Inflammatory Response	1/27 (78)	0.0979	0.0598
GenMapp//Hs_Inflammatory_Response_Pathway	2/29(29)	0.0004	0.0004

4.2 RT-PCR

4.2.1 Confirmation of gene expression on microarrays

Confirmation of gene expression patterns on microarrays was carried out through RT-PCR for a selected subset 24 genes altered by $SLR \geq 1.5$ in both replicates in at least one of the 6 NHMECs. Of these, 7 were immune response genes. The results of RT-PCR confirmation are presented in Tables 9A, 9B and 10. A more extensive version of Tables 9A and 9B can be found in Table E and Table F, respectively at (<http://www.cdc.gov/niosh/ext-supp-mat/MTP/index.htm>). For genes altered by BP, there was a very good correlation of FC as measured by GC and RT-PCR for all genes except for *CALB1* and *SECTM1* which exhibited only a moderate correlation and *MERTK* which exhibited a very poor correlation. A very good correlation of FC between GC and RT-PCR was also observed for all genes altered on pre-treatment with CHL followed by co-treatment except for *AKRIC2*, *DHRS3* and *MCM5* which exhibited only a moderate correlation.

As for the immune response genes there was a good correlation for some genes while the remaining correlated moderately to poorly (Table 10). Upon BP treatment there was a good correlation for *IL1B* and *CXCL2*, a moderate correlation for *PI3* but a poor correlation for the remaining genes (*SECTM1*, *IL1RN*, *CD86* and *MXI*). However, more genes had a better correlation on pre CHL + co-treatment. *IL1RN* and *PI3* also exhibited a good correlation of expression on GC versus RT-PCR apart from *IL1B* and *CXCL2* while *CD86* and *MXI* exhibited a moderate correlation.

Table 9A: Correlation of fold change (FC) on Hu-Gene 133A arrays/gene-chip (GC) and RT-PCR for a partial list of genes altered by SLR $\geq$ 1.5 in at least one of the 6 NHMECs when treated with BP alone. A more extensive version of this table can be found at (<http://www.cdc.gov/niosh/ext-supp-mat/MTP/index.htm>) [Table E].

Gene	Probe ID	Accession no.	BP alone vs vehicle control (avg. FC ± SD)												Correlation (r)
			1 (M98035)		2 (M99005)		3 (M98015)		4 (M98025)		5 (M99025)		6 (M99016)		GC-RT
			GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	BP alone
CYP1B1	202437_s_at	NM_000104	6.76 ± 0.99	10.14 ± 0.46	3.62 ± 0.53	7.61 ± 0.32	11.72 ± 0.57	32.10 ± 0.85	5.55 ± 1.35	7.52 ± 2.41	13.46 ± 0.66	21.58 ± 2.18	5.89 ± 0.86	9.28 ± 2.35	0.87
CYP1A1	205749_at	NM_000499	1.68 ± 0.87	10.48 ± 1.47	1.65 ± 0.92	14.50 ± 0.79	11.34 ± 1.11	47.62 ± 2.64	8.59 ± 0.84	15.92 ± 2.62	1.32 ± 0.13	9.83 ± 0.99	9.21 ± 0.90	95.29 ± 5.83	0.67
ALDH1A3	222168_at	NM_000693	3.23 ± 1.09	4.54 ± 0.44	3.03 ± 0.00	3.44 ± 0.53	14.43 ± 0.71	7.74 ± 0.59	-1.96 ± 5.80	3.14 ± 0.67	1.44 ± 0.42	3.84 ± 2.98	16.65 ± 2.44	14.42 ± 2.49	0.90
MERTK	206028_s_at	NM_006343	7.25 ± 1.06	0.67 ± 0.47	1.57 ± 0.60	1.59 ± 0.64	1.57 ± 0.60	2.06 ± 0.33	0.27 ± 2.25	1.94 ± 1.88	1.42 ± 0.14	NA ± -	-2.82 ± 5.86	0.92 ± 0.27	-0.30
CALB1	205625_s_at	NM_004929	-1.37 ± 3.35	0.48 ± 0.09	-1.27 ± 4.26	0.90 ± 0.04	-1.42 ± 0.14	0.53 ± 0.05	0.06 ± 2.55	0.38 ± 0.09	3.26 ± 0.32	1.68 ± 0.62	-1.80 ± 4.42	0.83 ± 1.17	0.20
SECTM1	213716_s_at	NM_003004	3.06 ± 0.60	3.07 ± 0.31	2.17 ± 0.93	4.28 ± 0.45	2.65 ± 0.26	7.48 ± 2.33	2.38 ± 0.12	6.55 ± 0.51	1.52 ± 0.15	5.97 ± 0.86	3.41 ± 0.83	10.94 ± 0.83	0.39
SGK	201739_at	NM_005627	-1.46 ± 0.07	0.61 ± 0.12	-1.41 ± 0.00	0.78 ± 0.16	-1.41 ± 0.00	0.62 ± 0.11	-1.94 ± 0.28	0.54 ± 0.02	-1.43 ± 0.28	0.86 ± 0.05	-2.93 ± 0.14	0.37 ± 0.04	0.83
CTGF	209101_at	NM_001901	-2.64 ± 0.00	0.48 ± 0.09	-1.63 ± 0.16	0.90 ± 0.04	-1.63 ± 0.16	0.53 ± 0.05	-2.81 ± 0.95	0.38 ± 0.09	-1.43 ± 0.28	1.05 ± 0.02	-3.56 ± 1.03	0.30 ± 0.13	0.85
PPP1R3C	204284_at	NM_005398	-1.47 ± 0.22	0.64 ± 0.08	1.11 ± 0.05	0.82 ± 0.08	-1.87 ± 0.00	0.52 ± 0.01	-1.28 ± 0.19	0.61 ± 0.14	-1.83 ± 0.44	0.33 ± 0.03	-3.26 ± 0.32	0.24 ± 0.06	0.89

NA= no amplification by RT-PCR

= only 5 donors with amplification considered for correlation analysis

Table 9B: Correlation of fold change (FC) on Hu-Gene 133A arrays/gene-chip (GC) and RT-PCR for a partial list of genes altered by SLR $\geq$ 1.5 in at least one of the 6 NHMECs when treated with pre-CHL followed by co-treatment. A more extensive version of this table can be found at (<http://www.cdc.gov/niosh/ext-supp-mat/MTP/index.htm>) [Table F].

Gene	Probe ID	Accession no.	Pre CHL + co-treatment vs vehicle control (avg. FC ± SD)												Correlation (r)
			1 (M98035)		2 (M99005)		3 (M98015)		4 (M98025)		5 (M99025)		6 (M99016)		GC-RT
			GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	PreCHL + co
AKR1C2	211653_x_at	NM_001354	3.04 ± 0.30	1.62 ± 0.13	1.74 ± 0.00	1.82 ± 0.06	2.07 ± 0.10	2.00 ± 0.16	5.10 ± 0.25	2.39 ± 0.08	2.08 ± 0.30	2.25 ± 0.22	2.22 ± 0.11	1.76 ± 0.48	0.50
CLDN4	201428_at	NM_001305	7.03 ± 1.37	4.87 ± 0.39	-6.06 ± 0.00	0.13 ± 0.01	1.88 ± 0.37	1.43 ± 0.08	15.13 ± 6.46	3.71 ± 0.14	-0.31 ± 2.59	0.46 ± 0.04	3.62 ± 0.53	0.35 ± 0.04	0.76
DHRS3	202481_at	NM_004753	1.80 ± 0.09	1.52 ± 0.17	-1.19 ± 0.06	0.96 ± 0.03	1.76 ± 0.34	2.17 ± 0.94	3.26 ± 0.32	1.53 ± 0.16	2.30 ± 0.00	3.35 ± 0.28	1.15 ± 0.00	1.52 ± 0.16	0.50
SOD2	216841_s_at	NM_000636	-1.15 ± 0.00	0.66 ± 0.02	-3.14 ± 0.15	0.38 ± 0.06	1.15 ± 0.00	1.28 ± 0.07	-1.33 ± 0.26	0.51 ± 0.05	-1.15 ± 0.11	0.87 ± 0.08	-1.81 ± 0.69	0.11 ± 0.02	0.83
CYR61	201289_at	NM_001554	-2.83 ± 0.00	0.23 ± 0.01	-1.11 ± 0.05	1.00 ± 0.05	-1.62 ± 0.00	0.53 ± 0.02	-2.30 ± 0.00	0.27 ± 0.02	-1.80 ± 0.09	0.62 ± 0.05	-0.22 ± 1.83	0.97 ± 0.15	0.92
SERPINB2	204614_at	NM_002575	9.52 ± 0.47	10.18 ± 1.55	-3.14 ± 0.15	0.25 ± 0.01	1.46 ± 0.07	1.75 ± 0.14	3.49 ± 0.34	2.20 ± 0.28	-4.00 ± 0.00	0.25 ± 0.01	4.46 ± 0.65	0.58 ± 0.03	0.81
MCM5	216237_s_at	NM_006739	-1.87 ± 0.18	0.33 ± 0.02	1.69 ± 0.25	1.84 ± 0.52	-1.52 ± 0.15	0.49 ± 0.02	-3.26 ± 0.32	0.23 ± 0.02	-0.07 ± 1.52	1.34 ± 0.32	-2.35 ± 0.68	1.08 ± 0.22	0.37
GADD45B	207574_s_at	NM_015675	-1.87 ± 0.18	0.30 ± 0.03	-1.23 ± 0.12	0.69 ± 0.07	-0.28 ± 1.91	0.79 ± 0.09	-3.38 ± 0.50	0.16 ± 0.03	-1.57 ± 0.08	0.83 ± 0.15	-1.37 ± 0.20	0.27 ± 0.02	0.71

A negative sign for FC by gene-chip (GC) refers to down-regulation of gene- expression by the corresponding value.

Table 10: Correlation of FC on gene-chip (GC) versus RT-PCR for a selected set of immune response genes altered by $SLR \geq 1.5$ in at least one of the 6 NHMECs on treatment with BP in the absence or presence of CHL

Gene	Cell strain	Treatment					
		BP versus control vehicle FC		Correlation (r)	Pre CHL + co versus control vehicle FC		Correlation (r)
		GC	RT-PCR	FC by GC vs RT	GC	RT-PCR	FC by GC vs RT
IL1B	M98035	2.00 ± 0.20	3.68 ± 0.56	0.97	3.03 ± 0.00	3.47 ± 3.03	0.88
	M99005	1.23 ± 0.00	1.68 ± 0.05		-1.46 ± 0.07	0.62 ± -1.46	
	M98015	2.83 ± 0.00	5.21 ± 0.73		3.73 ± 0.00	5.61 ± 3.73	
	M98025	2.02 ± 0.39	1.29 ± 0.05		2.55 ± 0.12	1.22 ± 2.55	
	M99025	1.94 ± 0.28	3.20 ± 0.51		1.32 ± 0.00	1.41 ± 1.32	
	M99016	5.89 ± 0.86	11.52 ± 1.20		5.89 ± 0.86	7.44 ± 5.89	
SECTM1	M98035	3.06 ± 0.60	2.87 ± 0.34	0.33	1.87 ± 0.00	0.65 ± 0.09	0.43
	M99005	2.17 ± 0.93	5.17 ± 1.15		1.64 ± 0.32	1.83 ± 0.29	
	M98015	2.65 ± 0.26	9.22 ± 1.19		1.88 ± 0.37	4.17 ± 0.53	
	M98025	2.38 ± 0.12	4.87 ± 0.96		0.05 ± 1.93	2.07 ± 0.70	
	M99025	1.52 ± 0.15	4.84 ± 0.77		1.23 ± 0.00	1.67 ± 0.18	
	M99016	3.41 ± 0.83	8.76 ± 0.56		3.49 ± 0.34	3.60 ± 0.50	
IL1RN	M98035	1.32 ± 0.00	1.60 ± 0.13	0.12	1.87 ± 0.00	1.33 ± 0.05	0.76
	M99005	1.04 ± 0.05	1.59 ± 0.16		-0.53 ± 1.55	0.53 ± 0.01	
	M98015	1.28 ± 0.06	1.77 ± 0.15		1.52 ± 0.15	1.47 ± 0.06	
	M98025	1.52 ± 0.00	0.93 ± 0.06		3.26 ± 0.32	1.39 ± 0.08	
	M99025	-1.15 ± 0.00	1.39 ± 0.16		-1.19 ± 0.06	1.01 ± 0.10	
	M99016	-0.17 ± 1.76	1.23 ± 0.44		2.25 ± 0.55	1.76 ± 0.36	
CD86	M98035	1.41 ± 0.00	1.66 ± 0.11	0.28	2.25 ± 0.55	1.64 ± 0.17	0.64
	M99005	1.19 ± 0.06	1.91 ± 0.11		-1.37 ± 0.07	0.73 ± 0.03	
	M98015	1.21 ± 0.29	1.57 ± 0.17		1.68 ± 0.08	2.28 ± 0.21	
	M98025	1.41 ± 0.00	1.17 ± 0.25		3.04 ± 0.30	1.62 ± 0.09	
	M99025	0.09 ± 1.75	1.94 ± 0.16		1.15 ± 0.11	1.13 ± 0.14	
	M99016	-0.12 ± 1.69	0.78 ± 0.25		1.83 ± 0.44	2.40 ± 1.05	
PI3	M98035	2.82 ± 0.60	1.43 ± 0.28	0.41	7.51 ± 0.87	5.63 ± 1.89	0.86
	M99005	1.54 ± 0.07	1.19 ± 0.06		0.48 ± 0.10	-2.08 ± 0.30	
	M98015	1.29 ± 0.09	1.11 ± 0.05		1.40 ± 0.10	1.37 ± 0.07	
	M98025	1.03 ± 0.08	1.42 ± 0.14		3.05 ± 0.17	5.47 ± 0.27	
	M99025	1.58 ± 0.97	0.05 ± 2.07		0.36 ± 0.13	-2.87 ± 1.22	
	M99016	2.95 ± 0.38	1.69 ± 0.25		5.44 ± 1.15	5.74 ± 3.19	
CXCL2	M98035	-1.93 ± 0.09	0.70 ± 0.09	0.79	-2.08 ± 0.30	0.26 ± 0.04	0.77
	M99005	-1.19 ± 0.06	1.62 ± 0.16		-2.38 ± 0.12	0.53 ± 0.06	
	M98015	1.46 ± 0.07	2.31 ± 0.19		1.04 ± 0.05	1.23 ± 0.12	
	M98025	0.00 ± 2.00	1.05 ± 0.55		-3.04 ± 0.30	0.17 ± 0.02	
	M99025	1.19 ± 0.06	1.97 ± 0.48		-1.37 ± 0.20	0.75 ± 0.04	
	M99016	1.21 ± 0.29	1.67 ± 0.29		1.23 ± 0.12	0.63 ± 0.02	
MX1	M98035	1.32 ± 0.13	1.34 ± 0.08	0.16	1.57 ± 0.60	0.55 ± 0.12	0.53
	M99005	1.19 ± 0.06	1.85 ± 0.37		-2.14 ± 0.00	0.19 ± 0.02	
	M98015	1.07 ± 0.11	0.96 ± 0.10		-4.46 ± 0.65	0.10 ± 0.04	
	M98025	1.99 ± 0.67	1.14 ± 0.10		1.28 ± 0.19	0.16 ± 0.04	
	M99025	0.12 ± 1.69	2.02 ± 0.46		-1.33 ± 0.12	0.51 ± 0.36	
	M99016	-1.80 ± 0.09	0.93 ± 0.32		-2.39 ± 0.91	0.20 ± 0.04	

4.2.2 *CYP1A1* and *CYP1B1* expression in 20 NHMECs on treatment with BP in the absence or presence of CHL

Wide inter-individual variations were observed both in the induction and modulation of *CYP1A1* (Table 11) and *CYP1B1* (Table 12) following treatment of 20 NHMECs with BP in the absence or presence of CHL. The basal expression of *CYP1A1* was highest in M98014 ($\Delta Ct = 11.18$) and lowest in M99005 ($\Delta Ct = 16.61$). The basal expression of *CYP1B1* was highest in M99016 ($\Delta Ct = 4.19$) and lowest in M98015 ($\Delta Ct = 9.77$). Overall, the average basal expression of *CYP1A* in the 20 NHMECs ($\Delta Ct = 13.44$) was 53 fold lower than that of *CYP1B1* ($\Delta Ct = 7.72$). The basal expression in terms of ΔCt for the 20 NHMECs is presented in Table 13.

All CHL treatments caused mitigation of *CYP1A1* and *CYP1B1* expression in 18/20 cell strains (4-88% reduction across the different CHL treatments) and 17/20 cell (4-83% reduction across the different CHL treatments) strains respectively, compared to those treated with BP alone.

CHL co-treatment mitigated *CYP1A1* and *CYP1B1* expression in all 20 cell strains. *CYP1A1* expression in the presence of CHL co-treatment ranged from 2-62 fold (4-75% reduction compared to cells treated with BP alone) while that of *CYP1B1* ranged from 1-36 fold (4-67% reduction).

Pre CHL + post BP treatment enhanced BP induced *CYP1A1* expression in 2/20 cell strains (3% and 81% in M98011 and M98014, respectively) while decreasing it in the remaining 18/20 cell strains (11-76% reduction). *CYP1B1* expression was also enhanced by pre CHL + post BP treatment in 3/20 cell strains (30%, 20% and 11% in M98019, M98011 and M99006, respectively) while decreasing it in the remaining 17/20 cell strains (8-60% reduction).

Table 11: Inter-individual variations in the induction and modulation of *CYP1A1* upon exposure of NHMECs to either BP alone or in combination with CHL

	<i>CYP1A1</i>			
	<i>Treatment</i>			
	1	2	3	4
Cell strain				
M98019	3.62 ± 1.04	1.85* ± 0.53	3.03 ± 0.63	2.38 ± 0.83
M98014	4.57 ± 1.52	2.79 ± 0.25	8.25 ± 1.91	5.90 ± 0.91
M98011	5.36 ± 1.98	5.12 ± 0.99	5.50 ± 0.56	4.99 ± 2.63
M99021	5.90 ± 1.46	3.73 ± 1.61	3.75* ± 0.36	3.59* ± 0.52
M99006	6.46 ± 1.54	4.21 ± 1.01	5.31 ± 0.61	3.49 ± 0.80
M99025	6.58 ± 2.94	4.06 ± 1.59	2.62 ± 0.48	1.65* ± 0.46
M00004	7.07 ± 2.53	4.02 ± 0.48	4.35 ± 0.81	3.79 ± 1.41
M98025	11.22 ± 3.22	6.01* ± 2.61	9.76 ± 1.62	6.94 ± 0.65
M98035	13.83 ± 2.89	12.82 ± 2.01	9.70 ± 1.93	5.29* ± 1.58
M99005	13.69 ± 1.35	7.89* ± 1.69	8.14* ± 1.11	4.11* ± 1.37
M99003	14.27 ± 5.02	10.21 ± 2.29	11.49 ± 1.60	13.02 ± 3.96
M00012	17.09 ± 2.89	11.00 * ± 1.72	12.19* ± 2.80	9.82* ± 2.68
M99004	20.25 ± 2.06	10.78* ± 3.96	10.54* ± 3.42	11.55* ± 1.58
M98016	32.50 ± 3.40	9.99* ± 2.25	13.2* ± 1.35	6.09* ± 2.02
M99015	35.88 ± 4.71	20.75* ± 3.02	31.78 ± 2.69	29.91 ± 9.71
M98030	44.32 ± 12.41	18.54* ± 1.53	25.50* ± 2.09	15.97* ± 4.05
M98015	49.95 ± 6.64	44.79 ± 12.10	42.59 ± 10.52	47.42 ± 9.35
M98026	52.71 ± 12.93	38.99 ± 10.16	31.34* ± 11.54	30.27* ± 5.99
M98013	66.72 ± 12.10	16.36* ± 6.40	14.72* ± 6.90	8.03* ± 4.95
M99016	95.84 ± 9.86	62.36* ± 20.75	62.36* ± 4.82	53.54* ± 5.38

Treatments - 1-BP alone, 2-co-treatment, 3-Pre CHL+ post BP treatment, 4-Pre CHL + co-treatment (Details of treatments in Materials and methods)

Numbers shown represent fold change ± standard deviation (n = 2) as measured by RT-PCR

* = statistically significant reduction in expression ($p \leq 0.05$) compared to expression induced by BP alone

Table 12: Inter-individual variations in the induction and modulation of *CYP1B1* upon exposure of NHMECs to either BP alone or in combination with CHL

	<i>CYP1B1</i>			
	<i>Treatment</i>			
	1	2	3	4
Cell strain				
M98019	6.09 ± 0.38	4.40 ± 1.95	7.90 ± 1.00	6.77 ± 1.79
M98014	3.49 ± 0.58	2.92 ± 0.90	1.93* ± 0.18	1.86* ± 0.25
M98011	4.74 ± 0.86	3.69 ± 0.63	5.69 ± 0.90	6.32 ± 1.53
M99021	8.75 ± 1.41	5.78* ± 1.01	6.27* ± 1.20	5.46* ± 0.86
M99006	8.92 ± 1.57	5.54* ± 0.44	9.88 ± 3.36	4.80* ± 0.76
M99025	22.86 ± 3.92	11.35 ± 3.09	9.19 ± 1.15	6.50 ± 1.26
M00004	7.92 ± 2.62	5.10 ± 0.54	7.31 ± 2.91	4.53 ± 1.95
M98025	8.41 ± 2.23	4.42* ± 1.81	4.81* ± 1.50	3.88* ± 0.54
M98035	9.46 ± 1.89	7.97 ± 1.84	7.31 ± 1.75	3.32 ± 0.40
M99005	9.16 ± 1.78	7.48 ± 0.42	5.82* ± 1.65	3.49* ± 1.30
M99003	19.66 ± 1.86	13.02* ± 1.22	15.27* ± 0.97	16.48 ± 5.15
M00012	17.54 ± 3.14	10.32* ± 1.65	14.67 ± 4.43	11.90* ± 3.29
M99004	12.13 ± 1.25	7.67* ± 1.34	6.96* ± 0.72	6.89* ± 0.83
M98016	14.65 ± 2.98	6.16* ± 0.61	10.04 ± 5.87	3.86* ± 1.12
M99015	33.88 ± 1.73	13.50* ± 2.25	22.59 ± 11.19	19.94* ± 3.73
M98030	16.00 ± 4.12	7.01* ± 1.35	6.45* ± 0.99	4.89* ± 1.77
M98015	43.41 ± 4.80	35.88* ± 3.30	39.74* ± 9.32	39.26 ± 6.89
M98026	31.67 ± 3.34	21.08* ± 1.69	24.21 ± 8.40	23.55 ± 6.40
M98013	42.01 ± 6.89	13.95* ± 1.46	11.49* ± 1.88	7.00* ± 3.24
M99016	8.94 ± 1.39	8.60 ± 1.61	8.27 ± 1.08	6.92 ± 1.26

Treatments - 1-BP alone, 2-co-treatment, 3-Pre CHL+ post BP treatment, 4-Pre CHL + co-treatment (Details of treatments in Materials and methods)

Numbers shown represent fold change ± standard deviation (n = 2) as measured by RT-PCR

* = statistically significant reduction in expression ($p \leq 0.05$) compared to expression induced by BP alone

Table 13: Basal *CYP1A1* and *CYP1B1* expression levels (ΔCt) in the 20 NHMECs normalized to that of GAPDH

Basal expression levels (ΔCt)		
Cell strain	<i>CYP1A1</i>	<i>CYP1B1</i>
M98019	15.39	8.67
M98014	11.18	6.75
M98011	12.92	7.80
M99021	11.30	7.62
M99006	12.26	8.32
M99025	15.05	8.63
M00004	12.26	7.03
M98025	12.10	6.35
M98035	15.05	7.48
M99005	16.61	6.06
M99003	13.48	8.70
M00012	12.78	7.92
M99004	12.46	7.65
M98016	13.43	7.60
M99015	12.34	9.29
M98030	15.60	6.62
M98015	12.18	9.77
M98026	12.99	8.81
M98013	13.93	8.84
M99016	15.86	4.19
Average	13.46	7.71

Pre CHL + post co-treatment enhanced *CYP1A1* expression in 1/20 cell strains (29% in M98014) while decreasing it in the remaining 19/20 cell strains (5-88% reduction). *CYP1B1* expression was enhanced in 2/20 cell strains (11% and 33% in M98019 and M98011 respectively) while decreasing it in the remaining 18/20 cell strains (10-83% reduction).

There was a poor correlation between *CYP1A1* and *CYP1B1* induction on treatment with BP alone ($r^2 = 0.28$) and by pre CHL + post BP treatment ($r^2 = 0.29$) but a moderate correlation on co-treatment ($r^2 = 0.41$) and pre CHL + post co-treatment ($r^2 = 0.47$) (Figure 13). The correlation between the percentage modulation in the expression of *CYP1A1* to that of *CYP1B1* on co-treatment as well as pre CHL + post co-treatment was moderate ($r^2 = 0.46, 0.41$, respectively) and absent on treatment with pre CHL + post BP ($r^2 = 0.09$) (Figure 14).

This was mainly due to the presence of several outliers in pre CHL + post BP treatment (M98011 and M98014 for *CYP1A1* and M98019, M98011 and M99006 for *CYP1B1*) and pre CHL + post co-treatment groups (M98014 for *CYP1A1* and M98019 and M98011 for *CYP1B1*). Removal of these outliers from the analysis significantly improved the correlation between the percentage modulation in the expression of *CYP1A1* to that of *CYP1B1* upon pre CHL + post co-treatment ($r^2 = 0.73$). However, the correlation between the modulation of *CYP1A1* to that of *CYP1B1* on pre CHL + post BP treatment improved only marginally ($r^2 = 0.34$).

The correlation (r^2) between *CYP1A1* expression by BP alone and in the presence of different CHL treatments was 0.78, 0.78 and 0.64 for co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively (Figure 15). The correlation (r^2) between *CYP1B1* expression by BP alone and in the presence of different CHL treatments was 0.72, 0.66 and 0.57 for co-treatment, pre CHL + post BP and pre CHL + post co-treatment, respectively (Figure 16).

Figure 13: Correlation of *CYP1A1* expression to that of *CYP1B1* in the 20NHMECs

% modulation of *CYP1B1* expression by different treatments

Figure 14: Correlation between the percentage modulation in the expression of *CYP1A1* to that of *CYP1B1*

CYP1A1 expression by different CHL treatments

Figure 15: Correlation of *CYP1A1* expression in the absence and presence of CHL

CYP1B1 expression by different CHL treatments

Figure 16: Correlation of *CYP1B1* expression in the absence and presence of CHL

4.2.3 *CYP1A1* and *CYP1B1* expression in 2 NHMECs on treatment with BP or B[e]P in the absence or presence of CHL

With respect to studies on comparison of BP versus B[e]P in the induction of *CYP1A1* and *CYP1B1* wide ‘inter’ cell strain differences were observed in the induction and modulation of both *CYP1A1* and *CYP1B1* in both M98015 and M98025 on treatment with BP or B[e]P in the absence or presence of CHL (Figures 17 and 18).

The induction of both *CYP1A1* and *CYP1B1* by BP was comparatively higher in M98015 than M98025 (35 fold versus 10 fold for *CYP1A1* and 23 fold versus 5 fold for *CYP1B1*). Though B[e]P was a very poor inducer of both *CYP1A1* and *CYP1B1* in M98015 (2 fold and 1 fold, respectively) and M98025 (0.7 fold and 1 fold, respectively) compared to BP, the induction of *CYP1A1* by B[e]P was slightly higher in M98015 compared to M98025 (2 fold versus 0.7 fold). The induction *CYP1B1* by B[e]P, however, showed little difference between M98015 and M98025.

CHL co-treatment caused a statistically significant decrease of BP induced *CYP1A1* expression in M98015 and M98025 (30% and 37% respectively, $p < 0.05$ for both). CHL co-treatment also caused a decrease of BP induced *CYP1B1* expression in both M98015 and M98025 (42% and 21%, respectively) with the reduction in M98015 being statistically significant ($p < 0.05$). In the case of B[e]P, all CHL co-treatments caused an increase in the expression of B[e]P induced *CYP1A1* and *CYP1B1* in both M98015 (148%, $p < 0.005$ and 219%, $p = 0.005$, respectively) and M98025 (75%, $p < 0.005$ and 132%, $p < 0.05$ respectively).

Pre CHL + post BP treatment did not mitigate BP induced expression of *CYP1A1* in M98015 (0.17% increase) but did mitigated it in M98025 (29%). The expression of BP induced *CYP1B1* was reduced only slightly in M98015 (5%) but significantly in M98025

(50%, $p < 0.05$). Pre CHL + post BP treatment caused a mitigation of B[e]P induced *CYP1A1* and *CYP1B1* expression in M98015 (3% and 4%, respectively) and M98025 (19% and 54%, respectively).

Pre CHL + co-treatment did not modulate the expression of BP induced *CYP1A1* in M98015 but caused a statistically significant decrease in the expression of *CYP1A1* in M98025 (40%, $p < 0.05$). The expression of *CYP1B1* was significantly reduced in both M98015 and M98025 (35% and 47% respectively, $p < 0.05$ for both). There was a statistically significant increase in the expression of B[e]P induced *CYP1A1* in M98015 (155%, $p < 0.005$) and M98025 (112%, $p < 0.05$) upon pre CHL + co-treatment. The expression of B[e]P induced *CYP1B1* was increased in M98015 (62%, $p < 0.05$) but decreased in M98025 (25%).

The correlation (r^2) of BP induced *CYP1A1* expression to that of *CYP1B1* expression across all treatments for M98015 and M98025 was 0.47 and 0.54, respectively. The correlation (r^2) of B[e]P induced *CYP1A1* expression to that of *CYP1B1* expression across all treatments for M98015 and M98025 was 0.97 and 0.71, respectively.

Figure 17: Comparison of *CYP1A1* expression by BP or B[e]P in (A) M98015 and (B) M98025 in the absence or presence of CHL.

Treatments: 1= BP alone, 2 = co-treatment, 3 = Pre CHL + post BP treatment, 4 = Pre CHL + post co-treatment.

* indicates a statistically significant reduction in *CYP1A1* expression compared to BP alone

Figure 18: Comparison of *CYP1B1* expression by BP or B[e]P in (A) M98015 and (B) M98025 in the absence or presence of CHL.
 Treatments: 1= BP alone, 2 = co-treatment, 3 = Pre CHL + post BP treatment, 4 = Pre CHL + post co-treatment.
 * indicates a statistically significant reduction in *CYP1B1* expression compared to BP alone

4.2.4 *CYP1A1* and *CYP1B1* expression in MCF-7 and M00012 on treatment with BP in the absence or presence of CHL

Comparison of MCF-7 with a NHMEC (M00012) across various CHL treatments (as detailed in Table 3 of Materials and Methods) revealed that though basal *CYP1A1* expression was comparable for both MCF-7 and M00012 ($\Delta Ct = 12.8$ for both), *CYP1B1* expression was much higher in MCF-7 ($\Delta Ct = 4.37$) as compared to M00012 ($\Delta Ct = 7.92$). The basal expression level of *CYP1B1* in MCF-7 cells was 348 times that of *CYP1A1* whereas that of M00012 was only 29 times that of *CYP1A1*. The basal expression of *CYP1B1* in MCF-7 was nearly 12 times that in M00012. The induction of *CYP1A1* by BP alone was 114 fold whereas that of *CYP1B1* was only 5 fold in MCF-7. All CHL treatments caused an increase of BP induced *CYP1A1* (16%, 22% and 23% increase on co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively) and *CYP1B1* (11%, 12% and 33% increase on co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment respectively) expression. In the case of M00012, all CHL treatments caused a mitigation of BP induced *CYP1A1* (36%, 29% and 43% decrease on co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively) and *CYP1B1* (41%, 16% and 32% decrease on co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively).

The overall correlation of *CYP1A1* expression to that of *CYP1B1* expression across all treatments was moderate for MCF-7 and good for M00012 ($r^2 = 0.61$ and 0.80 for MCF-7 and M00012, respectively).

A comparison of the extent of *CYP1* expression between MCF-7 and 20 NHMECs used in the above studies was also carried out in order to have a comparison against response

patterns of a broad panel of cell strains. Such a comparison against a broad panel would take into account inter-cell strains differences in induction and modulation. The basal *CYP1A1* and *CYP1B1* expression in MCF-7 was higher than the average basal *CYP1A1* and *CYP1B1* expression across all the 20 NHMECs ($\Delta C_t = 12.81$ and 4.37 for *CYP1A1* and *CYP1B1* in MCF-7 versus 13.46 and 7.71 for *CYP1A1* and *CYP1B1* in the 20 NHMECs). This corresponds to a 2 fold higher expression of *CYP1A1* and a 10 fold higher expression level of *CYP1B1* in MCF-7 versus the 20 NHMECs. The average basal *CYP1B1* expression in the 20 NHMECs was 53 fold that of *CYP1A1*. The expression patterns of *CYP1A1* and *CYP1B1* on treatment of the 20 NHMECs with BP alone or in the presence of different CHL treatments are presented in Tables 11 and 12, respectively. The induction of *CYP1A1* by BP alone in MCF-7 (114 fold) was much higher than even the highest inducer in the 20 NHMECs (96 fold in M99016). This held true for even the other CHL treatments (co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment). With regard to *CYP1B1* expression, the expression in MCF-7 by BP in the absence and presence of CHL was in the range of the 20 NHMECs. Figures 19 and 20 depict the expression patterns of *CYP1A1* and *CYP1B1*, respectively in MCF-7 and M00012 across the different treatment groups. In each case the inset shows the expression pattern of MCF-7 in comparison with the other 20 NHMECs.

Figure 19: (A) *CYP1A1* expression patterns on treatment of MCF-7 cells or M00012 (a NHMEC) with BP in the absence or presence of CHL. (B) *CYP1A1* expression patterns in MCF-7 versus 20 NHMECs. The expression of *CYP1A1* in MCF-7 cells is much higher and different compared to the NHMECs.

Treatments: 1= BP alone, 2 = co-treatment, 3 = Pre CHL + post BP treatment, 4 = Pre CHL + post co-treatment.

Figure 20: (A) *CYP1B1* expression patterns on treatment of MCF-7 cells or M00012 (a NHMEC) with BP in the absence or presence of CHL. (B) *CYP1B1* expression patterns in MCF-7 versus 20 NHMECs. The expression of *CYP1B1* in MCF-7 cells was in the range of the NHMECs.

Treatments: 1= BP alone, 2 = co-treatment, 3 = Pre CHL + post BP treatment, 4 = Pre CHL + post co-treatment.

4.2.5 *CYP1A1* and *CYP1B1* expression in MCF-7 and M00012 on treatment with BP in the absence or presence of different CHL concentrations

With regard to the effect of a range of CHL concentrations on BP induced *CYP1A1* and *CYP1B1* expression in MCF-7 cells, BP induced *CYP1A1* expression 38 fold while that of *CYP1B1* was induced only 3 fold. Pre CHL + post co-treatment enhanced BP induced *CYP1A1* expression of cells at low concentrations of CHL (2 μ M, 3 μ M and 4 μ M) in MCF-7 cells. The induction of *CYP1A1* was 100 fold, 260 fold and 48 fold on pre-treatment with CHL 2 μ M, 3 μ M or 4 μ M, respectively followed by co-treatment. However, higher concentrations of CHL (8 μ M and 16 μ M) mitigated BP induced *CYP1A1* expression on pre-treatment with CHL followed by the respective post co-treatment, though there did not appear to be a concentration dependent response. The fold induction of *CYP1A1* on pre 8 μ M CHL + post co-treatment and pre 16 μ M CHL + post co-treatment was 24 fold and 36 fold, respectively. The expression of *CYP1B1* however was not altered across the different CHL concentrations in MCF-7. All CHL concentrations caused a slight increase of BP induced *CYP1B1* expression. *CYP1B1* expression on treatment of MCF-7 cells with BP alone and in the presence of 2 μ M, 3 μ M, 4 μ M, 8 μ M and 16 μ M CHL in a pre CHL + post co-treatment were 3, 3, 4, 4, 4 and 3 fold, respectively.

In the case of M00012, highest *CYP1A1* expression was in the group treated with BP alone (23 fold) while all CHL treatments except pre 8 μ M + post co-treatment mitigated BP induced *CYP1A1* expression to different extents (21, 13, 19, 23 and 2 fold on pre-treatment with 2 μ M, 3 μ M, 4 μ M, 8 μ M and 16 μ M CHL followed by co-treatment, respectively). With respect to *CYP1B1* expression, again there did not appear to be a concentration dependent response. The induction of *CYP1B1* by BP was 8 fold whereas the induction in the

presence of 2 μ M, 3 μ M, 4 μ M, 8 μ M and 16 μ M CHL pre-treatment followed by co-treatment was 10, 5, 3, 16 and 3 fold, respectively. The correlation (r^2) of *CYP1A1* expression to *CYP1B1* expression by BP in the absence or presence of a range of CHL concentrations in MCF-7 cells was 0.24 while that in M00012 was 0.41. The expression patterns of *CYP1A1* or *CYP1B1* in MCF-7 and M00012 by BP in the absence or presence of a range of CHL concentrations are shown in Figures 21 and 22, respectively.

Figure 21: *CYP1A1* expression patterns in MCF-7 and M00012 by BP in the absence or presence of a range of CHL concentrations.

Figure 22: *CYP1B1* expression patterns in MCF-7 and M00012 by BP in the absence or presence of a range of CHL concentrations.

4.3 DNA adducts

4.3.1 BP-DNA adducts in 20 NHMECs on treatment with BP in the absence or presence of CHL

The levels of BP-DNA adducts across the 20 NHMECs exhibited considerable inter-individual variation with highest levels being in the BP treated group (Table 14). All CHL treatments lowered BP-DNA adducts in most cell strains except in M99021 (which exhibited an increase in adduct levels upon co-treatment and pre CHL + post BP treatment) and M99025 (which showed no reduction in adduct levels upon CHL co-treatment). The number of BP-DNA adducts in the BP treated group ranged from 2.5 adducts/ 10^8 nucleotides (in M99005) to 57.5 adducts/ 10^8 nucleotides (M99015).

CHL co-treatment caused a decrease in BP-DNA adduct levels in 18/20 cell strains (13-55% reduction) while increasing it in M99021 (5% increase) and having no effect in M99025. The average reduction in BP-DNA adducts by CHL co-treatment was 33%. Pre CHL + post BP treatment caused a reduction in BP- DNA adduct levels in 19/20 cell strains (3-67%) while increasing it in M99021 (12% increase). The average reduction in BP-DNA adducts by CHL pre-treatment followed by post BP treatment was 28%. Pre CHL + post co-treatment caused a reduction in BP-DNA adducts in all cell strains (28-87%). The average reduction in BP-DNA adducts by CHL pre-treatment followed by post co- treatment was 53%. As evident, the maximum reduction in BP-DNA adducts for most cell strains was by pre CHL + post co-treatment except in M99015, M98019 and M99005.

There was a good correlation between the levels of BP-DNA adducts in the presence of BP alone and by CHL co-treatment ($r^2=0.90$) as well as pre CHL + post BP treatment ($r^2= 0.89$). However, their correlation in the presence of pre CHL + post co-was only moderate ($r^2= 0.68$).

Table14: Inter-individual variations in BP-DNA adduct formation on exposure of NHMECs to either BP alone or in combination with CHL

Cell strain	BP-DNA adducts/10 ⁸ nt.			
	Treatment			
	1	2	3	4
M99005	2.45 ± 1.34	1.25 ± 1.20	2.20 ± 2.26	1.50 ± 1.70
M99025	3.15 ± 0.64	3.15 ± 0.21	2.50 ± 1.27	1.35 ± 0.07
M98014	4.67 ± 0.61	2.24* ± 0.35	2.87* ± 0.15	2.10* ± 0.73
M99004	4.75 ± 0.49	2.75 ± 0.64	2.30 ± 0.99	1.90 ± 0.85
M98019	5.09 ± 0.94	3.05* ± 1.29	3.22* ± 0.81	3.59 ± 1.11
M98035	5.85 ± 3.89	2.70 ± 1.13	5.70 ± 1.56	2.65 ± 1.34
M99003	7.25 ± 1.48	3.30 ± 0.28	3.45 ± 1.20	1.30 ± 0.42
M98015	8.30 ± 4.95	5.50 ± 3.25	5.45 ± 4.03	4.30 ± 4.10
M98030	9.47 ± 1.52	8.12 ± 3.26	5.53 ± 1.78	4.00 ± 1.63
M99021	13.66 ± 3.16	14.29 ± 1.49	15.32 ± 3.76	10.21 ± 5.40
M98025	15.10 ± 2.40	5.10 ± 0.57	4.95 ± 1.77	3.70* ± 2.40
M99006	17.32 ± 3.70	13.59 ± 3.25	15.49 ± 5.75	12.56 ± 4.14
M98011	20.83 ± 13.03	15.33 ± 13.06	17.84 ± 9.19	11.73 ± 9.25
M00012	21.0 ± 4.53	14.65 ± 1.77	16.35 ± 3.46	10.85 ± 3.32
M98013	23.13 ± 1.96	14.45* ± 2.75	12.18* ± 1.23	3.00* ± 0.91
M00004	33.41 ± 6.12	27.05 ± 13.72	28.33 ± 9.63	19.83 ± 12.34
M99016	34.00 ± 3.39	23.85 ± 3.61	25.45 ± 2.62	12.50* ± 1.41
M98016	53.38 ± 2.52	26.05* ± 2.32	25.59* ± 7.00	7.04* ± 1.37
M98026	54.46 ± 29.74	47.29 ± 26.00	42.49 ± 21.40	32.49 ± 18.19
M99015	57.49 ± 16.23	34.99 ± 15.88	51.40 ± 12.08	35.43 ± 10.79

Treatments - 1-BP alone, 2-co-treatment, 3-Pre CHL+ post BP treatment, 4-Pre CHL + co-treatment (Details of treatments in Materials and methods)

Numbers shown represent fold change ± standard deviation (n = 2) as measured by CIA

* = statistically significant reduction in expression (p ≤ 0.05) compared to expression induced by BP alone

4.3.2 BP-DNA and B[e]P-DNA adducts in 2 NHMECs on treatment with BP or B[e]P in the absence or presence of CHL

In the comparison between M98015 and M98025 on treatment with BP or B[e]P in the absence or presence of CHL, both cell strains had the highest number of BP-DNA adducts in cells treated with BP alone (8.3 and 15.1 adducts/ 10^8 nucleotides in M98015 and M98025, respectively). All CHL treatments reduced the levels of BP-DNA adducts in M98015 (34%, 34% and 48% reduction for co-treatment, pre CHL + post BP and pre CHL + post co-treatment, respectively) and M98025 (66%, 67% and 76% for co-treatment, pre CHL + post BP and pre CHL + post co-treatment, respectively). B[e]P-DNA adducts were not detectable by synchronous fluorescence spectrometry in B[e]P as well as the CHL treatment groups (B[e]P co-treatment, pre CHL + post B[e]P treatment and pre CHL + post B[e]P co-treatment) in both M98015 and M98025. Benzo(a)pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol was used as a standard to construct the standard curve and the synchronous spectra of one of the dilutions is shown in Figure 23. Similarly, the synchronous spectra of the B[e]P treated cells in M98015 and M98025 are shown in Figure 24.

Figure 23: Synchronous fluorescence spectra obtained from standard benzo(a)pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol. The spectra shown here was generated using 80fM of the standard

Figure 24: Synchronous spectra of the B[e]P treated cells (A) M98015 and (B) M98025

4.3.3 BP-DNA adducts in MCF-7 and M00012 or 20 NHMECs on treatment with BP in the absence or presence of CHL

Comparison of adducts formed in MCF-7 versus M00012 (NHMEC) following treatment with BP alone or in the presence of CHL, MCF-7 exhibited adduct levels greater than 25 orders of magnitude of that of M00012 for all the treatment groups (BP alone, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment). 609 BP-DNA adducts / 10^8 nucleotides were detected in MCF-7 on treatment with BP alone as opposed to only 21 BP-DNA adducts / 10^8 nucleotides in M00012. None of the CHL treatments mitigated BP-DNA adduct levels in MCF-7 (3% reduction on co-treatment, 17 and 6% increase, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively). M00012 on the other hand exhibited a reduction of BP-DNA adduct levels in all CHL treatment groups (30%, 22% and 48% reduction on co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively).

In comparison with 20 NHMECs, MCF-7 cells exhibited adduct levels greater than 10 orders of magnitude than the highest number of adducts formed in the 20 NHMECs. This was true across all treatment groups (BP, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment). The average reduction of BP-DNA adducts across different CHL treatments among the 20 NHMECs were 33%, 28% and 53% on co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively as opposed to no mitigation in BP-DNA adduct levels in MCF-7 across the different CHL treatments. The adduct levels in MCF-7 and M00012 on treatment with BP in the absence or presence of CHL are presented in Figure 25.

Figure 25: BP-DNA adduct levels/ 10^8 nucleotides in MCF-7 and M00012 on treatment with BP in the absence or presence of different CHL treatments. The inset on the right shows the BP-DNA adduct levels in MCF-7 versus that in 20 NHMECs. Treatments: 1= BP alone, 2 = co-treatment, 3 = Pre CHL + post BP treatment, 4 = Pre CHL + post co-treatment.

4.3.4 BP-DNA adducts in MCF-7 and M00012 on treatment with BP in the absence or presence of different CHL concentrations

When compared across a range of CHL concentrations, CHL did not imitate the level of BP-DNA adducts across different CHL concentrations in MCF-7 cells. There was no concentration dependent response and even the highest concentration of CHL (16 μ M) did not cause any reduction in the level of BP-DNA adducts. However, there was concentration dependent reduction in the levels of BP-DNA adducts in M00012 for all CHL concentrations except for pre-treatment with 2 μ M CHL followed by co- treatment (8% increase compared to cells treated with BP alone). The reduction in BP-DNA adduct levels across all the other CHL treatments were 19%, 25%, 36% and 66% for pre-treatment with 3 μ M, 4 μ M, 8 μ M and 16 μ M CHL followed by co-treatment respectively. The reduction in adduct levels at the higher concentrations of CHL (8 μ M and 16 μ M) was significant ($p < 0.05$). The levels of BP-DNA adducts in MCF-7 were more than 13 fold that in M00012 for BP at the lower concentrations of CHL (BP, 2 μ M and 3 μ M CHL pre-treatment followed by co-treatment), more than 24 fold for both 4 μ M and 8 μ M CHL pre-treatment followed by co-treatment) and 82 fold for the highest concentration of CHL (pre-treatment with CHL 16 μ M followed by co-treatment). The response patterns of MCF-7 and M00012 across a range of CHL concentrations are shown in Figure 26.

Figure 26: BP-DNA adduct levels/10⁸ nucleotides in MCF-7 and M00012 on treatment with BP in the absence or presence of a range of CHL concentrations. Inset shows a clearer picture of the response pattern of M00012.

* indicates statistically significant reduction compared to cells treated with BP alone
Treatments: 1= BP alone, 2 = Pre 2 μ M CHL + post co-treatment, 3 = Pre 3 μ M CHL + post co-treatment, 4 = Pre 4 μ M CHL + post co-treatment, 5 = Pre 8 μ M CHL + post co-treatment, 6 = Pre 16 μ M CHL + post co-treatment

4.4 Correlation of *CYP1* gene expression to BP-DNA adducts

4.4.1 Correlation of cell strain origin with *CYP1* gene expression and DNA adduct formation

To examine the existence of a relationship, if any, between age of the donors and inter-individual variability in the induction of *CYP1* enzymes and DNA adduct formation, a correlation analysis of age versus the *CYP1* expression and DNA adduct levels was carried out across the 20 NHMECs used in the study. There was no correlation between age of the donors and *CYP1A1* expression ($r^2 = 0, 0.02, 0.02$ and 0.07 for BP, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively), *CYP1B1* expression ($r^2 = 0.04, 0.15, 0.13$, and 0.22 for BP, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively) and BP-DNA adduct formation ($r^2 = 0.10, 0.11, 0.10$ and 0.07 for BP, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment, respectively).

4.4.2 Correlation of *CYP1* expression to DNA adducts in 20 NHMECs on treatment with BP in the absence or presence of CHL

In order to understand the contribution and relevance of the Ahr mechanism to BP metabolism, a correlation of *CYP1A1* and/or *CYP1B1* expression to the level of BP-DNA adducts was carried out. Also, the correlation (r^2) of *CYP1A1* expression in the presence of BP alone, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment to the levels of BP-DNA adducts by the corresponding treatments was $0.19, 0.16, 0.16$ and 0.13 , respectively. Similarly, the correlation (r^2) of *CYP1B1* expression in the presence of BP alone, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment to the

levels of BP-DNA adducts by the corresponding treatments were 0.09, 0.03, 0.11 and 0.12, respectively. Also, since both *CYP1A1* and *CYP1B1* are involved in the metabolism of BP, a correlation analysis of the extent of *CYP1A1* + *CYP1B1* expression by BP alone, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment to the levels of BP-DNA adducts by the corresponding treatments was also carried out. The correlation (r^2) *CYP1A1* + *CYP1B1* expression on treatment with BP alone, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment to the levels of BP-DNA adducts by the corresponding treatments were 0.17, 0.13, 0.18 and 0.15, respectively. The basal expression of *CYP1B1* was higher than that of *CYP1A1*.

4.4.3 Correlation of *CYP1* expression to DNA adducts in 2 NHMECs on treatment with BP or B[e]P in the absence or presence of CHL

In a comparative study between BP and B[e]P across 2 NHMECs (M98015 and M98025), there was no correlation between BP induced *CYP1A1* expression and the level of BP-DNA adducts in the absence and presence of CHL in M98015 ($r^2 = 0.02$). However, there was a very good correlation in M98025 ($r^2 = 0.94$). BP induced *CYP1B1* expression in the absence and presence of CHL correlated moderately with the level of BP-DNA adducts in M98015 ($r^2 = 0.47$) and well in M98025 ($r^2 = 0.74$). Correlation of BP induced *CYP1A1* + *CYP1B1* expression with the level of BP-DNA adducts was poor ($r^2 = 0.20$).

Although B[e]P induced a low level of *CYP1* expression, as mentioned in section 4.3.2 it did not form a detectable level of DNA adducts.

4.4.4 Correlation of *CYP1* gene expression to DNA adducts in MCF-7 and M00012 or 20 NHMECs on treatment with BP in the absence or presence of CHL

Comparison of *CYP1* expression with the level of BP-DNA adducts in MCF-7 versus M00012 across different treatments (BP, co-treatment, pre CHL + post BP treatment and pre CHL + post co-treatment) showed a poor correlation for *CYP1A1* expression versus BP-DNA adducts ($r^2 = 0.33$). There was no correlation between *CYP1B1* expression versus BP-DNA adducts ($r^2 = 0.06$). The expression of *CYP1A1* + *CYP1B1* across all treatment groups versus BP-DNA adducts by the corresponding treatments was 0.32 and 0.92 for MCF-7 and M00012, respectively. When compared against all the 20 NHMECs, the correlation (r^2) of *CYP1A1* with BP-DNA adducts and *CYP1B1* with BP-DNA adducts across all treatment groups were 0.20 and 0.12, respectively. The expression of *CYP1A1* + *CYP1B1* across all treatment groups versus BP-DNA adducts by the corresponding treatments for all 20 NHMECs was 0.21.

4.4.5 Correlation of *CYP1* gene expression to DNA adducts in MCF-7 and M00012 on treatment with BP in the absence or presence of different CHL concentrations

The correlation of *CYP1A1* expression versus levels of BP-DNA adducts in MCF-7 cells on treatment with BP in the absence or presence of different CHL concentrations was poor ($r^2 = 0.16$) but moderate in M00012 ($r^2 = 0.54$). There was no correlation between *CYP1B1* expression versus levels of BP-DNA adducts in both MCF-7 cells ($r^2 = 0.02$) and M00012 ($r^2 = 0.07$). The overall correlation of *CYP1A1* + *CYP1B1* expression to DNA adducts was poor for both MCF-7 ($r^2 = 0.16$) and M00012 ($r^2 = 0.37$).

5.0 DISCUSSION

5.1 Gene expression by microarrays

The development of cancer is a ‘multi-hit’ process requiring perturbation of a number of normal cellular processes and in turn the function of a multitude of genes that orchestrate these processes. In this study, we observed alterations both in the extent and patterns of expression of many genes belonging to different functional categories, on exposure to BP in the absence or presence of CHL. A complete list of all the genes discussed here can be found in Tables A, B, C, D at (<http://www.cdc.gov/niosh/ext-supp-mat/MTP/index.htm>).

5.1.1 Genes altered by BP treatment

Various members of the Phase I and Phase II battery of enzymes were up-regulated on treatment with BP. The highly consistent increase in the expression of *CYP1B1* across all the 6 NHMEC strains may suggest its possible role as the predominant CYP isoform involved in the metabolism of BP to DNA binding electrophiles in most individuals. These results are in agreement with previous studies from our laboratory (45). It also parallels another study which sought to examine ‘expression signatures’ of tobacco smoke exposure in a population of 85 individuals using peripheral blood leukocytes. In this study, *CYP1B1* was found to be one of the genes which consistently and significantly correlated with plasma cotinine concentrations (Lampe et al. 2004). The remaining members of the Phase I cluster of enzymes up-regulated by BP in this study (*CYP3A1*, *FMO*, and *CYP1A1*) are known to be involved in the metabolism of steroids and various endogenous substrates in addition to toxicants. The aldo-keto reductase family 1, member D1 (*AKR1D1*), an enzyme up-regulated by BP has been documented to be involved in prevention of oxidative free radical damage

apart from its role in endogenous aldehyde metabolism (Pappa et al. 2003). Its up-regulation may indicate to its probable role in the scavenging of any free radicals generated by catalytic cycling of BP (Porter et al. 1991). The aldehyde dehydrogenases ALDH1A3 and ALDH3A1 have been reported to be involved in aldehyde detoxification generated from alcohol metabolism and lipid peroxidation.

Given some of the hallmarks of cancer being enhanced uncontrolled cell proliferation and dysregulation of normal cellular controls it may not be surprising that various genes possessing diverse roles in transcriptional regulation, cell signaling, cellular proliferation, cell matrix adhesion and other critical cellular processes may be targets of attack by carcinogens in different individuals. This might explain the alteration in the expression patterns of various genes belonging to these categories. They may also represent a response pattern of the cell to carcinogenic stress as induced by exposure to BP. The up-regulation of genes like the son of sevenless (SOS) (a guanine exchange factor well known for its role in Ras activation), STK4 (a serine threonine kinase up-regulated in certain inflammatory breast cancers (Bieche et al. 2004)), EREG (a member of the epidermal growth factor family found to exhibit enhanced expression in various epithelial tumors (Yamamoto et al. 2004)) strongly point to this possibility. In fact, BP has been shown to be capable transforming and immortalizing cells *in vitro* (Gudjonsson et al. 2004).

BP is also an immunotoxicant and has been found to lead to T cell suppression (Rodriguez et al. 1999) and inhibition of B cell lymphopoiesis (Hardin et al. 1992). Among the battery of immune response genes altered by BP, interleukin 1 beta (IL1B), a proinflammatory cytokine with a proangiogenic role *in vivo*, was consistently up-regulated in all the 6 cell strains on exposure to BP. This may suggest that among the many changes

occurring following exposure to BP, one of them could be enhanced cell proliferation and angiogenesis. Also, exposure to BP has been found to affect oxidative damage and immune system injuries probably necessitating up-regulation of inflammatory cytokines such as IL1B as a defense mechanism. This is similar to a study where exposure of rats to Fe₂O₃ or BP or in combination to study their role in lung cancer resulted in enhanced production of various inflammatory cytokines including IL1B (Garcon et al. 2001). Of the other immune response genes up-regulated by BP, MAL, a T cell differentiation proteolipid has been found to behave as a tumor suppressor (Kazemi-Noureini et al. 2004) and also has a role in apoptosis (Kohler et al. 1999). Any BP induced cellular damage may be triggering its up-regulation. Not much is known at this time about the human T-cell leukemia virus enhancer factor (HTLF). SECTM1 (K12), another immune response gene, is protease inhibitor thought to have a role in hematopoiesis and in immunogenic processes by acting as CD7 ligand (Slentz-Kesler et al. 1998). Though initially unknown, CD7 is now known to be involved in T and NK cell activation and cytokine production. K12 by interacting with CD7 is, therefore, thought to play a role in regulation of thymocyte signaling and cytokine release (Lam et al. 2005).

Various other genes up-regulated by BP share an unknown association with carcinogenesis. While some are well characterized genes others appear to be mostly ESTs or sequences coding for hypothetical proteins. BEX1 and LBH are genes having a role in developmental regulation being involved in neurogenesis and early limb development, respectively (Briegel et al. 2001; Koo et al. 2005). Recently, BEX1 has been reported to be overexpressed in patients with acute myeloid leukemia (Quentmeier et al. 2005). These

might provide cues to the manner in which BP manifests some of its developmental toxicities.

Alterations in expression profiles induced by BP appear to be mainly mediated through up-regulation of genes rather than down-regulation given that only one cell strain exhibited down-regulation of genes by $SLR \geq 1.5$.

Only one gene with a potential role in immune responses (CXCL14) was down-regulated by BP. Contradictory reports exist regarding the role of CXCL14 (BRAF) with some studies pointing more to a role of a tumor suppressor inhibiting tumor growth (Schwarze et al. 2002; Shellenberger et al. 2004) and yet others reporting a role for it in enhanced proliferation, migration, and invasion of myoepithelial cells and myofibroblasts (Allinen et al. 2004).

Taken together, there appears to be a dual role of BP by either enhancing (in most cases) the expression of genes which may aid in the various aspects of carcinogenesis or in parallel also modulating the expression of genes which may serve to have a protective effect by maintaining cellular integrity. Considerable inter-individual variations in response patterns are also apparent with most genes not being consistently altered in all individuals except *CYP1B1*.

5.1.2 Genes altered by Pre CHL + co-treatment

CHL has been documented to modulate various aspects of carcinogenesis and mutagenesis either through non-specific inhibition of CYPs (Waters et al. 1996), up-regulation of various members of the detoxification battery (Singh et al. 1996; Fahey et al. 2005), enhancement of apoptosis (Chiu et al. 2005), modulation of carcinogen transport

(Mata et al. 2004), mitigation of oxidative stress (Kumar et al. 2004) or various combinations of these. Alteration in the expression profiles of genes belonging to various functional categories as seen in this study may point to additional targets of CHL. The large number of genes modulated by pre CHL + co-treatment may be due a 'synergistic interaction' between BP and CHL.

CHL modulated similar functional classes of genes as those altered by BP though some of the individual members within each functional class were different. Most members of the Phase I group of enzymes are common to BP treated cells indicating that their expression may predominantly be affected by carcinogenic stress (BP) or the fact that CHL may be a 'bifunctional inducer' inducing Phase I enzymes too in addition to Phase II enzymes or both. Again, only *CYP1B1* was consistently up-regulated by $SLR \geq 1.5$ (except M99005) in all the donors while considerable inter-individual variations existed in expression patterns of the other genes. Several other members of the aldehyde dehydrogenase and aldo-keto reductase (AKR1) families, apart from the ones altered by BP treatment were up-regulated by CHL compared to cells treated with BP alone further indicating a 'bifunctional' role of CHL. Most of these members were also up-regulated in a consistent fashion though not by $SLR \geq 1.5$ in all 6 donors. Many members of the AKR1 family have been implicated for their role in the carcinogenesis of organs such as the breast and endometrium by playing a vital role in progesterone signaling and in turn also regulating the estrogen: progesterone balance (Bauman et al. 2004; Ji et al. 2004).

Various other genes involved in phenomena as diverse as cell cycle control, apoptosis and developmental regulation were differentially modulated in different individuals. A multitude of genes coding for proteins such as kinases, Ca binding proteins, cell junction

proteins, growth factors, G proteins, various receptors, ligands and other regulatory proteins involved in various facets of cell signaling were differentially regulated by CHL being 'up' in some donors and 'down' in others thereby indicating that modulatory effects of CHL may partly be mediated through modulation of cellular communication processes.

Various immune response genes were also altered differentially by CHL. Genes such as SLP1, a serine protease (Devoogdt et al. 2003), interleukin 8 (Huang et al. 2002; Mian et al. 2003), IL1B and several other altered immune response genes have been found to enhance the risk of different cancers. Their up-regulation by CHL treatment may be explained as the response possibly mediated by the interactive action of BP +CHL together. Interferon alpha (IFNA), a gene with the highest degree of up-regulation compared to all other genes (in one cell strain M99016) has at times been shown to exhibit antitumor effects (Zella et al. 1999). Its differential regulation in the 6 cell strains could be due to polymorphisms in the gene for this cytokine or other down stream modulators in the signaling pathway thereby ultimately leading to differential expression extents and differential effects. CD80 didn't exhibit up-regulation in most of the cell strains except M99016 and has been reported to activate cytotoxic T lymphocytes (Chan et al. 2004) and also provide negative signals for the proliferation and IgG secretion of normal B cells and B cell lymphomas (Suvas et al. 2002). CD86 up-regulated in most of the cell strains has been found to have a role in priming and activation of naive and memory T cells, respectively (Jeannin et al. 2000) and also for enhanced activity of B cells (Suvas et al. 2002). PI3, protease inhibitor, up-regulated by CHL treatment in most of the individuals (except M99005 and M99025) has been reported to have a role in cell differentiation and apoptosis (Yamamoto et al. 1997) and possibly in the control of tumor cell invasion (Alkemade et al. 1993).

A number of other genes with diverse functions, ORF's and sequences coding for hypothetical proteins were differentially modulated by CHL and are presently not very well characterized in relation to cancer. However, many of these are involved in several developmental processes indicating that developmental processes may also form some key targets of attack several by some PAHs.

Genes down-regulated by the interactive action of BP + CHL included those whose enhanced expression has been documented to aid either directly or indirectly the progression of various cancers, e.g.:SOD2 (Drane et al. 2001), CRY61 (Lin et al. 2005), RAI3 (Wu et al. 2005) and several others. However, various other genes whose enhanced expression is known to inhibit various facets of carcinogenesis were also down-regulated by the interactive action of BP + CHL. These included AFT3 (Yan et al. 2005), OCLN (Tobioka et al. 2004), GADD45B (Qiu et al. 2003), ADAMST1 (Iruela-Arispe et al. 2003), MEST1 (Pedersen et al. 1999) and DOC1 (Schwarze et al. 2002).

Nine genes with a role in immune responses were down- regulated by BP + CHL. Of these, some like the interferon alpha inducible protein (IFI27) down-regulated in all 6 cell strains, is a protein suggested to be a novel biomarker of epithelial proliferation and cancer (Suomela et al. 2004). GIP2 (INSIG1) consistently down-regulated in all the cell strains has previously been suggested to function intracellularly as a ubiquitin homologue and a cytokine that induces production of IFN-gamma to augment NK/lymphokine-activated killer cell proliferation and function, possibly playing a vital role in antiviral response (Zhao et al. 2005). CXCL2 has been implicated in inflammatory responses along with other chemokines and cytokines (Yamagami et al. 2003) in human corneal endothelium and human melanoma cells (Gallagher et al. 2005).

Not much is known about the role of the remaining genes in relation to cancer and disease.

5.2 Correlation of gene expression on microarrays versus RT-PCR

Overall, there was a good correlation between the magnitude of expression for different genes as measured by GC and by RT-PCR. However, there were a few genes which correlated only moderately to poorly. In general, RT-PCR was more sensitive than GC in measuring gene expression changes. The lack of good correlation for some genes may be due to the comparatively lower sensitivity of GC in relation to RT-PCR in addition to differences in the exact regions being probed by GC and RT-PCR. A low level of expression of particular gene in only some individuals may cause GC to ‘miss’ it or at most exhibit its expression at an extremely low level, whereas RT-PCR being more sensitive may be able to more realistically track its expression giving a higher FC compared to GC culminating in a poor correlation.

CHL clearly appears to be a ‘bifunctional inducer’ modulating the expression of both bioactivating and detoxication enzymes. Various other functional categories of genes modulated by the interactive action of BP + CHL may point to various other presently unknown targets and mechanisms of action of CHL though these will need to be confirmed. Some genes (*AKR1C1*, *SECTM1*, *CRY61* and a few others) following a similar trend of modulation in most donors though not necessarily by $SLR \geq 1.5$ in all of them suggest that these could be good putative ‘biomarkers of exposure’ to be confirmed by further validation. Many other genes not consistently altered in a similar direction across all donors suggest the presence of extensive inter-individual variability in response to carcinogenic stress and

intervention strategies, an issue addressed earlier in our laboratory regarding *CYP1A1* and *CYP1B1* (John et al.). However, despite inter-individual differences, *CYP1B1* followed by *ALDH1A3* were consistently up-regulated in most individuals following treatment with BP as well as BP + CHL indicating that these could be definite biomarkers of BP exposure. The consistent up-regulation of *CYP1B1* by BP has been previously reported from our laboratory (Keshava et al. 2005) and was further confirmed by RT-PCR studies of the 20 NHMEC strains as discussed below.

5.3 Genetic damage in terms of *CYP1* induction and DNA adduct formation in 20 NHMECs on treatment with BP in the absence or presence of CHL

PAHs, including BP, are known to bring about their own metabolism through the Ahr pathway (Ma et al. 2003). *CYP1A1*, a substrate inducible enzyme expressed predominantly in extra hepatic tissues including the breast (Whitlock 1999; Masson et al. 2005) was highly induced in our studies (96 fold variation). Its induction, however, correlated poorly with donor age. This is in agreement with another study which also found an age independent variation in the expression of *CYP1A1* in normal breast tissue samples (n=58) obtained from breast cancer patients and normal cancer free individuals (Goth-Goldstein et al. 2000). The induction of *CYP1B1* was much less (43 fold variation) as compared to *CYP1A1* and also correlated poorly with the age of the donor. In contrast to *CYP1A1*, *CYP1B1* is expressed constitutively in various extra hepatic tissues including the breast but exhibits weak induction by Ahr ligands which may explain its lower induction in our study compared to *CYP1A1* (Ma et al. 2003).

The wide inter-individual differences among the cell strains in the induction of both *CYP1A1* and *CYP1B1* could be explained at least in part due to polymorphisms in the genes coding for CYPs and other Phase I enzymes, AhR-Arnt as well as detoxication and repair enzymes (Ma et al. 2003; Weston et al. 2005). In addition, physiological factors (such as the presence of endogenous agonists or antagonists), environmental and dietary exposures of the donors could also play a role although these are much less likely, since the cells have been in culture for long. Given the fact that majority of the cell strains were of Caucasian origin, variability due to ethnic differences though low cannot be ruled out completely. The presence of only a moderate to poor correlation between the induction of both *CYP1A1* and *CYP1B1* both in the absence and presence of CHL may suggest that both of these isoforms could be differentially regulated in response to BP or the fact that one isoform may be involved to a greater extent in carcinogen metabolism than the other or both. In fact, *CYP1B1* has been found to be catalytically more active than *CYP1A1* in the bioactivation of different PAHs (Shimada et al. 1996).

The universal reduction in the induction of both *CYP1A1* and *CYP1B1* in all the cell strains upon co-treatment as well as the existence of a good correlation between the fold induction upon treatment with BP alone and upon co-treatment (when CHL is present along with the carcinogen) suggests the action of CHL as an ‘interceptor molecule,’ reducing the availability of BP to trigger the Ahr pathway. Other *in vivo* studies have also found highly effective inhibition when CHL was co-administered with the carcinogen (Dashwood 1997a). A reduction in *CYP1* expression in most individuals upon treatment with CHL prior to carcinogen exposure (pre CHL + post BP) suggests mitigation of *CYP1* expression by mechanisms other than just carcinogen sequestration. Prior exposure to CHL may in some

manner alter specific cell signaling circuitry or modulate the abundance of Phase I enzymes directly or indirectly by altering the genes/proteins involved in the 'Ahr' battery.' This however, might not be a universal phenomenon and the 'bifunctional' nature of CHL may be coming into play to different extents in different individuals which might in part explain the increase in *CYP1* expression in some individuals upon CHL pretreatment. However, the exact molecular mechanism of CHL mediated modulation of *CYP1* gene expression upon its administration prior to carcinogen exposure is currently unknown. Pre CHL treatment followed by co-treatment enables CHL to act both as a chelator and as a modulator of *CYP1* gene expression thereby causing a reduction in *CYP1* expression in the majority of individuals and also causing a greater reduction in expression compared to other CHL treatments where CHL acts predominantly by any one of the above mechanisms. This is exemplified by the presence of a good correlation between the percentage reduction in the expression of *CYP1A1* to *CYP1B1* upon pre-treatment with CHL followed by co-treatment in the absence of outliers.

The reduction in adduct levels in most of the cell strains on co-treatment again points to the role of CHL as an 'interceptor' molecule sequestering the carcinogen and preventing it from interacting with DNA. This mechanism, however, requires CHL to be present in large molar excess compared to the carcinogen (Dashwood 1997a). Non-specific inhibition of CYPs may also lead to reduced metabolism of the carcinogen in turn leading to reduced availability of the carcinogen to interact with DNA as in a pre CHL + post BP treatment. However, the poor correlation between *CYP1* expression and DNA-adduct levels may suggest interplay of Ahr independent mechanisms of activation along with cross-talks with various other cellular pathways and/or factors. Aldo keto reductases (AKRs) have also been

shown to have a role in the metabolism of BP. In this respect, human AKR1A1, AKR1C1–AKR1C4 have been found to be particularly active in metabolizing BP to reactive quinone metabolites and also generating of reactive oxygen species. AKR1A1 has been found to serve a dual role in directly metabolizing the BP-7,8-diols to reactive quinones and also indirectly trans-activating *CYP1B1* (Jiang et al. 2004). A peroxidase pathway for BP metabolism is also documented to exist (Cavalieri et al. 1995). Though there are hardly any studies on the role of flavin monooxygenases in BP metabolism it may not be misleading to assume that these monooxygenases along with various other Phase I enzymes may potentially play at least a minor role in the metabolism of BP in some individuals. Many of these processes compete with the CYP pathway for BP metabolism. In addition, basal expression along with induced CYPs is responsible for metabolism of the compound to toxic metabolites. Therefore, trying to correlate only induced expression with the extent of BP-DNA adducts may give poor correlation. The basal expression levels in terms of RNA copy number need to be determined in future studies. Also, should CHL be triggering Phase II metabolic reactions (Fahey et al. 2005) or DNA repair process some of the reactive metabolites/ adducts formed may either be prevented from interacting with DNA or repaired, respectively leading to the lack of a good correlation.

5.4 Genetic damage in terms of *CYP1* induction and DNA adduct formation in 2 NHMECs on treatment with BP or B[e]P in the absence or presence of CHL

With respect to the studies on comparison of BP versus B[e]P, the lower potency of B[e]P in inducing CYPs and forming adducts was apparent. BP is a strong PAH receptor (8S) ligand but a weak PAH-binding protein (4S) ligand whereas B[e]P is mainly a 4S ligand

but a poor AH receptor ligand (Houser et al. 1992; Sterling et al. 1994). This explains the overall poor induction of *CYP1* by B[e]P compared to BP in both cell strains. However, an explanation for the differential action of CHL towards B[e]P induced *CYP1* expression is unknown. The enhancement in *CYP1* expression in most cases involving a co-treatment of CHL with the carcinogen may suggest a lack of efficient complex formation between B[e]P and CHL probably due to some change in carcinogen structure and subsequent alteration of stabilizing forces. In fact CHL has been found to form tight molecular complexes with planar aromatic hydrocarbons possessing at least partial ring structure when present in large molar excess to the carcinogen and administered simultaneously with the carcinogen (Dashwood 1997a). CHL in low concentrations has been shown to have the potential to act as an NADPH generating system by replacing glucose 6-phosphate (Waters et al. 1996). This was a mechanism suggested for the ‘potentiator effect’ of low concentration of CHL against nitrosamines (Waters et al. 1996) which was, however, reversed at higher CHL concentrations. Here, though the concentration of CHL used for intervention of BP and B[e]P was the same, in the absence of effective complex formation as in the case of B[e]P, the low concentration of free CHL may have the potential to bring about ‘potentiation’ through the above mechanism. This suggests that though some carcinogens may be only weakly carcinogenic they may be rendered more active in the presence of certain chemopreventive agents which are generally administered to mitigate the ill effects of more potent carcinogens depending on the concentration and treatment protocol involved. The mechanisms of BP-DNA adduct reduction by CHL may occur through some of the above discussed mechanisms. However, the lack of any detectable adducts by B[e]P in the absence or presence of CHL, suggests that there may be a very low level of B[e]P-DNA adducts given

its much lower carcinogenicity compared to BP. In addition, synchronous fluorescence spectrometry (SFS) may not be sensitive enough to detect rather low levels of adducts formed by B[e]P (detection limit of 1 in 10^7 adducts). A more sensitive assay will be needed to detect these low levels of adducts. The presence of a good correlation between *CYP1* gene expression and BP-DNA adduct formation in M98025 but only a moderate to poor correlation in M98015 again points to wide inter-individual variation in induction and response to intervention.

5.5 Genetic damage in terms of *CYP1* induction and DNA adduct formation in MCF-7 cells versus an NHMEC (M00012) on treatment with BP or B[e]P in the absence or presence of CHL

Cancerous cells are often used to study various aspects of carcinogenesis. Their relative ease of maintenance and faster generation time are some factors which favor their use. Here, MCF-7, an estrogen receptor positive breast cancer cell line used in comparison with normal breast cells exhibited a response pattern sharply different from those of normal cells. CHL failed to mitigate BP induced *CYP1* expression and DNA adduct across a range of CHL treatments and CHL concentrations suggesting that CHL neither behaves as a CYP inhibitor nor an interceptor molecule in these cells. However, it remains to be determined whether this is an exclusive response exhibited by MCF-7 cells or is commonly exhibited by other cancerous cells including other breast cancer cells, some of which may be estrogen receptor negative. Though the induction of *CYP1B1* was low compared to *CYP1A1* its basal expression was much higher than that of *CYP1A1*. This has been shown even in a few other studies (McKay et al. 1995; Spink et al. 1998). Nevertheless, it shows that cancerous cells

exhibit different response patterns compared to normal cells and therefore cannot be used as surrogates to predict responses of normal cells to carcinogens and chemopreventive agents.

BP is a widely distributed environmental carcinogen and a high risk factor for a large section of the world's population whose dietary lifestyle involves use of tobacco. Smoking alone has been linked to about 30% of all cancer deaths and more than 400,000 premature deaths/year in the U.S. alone (http://www.cancer.org/docroot/PED/ped_10.asp; <http://www.cdc.gov/HealthyYouth/tobacco>). A greater understanding of the 'molecular changes' that ensue following PAH exposure can help better design exposure monitoring and intervention strategies. This study has revealed at least a few potential biomarkers of exposure some of which could be potential targets for intervention strategies. Clearly, CYP1B1 is one such target as seen from this study. Additionally, CHL appears to function as a bifunctional inducer differentially modulating the expression of most genes except a few where most individuals seem to respond in a similar fashion. Wide inter-individual variations will need to be accounted for during biomarker validation as well as while designing intervention strategies.

6.0 SUMMARY AND CONCLUSIONS

These studies were intended to extend our understanding of human chemical carcinogenesis, potential biomarkers of carcinogen exposure and strategies for chemoprevention. To do this a human mammary cell model system was exposed *in vitro* to benzo[a]pyrene a known polycyclic aromatic hydrocarbon carcinogen, with or without chlorophyllin, a potential chemopreventive agent. The effects of these exposures on gene expression and carcinogenesis precursor events were evaluated.

Global gene expression analysis was carried out to seek for biomarkers of carcinogen exposure and chemopreventive intervention. Immune response genes altered on exposure to polycyclic aromatic hydrocarbons, with or without the modulating agent, chlorophyllin, were analyzed in the context of having cross-talks between themselves as well as interacting with several other cellular pathways. The expression of cytochrome P450 (*CYP1*) enzymes involved in polycyclic aromatic hydrocarbon metabolism was examined across a spectrum of individuals in relation to their ability to cause DNA damage, in the absence and presence of chlorophyllin.

CYP1B1 appeared to be a potential biomarker of polycyclic aromatic hydrocarbon exposure. Chlorophyllin served as an effective chemopreventive agent in mitigating genetic damage by reducing polycyclic aromatic hydrocarbon metabolism and the extent of DNA damage.

The main conclusions from the study are that:

- Genes from a broad spectrum of functional categories were altered by exposure to BP in the absence or presence of chlorophyllin.

- Only *CYP1B1* was altered in a consistent manner across all donors, and therefore appeared to be good biomarker of polycyclic aromatic hydrocarbon exposure and chemopreventive intervention. All other genes were altered in a less consistent in one or more donors indicating inter-individual variations in responses.
- Extensive inter-individual variations were observed in the extent of induction and modulation of *CYP1A1* and *CYP1B1* expression as well as benzo[a]pyrene-DNA adduct formation on exposure to benzo[a]pyrene in the absence or presence of chlorophyllin.
- The reduction of *CYP1* gene expression and BP-DNA adduct formation in most normal human mammary epithelial cells suggests the role of chlorophyllin acts as a ‘cytochrome P450 inhibitor’ as well as a ‘chelator’ in mitigating genetic damage.
- Chlorophyllin may enhance the carcinogenicity of even weakly carcinogenic polycyclic aromatic hydrocarbons. The extent of enhancement depends on the nature of the carcinogen as well as the time of chlorophyllin administration as seen from the enhancement of benzo[e]pyrene induced *CYP1* gene expression by pre chlorophyllin + post benzo[e]pyrene treatment but not any of the other chlorophyllin treatments.
- Cancerous MCF-7 cells exhibited high levels of *CYP1A1* induction and benzo[a]pyrene-DNA adduct formation on exposure to benzo[a]pyrene, both in the absence and presence of chlorophyllin, suggesting probably a different mechanism of chlorophyllin action in cancerous cell as opposed to normal cells and/or the fact that MCF-7 may be an inappropriate surrogate to normal mammary cells.
- Only chlorophyllin concentrations higher than those used in other experiments (8 μ M, 16 μ M) mitigated BP induced *CYP1* gene expression in MCF-7 cells. However, none

of the chlorophyllin concentrations mitigated BP-DNA adduct formation in MCF-7 cells supporting the previous conclusion.

- The lack of reduction in the extent of *CYP1* gene expression and benzo[a]pyrene-DNA adduct formation in MCF-7 cells suggests that chlorophyllin performs poorly as a ‘*CYP* inhibitor’ and ‘chelator’ in MCF-7 cells.

7.0 FUTURE DIRECTIONS

- In order to gain a better understanding of the correlation between *CYP1* gene expression and BP-DNA adduct formation an assessment of gene copy number needs to be determined. This will also help reveal the possible interplay of Ahr independent mechanisms in the metabolism of BP.
- The balance between toxicity and detoxication can be better understood if the effects of CHL on other phase I enzymes, e.g., other monooxygenases, in addition to the phase II enzyme battery are determined.
- The effect of chlorophyllin alone on global gene expression would be a valuable asset to the scientific community involved in screening for new cancer chemopreventive agents.
- Studies on functional CYP1 enzyme abundance and activity following exposure of cells to BP in the absence or presence of CHL will help reveal whether CHL mediated changes are transcriptionally or translationally regulated and in turn will help gain a better understanding of the mechanistic aspects of CHL action and BP metabolism.
- Studies on a wider spectrum of cancerous cells will help determine whether the large extent of genetic damage as seen in MCF-7 cells (as opposed to NHMECs) is a common feature of most cancerous cells and in turn point to the feasibility of using cancerous cells as surrogates to normal cells for studying the carcinogenic process.

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9.0 APPENDIX

Tissue Mix media

Penicillin Streptomycin mix (10,000U/ml Penicillin G sodium and 10,000U/ml Streptomycin sulfate)	10 ml
Fungizone (250µg/ml Amphotericin and 250µg/ml sodium deoxycholate)	20 ml
Insulin (10mg/ml)	1 ml
Hanks buffered saline	<u>969 ml</u>
Total	1000 ml

Filter through 0.2µM filter.

Tissue Digestion media

Tissue mix media	70 ml
Collagenase (2000U/ml)	10 ml
Hyaluronidase	10 ml
Fetal Bovine Serum	<u>10 ml</u>
Total	100 ml

Filter through 0.2µM filter.

Freezing media

MEBM media	80 ml
DMSO	10 ml
Fetal Bovine Serum	<u>10 ml</u>
Total	100 ml

Filter through 0.2µM filter.

MCF-7 media

DMEM (Dulbecco's modified Eagle's medium)	894.5 ml
Fetal calf serum (FCS)*	100 ml
Penicillin (5000U/ml)-Streptomycin mix (5000µg/ml) mix	2 ml
Amphotericin B (250µg/ml)	1 ml
Insulin (1000 µg/ml)	<u>2.5 ml</u>
Total	1000 ml

Filter through 0.2µM filter.

* FCS is to be heated (56°C for 50 min) to inactivate components of the complement system

5X RNA Fragmentation buffer

200 mM Tris acetate, pH 8.1
500 mM potassium acetate
150 mM magnesium acetate