UNDERSTANDING THE EFFECTS OF DIETARY CHEMOPREVENTIVE AGENTS ON METABOLISM AND MUTAGENICITY OF POLYCYCLIC AROMATIC HYDROCARBONS BY HUMAN CYTOCHROME P450 AND HUMAN GLUTATHIONE S-TRANSFERASE ENZYMES

By

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DEDICATION

This work is dedicated to the loving memory of Mrs. Josephine A. Durant and Mr. Maxie M. Durant, my mother and father. My mother has been looking down on me from Heaven and protecting me all my life, of this I am sure. My father did not live to see a completed work, but he had faith that this day would come. I thank him for loving me, nurturing me, and teaching me about the power of prayer. It is because of both of them and our faith in God that this dream has been realized.
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To my wonderful son, Brandon A. Durant (excuse me, Mr. Brandon A. Durant), I hope you are half as proud of me as I am of you. You have been the reason for it all, you are my main inspiration. No mother could love a child more. I love you more and more each day. I look forward to when you become “Dr. Durant”, as well…but if not, I will still love you no matter what! (smile)

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>6-TG</td>
<td>6-thioguanine</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
</tr>
<tr>
<td>CP</td>
<td>chemopreventive</td>
</tr>
<tr>
<td>AITC</td>
<td>allyl isothiocyanate</td>
</tr>
<tr>
<td>B[a]P</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid solution</td>
</tr>
<tr>
<td>BITC</td>
<td>benzyl isothiocyanate</td>
</tr>
<tr>
<td>BOMCC</td>
<td>benzyloxmethyloxy-3-cyanocoumarin</td>
</tr>
<tr>
<td>BPD</td>
<td>(-) benzo[a]pyrene-7,8-dihydriodiol</td>
</tr>
<tr>
<td>BPDE</td>
<td>benzo[a]pyrene-7,8-dihydriodiol-9,10-epoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>CAPE</td>
<td>caffeic acid phenethyl ester</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EROD</td>
<td>ethoxyresorufin-o-deethylase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GGT</td>
<td>gamma glutamyl transpeptidase</td>
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<tr>
<td>GSH</td>
<td>reduced glutathione</td>
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<td>GSNO</td>
<td>S-nitrosoglutathione</td>
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<tr>
<td>GSR</td>
<td>glutathione reductase</td>
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<td>glutathione disulfide</td>
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<td>glutathione-S-transferase</td>
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<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>hCYP1A1</td>
<td>human cytochrome P450 1A1</td>
</tr>
<tr>
<td>hCYP1B1</td>
<td>human cytochrome P450 1B1</td>
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<td>human glutathione-S-transferase alpha</td>
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<td>hGST-M1</td>
<td>human glutathione-S-transferase mu</td>
</tr>
<tr>
<td>hppt</td>
<td>hypoxanthine-phosphoribosyltransferase</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>a concentration which inhibits 50% of cell survival</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein</td>
</tr>
<tr>
<td>MEOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyl transferase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PE-ITC</td>
<td>phenethyl isothiocyanate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SRB</td>
<td>sulforhodamine-B</td>
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ABSTRACT

Patricia Denise Durant

Understanding the Effects of Dietary Chemopreventive Agents on Metabolism and Mutagenicity of Polycyclic Aromatic Hydrocarbons by Human Cytochrome P450 and Human Glutathione S-Transferase Enzymes

Dissertation under the direction of Alan J. Townsend, Ph.D., Professor of Biochemistry

Some of the more widely studied and effective chemopreventive (CP) agents in the current literature include isothiocyanates (ITCs), flavonoids (in soy products), polyphenols such as resveratrol, and flavonols such as galangin. Polycyclic aromatic hydrocarbons (PAHs) such as the classic model compound benzo[a]pyrene (B[a]P) are ubiquitous environmental pollutants that have been implicated as potent lung carcinogens. They are activated to their more carcinogenic metabolites by cytochrome P450 (CYP) Phase I enzymes, rendering them more reactive towards cellular DNA. This can lead to genetic damage, changes in function such as loss of tumor suppressor activity, and possibly cancer. However, glutathione S-transferase (GST) Phase II enzymes have the potential to prevent such mutations by conjugating carcinogenic metabolites to glutathione. The goal of these studies is to develop an understanding of how the various CP agents directly affect the activity of Phase I and Phase II enzymes in their metabolism of potent carcinogens such as B[a]P and benzo[a]pyrene-7,8-di hyd rodiol (BPD).

Purified human GST-M1 and GST-A1 isozymes were initially used to examine the direct effects of CP agents on Phase II enzymes. We employed V79 Chinese hamster lung fibroblast cell lines that were modified by stable transfection to express human CYP1A1 (hCYP1A1) or human CYP1B1 (hCYP1B1) to examine the direct
effects of CP agents on the CYP activity. We examined the endpoints of cytotoxicity, mutagenicity, and \(^3\)H-protein adducts to determine how these CP agents affected the toxicity of B[a]P and BPD. We also examined metabolic distribution and consumption profiles using HPLC analysis.

We observed that various CP agents affect GST and hCYP differently in an isoform-specific manner. There was no direct inhibition of GST activity by any of the CP agents when using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate with purified enzyme, except at supraphysiological concentrations of the CP agents (1 – 10mM). We observed direct inhibition of hCYP1A1 or hCYP1B1 activity by some, but not all CP agents, using an intact cell assay. This inhibition was CYP isoform specific, dose-dependent and mostly stable for acute time points. Resveratrol was a potent inhibitor of both CYPs, and galangin inhibited hCYP1B1 more effectively. Neither PE-ITC nor genistein were as potent in their inhibition of hCYP1A1 and genistein proved to be more effective at inhibiting hCYP1B1 than PE-ITC. However, direct inhibition of CYP by the CP agents did not always predict their ability to inhibit the activation and resulting cytotoxicity and/or mutagenicity of B[a]P or its metabolite, BPD. While resveratrol reduced the cytotoxicity and mutagenicity of B[a]P, it did not reduce the toxicity or mutagenicity of BPD. Metabolic distribution and consumption profiles examining the consumption of B[a]P and BPD by hCYP showed that galangin blocks hCYP1A1 and hCYP1B1 metabolism of B[a]P and BPD, while the consumption of each PAH was slower in the presence of resveratrol, but not inhibited. \(^3\)H-B[a]P and \(^3\)H-BPD protein adduct data in cells expressing both hCYP1A1 and hCYP1B1 revealed fewer protein adducts in the presence of resveratrol, and a significant reduction in protein adducts in the
presence of galangin, as compared to $^3$H-PAH in the absence of the CP agent. What we can conclude from these studies is that direct inhibition of a substrate by a CP agent may not always predict the effect of the agent on other biological points such as cytotoxicity and mutagenicity.
CHAPTER 1

INTRODUCTION

Chemoprevention, as defined by the National Cancer Institute, is the use of drugs, vitamins, or other agents to try to reduce the risk of, or delay the development or recurrence of cancer. Wattenburg went on to further say that the agents used to block or suppress carcinogenesis could be natural or synthetic dietary compounds (1). It has been established in many epidemiological studies that populations that consume large quantities of fruits and vegetables in their diet lower their risk of developing cancer. Abundant evidence from epidemiological studies has established an inverse association between the consumption of a wide variety of vegetables and fruits and the risk of cancer at most sites (2). Therefore, the biologically active compounds found in these fruits and vegetables have been classified as chemopreventive (CP) agents. Some of the more efficacious CP agents that are being studied in the current literature include the isothiocyanates (ITCs) (3), polyphenols such as resveratrol and curcumin (4, 5), flavonoids (in soy products) (6), quercetin (7), and galangin (8). They are discussed more thoroughly for their chemopreventive properties below.

Pathology of Cancer

Cancer is believed to be caused by damage to the genetic material of a cell, resulting in a change in normal function. It is believed that the genesis of human malignancies is caused by damage to DNA by chemicals or endogenous reactive oxygen species (ROS). This can alter the normal, functional sequence of DNA. The modification of DNA can mutate genes that code for components of pathways that
stimulate cell growth (oncogenes) and genes that inhibit cell growth (tumor suppressor genes). Mutations that delete the function of tumor suppressor genes would be expected to enhance tumor promotion. This would lead to uncontrolled growth of cells (9).

Polycyclic aromatic hydrocarbons (PAH) are among the most widely studied carcinogens (10). They are produced by the incomplete combustion of organic matter and are ubiquitous in our environment. Human exposure to these compounds is widespread and practically unavoidable. Some sources of PAH are cigarette smoke, automobile exhaust, and deposition on cooked foods.

There is no doubt of the presence of PAH in mainstream and sidestream smoke by both non-filter and filter cigarettes; the concentrations have been studied and reported (11, 12, 13, 14). There is also no doubt that PAHs are effective pulmonary carcinogens in rodents. Also, their uptake by smokers has been clearly demonstrated (15). Human lung metabolically activates B[a]P, in part by cytochrome P450 1A1 (CYP1A1), which is induced by cigarette smoking (15). Benzo[a]pyrene diol epoxide (BPDE)-DNA adducts have been detected in human lung (15). Collectively, these findings provided strong evidence for a role of PAHs as an exogenous source of insult to DNA and likely causative agents for lung cancer in smokers (15).

PAH such as benzo[a]pyrene (B[a]P) are activated to their more carcinogenic epoxide metabolites by cytochrome P450 (CYP) enzymes (16). The primary enzymes involved are CYP isozymes 1A1 (17) and epoxide hydrolase (EH) (18). The reactive electrophilic metabolites that are formed are capable of binding covalently to DNA. This could alter DNA structure and result in erroneous base pairing when DNA undergoes
replication. Such a mis-match can end up in base substitutions (point mutations), or deletion or addition of a nucleotide (frameshift mutations).

Normally, the body safeguards against cancer, via methods such as apoptosis. However, these safeguards can fail, especially in environments that make errors more likely to arise and propagate, such as those that include disruptive substances like carcinogens. These errors, left uncorrected, can become genetic abnormalities that affect oncogenes or tumor suppressor genes. The activated oncogene in cancer cells gives those cells new properties, such as hyperactive growth and division, protection against apoptosis, and loss of respect for normal tissue boundaries. Inactivated tumor suppressor genes in cancer cells can result in loss of accurate DNA replication, as well as loss of control over the cell cycle. These changes in function are what ultimately can lead to cancer.

The formed epoxides can be detoxified via conjugation, primarily by GST. This process reduces damage to the genetic material, and therefore serves to prevent the potential carcinogenic effects of PAH. While the cell has effective repair mechanisms, some damage is irreversible and can lead to disease progression; therefore, preventing any damage to the cell’s DNA before it occurs affords the greatest impact for attenuating and/or suppressing oncogenic transformation. Cancer chemoprevention is a novel and rapidly growing area of research focusing on the use of natural or synthetic substances to inhibit, delay or reverse carcinogenesis.
Cytochrome P450 Enzymes Are Involved in Phase I Reactions

The body metabolizes (metabolize in this context meaning chemically modify or degrade) environmental toxins, or xenobiotics, through a process that consists of activation, deactivation, and excretion of the product. The CYP system is the primary pathway of metabolic bioactivation of xenobiotics. The CYPs are a superfamily of enzymes that catalyze oxidation of drugs, pesticides, and carcinogens and endobiotic chemicals such as steroids, fatty acids, vitamins, and prostaglandins (19). The most common reaction catalyzed by CYP enzymes is the monooxygenase reaction, but they can also catalyze other reactions such as hydroxylation and epoxidation reactions. They are found in all prokaryotic and eukaryotic species. Most organisms have multiple isoforms and many have broad substrate specificity.

The discovery and initial characterization of the CYP system involved studies investigating the metabolism of chemical carcinogens, identifying NADPH-dependent microsomal enzymes that catalyzed the biotransformation of azo dyes (20, 21). It is a monooxygenase system that has the ability to incorporate one atom of molecular oxygen into its substrates and the second oxygen atom into water. The reaction is shown by the following chemical equation, where the substrate is represented by RH:

$$\text{RH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+$$

They are a diverse group of hemoproteins, which is a metalloprotein containing a heme prosthetic group. The iron in the heme is capable of undergoing oxidation and reduction. The enzyme as a whole is shaped like a triangle with the heme in the center. It is composed of half α-helix and half β-sheet, or non-repetitive segments. The protein
has an N-terminal membrane anchor, as it is found strictly bound to the membranes of mitochondria and endoplasmic reticulum. The CYPs have an unusual spectrum, in that they strongly absorb light at 450nm, which contributes to their nomenclature. The unusual spectrum is the result of the heme ring at the active site of the enzyme.

Only a small subset of CYP enzymes in humans is involved in the activation of procarcinogens and promutagens, including CYP1A1 and CYP1B1 isoforms. CYP1A1 is poorly expressed in human liver, although its synthesis can be markedly induced in many extrahepatic tissues, expressly the lung (22). The induction of CYP1A1 by PAH is believed to contribute to pulmonary carcinogenesis, because increased lung CYP1A1 expression and activity are associated with a high risk of lung cancer (22). A low level of CYP1B1 mRNA was detected by Northern blotting in several normal human tissues including kidney, liver, intestine, eye tissue, and brain (23, 24, 25, 26). However, the level of CYP1B1 protein expressed (as opposed to mRNA) in normal human tissues is unclear. It has been proposed, based on previously mentioned Northern blots, that CYP1B1 has widespread expression in extra-hepatic tissues. Native CYP1B1 protein has not, however, been isolated and purified from any normal human tissue, suggesting that constitutive expression in normal human tissues is, at best, very low. CYP1B1, however, is inducible after exposure to PAH (27). CYP1B1 has also been shown to be overexpressed in breast, lung, liver, gastrointestinal tract, prostate, and bladder tumors (28).
Glutathione and Glutathione-S-Transferase Are Involved in Phase II Reactions

**Glutathione.** Reactive oxygen species (ROS) generated in oxidative metabolism inflict damage on all classes of macromolecules. Environmental toxins that are metabolized to reactive electrophiles can aggravate the problem by binding covalently to DNA, ultimately leading to cell death. Reduced glutathione (GSH) is required to combat oxidative stress and maintain the normal reduced state in the cell. GSH is present in plants, animals, and some bacteria. It is a nucleophilic tripeptide that is not an essential nutrient since it can be synthesized from the amino acids glycine, glutamate, and cysteine (Figure 1). Cysteine is relatively rare in the diet; therefore, availability of this amino acid is the rate-limiting factor in GSH synthesis by the cells. GSH is synthesized in two adenosine triphosphate (ATP)-dependent steps. First, γ-glutamylcysteine is synthesized from glutamate and cysteine via the enzyme γ-glutamylcysteine synthetase (γ-GCS). This reaction is the rate-limiting step in GSH synthesis. Second, glycine is added to the C-terminal of γ-glutamylcysteine via the enzyme glutathione synthetase (GS).

![Figure 1. Reduced Glutathione](image-url)
The sulfhydryl (thiol) group (SH) of cysteine is nucleophilic following deprotonation and is responsible for the biological activity of reduced glutathione (GSH). By acting as an electron donor, GSH is converted to its oxidized form, glutathione disulfide (GSSG). The enzyme that catalyzes the reduction of GSSG, glutathione reductase (GSR), is constitutively active and inducible when the cell is in a state of oxidative stress. The nucleotide cofactor of GSR, NADPH, is kept highly reduced (>95%) in cells by the pentose shunt pathway. Therefore, GSH is efficiently maintained in its reduced state. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent to other unstable electrophiles in the cell, such as ROS. GSH is oxidized to a thyl radical intermediate which readily reacts with another thyl-GSH to form GSSG. Such a reaction is possible due to the relatively high concentrations of GSH in the cell. Thiol groups are kept in a reduced state at a concentration of approximately 15mM in the lens, 5–10mM in animal liver cells, and 1-5mM in other tissues (29). The redox state of the tissue also affects the GSH concentration (30, 31). In healthy cells and tissue, more than 90% of the total glutathione pool exists as GSH, and less than 10% exists as GSSG. An increased GSSG-to-GSH ratio is considered an indication of oxidative stress, with attendant cytotoxic effects.

**Glutathione-S-Transferase.** Enzymes of the glutathione S-transferase (GST) superfamily catalyze nucleophilic attack by the GSH sulphydryl group on nonpolar compounds that contain an electrophilic site. The GSH serves to lower the pKₐ of the thiol, and the deprotonated thiolate is the reactive nucleophile. The conjugation of these compounds with GSH inactivates their electrophilic centers and renders them more water soluble, where they can be easily excreted from the cell by the transmembrane multidrug
resistance-associated protein (MRP) (32). This is an important defense mechanism for the cell. The conjugation reaction of GSH to electrophilic substrates is shown by the following chemical equation, where the reactive electrophile is represented by R and X represents a leaving group on the reactive electrophile:

\[ \text{GSH} + \text{R-X} \rightarrow \text{GSR} + \text{HX} \]

GSH conjugates (represented above by GSR) are more readily excreted than electrophilic substances, due to their hydrophilic nature. They are more soluble in an aqueous medium, thereby facilitating the elimination of hydrophobic electrophiles. The conjugate can also be excreted by ATP-dependent transporters through the bile or urine (33). In addition, GSH conjugates can be further metabolized via the mercapturic acid pathway. The \( \gamma \)-glutamate and glycine residues in the GSH molecule are removed by gamma-glutamyl transpeptidase (GGT) and dipeptidases. While in the kidney or gut, the resulting cysteine S-conjugates can be acetylated on the amino group of the cysteinyl residue by N-acetyl-transferases (NAT). The products, mercapturic acids, can then be released into the circulation and delivered to the kidney for excretion in urine. The detoxification of genotoxic electrophiles by GSH does not occur efficiently under spontaneous conditions. Glutathione \( S \)-transferase (GST) catalyzes the conjugation of GSH to these electrophiles.

GSTs are ubiquitous in nature and are found throughout the phylogenetic tree. They are expressed in humans in a tissue- and cell-specific manner (34, 35). They are cytosolic, mitochondrial, and microsomal proteins. The cytosolic and mitochondrial
proteins are soluble enzymes. The microsomal proteins, designated as membrane-associated proteins in eicosanoid and glutathione (MAPEG), are transmembrane proteins.

Cytosolic GSTs are the largest family of these transferases. There are seven classes of cytosolic GSTs, with five major classes recognized in mammalian species. These include GST Alpha, Mu, Omega, Pi, and Theta. Cytosolic GSTs exist as a 50kDa homodimer or heterodimer of subunits within the same class. The structure seems to be consistent among the classes, with the same basic protein fold consisting of two domains. The N-terminal domain consists of four β-sheets that are flanked by two α-helices, and makes up approximately one-third of the protein (36). The C-terminal domain is a tightly arranged bundle of α-helices that make up the other two-thirds of the protein (36). The interface between the N- and C-terminal domains has the appearance of a V-shape (36). The dimer itself is a globular molecule.

The GST dimer must be intact in order for the enzyme to be active. Each dimer consists of two monomers with substrate binding sites for glutathione and the electrophilic substrate (37). The active sites function independently of each other (38). The N-terminal domain provides the binding site for GSH. The hydrogen-bonding interaction between GSH and the GSH-binding site occurs through a conserved tyrosine (in classes α, μ, π, σ) or serine (class σ), thereby suggesting the involvement of each in the catalytic activity of the enzyme. The cytosolic family of GSTs is involved in xenobiotic metabolism. When GSH forms a conjugate with a xenobiotic, GSH binds the active site of the enzyme at the end of the β-sheet (G-site or glutathione binding site) and the xenobiotic binds the active site between the N- and C-terminal domains (H-site or hydrophobic binding site). The G-site is conserved, while the H-site is not.
Figure 2. Metabolism of Benzo[a]pyrene (B[a]P) by Phase I and Phase II enzymes, and possible Chemopreventive Agent (CPA) Inhibition Site. The parent compound, B[a]P, is metabolized by CYP (denoted “p450” in the figure) to B[a]P phenols and oxides. The first step in this metabolic activation is CYP catalyzed formation of B[a]P-7,8-epoxide followed by hydrolysis by epoxide hydrolase (EH) to B[a]P-7,8-dihydrodiol metabolite. This metabolite is further metabolized by CYP to the reactive and mutagenic (±)-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) species. The (+)-anti-BPDE metabolite is the most reactive towards DNA, proteins and other macromolecular structures in the cell, making it the most mutagenic an carcinogenic (39) Chemopreventive agents may inhibit activation of the parent compound or further activation of diols to the diol epoxides. Another mode of action is induction of phase II
enzymes, such as glutathione S-transferase (GST), that modify PAH diol-epoxides via conjugation with reduced glutathione (GSH). Figure modified (40).

**Chemopreventive Agents Employed In These Studies**

**Isothiocyanates.** Organic isothiocyanates (ITCs) (R—N==C==S) are an important group of breakdown products that occur as their glucosinolate conjugates in a wide variety of cruciferous vegetables (*Cruciferae* family, including *Brassica*) such as broccoli, cabbage, cauliflower, and Brussels sprouts (3). Cellular damage such as chewing, which occurs as the foods are consumed, releases the enzyme myrosinase, which then catalyzes the hydrolysis of glucosinolates to form isothiocyanates (41). Myrosinase activity can be deactivated by high temperatures (such as cooking the food); however, hydrolysis of glucosinolates has been demonstrated in the gastrointestinal (GI) tract, where ITCs undergo biotransformation by human gut microflora and are rapidly absorbed (42, 43). The mechanism by which ITCs inhibit carcinogen activation is generally to induce the activity of Phase II enzymes (44), at a transcriptional level, with some evidence that they also inhibit Phase I enzymes (46). ITCs have also been shown to inhibit proliferation of cultured cancer cells (47), and cause cell cycle arrest (48). The ITCs examined in these studies are the more effective ITCs studied in the current literature and include allyl-ITC, benzyl-ITC, sulforaphane, and phenethyl-ITC.

Allyl-ITC (AITC) is a chemical compound responsible for the pungent taste of mustard, horseradish, and wasabi. It serves the plant as a defense against herbivores. AITC itself does not occur in the plant, as it is harmful to the plant. Therefore, it is stored
in the harmless form of sinigrin, a glucosinolate. Sinigrin is hydrolyzed in a reaction catalyzed by myrosinase, which is stored in another part of the plant. This reaction occurs when the plant tissue is somehow disrupted or damaged, such as grinding the mustard seed or chewing the plant. There have been conflicting reports on the biological effects of AITC. It has been reported as a chemopreventive agent, selectively inducing Phase II enzymes (48), and inhibiting the growth of human prostate cancer cells implanted in nude mice (47); however, AITC was also reported to be genotoxic and carcinogenic (49).

Benzyl-ITC (BITC) is a product of the enzymatic hydrolysis of the glucosinolate, glucotropaeolin, catalyzed by myrosinase. It is found naturally in relatively high amounts in garden cress (*Lepidium sativum*), papaya (50), and in common *Brassica* vegetables. Both Lin *et al.* and Hecht *et al.* showed that BITC significantly inhibits lung tumorigenesis produced by B[a]P in A/J mice (51, 52). However, Knasmuller *et al.* found that BITC causes DNA damage in human hepatoma cells (53). BITC has been reported to activate mitogen activated protein kinase pathways, thereby leading to apoptosis in PC-3, HL-60, and Jurkat cells (54). Al-Dosari *et al.* found BITC to significantly reduce micronucleus formation in mouse bone marrow cells induced by B[a]P (55). They attributed this protective effect to BITC interfering with B[a]P metabolism and / or accelerating the detoxification process of the ultimate metabolites, or affecting cell differentiation and proliferation (55).

Sulforaphane is the product of the enzymatic hydrolysis of the glucosinolate glucoraphanin. It is found in high concentrations in *Brassica* vegetables, particularly broccoli. Sulforaphane acts as a potent monofunctional inducer of detoxification
enzymes, including GST (56). Enzyme induction has been observed in vitro as well as in organs (liver, stomach, small intestines, and lung) of mice fed sulforaphane (56). It has also been shown to induce GSTs and other protective enzymes in rat models (55). Gamet-Payrastre et al. found that sulforaphane induces apoptotic cell death in human colon cancer cells through upregulation of the proapoptotic protein bax and release of cytochrome c (57). Mouse studies have shown that sulforaphane prevents certain cancers, as reported by Singh et al. (59). It was shown that oral gavage of sulforaphane significantly prevented prostate cancer progression and pulmonary metastasis in a transgenic mouse model of prostate cancer (59). Another study done by Fahey et al. showed that sulforaphane protected the mouse forestomach against the neoplastic effects of B[a]P, and that this effect depended on the induction of phase II enzymes (60). While sulforaphane has been shown to be an inducer of Phase II enzymes, its glucosinolate precursor, glucoraphanin, has been shown to induce Phase I enzymes, particularly CYP1A1 (61).

Gluconasturtiin is a glucosinolate that, when hydrolyzed in a reaction involving myrosinase, forms phenethyl ITC (PE-ITC). It is found in horseradish, cabbage, and in high concentrations in watercress. It has been suggested that PE-ITC is a chemopreventive agent against lung cancer. Chung et al. showed that PE-ITC inhibited tobacco-specific nicotine-derived nitrosamine ketone (NNK)–induced lung carcinogenesis in laboratory animals (62), and Hecht et al. later supported this finding in humans by doing a study using healthy smokers on a diet consisting of no cruciferous vegetables except watercress (63). He collected urine and analyzed for NNAL-Gluc (a detoxification product of NNK) and observed that NNAL-Gluc levels decreased after
watercress consumption. He therefore concluded, from this study, that PE-ITC decreased the metabolic activation of NNK by inhibiting the CYP-dependent $\alpha$-hydroxylation of NNK. PE-ITC has also been shown to induce apoptosis in both p53-dependent and – independent manner in JB6 and PC-3 cells (64, 65). Similarly, activation of mitogen activated protein kinase pathways such as ERK, JNK, and p38 by PE-ITC were reported to be the possible mechanisms of growth arrest and apoptosis in PC-3, HL-60, and Jurkat cells (65, 66).

**Polyphenols.** Polyphenols are a group of chemical substances found in plants and are characterized by the presence of more than one phenol group per molecule. Sources of polyphenols in the diet include berries, tea, beer, grapes/wine, olive oil, chocolate/cocoa, coffee, walnuts, peanuts, and other fruits and vegetables. Studies have shown that polyphenols have antioxidant characteristics (67) and may reduce the risks of cardiovascular disease and cancer, although the mechanisms by which they achieve these biological activities are not completely understood (68).

The most common and studied polyphenolic compounds in the human diet are the bioflavonoids (69). They are found ubiquitously in plants and are important pigments for flower coloration producing yellow, red, or blue pigmentation in petals designed to attract animal pollinators. They also protect plants from insects and fungi (70). *In vitro* studies have shown flavonoids to have biological activities that include anti-allergic, anti-inflammatory, anti-oxidative (71), and anti-microbial (72). However, other studies have suggested that, following dietary intake, flavonoids themselves have little or no direct antioxidant value (73, 74). Flavonoids and other polyphenols are poorly absorbed (less than 5%), with most of what is absorbed being quickly metabolized and excreted (75).
The increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is not caused by flavonoids themselves, but most likely is due to increased uric acid levels that result from metabolism of flavonoids (75). Uric acid is a water-soluble biological antioxidant with the ability to scavenge free radicals. It is involved in a one-electron oxidation reaction, where it serves as a reducing agent towards radical species.

Flavonoids have also been viewed as anti-cancerous because of their ability to directly inhibit Phase I enzymes (76) and indirectly induce Phase II enzymes. Physiological processing of flavonoid compounds induces Phase II enzymes, as the body reacts to flavonoids as foreign and tries to eliminate them. This induction of Phase II enzymes also helps to detoxify mutagens and carcinogens, and therefore is beneficial in cancer prevention. Studies have found that populations that consume foods containing certain flavonoids, such as catechins found in strawberries and green and black teas, kaempferol found in brussel sprouts and apples, and quercetin found in beans, onions, and apples, may have reduced risk of developing lung cancer (77).

The bioflavonoids are further divided into subgroups based on their chemical structure, and include flavonols, flavones, and isoflavones. The polyphenol resveratrol is a flavonol found in many plants, including peanuts and grapes. The skin of grapes is a rich source of resveratrol, with fresh grape skins containing about 50 to 100 µg of resveratrol per gram (78); therefore, resveratrol is present at relatively high levels in red wine. It has been shown to be an anti-oxidant that inhibits cyclooxygenase (COX) action (79), to have anti-angiogenic properties (80), and to trigger apoptosis (79). It is a known inhibitor of Phase I enzymes (81).
Galangin is a dietary flavonoid that is present in high concentrations in honeybee propolis, which is a natural mixture produced by honeybees from the gum of various plants (82). It is also found in *Alpinia officinarum* (common name, India or China root), a plant which has been used as a spice and as an herbal medicine for a variety of ailments in Asia. The biological activities shown for galangin are numerous, including anti-mutagenic (83), anti-clastogenic (84), anti-oxidative (85), radical scavenging (86), and COX-2 inhibiting (87). Galangin is also a known inhibitor of Phase I cytochrome P450 (CYP) enzymes (86).

**Flavonoids (In Soy Products).** Flavonoids and isoflavones have been shown to act as phytoestrogens (88), termed this because the chemical structure is similar to that of estrogen. Soybeans are a rich source of isoflavones (89). The soy isoflavones are rapidly absorbed upon hydrolysis in the GI tract, and are transformed by the gut microflora to several metabolites (90, 91).

Genistein is a major component isoflavone in soy, and is the aglycone of the glycoside genistin. Genistein must be released from genistin in a hydrolysis reaction that can occur in the stomach (acid hydrolysis) and intestine (action of bacterial enzymes) in order to be biologically active. Genistein has been shown to induce the expression of GSTs in human breast cells (92), thus conferring protection towards genotoxic carcinogens, which are GST substrates. Genistein was also shown to inhibit metastasis of human prostate cancer cells implanted in mice by inhibiting detachment of the cancer cells (93). Genistein was shown to block phosphorylation of “promotility” proteins, such as FAK, p38 MAPK, and HSP27, thereby inhibiting their mobility (93). Genistein also works as an antioxidant (94). Aside from it acting as a phytoestrogen, it has been
suggested that genistein promotes its anticancer actions through other mechanisms that could include inhibition of angiogenesis (95) and inhibition of tyrosine kinases (96), which play an important role in cell growth. Conversely, some studies have reported genistein to be genotoxic due to its effect to “poison” cellular DNA topoisomerase II (topo II) resulting in stable chromosome breakage and mutation in vitro at concentrations that can be achieved in vivo (97).

Daidzein is another predominant form of an isoflavone phytoestrogen (98) and is a naturally occurring component of soy food products. Daidzein can be further metabolized to equol by intestinal microflora (90). Daidzein and equol are both structurally similar to estradiol and there have been several reports identifying the two compounds as having estrogen-like properties (99, 100, 101). Xu et al. reported that daidzein is the more bioavailable isoflavone compared to genistein in adult women (102). In that study, 12 females were given 1 meal a day consisting of soymilk powder reconstituted in distilled water. There were three feeding days with two weeks in between. The average urinary recoveries of daidzein and genistein were 21% and 9%, respectively (102). Not much more is known about daidzein and its biological effects. The question of chromosomal genotoxicity of daidzein has been previously raised by Kulling and Metzler et al.; however, data showed genistein to be more genotoxic than daidzein (97).

Equol, first described by Setchell et al. (103), is an isoflavone metabolite that is derived from daidzein and is more estrogenic than the parent compound (104). It has been reported in high levels in certain hormone-dependent tissues, with Maubach et al. reporting an equol concentration in breast tissue that exceeded that in plasma (105). It is
interesting to note that only a certain percentage, about one-third, of the human population has the ability to convert daidzein into equol (106). These persons, known as “responders” or “converters” have the necessary intestinal conditions (bacterial enzymes) to affect this biotransformation.

**Additional Agents.**

Curcumin is the main component of the spice turmeric and is a known antioxidant (107). Curcumin also has anti-tumor properties that are not fully understood, but it has been shown to inhibit NF-κB (5). Inhibiting NF-κB is important because it has been shown to be a mediator of inflammation and of inflammation associated with cancer (5). Inflammation acts as a key regulator in promotion of cancer cells, possibly by providing them with proliferating signals and by preventing apoptosis (5). Inhibiting NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Curcumin has been shown to increase the levels of various mouse GST isozymes in hepatic tissue, with maximum induction being observed in the Pi class (mGSTP-1) (109). Also, curcumin has been shown to directly inhibit CYP1A1 activity (109). There are multiple molecular targets for curcumin, and the biological activity, such as induction of apoptosis, antioxidant actions, and the effects on phase I and phase II enzymes has been reviewed extensively (5).

Quercetin is a flavonoid and more specifically, a flavonol. It is the aglycone form of a number of other flavonoid glycosides, such as rutin and quercitrin, found in citrus fruit, buckwheat apples, and onions. It is readily absorbed in humans and can reach micromolar concentrations in plasma and urine (110, 111). It has also been shown to be
eliminated slowly from the blood, with an elimination half-life of approximately 24 hours (110), suggesting that repeated intake would lead to a build-up of quercetin to reach even higher levels. Quercetin was shown to suppress CYP1A1 activity and gene expression in human HepG2 (hepatocellular liver carcinoma) cells, thereby inhibiting B[a]P-induced DNA adducts (7). Commercial preparations of *Ginkgo biloba* extract are formulated to contain about 24% flavonol glycosides, including quercetin (112). Chang, *et al.* did a study on the effects of *Ginkgo biloba* extract, and some of its components (including quercetin) on the activity of CYP1A1, CYP1B1, and CYP1A2 in human liver microsomes (113). What they found was that quercetin was a relatively potent inhibitor of each CYP, with the most potent inhibition being of CYP1B1.

Caffeic acid phenethyl ester (CAPE) is a polyphenol and an active component of propolis from honeybee hives and honey. It has a broad spectrum of biological activities, including antioxidant (114, 115, 116), anti-inflammatory (117, 118), and antiviral action (119). It also has suspected anticancer activity (120). Lin *et al.* showed that CAPE interfered with cell proliferation of human medulloblastoma cells, through cell cycle arrest and not through apoptosis (120). They found cell cycle arrest at S phase and delay of cell progression to G2/M phase through down-regulation of cyclin B1 (120). CAPE has been shown to block the production of free oxygen radicals (121), and interfere with several types of intracellular signaling including being a specific inhibitor of the nuclear transcription factor, NF-κB (122), which is involved in most of the aforementioned biological activities. The mechanisms of inhibition of NF-κB by CAPE are not clear.
**Figure 3. Chemical Structures of Chemopreventive Agents**

- Allyl Isothiocyanate
- Benzyl Isothiocyanate
- Phenethyl Isothiocyanate
- Sulforaphane
- Resveratrol
- Galangin
- Genistein
- Daidzein
- Equol
- Quercetin
- Curcumin
- Caffeic Acid Phenethyl Ester
**V79 Chinese Hamster Lung Fibroblast Cell Model**

Previous studies looking at the metabolism of PAH, particularly B[a]P, were initially done using microsomes isolated from mammalian tissues (123). The development of recombinant bacterial enzyme systems furthered the field, and were used to study the kinetics of CYP-mediated reactions of B[a]P (124). V79 Chinese hamster cells engineered to express human or rat CYPs were later used to study the metabolite profile of B[a]P (125). This system allowed a better understanding of B[a]P metabolism in a eukaryotic cell model. Our lab employed this unique cell model to perform the assays for these studies.

Parental V79MZ Chinese hamster lung fibroblast cells were engineered to express human CYP (hCYP) (125). There are several reasons why this novel system is favorable over cell lines or microsomes used in previous studies. First, the parent cells express no constitutive or inducible CYP activity (126). They do express an endogenous Pi-class hamster GST at low levels (127), but this GST is not active for the conjugation of reactive electrophiles of B[a]P or BPD with GSH (128). Second, expression of the transfected CYP is stable and constitutive, rather than variable due to carcinogen induction. Next, expression of the enzyme is unhindered by background interference because each CYP is expressed against the same V79 cell background; therefore, each isozyme or enzyme combination is comparable. Last, the endpoints can be analyzed in live and intact cells, all the while maintaining cell architecture, enzyme compartmentalization, and biological endpoints.
Epithelial cells, such as hepatocytes, are the major cell type for cancer in most organs. The use of primary hepatocytes for studies such as these has some disadvantages. They have high enzyme (i.e. CYP) levels \textit{in vivo}, but the total CYP levels fall rapidly, and the profile of the expressed CYP changes constantly (129), as opposed to being stable and constitutive in the genetically engineered V79 cells. V79 cells have a low chromosome number (21 chromosomes), a quick multiplication rate, and a stable karyotype as compared to hepatic cells (126). Also, previous studies in the lab examining the metabolism of PAHs were done using V79 cells; therefore, it was logical to examine the effects of CP agents on the metabolism of the same PAHs in these same cells.

\textbf{Rationale for the Proposed Studies}

Phytochemicals found in fruits and vegetables can significantly reduce the risk of cancer (2). \textit{In vitro} and animal model studies suggest that the chemopreventive potential of these agents is the result of two major actions: 1) their inhibition of Phase I enzymes leading to decreased formation of the carcinogenic derivatives of xenobiotics, and / or 2) their induction of Phase II enzymes leading to increased excretion of the metabolites (130, 57). For instance, resveratrol is a known inhibitor of CYP1A1 and CYP1B1, suggesting that the mechanism for this protection is decreased activation of carcinogens by inhibition of CYP (4). Other reports have shown that ITCs (or their glucosinolate precursors) are inducers of Phase I enzymes (131) and are metabolized to GSH conjugates (132) that could inhibit GST. However, the effects of these agents on Phase I
and Phase II enzymes is often debated in the literature, and at times, can produce conflicting data.

CP agents may affect xenobiotic metabolism through mechanisms independent of direct CYP and GST inhibition. Previous work in rodent and human prostate cancer cell has shown that the ITCs act on signal transduction pathways within the cell and induce apoptosis and inhibit cell growth (133, 134). CP agents can also affect proteosomal turnover of key proteins involved in cell cycling, which could modulate DNA repair pathways. Curcumin was shown to inhibit the proteasome activity in human colon cancer cells (135). Sulforaphane and PE-ITC have been shown to directly affect the Nrf2-KEAP1 complex (136) which regulates the induction of Phase II enzymes (137). Induction of GST could lead to increased conjugation of GSH with PAH metabolites, thereby decreasing mutagenicity.

The transcription of anti-oxidant response element (ARE)-driven genes is regulated, at least in part, by nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) (138). Nrf2, a 66-kDa protein induces Phase II enzymes by binding to the ARE region of the promotor (137). Under basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (KEAP1), a 69-kDa cytoplasmic protein anchored to the actin cytoskeleton. Nrf2 is actively targeted by KEAP1 for ubiquitination and degradation by the proteosome (139). The Kelch repeat of Keap1 combines with Nrf2 through the Neh2 domain of Nrf2, making it possible to retain Nrf2 in the cytoplasmic compartment. Exposure of cells to ARE inducers results in the dissociation of Nrf2 from KEAP1 and facilitates translocation of Nrf2 to the nucleus, where it heterodimerizes with small Maf protein and binds to ARE (137). This leads to
activation and gene transcription. Sulforaphane interacts with the cysteine-rich intervening region of KEAP1 located between the BTB region, an actin-binding site and the Kelch repeat (double glycine region, DGR), for interaction with Nrf2 (136).

Not having a clear understanding of the mechanisms of these agents could be problematic when employing them as chemopreventive agents. Therefore, a key gap in knowledge is in understanding the mechanisms of how CP agents directly affect xenobiotic metabolism in an intact cell system. Understanding their effects on Phase I and Phase II enzymes, and particularly their direct effects on metabolism of potent carcinogens such as B[a]P and BPD, will offer a clearer picture of how CP agents can be used to reduce carcinogenic genetic damage in humans.
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CHAPTER 2

Effects of Resveratrol on hCYP1A1 and hCYP1B1 Activation of Benzo[a]pyrene (B[a]P) and Benzo[a]pyrene-7,8-dihydrodiol (BPD) to Toxic and Mutagenic Metabolites

Abstract:

Benzo[a]pyrene (B[a]P) and its metabolite benzo[a]pyrene-7,8-diol (BPD) are polycyclic aromatic hydrocarbons (PAH) that have been implicated as potent lung carcinogens. They are activated in the cell to their more carcinogenic metabolites by cytochrome P450 (CYP) Phase I enzymes, causing them to be more reactive towards cellular DNA and possibly leading to cancer. Resveratrol, found in grapes and as a minor component of wine, is a known inhibitor of CYP1A1 and CYP1B1. We investigated whether direct inhibition of human cytochrome P450 (hCYP) activity, as measured by cleavage of the fluorescent VIVID BOMCC substrate in intact cells, was predictive of resveratrol’s effects on the cytotoxicity and/or mutagenicity of B[a]P or BPD. We employed V79MZ cells stably transfected to express hCYP1A1 or hCYP1B1. Inhibition of CYP activity by 30µM resveratrol was 94% in hCYP1B1 cells as compared to 90% inhibition in hCYP1A1 cells. The CYP activity inhibition assay predicted protection by resveratrol against the parent compound B[a]P, reflecting a significant 3.4-fold and 2-fold reduction in cytotoxicity in hCYP1B1 and hCYP1A1 cell lines, respectively. Similarly, resveratrol significantly reduced mutagenicity of B[a]P at the hprt locus of the V79 cell in hCYP1A1 cell line (2.1-fold). Based on data from the enzyme inhibition assay, we hypothesized inhibition of BPD metabolism by resveratrol and therefore, protection against BPD
cytotoxicity and mutagenicity, similar to that of the parent compound. However, resveratrol unexpectedly had no effect at preventing hCYP1A1 or hCYP1B1 activation of the intermediate metabolite BPD when measuring the endpoints of cytotoxicity and mutagenicity. Consumption profiles examining the metabolism of B[a]P and BPD by hCYP showed that the rate of consumption of each PAH was slower in the presence of resveratrol, but not inhibited. This may explain why resveratrol had no effect on BPD cytotoxicity or mutagenicity, but doesn’t explain the reduction of B[a]P cytotoxicity and mutagenicity by resveratrol. This suggests that resveratrol may not be acting as a direct inhibitor of PAH metabolism, but is somehow modifying metabolism. Measurement of $^{3}$H-B[a]P total protein adducts in cells expressing hCYP1A1 and hCYP1B1 revealed a 37% (1.6-fold) and 31% (1.4-fold) trend towards reduction in adducts, respectively, in the presence of resveratrol. We observed a less than 45% (1.7-fold) trend towards reduction in hCYP1B1 cells. This indicates that resveratrol is not blocking consumption of B[a]P or BPD, nor formation of protein adducts; therefore, resveratrol could possibly be affecting toxicity and mutagenicity of B[a]P and BPD through metabolite distribution.
**Introduction**

There is an inverse association between the consumption of a wide variety of fruits and vegetables and the risk of cancer at most sites (1); therefore, populations that consume large quantities of fruits and vegetables in their diet reduce their risk of developing cancer. The biologically active compounds found in these fruits and vegetables have been classified as dietary chemopreventive (CP) agents. There is a gap in knowledge in understanding the mechanisms of how CP agents directly affect xenobiotic metabolism in an intact cell system. It has been suggested that the chemopreventive potential of these agents is largely the result of two major actions: 1) their inhibition of Phase I enzymes leading to decreased formation of the carcinogenic derivatives of xenobiotics, and/or 2) their induction of Phase II enzymes leading to increased excretion of the metabolites (2,3).

One of the more effective CP agents in the current literature is the polyphenol resveratrol (4). Carbo *et al.* administered resveratrol to rats inoculated with fast-growing hepatoma cells, and found that resveratrol significantly reduced the tumor cell count (5). The observed antitumor effects were associated with an increase in the number of cells in the G2/M phase of the cycle and apoptosis of the tumor cell population. In another study by Yu *et al.*, the effects of resveratrol were investigated against the growth of H22 tumors in liver (6). The investigators first developed external tumors by injecting H22 cells into the groin of BALB/c mice. Subsequently, tumor tissue was xenografted into the liver and allowed to grow. Resveratrol treatment was found to reduce hepatic tumor growth through reduced expression of cell cycle proteins.
Resveratrol is found in many plants, including peanuts and grapes. The skin of grapes is a rich source of resveratrol, with fresh grape skins containing about 50 to 100 μg of resveratrol per gram (7); therefore, resveratrol is present at relatively high levels in red wine. It has been shown to be an anti-oxidant that inhibits cyclooxygenase (COX) action (8), to have anti-angiogenic properties (9), and to trigger apoptosis (8). It is also a known inhibitor of Phase I cytochrome P450 (CYP) enzymes, specifically CYP1A1 (10) and CYP1B1.

The CYP system is the primary pathway of metabolic activation of xenobiotics, particularly 1A1 and 1B1 isozymes. Human CYP1A1 (hCYP1A1) can be expressed in the liver but expression is induced predominantly in extrahepatic tissues (11,12). In addition, hCYP1A1 is induced in lung tissue after exposure to cigarette smoke (13). Human CYP1B1 (hCYP1B1) is expressed at low levels in numerous tissues including the kidney and liver (14, 15) and is also induced after exposure to polycyclic aromatic hydrocarbons (PAH) (15). CYP1B1 has also been shown to be overexpressed in breast, lung, liver, gastrointestinal tract, prostate, and bladder tumors (15, 16).

PAH are among the most widely studied carcinogens (17) and are ubiquitous in our environment. They are produced by the incomplete combustion of organic matter and some sources include cigarette smoke, automobile exhaust, and deposition on cooked foods. They are likely causative agents for lung cancer (18). Benzo[a]pyrene (B[a]P) is a PAH compound and a pro-mutagen that is bioactivated to more reactive metabolite, B[a]P-7,8-diol (BPD) by several CYP (19). In humans and rodents, both CYP1A1 and CYP1B1 stereoselectively activate B[a]P at the first step in the pathway to its epoxide metabolites by formation of the 7,8-diol metabolite (20). BPD can be further
metabolized by CYP to form (+)-anti-B[a]P-7,8-diol-9,10-epoxide [(+)-anti-BPDE)], which is the most carcinogenic B[a]P metabolite (19) and is highly reactive towards nucleophilic sites in DNA and other macromolecular structures in the cell.

Previous studies have often focused on extracellular activation of PAH using a microsomal preparation or in intact cells with a lack of ability to control for enzyme expression. Our lab has developed transgenic cell models, employing V79 Chinese hamster lung fibroblast cells that have been stably transfected to express hCYP1A1 and hCYP1B1 isozymes. This novel system allows us to study metabolism of PAH in the absence and presence of resveratrol under tightly controlled conditions, while maintaining cell architecture, enzyme compartmentalization, and biological responses. We investigated whether inhibition of hCYP1A1 or hCYP1B1 activity, as measured by cleavage of the fluorescent VIVID BOMCC substrate in intact cells, was predictive of resveratrol’s effects on the biological endpoints of cytotoxicity and / or mutagenicity of B[a]P or BPD.
Experimental Procedures

Materials and Chemicals

All chemicals used in these studies were of analytical grade and were obtained from Fisher (Atlanta, GA), Sigma (St. Louis), or Invitrogen (Carlsbad, CA). Advanced Dulbecco’s modified Eagle’s medium (Adv. DMEM) was purchased from Gibco/BRL (Grand Island, NY). B[a]P and BPD were purchased from Midwest Research Institute (Lexena, KS). \(^3\)H-B[a]P was purchased from American Radiolabeled Chemicals (ARC, St. Louis, MO). \(^3\)H-BPD was purchased from Midwest Research Institute (Lexena, KS).

Cell Lines and Culture

Parental V79MZ Chinese hamster lung fibroblast cells were modified to express either hCYP1A1 or hCYP1B1, as previously described (21). Cells were grown and maintained in Advanced DMEM supplemented with 2% fetal bovine serum (FBS) at 37°C and 5% CO\(_2\). Cells were selected with 400 µg/ml G-418 and passaged every 2-3 days. Activities of hCYP450’s were assayed at least once a week to maintain and verify stable expression levels.

CYP Fluorescent Enzyme Activity Assays

VIVID® benzyloxymethoxy-3-cyanocoumarin (BOMCC) substrates are blocked dyes that yield minimal fluorescence signal until cleaved at either of two potential cleavage sites, releasing a highly fluorescent product. This assay is performed in an intact cell system, as both substrate and product are membrane permeable. Cells expressing either hCYP1A1 or hCYP1B1 were plated on 6-well plates at 1.8 x 10^5 in 2
ml Advanced DMEM (2% FBS) and allowed to attach and grow for 48 hours. At the
time of the assay, 1 ml of serum-free media containing a 5µM concentration of BOMCC
substrate was replaced on each plate and incubated at 37°C for 30 minutes. To determine
the activity of the enzyme, the highly fluorescent product was then measured
spectrofluorometrically in the medium at 409 nm excitation / 460 nm emission.
Activities of hCYP1A1 and hCYP1B1 were measured using a known VIVID blue
standard curve (0-100µM). Protein was determined by the Coomassie dye method (Bio-
Rad, Hercules, CA). Final enzyme activity was determined by comparison to the
standard curve and normalized to protein content on each plate and expressed as pmol /
min / mg protein. It is important to note that hCYP1A1 has a higher specific activity with
the BOMCC substrate than hCYP1B1.

In the direct enzyme inhibition studies, the assay was plated as above. At the time
of the assay for the dose-dependent studies, 1 ml of media containing a range of
concentrations of resveratrol was replaced on the plates for a 30 minute pre-incubation at
37°C prior to adding the substrate. For the time-dependent studies, 1 ml of serum-free
medium containing a fixed concentration of resveratrol (30µM) was replaced on each
plate for pre-incubation times ranging from 0 minutes – 6 hours at 37°C prior to adding
the substrate. Incubation for an additional 30 minutes at 37°C was allowed and enzyme
activity was determined as described above.

Cytotoxicity Assays

Cytotoxicity was assayed using the sulforhodamine-B (SRB) method, as
previously described (22). The principle of the assay is based on the ability of the protein
dye (SRB) to bind protein basic amino acid residues of trichloroacetic acid-fixed cells. Cells were plated in Advanced DMEM (2% FBS) on 96 well plates at a density of 250 cells / well. This density has been optimized for a growth interval of up to 4 days. Cells were allowed to attach and grow for 16 – 24 hours prior to being dosed. At the time of dosing (for non-continuous exposure), media was removed and 150 µl of serum-free media was added to each well. A concentration calculated to yield 30µM resveratrol in serum-free media (25 µl) was added for a 30 minute pre-incubation at 37ºC prior to adding the PAH. An 8x concentration of B[a]P or BPD (25 µl) was added and incubated for 6 hours. At the end of the exposure period, medium was removed and 200 µl Adv DMEM (2% FBS) was added back to each well for the remainder of the 4 day growth interval. Following incubation for 4 days, cells were harvested by decanting the medium and fixing the cells in 100 µl cold 5% TCA for a minimum of 1 hr at 4ºC. Cells were rinsed with a gentle stream of tap water, allowed to dry, and then stained for 10 minutes with 100 µl 0.4% SRB dye in 1% glacial acetic acid. Cells were then washed 4-5 times with 1% glacial acetic acid, dried, and the dye solubilized in 100 µl 10mM non-pH adjusted Tris-Base. Cell density was determined by measuring absorbance with a microplate reader (Molecular Devices, Sunnyvale, CA) at 560 nm to quantitate protein as an indirect measure of cell number.

**hprt Mutagenicity Assays**

Cells were plated in Advanced DMEM (2% FBS) at a density of 5 x 10⁵ cells per 100 mm plate and allowed to adhere for 16 – 24 hours prior to being dosed. At the time of dosing, media was removed and plates were rinsed twice with 2 ml serum-free media. Cells were pre-incubated with 30µM resveratrol in serum-free media for 30 minutes at
37°C prior to adding the PAH. Cells were then exposed to 0.3µM B[a]P or 0.15µM BPD in DMSO (co-exposure with resveratrol) for 6 hours, media was removed, and cells were rinsed twice with 2 ml Adv DMEM (2% FBS). Following incubation for an additional 3 hours with 30µM resveratrol in 2% serum media, 10 ml fresh serum-containing media was added to each plate and cells were allowed to recover for 72 hours. Cells were then subcultured at 5 x 10^5 cells / 100 mm plate and grown for 6 days, with one subculture at 3 days, to allow for phenotypic development. Cells were subcultured once more at the same density, and the next day 6-thioguanaine (6-TG) was added at a concentration of 10µg 6-TG / ml medium in order to select for *hprt* mutants. Cells were maintained under 6-TG selection for 10 – 12 days, with two changes of serum media + 6-TG during that time, after which mutant *hprt* mutant colonies were stained with 5mM methylene blue in methanol. Mutant colonies were scored manually and are expressed as colonies per million cells.

**HPLC Analysis of PAH Consumption**

Cells were plated in Advanced DMEM (2% FBS) at 5 x 10^5 per 100 mm plate 16-24 hours prior to dosing. At the time of dosing, media was removed and plates were rinsed twice with 2ml serum-free media. Cells were pre-incubated with 30µM resveratrol in serum-free media for 30 minutes at 37°C prior to adding the PAH. Cells were then exposed to 0.3µM B[a]P or 0.15µM BPD in DMSO (co-exposure with resveratrol). Medium was harvested at the indicated times, chrysene was added as internal standard, and frozen at -80°C until analysis. Metabolites were extracted by gravity flow through C18 solid phase extraction columns (50mg MFC18 columns, Biotage AB, Sweden) that were preconditioned with 1 ml methanol and then 1 ml serum-free medium. Following
loading with 12 ml sample, columns were washed with 1 ml 10% acetonitrile (ACN) in dH₂O and eluted with 2 ml 100% ACN. 1ml dH₂O was added to the eluate, which was frozen at -80°C for at least one hour before drying on a lyophilizer. The sample was redissolved in 120 µL ACN, and 25µL was injected onto a C18 column (Onyx Monolithic, 100 x 4.6 mm: Phenomenex Inc., Torrance, CA) attached to a Shimadzu VP HPLC system. The solvent gradient program started at 25% ACN for 0-2 minutes, then a linear gradient to 40% ACN for 2-8 minutes, followed by a linear gradient to 100% from 8-18 minutes. The 100% ACN was maintained for 3 minutes, and then returned to 25% in preparation for the next sample. Peak areas were quantified using a fluorescence detector. Retention times for B[a]P and BPD were determined with standards. These standards were also used to calculate the absolute amount of the PAH in each sample, after correction for recovery using the chrysene internal standard peaks.

**Total Cellular Protein Adducts**

Cells were plated in Advance DMEM (2% FBS) at 1 x 10⁶ per 100 mm plate 16-24 hours prior to dosing. Three 100 mm plates, at this density, were used for each time exposure condition. At time of dosing, media was removed and plates were rinsed twice with 2ml serum-free media. Cells were pre-incubated with 30µM resveratrol in 5 ml serum-free media for 30 minutes at 37°C prior to adding 0.3µM ³H-B[a]P (50 Ci/nmol) or 0.15µM ³H-BPD (4.25 mCi/ml). ³H-PAH (co-exposure with resveratrol) for 6 hours. At the time of harvest, media was removed and cells were scraped in 1X PBS. Cell pellets were initially resuspended in 200 µl 10 mM Tris (pH 7.6) + 1mM EDTA, then sonicated for 20 seconds, and 20µl taken for protein concentration analysis. To the remaining volume, 500 µl cold MeOH/1% perchloric acid (PCA) was added. The suspension was
incubated on ice for 10 minutes. 700 µl hexane was then added, and the hexane/MeOH/1% PCA suspension mixed by inversion for 15 minutes. Cells were pelleted at 14,000 x g for 5 minutes and pellets resuspended in 600 µl 70% MeOH/1% PCA, followed by a 20 second sonication. 600 µl hexane was then added and the suspension once again mixed by inversion for 15 minutes. The cells were pelleted at 14,000 x g for 5 minutes and resuspended in 500µl 70% MeOH, followed by a 20 second sonication. Suspensions were analyzed by scintillation counting and final CPM normalized to protein (as determined by BCA assay).

Statistical Methods

Descriptive statistics (median, mean, S.D., range) were summarized by groups and also graphically. Analysis of Variance (ANOVA) was performed for the analysis of experiments other than for cytotoxicity. Where applicable, Dunnett’s method was used for specific pairwise comparisons to control (e.g., no inhibitor, B[a]P only, etc.) or to baseline (e.g., 5 min time point) to control Type I error. The homogeneity of variances assumption was checked with an O’Brien test and if violated, comparisons were performed using Kruskal-Wallis tests. Cytotoxicity data were analyzed using regression analysis, where concentration was modeled using restricted cubic splines with four knots based on available sample size and Harrell’s recommendations. The goal of this analysis was to get an appropriate test of the chemopreventive agent compared to no agent for each experiment, while appropriately accounting for survival vs. concentration relationships. Analyses were performed using SAS v9.2 (SAS Institute, Cary, NC) and Stata v10.1 (StataCorp, College Station, TX). A two-sided p-value<0.05 was considered statistically significant. A p-value between 0.05 and 0.1 was considered a trend.
Results

*hCYP Enzyme Activity and Inhibition by Resveratrol*

The parental V79MZ control cells have no significant or inducible CYP activity. CYP enzyme activities in transfected cells were measured to ensure that expression levels were adequate and stable, and that CYP activity was confirmed at background levels in the control cells. The CYP activity was greatly increased in cell lines transfected with hCYP1A1 (101-113 pmol / min / mg protein) or hCYP1B1 (8-10 pmol / min / mg protein), as compared to background levels in the control cells (0.16 pmol / min / mg protein) (Table 1). Therefore, adequate CYP activity was obtained in transfected cells. CYP activity was measured after each passage of cells to insure expression of the transfected CYP was stable and constitutive.

Resveratrol (30µM) effectively inhibited hCYP1B1 by greater than 90% (p<0.0001) and hCYP1A1 by greater than 85% (p<0.0001), compared to no inhibitor, in a dose-dependent manner when measuring cleavage of VIVID® BOMCC substrate (Figure 4). Results were similar and stable for timed experiments, where 30µM resveratrol inhibited hCYP1B1 by 100% (p<0.0001) and hCYP1A1 by 95% (p<0.0001) at 6 hour pre-incubation time (Figure 5). Results are presented as the mean ± SD of at least three independent assays.

*Cytotoxicity of B[a]P and BPD +/- Resveratrol*

A dose-response of the cytotoxicity of the PAH in the absence or presence of 30µM resveratrol was determined after a 30-minute pre-incubation with resveratrol followed by a 6 hour exposure to the PAH in the presence of resveratrol. The results
indicated a significant (p<0.001) 3.4-fold reduction of B[a]P cytotoxicity in hCYP1B1 cells by resveratrol (IC$_{50}$ = 1.4 ± 0.3 µM as compared to IC$_{50}$ = 4.8 ± 0.3 µM) (Table 2). Resveratrol significantly (p<0.001) reduced the cytotoxicity of B[a]P (IC$_{50}$ = 0.4 ± 0.04 µM) by 2-fold in hCYP1A1 cells (IC$_{50}$ = 0.8 ± 0.2 µM) (Table 2). Conversely, resveratrol did not protect against cytotoxicity of exposure to BPD for 6 hours in either hCYP1A1 or hCYP1B1 cell lines (Table 3). The V79MZ control cell line showed no cytotoxicity to PAH or resveratrol (not shown).

**Mutagenicity of B[a]P and BPD +/- Resveratrol**

Mutagenicity was measured by frequency of mutation at the hprt locus following exposure of cells to 0.3µM B[a]P or 0.15µM BPD in the absence or presence of 30µM resveratrol for 6 hours. Resveratrol significantly (p=0.05) reduced the mutagenicity of B[a]P in hCYP1A1 cells by 2-fold (Figure 6a). Conversely, resveratrol did not protect against the mutagenicity of BPD in hCYP1A1 cells. A 1.4-fold trend towards reduction (p=0.1) of BPD mutagenicity was observed in hCYP1B1 cells (Figure 6b). The V79MZ control cell line showed no mutagenicity to PAH or resveratrol. The hCYP-transfected cells showed no mutagenicity with resveratrol alone.

**Consumption of B[a]P and BPD +/- Resveratrol**

Consumption of PAH was analyzed by reverse-phase HPLC following exposure of cells to 0.3µM B[a]P or 0.15µM BPD in the absence or presence of 30µM resveratrol for 6 hours. Resveratrol reduced the rate of consumption of B[a]P in both hCYP1A1 and hCYP1B1 cell lines. Consumption of B[a]P in both cell lines in the absence of resveratrol was >80% during the six hour exposure, 83% in hCYP1A1 cells (p=0.01) and
85% in hCYP1B1 (p=0.001). Consumption was 65% in the presence of resveratrol in hCYP1A1 cells (p=0.02) and 63% in hCYP1B1 cells (p=0.02) (Figure 7a, b). When comparing consumption of B[a]P in the presence vs absence of resveratrol within each time point, there was a significant difference (p=0.01) in B[a]P consumption at 6 hour in hCYP1A1 cells. Consumption of BPD in the absence of resveratrol in hCYP1A1 cells during the two hour exposure was >81% (p=0.01), and 49% (p=0.001) in hCYP1B1 cells. Consumption of BPD was 64% (p=0.09) in the presence of resveratrol in hCYP1A1 cells and 14% in hCYP1B1 cells, at five minute exposure vs 2 hour exposure (Figure 7c, d).

**Total Protein Adducts of \(^3\)H-B[a]P and \(^3\)H-BPD +/- Resveratrol**

The effect that 30µM resveratrol would have on the formation of \(^3\)H-B[a]P and \(^3\)H-BPD protein adducts was measured after a 6 hour exposure to 0.3µM \(^3\)H-B[a]P (50 Ci/nmol) (Figure 8a) or 0.15µM \(^3\)H-BPD (4.25 mCi/ml). There was a trend towards reduction (1.7-fold, p=0.1) of \(^3\)H-BPD total protein adducts by resveratrol in hCYP1B1 cells (Figure 8b). The V79MZ control cell line showed no \(^3\)H-PAH protein adduct formation in the absence or presence of resveratrol.

A summation table on the effects of resveratrol on CYP inhibition and on B[a]P and BPD cytotoxicity, mutagenicity, consumption and total protein adduct formation can be found in Chapter 5, Summary.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Genes Expressed</th>
<th>Human CYP Specific Activity (pmol / mg / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZ control</td>
<td>No transgenes</td>
<td>0.16 ± 0.2</td>
</tr>
<tr>
<td>V79MZh1A1</td>
<td>hCYP4501A1</td>
<td>101.2 ± 11.6</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>hCYP4501B1</td>
<td>7.9 ± 1.6</td>
</tr>
</tbody>
</table>

**Table 1. Human Cytochrome P450 specific activities in parental and transfected cell lines.** The CYP fluorescent enzyme activity assay was performed as described in *Experimental Procedures*. Results are presented as the mean ± S.D. of at least 3 independent assays.
Table 2

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>B[a]P IC₅₀ (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.4 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + Resveratrol</td>
<td>0.8 ± 0.2</td>
<td>2.0*</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>1.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>V79MZh1B1 + Resveratrol</td>
<td>4.8 ± 0.3</td>
<td>3.4*</td>
</tr>
</tbody>
</table>

**Table 2.** Cytotoxicity of B[a]P ± 30µM resveratrol in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in *Experimental Procedures*. Results are presented as the mean ± S.D. of 3 or more independent assays. Asterisk (*) indicates significance (p<0.001 for V79MZh1A1 vs V79MZh1A1 + resveratrol; p<0.001 for V79MZh1B1 vs V79MZh1B1 + resveratrol).
Table 3

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>BPD IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZhlA1</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZhlA1 + Resveratrol</td>
<td>0.03 ± 0.01</td>
<td>None</td>
</tr>
<tr>
<td>V79MZhlB1</td>
<td>0.09 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZhlB1 + Resveratrol</td>
<td>0.09 ± 0.02</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 3. Cytotoxicity of BPD + 30µM resveratrol in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in Experimental Procedures. Results are presented as the mean ± S.D. of 3 or more independent assays.
**Figure 4a.** Resveratrol dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP intact cell enzyme activity assay was performed as described in *Experimental Procedures*. Results are expressed as pmol/min/mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), black bars represent V79MZh1A1. p values are for comparison of activity in the presence of resveratrol to activity in the control (no inhibitor).
Figure 4b. Resveratrol dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP intact cell enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), gray bars represent V79MZh1B1. p values are for comparison of activity in the presence of resveratrol to activity in the control (no inhibitor).
Figure 5a. Stability of 30μM resveratrol inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP intact cell enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), black bars represent V79MZh1A1. p values are for comparison of activity in the presence of resveratrol to activity in the control (no inhibitor).
Figure 5b. Stability of 30μM resveratrol inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP intact cell enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), gray bars represent V79MZh1B1. p values are for comparison of activity in the presence of resveratrol to activity in the control (no inhibitor).
Figure 6a. Mutagenicity of 0.3µM B[a]P ± 30µM resveratrol in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the B[a]P exposure condition to the mutant colonies in the B[a]P + resveratrol exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P only, light bars represent exposure to B[a]P + resveratrol. p values are for B[a]P only compared to B[a]P + resveratrol, within the same cell line.
Figure 6b. Mutagenicity of 0.15µM BPD ± 30µM resveratrol in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the BPD exposure condition to the mutant colonies in the BPD + resveratrol exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD only, light bars represent exposure to BPD + resveratrol.
**Figure 7a.** Consumption of B[a]P in V79MZ cells engineered to express hCYP1A1 exposed to 0.3µM B[a]P + 30µM resveratrol, 6 hour exposure. B[a]P consumption was analyzed using HPLC, as described in *Experimental Procedures*. Percent consumption is the ratio of B[a]P at 5 minute to B[a]P at 6 hour, or B[a]P + resveratrol at 5 minute to B[a]P + resveratrol at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + resveratrol. p values are a pairwise comparison to 5 minute exposure. p values shown in parentheses are B[a]P compared to B[a]P + resveratrol within the same exposure time.
Figure 7b. Consumption of B[a]P in V79MZ cells engineered to express hCYP1B1 exposed to 0.3μM B[a]P + 30μM resveratrol, 6 hour exposure. B[a]P consumption was analyzed using HPLC, as described in Experimental Procedures. Percent consumption is the ratio of B[a]P at 5 minute to B[a]P at 6 hour, or B[a]P + resveratrol at 5 minute to B[a]P + resveratrol at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + resveratrol. p values are a pairwise comparison to 5 minute exposure.
**Figure 7c.** Consumption of BPD in V79MZ cells engineered to express hCYP1A1 exposed to 0.15μM BPD ± 30μM resveratrol, 6 hour exposure. BPD consumption was analyzed using HPLC, as described in *Experimental Procedures*. Percent consumption is the ratio of BPD at 5 minute to BPD at 6 hour, or BPD + resveratrol at 5 minute to BPD + resveratrol at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + resveratrol. p values are a pairwise comparison to 5 minute exposure.
Figure 7d. Consumption of BPD in V79MZ cells engineered to express hCYP1B1 exposed to 0.15µM BPD ± 30µM resveratrol, 6 hour exposure. BPD consumption was analyzed using HPLC, as described in Experimental Procedures. Percent consumption is the ratio of the BPD at 5 minute to BPD at 6 hour, or BPD + resveratrol at 5 minute to BPD at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + resveratrol. p values are a pairwise comparison to 5 minute exposure.
Figure 8a. Total protein adducts in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 exposed to $^{3}$H-B[a]P $\pm$ 30µM resveratrol, 6 hour exposure. The total protein adduct assay was performed as described in Experimental Procedures. Fold-protection is the ratio of CPM in the $^{3}$H-B[a]P exposure condition to the CPM in the $^{3}$H-B[a]P + resveratrol exposure condition. Results are presented as the mean $\pm$ S.D. of 3 independent assays. Black bars represent exposure to $^{3}$H-B[a]P, light bars represent exposure to $^{3}$H-B[a]P + resveratrol.
Figure 8b. Total protein adducts in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 exposed to $^3$H-BPD ± resveratrol, 6 hour exposure. The total protein adduct assay was performed as described in Experimental Procedures. Fold-protection is the ratio of CPM in the $^3$H-BPD exposure condition to the CPM in the $^3$H-BPD + resveratrol exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to $^3$H-BPD, light bars represent exposure to $^3$H-BPD + resveratrol.
Discussion

One of the more effective CP agents in the current literature is the polyphenol resveratrol (4) found in many plants, including peanuts and grapes. It is also a known inhibitor of Phase I cytochrome P450 (CYP) enzymes, specifically CYP1A1 (10) and CYP1B1. We investigated whether direct inhibition of human cytochrome P450 (hCYP) activity, as measured by cleavage of the fluorescent VIVID BOMCC substrate in intact cells, was predictive of resveratrol’s effects on the cytotoxicity and/or mutagenicity of B[a]P or BPD. The CYP activity inhibition assay predicted protection by resveratrol against the parent compound B[a]P; however, resveratrol was unexpectedly less effective for prevention of hCYP1A1 or hCYP1B1 activation of the intermediate metabolite BPD when measuring the endpoints of cytotoxicity and mutagenicity.

Parental V79MZ Chinese hamster lung fibroblast cells, modified to express either hCYP1A1 or hCYP1B1, were used in this study to examine the effects of resveratrol on hCYP1A1 and hCYP1B1 activation of B[a]P and BPD to toxic and mutagenic metabolites. There are several reasons why this novel model system is favorable over the cell lines or microsomes used in earlier studies. First, the parent cells express no constitutive or inducible CYP activity (23). They do express an endogenous Pi-class hamster GST at low levels (24), but this GST is not active for the conjugation of reactive electrophiles of B[a]P or BPD with glutathione (GSH) (25). Second, expression of the transfected CYP is stable and constitutive, rather than variable due to carcinogen induction. Next, expression of the enzymes is unhindered by background interference because each CYP is expressed against the same V79 cell background; therefore, each
isozyme is comparable. Last, the endpoints of cytotoxicity and mutagenicity can be analyzed in live and intact cells.

The CYP isozymes we employed in these studies, hCYP1A1 and hCYP1B1, are key in the metabolic activation of PAHs and there is high specific activity of these isozymes towards the PAH being examined. In addition, resveratrol is a known inhibitor of CYP1A1 (10) and CYP1B1. This suggests that one likely mechanism for protection by resveratrol is decreased activation of carcinogens by inhibition of CYP (4); therefore we performed an enzyme inhibition assay to determine if resveratrol was a potent inhibitor of these CYP isozymes in our cell system. We first determined a working range of concentrations of resveratrol that were below the IC$_{50}$ value for cytotoxicity, to minimize toxicity due to resveratrol. We examined inhibitory concentrations of resveratrol up to 50µM in our dose-dependent enzyme assay. We saw almost complete inhibition of hCYP1B1 and hCYP1A1 by resveratrol (30µM), greater than 90% and 85%, respectively. It was important to know if this inhibition was maintained over a period of time, so we examined time-dependent inhibition up to 6 hours. This acute time point was chosen because resveratrol appears to be metabolized at a significant rate and may not be stable over long periods (26). Results were similar for timed experiments, where 30µM resveratrol inhibited hCYP1B1 and hCYP1A1 initially by 100% and up to 95%, respectively, at 6 hour pre-incubation time. Thus, inhibition by resveratrol, when measuring cleavage of the fluorescent VIVID® BOMCC substrate, was both dose-dependent and stable for up to six hours. Also, inhibition of CYP activity was slightly higher in cells expressing hCYP1B1 than in cells expressing hCYP1A1.
In order to determine if this direct inhibition of CYP’s cleavage of the VIVID® BOMCC substrate by resveratrol would predict the polyphenol’s effects on the activation and toxicity of B[a]P or BPD, cytotoxicity was assayed using the sulforhodamine B (SRB) method, as previously described (22). An assessment of cytotoxicity over an acute (6 hour) interval was chosen because, as previously stated, resveratrol may not be stable over long periods of time (26). Also, concentrations of both PAH and resveratrol are highest in the media during shorter exposure times. In addition, this time frame allows for a higher concentration range, which is physiologically relevant to exposures of both resveratrol and PAH in the gastrointestinal tract after consuming a meal consisting of cooked meat (with PAH deposits) and wine. Resveratrol significantly reduced the cytotoxicity of B[a]P in cells expressing either hCYP1B1 or hCYP1A1 (3.4-fold and 2-fold, respectively). The V79MZ control cell line showed no cytotoxicity to PAH or resveratrol (data not shown). It is possible that we see a higher fold protection by resveratrol against B[a]P toxicity in cells expressing hCYP1B1 because these cells are less sensitive to B[a]P cytotoxicity (IC$_{50}$ = 1.4µM) than cells expressing hCYP1A1 (IC$_{50}$ = 0.4µM). The higher fold protection of B[a]P cytotoxicity by resveratrol in hCYP1B1 cells is also supported by our enzyme inhibition data, with more potent inhibition by resveratrol in cells expressing hCYP1B1. While we observed protection by resveratrol against B[a]P toxicity, protection against BPD cytotoxicity was not observed. The lack of protection was unexpected, given that BPD is a known substrate for hCYP1A1 and hCYP1B1 and resveratrol is such a potent inhibitor of both isozymes. One potential explanation is that B[a]P is a weaker substrate for CYP than BPD. BPD, being the better substrate, binds CYP more efficiently, making it harder for resveratrol to compete for the
CYP active site. Chun et al. reported mixed-type inhibition by resveratrol, showing that it can compete for the enzyme active site but still bind at a different site from the substrate (27). Therefore, resveratrol may bind allosterically to the CYPs and alter the conformation in a manner that affects B[a]P, but not BPD, metabolism.

Cells expressing hCYP1B1 were also moderately less sensitive to B[a]P mutagenicity than cells expressing hCYP1A1. Resveratrol reduced B[a]P mutagenicity after a 6 hour exposure in cells expressing either hCYP1A1 (2.1-fold, p=0.05) or hCYP1B1 (1.3-fold). Thus, there was a lower fold protection against B[a]P mutagenicity by resveratrol in hCYP1B1 cells, where we observed a higher fold protection of B[a]P when looking at the endpoint of cytotoxicity. While we did not observe protection against BPD cytotoxicity by resveratrol in either cell line, we did, however, see a 1.4-fold trend towards reduction (p=0.1) in BPD mutagenicity in the presence of resveratrol in hCYP1B1 cells, though this reduction was not statistically significant. We did not observe this reduction in hCYP1A1 cells. These results suggest that the endpoints of cytotoxicity and mutagenicity may be mediated by different metabolites that are formed from activation of the parent compound as compared to further activation of the intermediate metabolite. Effective inhibition by resveratrol of the metabolic activation of these potent carcinogens via CYP pathway should decrease cytotoxicity and/or mutagenicity caused by reactive electrophiles. However, the presence of resveratrol may not inhibit CYP, but modulating the metabolite distribution of B[a]P or BPD in a CYP isozyme-specific manner. Resveratrol could affect conformation of the enzyme, therefore causing the PAH to bind the active site differently (27). Therefore, we looked at the effects of resveratrol on CYP-mediated metabolism of B[a]P and BPD.
Analysis of B[a]P or BPD depletion showed that resveratrol slows the metabolism of each PAH but does not completely block it, with the exception of BPD consumption by hCYP1B1 cells. This is contrary to the direct CYP inhibition data, where resveratrol proved to be a strong inhibitor. However, this inhibition blocks cleavage of the fluorescent substrate, rather than oxidation of B[a]P or BPD. The consumption profiles don’t explain why we see a reduction in B[a]P cytotoxicity and mutagenicity but no effect on BPD cytotoxicity or mutagenicity. We must consider metabolite distribution, suggesting that resveratrol could be acting as a modifier instead of a pure inhibitor.

Both hCYP1A1 and hCYP1B1 metabolize B[a]P to yield BPD, a pathway leading to diol epoxide formation (19). The slower consumption of B[a]P in the presence of resveratrol could lend to fewer BPD metabolites from B[a]P. Also CYP metabolism of B[a]P yields both (+) and (-) BPD enantiomers. It is the (-) BPD that goes on to form the (+)-anti-BPDE, the most mutagenic metabolite of B[a]P. It is possible that metabolism of B[a]P in the presence of resveratrol yields more of the (+)-syn-BPDE, which is less reactive, more readily conjugated to GSH, and excreted from the cell. It is also possible that BPD is not the dominant metabolite formed, but the presence of resveratrol shifts distribution to other, less reactive metabolites of B[a]P. Phenol metabolites are much less toxic, as compared to BPD (20). Quinone metabolites are non-mutagenic; however, they are able to form adducts with DNA (21). The fact that we saw no significant reduction in 3H-B[a]P protein adducts in the presence of resveratrol, but observed a decrease in toxicity and mutagenicity, might be explained by a shift to more quinone metabolites in the presence of resveratrol.
This still does not explain why we did not see these same effects by resveratrol against BPD toxicity and mutagenicity. BPD is a better substrate for CYP than B[a]P, and possibly a better substrate than resveratrol. BPD could get metabolized more efficiently. While BPD is bound to the active site, resveratrol may still affect the conformation of the enzyme by binding a site different than the active site (27). This conformational change may shift metabolite formation to yield more (+)-anti-BPDE.

Resveratrol could have effects through mechanisms independent of direct CYP inhibition or metabolite distribution. Resveratrol has been shown to affect key proteins involved in signal transduction such as AKT (8) and nuclear factor kappa-B (NF-κB) (28). It has been reported that resveratrol interferes with AKT activity. When activated, AKT allows cell cycle progression to occur by overcoming cell cycle arrest in G1/S (29) and G2 (30) phases. This enables proliferation and survival of cells that have sustained a potentially mutagenic insult, thereby contributing to acquisition of mutations in other genes. Therefore, it is possible that attenuation in B[a]P mutagenicity was due to resveratrol inhibition of AKT, which inhibited cell cycle progression and the proliferation of cells with a high mutation load. Also, by altering cell cycle progression, this may have afforded more time for DNA repair, resulting in decreased mutagenicity.

AKT also plays a role in inhibition of apoptosis pathways via NF-κB (28). AKT activates NF-κB by phosphorylating inhibitor of kappa-B kinase (IKK), leading to inactivation and degradation of the kinase. IKK can no longer sequester the transcription factor. This allows NF-κB to translocate to the nucleus and turn on transcription of many genes, including survival genes. It also turns on the cell’s apoptosis pathways. By inhibiting AKT, and thus NF-κB activation, resveratrol could also inhibit activation of the
cell’s suicide program. Therefore, it is possible we saw a reduction in B[a]P toxicity because resveratrol inhibited apoptosis, e.g., via scavenging of ROS. There have been reports contradicting the ability of resveratrol to inhibit phosphorylation (and inactivation) of IKK (31). Sexton et al. reported that resveratrol actually triggered apoptosis in human uterine cancer cells (8). Although this is a possible reason why resveratrol did not appear to reduce toxicity of BPD, this does not explain why resveratrol had no significant effect on BPD mutagenicity, nor does it explain the different effects on B[a]P vs BPD toxicity.

Another chemopreventive mechanism of resveratrol, independent of direct inhibition of CYP, is its ability to directly bind and protect DNA. While PAH provide an exogenous source of insult to DNA, low levels of reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^-$) and hydroxyl radical (OH) are formed by peroxisomes and CYP within the cell. These endogenously generated free radicals can cause additional damage to DNA, including base modifications, base loss, single- and double-strand DNA breaks (32). It has been shown that resveratrol directly binds DNA (33, 34). While resveratrol itself is not a DNA intercalator, it has been shown to stabilize double-stranded DNA, thereby providing a protective effect (35). By inducing DNA repair pathways, resveratrol would decrease toxicity and mutagenicity, which is what we observed with B[a]P. The reason we did not observe this effect with BPD is unclear.

Oral administration in animal models showed that resveratrol inhibited the development of several cancers induced by chemical carcinogens, including esophageal (35) and mammary cancer (36, 37). However, this effect was not seen in lung cancer induced by carcinogens found in cigarette smoke (38, 39). It is unknown whether high
intake of resveratrol by humans will help in preventing cancer. Studies done on the bioavailability and metabolism of resveratrol suggest that even very high intake of resveratrol in the diet may not reach levels in tissue to achieve the same protective effects seen in cell culture studies (26, 40).

Oral intake of *trans*-resveratrol by humans has been reported to be well absorbed; however, the bioavailability is low because it is metabolized and eliminated so quickly (26, 41). In studies where healthy adults were administered oral doses of 25 mg of *trans*-resveratrol, only traces of resveratrol were detected in plasma. Plasma concentrations of resveratrol and metabolites peaked after 60 minutes at concentrations of 2 µM (491 µg/L) (26). Another study suggests that resveratrol from grape juice, which contains mostly glucosides of resveratrol, may have a lower bioavailability than *trans*-resveratrol (42).

Studies on the bioavailability of resveratrol in humans are important because most previous research has been done in cultured cells that have been exposed to unmetabolized resveratrol at concentrations up to 100 times greater than peak concentrations observed in human plasma after oral consumption (40). Cells that line the digestive tract are exposed to unmetabolized resveratrol; however, research suggests that other tissues are exposed to resveratrol metabolites and not much is known about the effects of these metabolites (26).

In this current study, we have shown that resveratrol inhibits hCYP1A1- and hCYP1B1-catalyzed cleavage of the VIVID® BOMCC substrate in both a dose- and time-dependent manner. We have also shown that while this inhibition was predictive of protection by resveratrol against the parent compound B[a]P, it did not accurately predict the ability of resveratrol to inhibit activation of the intermediate metabolite BPD when
measuring cytotoxicity and/or mutagenicity as endpoints. When examining xenobiotic metabolism in an intact cell system and the biological endpoints that are a result of that metabolism, it appears that resveratrol may exert its effects through CYP-independent as well as CYP-dependent mechanisms. This pleiotropic agent has the potential to interact with several biological and biochemical processes, including but not limited to CYP inhibition, signal transduction, cell cycle progression, apoptotic pathways, and DNA repair. Therefore, its activity should not be assigned to a single mechanism of action at this time. Lacking a clear understanding of the mechanisms of resveratrol could be problematic when employing it as a chemopreventive agent and it is clear that in vivo observations do not always agree with in vitro results. Additional studies will be required to reconcile these opposing results and controversies among published data.
References:


CHAPTER 3

Galangin Attenuates Cytotoxicity and Mutagenicity of Benzo[a]pyrene (B[a]P) and Benzo[a]pyrene-7,8-dihydrodiol (BPD) in Intact V79 Cells Expressing Either hCYP1A1 or hCYP1B1

Abstract:

Genetic damage, which can result in functional loss of tumor suppressor gene activity or gain in function of oncogenes, is believed to lead to a wide range of diseases, including cancer. Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmental carcinogen that is metabolized to its intermediate metabolite, benzo[a]pyrene-7,8-diol (BPD), by cytochrome P450 (CYP) Phase I enzymes. This leads to activation of these PAH to reactive electrophiles that are capable of binding covalently to protein and DNA, leading to changes in function, genetic damage, mutation, and possibly cancer. The flavonoid galangin is a component of propolis and Alpina officinarum (common name, India or China root) and a known inhibitor of CYP. We investigated whether inhibition of human cytochrome P450 (hCYP), as measured by cleavage of the VIVID® BOMCC substrate to a fluorescent product in intact cells, was predictive of galangin’s effects on cytotoxicity and / or mutagenicity of B[a]P or BPD. We employed V79MZ cells stably transfected to express hCYP1A1 or hCYP1B1. Inhibition of CYP activity by 3μM galangin was more potent in cells expressing hCYP1B1 than in cells expressing hCYP1A1, 99% as compared to 49%. The CYP activity inhibition assay predicted protection by galangin against B[a]P and BPD. Galangin (60μM) significantly reduced cytotoxicity of B[a]P by 1.6-fold in hCYP1A1
cells, and 1.1-fold in hCYP1B1 cell lines. We also observed significant protection against BPD cytotoxicity by galangin in hCYP1A1 (2.7-fold) and hCYP1B1 (2.4-fold) cell lines. Similarly, galangin significantly reduced mutagenicity of B[a]P at the hprt locus in hCYP1A1 cells by 3.3-fold. There was a 1.2-fold trend towards reduction of B[a]P mutagenicity in hCYP1B1 cells. Galangin also significantly reduced mutagenicity of BPD at the hprt locus in both hCYP1B1 and hCYP1A1 cell lines (4.8-fold and 1.7-fold, respectively). Consumption profiles examining the metabolism of B[a]P by hCYP450 shows that galangin blocks the consumption of B[a]P at 2 and 4 hour in hCYP1A1 cells, and at 6 hour in hCYP1B1 cells. Galangin blocks the consumption of BPD in hCYP1A1 cells at all exposure times. Measurement of $^3$H-B[a]P total protein adducts in cells expressing hCYP1A1 and hCYP1B1 revealed a 75% (4.1-fold) and 95% (21.6-fold) trend towards reduction in adducts, respectively, in the presence of galangin. We observed a 91% (11.1-fold) and 90% (10.2-fold) significant reduction in $^3$H-BPD adducts in both hCYP1A1 and hCYP1B1 cell lines, respectively.
Introduction

The goal and primary focus of chemoprevention is to block or suppress carcinogenesis using natural or synthetic compounds (1). Fruits and vegetables are major sources of biologically active compounds called phytochemicals. These have been shown to lower the risk of cancer in animal models (2); therefore, these compounds can be classified as chemopreventive (CP) agents. However, the mechanisms whereby CP agents reduce cancer risk and their relative importance remain incompletely understood. Agents that selectively inhibit Phase I enzymes, or preferentially activate Phase II more than Phase I enzymes, would likely have the most promise in chemoprevention. Understanding their effects on Phase I and Phase II enzymes, and particularly their direct effects on metabolism of potent environmental carcinogens, will offer a clearer picture of how CP agents can be used to reduce carcinogenic genetic damage in humans.

Galangin is a dietary flavonoid (more specifically, a flavonol) that is present in high concentrations in honeybee propolis, which is a natural mixture produced by honeybees from the gum of various plants (2). It is also found in *Alpinia officinarum* (common name, India or China root), a plant which has been used as a spice and as an herbal medicine for a variety of ailments in Asia. The biological activities shown for galangin are numerous, including anti-mutagenic (3), anti-clastogenic (4), anti-oxidative (5), radical scavenging (6), and COX-2 inhibiting (7). Galangin is also a known inhibitor of Phase I cytochrome P450 (CYP) enzymes (8).

The CYP system is the primary pathway through which the body metabolizes xenobiotics, through a process that consists of activation, deactivation and secretion of
the product. Human CYP1A1 (hCYP1A1) is poorly expressed in human liver; however, it can be induced in many extrahepatic tissues including the lung, after exposure to cigarette smoke (9). Human CYP1B1 (hCYP1B1) is expressed in numerous tissues and is also induced after exposure to polycyclic aromatic hydrocarbons (PAH) (10). The PAH, benzo[a]pyrene (B[a]P), is a ubiquitous environmental carcinogen found in tobacco smoke, charbroiled foods, and incomplete combustion effluents. It is activated to its more reactive metabolite, B[a]P-7,8-diol (BPD) by Phase I enzymes, primarily CYP1A1 and CYP1B1 (11).

We investigated whether inhibition of hCYP1A1 or hCYP1B1 activity, as measured by cleavage of the VIVID® BOMCC substrate in intact cells, was predictive of galangin’s effects on the cytotoxicity and/or mutagenicity of B[a]P and BPD. We employed V79 Chinese hamster lung fibroblast cells stably transfected to express hCYP1A1 or hCYP1B1. This novel system allows us to study the metabolism of PAH in the absence or presence of galangin under tightly controlled conditions, while maintaining cell architecture, enzyme compartmentalization, and biological responses.
Experimental Procedures

Materials and Chemicals

All chemicals used in these studies were of analytical grade and were obtained from Fisher (Atlanta, GA), Sigma (St. Louis), or Invitrogen (Carlsbad, CA). Advanced Dulbecco’s modified Eagle’s medium (Adv. DMEM) was purchased from Gibco/BRL (Grand Island, NY). B[a]P and BPD were purchased from Midwest Research Institute (Lexena, KS). \(^3\)H-B[a]P was purchased from American Radiolabeled Chemicals (ARC, St. Louis, MC). \(^3\)H-BPD was purchased from Midwest Research Institute (Lexena, KS).

Cell Lines and Culture

Parental V79MZ Chinese hamster lung fibroblast cells were modified to express either hCYP1A1 or hCYP1B1, as previously described (11). Cells were grown and maintained in Advanced DMEM supplemented with 2% fetal bovine serum (FBS) at 37°C and 5% CO\(_2\). Cells were selected with 400 µg/ml G-418 and passaged every 2-3 days. Activities of hCYP were assayed at least once a week to maintain and verify stable expression levels.

The parent cells express no constitutive or inducible CYP450 activity (12). They do express an endogenous Pi-class hamster GST at low levels (13), but this GST is not active for the conjugation of reactive electrophiles of the PAH in these studies with glutathione (GSH) (14). Expression of the transfected CYP450 is stable and constitutive, rather than variable due to carcinogen induction. Also, expression of the enzymes is unhindered by background interference because each CYP450 is expressed against the same V79 cell background; therefore, each isozyme is comparable.
CYP Fluorescent Enzyme Activity Assays

VIVID® benzyloxymethyloxy-3-cyanocoumarin (BOMCC) substrates are blocked dyes that yield minimal fluorescence signal until cleaved at either of two potential cleavage sites, releasing a highly fluorescent product. This assay is performed in an intact cell system, as both substrate and product are membrane-permeable. Cells expressing either hCYP1A1 or hCYP1B1 were plated on 6-well plates at $1.8 \times 10^5$ in 2 ml Advanced DMEM (2% FBS) and allowed to attach and grow for 48 hours. At the time of the assay, 1 ml of serum-free media containing a 5µM concentration of BOMCC substrate was replaced on each plate and incubated at 37°C for 30 minutes. To determine the activity of the enzyme, the highly fluorescent product was then measured spectrofluorometrically in the medium at 409 nm excitation / 460 nm emission. Activities of hCYP1A1 and hCYP1B1 were measured using a known VIVID blue standard curve (0-100µM). Protein was determined by the Coomassie dye method (Bio-Rad, Hercules, CA). Final enzyme activity was determined by comparison to the standard curve and normalized to protein content on each plate and expressed as pmol / min / mg protein. It is important to note that hCYP1A1 has a higher specific activity with the BOMCC substrate than hCYP1B1.

In the direct enzyme inhibition studies, the assay was plated as above. At the time of the assay for the dose-dependent studies, 1 ml of media containing a range of concentrations of galangin was replaced on the plates for a 30 minute pre-incubation at 37°C prior to adding the substrate. For the time-dependent studies, 1 ml of serum-free medium containing a fixed concentration (3µM galangin) was replaced on each plate for pre-incubation times ranging from 0 minutes – 6 hours at 37°C prior to adding the
substrate. Incubation for an additional 30 minutes at 37°C was allowed and enzyme activity was determined as described above.

**Cytotoxicity Assays**

Cytotoxicity was assayed using the sulforhodamine-B (SRB) method, as previously described (15). The principle of the assay is based on the ability of the protein dye (SRB) to bind protein basic amino acid residues of trichloroacetic acid-fixed cells. Cells were plated in Advanced DMEM (2% FBS) on 96 well plates at a density of 250 cells/well. This density has been optimized for a growth interval of up to 4 days. Cells were allowed to attach and grow for 16–24 hours prior to being dosed. At the time of dosing (for non-continuous exposure), media was removed and 150 µl of serum-free media was added to each well. A concentration calculated to yield 60µM galangin in serum-free media (25 µl) was added for a 30 minute pre-incubation at 37°C prior to adding the PAH. An 8x concentration of B[a]P or BPD (25 µl) was added and incubated for 6 hours. At the end of the exposure period, medium was removed and 200 µl Adv DMEM (2% FBS) was added back to each well for the remainder of the 4 day growth interval. Following incubation for 4 days, cells were harvested by decanting the medium and fixing the cells in 100 µl cold 5% TCA for a minimum of 1 hr at 4°C. Cells were rinsed with a gentle stream of tap water, allowed to dry, and then stained for 10 minutes with 100 µl 0.4% SRB dye in 1% glacial acetic acid. Cells were then washed 4-5 times with 1% glacial acetic acid, dried, and the dye solubilized in 100 µl 10mM non-pH adjusted Tris-Base. Cell density was determined by measuring absorbance with a microplate reader (Molecular Devices, Sunnyvale, CA) at 560 nm to quantitate protein as an indirect measure of cell number.
**hprt Mutagenicity Assays**

Cells were plated in Advanced DMEM (2% FBS) at a density of $5 \times 10^5$ cells per 100 mm plate and allowed to adhere for 16 – 24 hours prior to being dosed. At the time of dosing, media was removed and plates were rinsed twice with 2 ml serum-free media. Cells were pre-incubated with 60µM galangin in serum-free media for 30 minutes at 37°C prior to adding the PAH. Cells were then exposed to 0.3µM B[a]P or 0.15µM BPD in DMSO (co-exposure with galangin) for 6 hours, media was removed, and cells were rinsed twice with 2 ml Adv DMEM (2% FBS). Following incubation for an additional 3 hours with 60µM galangin in 2% serum media, 10 ml fresh serum-containing media was added to each plate and cells were allowed to recover for 72 hours. Cells were then subcultured at $5 \times 10^5$ cells / 100 mm plate and grown for 6 days, with one subculture at 3 days, to allow for phenotypic development. Cells were subcultured once more at the same density, and the next day 6-thioguanine (6-TG) was added at a concentration of 10µg 6-TG / ml medium in order to select for hprt mutants. Cells were maintained under 6-TG selection for 10 – 12 days, with two changes of serum media + 6-TG during that time, after which mutant hprt mutant colonies were stained with 5mM methylene blue in methanol. Mutant colonies were scored manually and are expressed as colonies per million cells.

**HPLC Analysis of PAH Consumption**

Cells were plated in Advanced DMEM (2% FBS) at $5 \times 10^5$ per 100 mm plate 16-24 hours prior to dosing. At the time of dosing, media was removed and plates were rinsed twice with 2ml serum-free media. Cells were pre-incubated with 60µM galangin
in serum-free media for 30 minutes at 37°C prior to adding the PAH. Cells were then exposed to 0.3µM B[a]P or 0.15µM BPD in DMSO (co-exposure with galangin). Medium was harvested at the indicated times, chrysene was added as internal standard, and frozen at -80°C until analysis. Metabolites were extracted by gravity flow through C18 solid phase extraction columns (50mg MFC18 columns, Biotage AB, Sweden) that were preconditioned with 1 ml methanol and then 1 ml serum-free medium. Columns were washed with 1 ml 10% acetonitrile (ACN) in dH₂O and eluted with 2 ml 100% ACN. 1ml dH₂O was added to the eluate, which was frozen at -80°C for at least one hour before drying on a lyophilizer. The sample was redissolved in 120 µL ACN, and 25µL was injected onto a C18 column (Onyx Monolithic, 100 x 4.6 mm: Phenomenex Inc., Torrance, CA) attached to a Shimadzu VP HPLC system. The solvent gradient program started at 25% ACN for 0-2 minutes, then a linear gradient to 40% ACN for 2-8 minutes, followed by a linear gradient to 100% from 8-18 minutes. The 100% ACN was maintained for 3 minutes, and then returned to 25% in preparation for the next sample. Peak areas were quantified using a fluorescence detector. Retention times for B[a]P and BPD were determined with standards. These standards were also used to calculate the absolute amount of the PAH in each sample, after correction for recovery using the chrysene internal standard peaks.

**Total Cellular Protein Adducts**

Cells were plated in Advance DMEM (2% FBS) at 1 x 10⁶ per 100 mm plate 16-24 hours prior to dosing. Three 100 mm plates, at this density, were used for each time exposure condition. At time of dosing, media was removed and plates were rinsed twice with 2ml serum-free media. Cells were pre-incubated with 60µM galangin in 5 ml
serum-free media for 30 minutes at 37°C prior to adding 0.3µM ³H-B[a]P (50 Ci/nmol) or 0.15µM ³H-BPD (4.25 mCi/ml) (co-exposure with galangin) for 6 hours. At the time of harvest, media was removed and cells were scraped in 1X PBS. Cell pellets were initially resuspended in 200 µl 10 mM Tris (pH 7.6) + 1mM EDTA, then sonicated for 20 seconds, and 20µl taken for protein concentration analysis. To the remaining volume, 500 µl cold MeOH/1% perchloric acid (PCA) was added. The suspension was incubated on ice for 10 minutes. 700 µl hexane was then added, and the hexane/MeOH/1% PCA suspension mixed by inversion for 15 minutes. Cells were pelleted at 14,000 x g for 5 minutes and pellets resuspended in 600 µl 70% MeOH/1% PCA, followed by a 20 second sonication. 600 µl hexane was then added and the suspension once again mixed by inversion for 15 minutes. The cells were pelleted at 14,000 x g for 5 minutes and resuspended in 500µl 70% MeOH, followed by a 20 second sonication. Suspensions were analyzed by scintillation counting and final CPM normalized to protein (as determined by BCA assay).

Statistical Methods

Descriptive statistics (median, mean, S.D., range) were summarized by groups and also graphically. Analysis of Variance (ANOVA) was performed for the analysis of experiments other than for cytotoxicity. Where applicable, Dunnett’s method was used for specific pairwise comparisons to control (e.g., no inhibitor, B[a]P only, etc.) or to baseline (e.g., 5 min time point) to control Type I error. The homogeneity of variances assumption was checked with an O’Brien test and if violated, comparisons were performed using Kruskal-Wallis tests. Cytotoxicity data were analyzed using regression analysis, where concentration was modeled using restricted cubic splines with four knots.
based on available sample size and Harrell’s recommendations. The goal of this analysis was to get an appropriate test of the chemopreventive agent compared to no agent for each experiment, while appropriately accounting for survival vs. concentration relationships. Analyses were performed using SAS v9.2 (SAS Institute, Cary, NC) and Stata v10.1 (StataCorp, College Station, TX). A two-sided p-value $< 0.05$ was considered statistically significant. A p-value between 0.05 and 0.1 was considered a trend.
**Results**

*hCYP enzyme activity and inhibition by galangin*

The parental V79MZ control cells have no significant or inducible CYP activity. CYP enzyme activities in transfected cells were measured to ensure that expression levels were adequate and stable, and that CYP activity was confirmed at background levels in the control cells. The CYP activity was greatly increased in cell lines transfected with hCYP1A1 (101-113 pmol / min / mg protein) or hCYP1B1 (8-10 pmol / min / mg protein), as compared to background levels in the control cells (0.16 pmol / min / mg protein) (Table1). Therefore, adequate CYP activity was obtained in transfected cells. CYP activity was measured after each passage of cells to insure expression of the transfected CYP was stable and constitutive.

Galangin (3µM) effectively inhibited hCYP1B1 by 99% (p<0.0001) and hCYP1A1 by greater than 45% (p<0.0001), compared to no inhibitor, in a dose-dependent manner when measuring cleavage of VIVID® BOMCC substrate (Figure 9). Results were similar for timed experiments, where 3µM galangin inhibited hCYP1B1 by 100% (p<0.0001) at 6 hour pre-incubation time (Figure 10). 3µM galangin inhibited hCYP1A1 by 84% (p<0.0001) at 120 minutes pre-incubation time, with activity returning after 4 hours, presumably due to metabolism of the galangin. At six hours pre-incubation time, we only observed 38% inhibition by 3µM galangin in hCYP1A1 cells (Figure 10). Results are presented as the mean ± SD of at least three independent assays.
Cytotoxicity of B[a]P and BPD +/- Galangin

A dose-response of the cytotoxicity of the PAH in the absence or presence of 60µM galangin was determined after a 30-minute pre-incubation with galangin followed by a 6 hour exposure to the PAH in the presence of galangin. The results indicated a significant (p=0.01) 1.6-fold reduction of B[a]P cytotoxicity in hCYP1A1 cells by galangin (IC$_{50}$ = 0.4 ± 0.04 µM as compared to IC$_{50}$ = 0.59 ± 0.1 µM) (Table 4). Galangin significantly (p=0.04) reduced the cytotoxicity of B[a]P (IC$_{50}$ = 1.4 ± 0.3 µM) by 1.1-fold in hCYP1B1 cells (IC$_{50}$ = 1.6 ± 0.9 µM) (Table 4). Similarly, galangin significantly (p<0.001) reduced BPD cytotoxicity in hCYP1A1 cells by 2.7-fold (IC$_{50}$ = 0.08 ± 0.02 µM as compared to IC$_{50}$ = 0.03 ± 0.01 µM, in untreated cells) (Table 5). There was a significant (p<0.001) 2.4-fold reduction of BPD cytotoxicity (IC$_{50}$ = 0.22 ± 0.2 µM as compared to IC$_{50}$= 0.09 ± 0.01 µM) in untreated hCYP1B1 cells (Table 5). The V79MZ control cell line showed no cytotoxicity to PAH or galangin (not shown).

Mutagenicity of B[a]P and BPD +/- Galangin

Mutagenicity was measured by frequency of mutation at the hprt locus following exposure of cells to 0.3 µM B[a]P or 0.15 µM BPD in the absence or presence of 60 µM galangin for 6 hours. There was a highly significant (p<0.0001) reduction in the mutagenicity of B[a]P in the presence of galangin in hCYP1A1 cells by 3.3-fold and a 1.2-fold trend towards reduction (p=0.07) in hCYP1B1 cells (Figure 11a). We also observed a highly significant (p=0.0002) reduction of BPD mutagenicity in the presence of galangin in hCYP1B1 cells by 4.8-fold and a significant (p=0.008) reduction in hCYP1A1 cells by 1.7-fold (Figure 11b). The V79MZ control cell line showed no
mutagenicity to PAH or galangin. The hCYP-transfected cells showed no mutagenicity when treated with only galangin.

*Consumption of B[a]P and BPD +/- Galangin*

Consumption of PAH was analyzed by reverse-phase HPLC following exposure of cells to 0.3µM B[a]P or 0.15µM BPD in the absence or presence of 60µM galangin for 6 hours. Galangin inhibited the consumption of B[a]P in both hCYP1A1 and hCYP1B1 cell lines. Consumption of B[a]P in both cells lines in the absence of galangin was >80% over the six hour exposure interval, 83% in hCYP1A1 cells (p=0.01) and 85% in hCYP1B1 (p=0.001). Consumption was 20% in the presence of galangin in hCYP1A1 cells and 24% in hCYP1B1 cells (Figure 12a, 12b). When comparing consumption of B[a]P in the presence vs absence of resveratrol within each time point, there was a significant difference in B[a]P consumption at 4 hour and 6 hour in hCYP1A1 cells (p=0.02 and p<0.0001, respectively) and in hCYP1B1 at 6 hours (p=0.03). Consumption of BPD in the absence of galangin in hCYP1A1 cells (5 min exposure vs 2 hour exposure) was 81% (p=0.001), and 49% (p=0.001) in hCYP1B1 cells. Galangin inhibited the consumption of BPD in hCYP1A1 cells from >80% in the absence of galangin to 16% in the presence of galangin during the 2 hours of exposure. That consumption was also inhibited in hCYP1B1 cells, from 49% without galangin to less than 10% in the presence of galangin, at the two hour exposure time point (Figure 12c, 12d). When comparing consumption of BPD in the presence vs absence of resveratrol within each time point, there was a significant difference in BPD consumption at 5 minute, 20 minute, 1 hour and 2 hour in hCYP1A1 cells (p=0.001, p=0.002, p<0.0001, and p<0.0001, respectively).
Total Protein Adducts of $^{3}H$-B[a]P and $^{3}H$-BPD +/- Galangin

The effect of 60µM galangin on the formation of $^{3}H$-B[a]P and $^{3}H$-BPD protein adducts was measured after a 6 hour exposure to 0.3µM $^{3}H$-B[a]P (50 Ci/nmol) or 0.15µM $^{3}H$-BPD (4.25 mCi/ml). There was a trend towards reduction of $^{3}H$-B[a]P total protein adducts in the presence of galangin by 75% (4.1-fold, p=0.1) and 95% (21.6-fold, p=0.07) in hCYP1A1 and hCYP1B1 cells, respectively (Figure 13a). Galangin significantly reduced (p=0.0002) $^{3}H$-BPD total protein adducts by 91% (11.1-fold) in hCYP1A1 cells and by 90% (10.2-fold) in hCYP1B1 cells (p=0.01) (Figure 13b). The V79MZ control cell line showed no $^{3}H$-PAH protein adduct formation in the absence or presence of galangin.

A summation table on the effects of galangin on CYP inhibition and B[a]P and BPD cytotoxicity, mutagenicity, consumption and total protein adduct formation can be found in Chapter 5, Summary.
Table 1. Human Cytochrome P450 specific activities in parental and transfected cell lines. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are presented as the mean ± S.D. of at least 3 independent assays.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Genes Expressed</th>
<th>Human CYP Specific Activity (pmol / mg / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZ control</td>
<td>No transgenes</td>
<td>0.16 ± 0.2</td>
</tr>
<tr>
<td>V79MZh1A1</td>
<td>hCYP4501A1</td>
<td>101.2 ± 11.6</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>hCYP4501B1</td>
<td>7.9 ± 1.6</td>
</tr>
</tbody>
</table>
Table 4

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>B[a]P IC₅₀ (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.4 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + Galangin</td>
<td>0.59 ± 0.1</td>
<td>1.6*</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>1.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>V79MZh1B1 + Galangin</td>
<td>1.6 ± 0.9</td>
<td>1.1*</td>
</tr>
</tbody>
</table>

Table 4. Cytotoxicity of B[a]P ± 60µM galangin in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in Experimental Procedures. Results are presented as the mean ± S.D. of 3 or more independent assays. Asterisk (*) indicates significance (p=0.01 for V79MZh1A1 vs V79MZh1A1 + galangin; p=0.04 for V79MZh1B1 vs V79MZh1B1 + galangin).
Table 5. Cytotoxicity of BPD + 60μM galangin in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in Experimental Procedures. Results are presented as the mean ± S.D. of 3 or more independent assays. Asterisk (*) indicates significance (p<0.001 for V79MZh1A1 vs V79MZh1A1 + galangin; p<0.001 for V79MZh1B1 vs V79MZh1B1 + galangin).

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>BPD IC₅₀ (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + Galangin</td>
<td>0.08 ± 0.02</td>
<td>2.7*</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>0.09 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1B1 + Galangin</td>
<td>0.22 ± 0.2</td>
<td>2.4*</td>
</tr>
</tbody>
</table>
Figure 9a. Galangin dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), black bars represent V79MZh1A1. p values are for comparison of activity in the presence of galangin to activity with no inhibitor.
Figure 9b. Galangin dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), gray bars represent V79MZh1B1. p values are for comparison of activity in the presence of galangin to activity with no inhibitor.
Figure 10a. Stability of 3μM galangin inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP intact cell enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), black bars represent V79MZh1A1. p values are for comparison of activity with galangin to activity without galangin.
Figure 10b. Stability of 3μM galangin inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP intact cell enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes), black bars represent V79MZh1A1. p values are for comparison of activity with galangin to activity without galangin.
Figure 11a. Mutagenicity of 0.3μM B[a]P ± 60μM galangin in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the B[a]P exposure condition to the mutant colonies in the B[a]P + galangin exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + galangin. p values are for B[a]P only compared to B[a]P+galangin, within the same cell line.
**Figure 11b.** Mutagenicity of BPD + galangin in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The *hprt* mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the BPD exposure condition to the mutant colonies in the BPD+ galangin exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + galangin. p values are for BPD only compared to BPD + galangin, within the same cell line.
Figure 12a. Consumption of B[a]P in V79MZ cells engineered to express hCYP1A1 exposed to 0.3μM B[a]P ± 60μM galangin, 6 hour exposure. B[a]P consumption was analyzed using HPLC, as described in Experimental Procedures. Percent consumption is the ratio of B[a]P at 5 minute to B[a]P at 6 hour, or B[a]P + galangin at 5 minute to B[a]P + galangin at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + galangin. p values are a pairwise comparison to 5 minute exposure. p value shown in parentheses are B[a]P compared to B[a]P + galangin within the same exposure time.
Figure 12b. Consumption of B[a]P in V79MZ cells engineered to express hCYP1B1 exposed to 0.3µM B[a]P ± 60µM galangin, 6 hour exposure. B[a]P consumption was analyzed using HPLC, as described in Experimental Procedures. Percent consumption is the ratio of B[a]P at 5 minute to B[a]P at 6 hour, or B[a]P + galangin at 5 minute to B[a]P + galangin at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + galangin. p values are a pairwise comparison to 5 minute exposure. p value shown in parentheses are B[a]P compared to B[a]P + galangin within the same exposure time.
Figure 12c. Consumption of BPD in V79MZ cells engineered to express hCYP1A1 exposed to 0.15µM BPD + 60µM galangin, 6 hour exposure. BPD consumption was analyzed using HPLC, as described in Experimental Procedures. Percent consumption is the ratio of BPD at 5 minute to BPD at 6 hour, or BPD + galangin at 5 minute to BPD + galangin at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + galangin. p values are a pairwise comparison to 5 minute exposure. p value shown in parentheses are BPD compared to BPD + galangin within the same exposure time.
Figure 12d. Consumption of BPD in V79MZ cells engineered to express hCYP1B1 exposed to 0.15μM BPD + 60μM galangin, 6 hour exposure. BPD consumption was analyzed using HPLC, as described in Experimental Procedures. Percent consumption is the ratio of BPD at 5 minute to BPD at 6 hour, or BPD + galangin at 5 minute to BPD + galangin at 6 hour. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + galangin. p values are a pairwise comparison to 5 minute exposure.
**Figure 13a.** Total protein adducts in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 exposed to $^{3}$H-B[a]P + 60µM galangin, 6 hour exposure. The total protein adduct assay was performed as described in *Experimental Procedures*. Fold-protection is the ratio of CPM in the $^{3}$H-B[a]P exposure condition to the CPM in the $^{3}$H-B[a]P + galangin exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to $^{3}$H-B[a]P, light bars represent exposure to $^{3}$H-B[a]P + galangin.
Figure 13b. Total protein adducts in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 exposed to $^3$H-BPD ± 60µM galangin, 6 hour exposure. The total protein adduct assay was performed as described in *Experimental Procedures*. Fold-protection is the ratio of CPM in the $^3$H-BPD exposure condition to the mutant colonies in the $^3$H-BPD + galangin exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to $^3$H-BPD, light bars represent exposure to $^3$H-BPD + galangin. *p* values are $^3$H-BPD + galangin compared to $^3$H-BPD, within the same cell line.
Discussion

The concept of chemoprevention, using natural or synthetic compounds to block, reverse, or prevent the development of cancers, has great appeal. Galangin, a dietary flavonoid found in honeybee propolis and India / China root (2), has been shown to have numerous biological activities, including anti-genotoxic (16), anti-mutagenic (3), and anti-oxidative (5). Galangin has also been shown to be a potent inhibitor of Phase I enzyme CYP (8). This would suggest galangin as a promising CP agent, implying that a primary mechanism for protection may be decreased activation of carcinogens by inhibition of CYP (8).

In this current study, we observed direct inhibition by galangin of CYP-catalyzed cleavage of the VIVID® BOMCC substrate to a fluorescent product. Using parental V79MZ Chinese hamster lung fibroblast cells, modified to express either hCYP1A1 or hCYP1B1, we measured CYP activity in an intact cell system and showed that galangin inhibits both isozymes in a dose- and time-dependent manner. We observed complete inhibition of hCYP1B1 by 3µM galangin (99%) and close to 50% inhibition in hCYP1A1 cells. This inhibition was maintained for up to six hours in hCYP1B1 cells. However, in hCYP1A1 cells, after causing more than 80% inhibition in the first two hours, 62% of activity was restored at six hours.

Flavonoids have been reported to inhibit the carcinogenic activity of B[a]P (17) and the mutagenicity of bay region diol-epoxides of PAHs (18). Buening et al. (8) showed that galangin inhibits the CYP-dependent hydroxylation of B[a]P in human liver microsomes. We wanted to determine if galangin’s direct inhibition of CYP cleavage of
the VIVID® BOMCC substrate that we observed would parallel its effects on the toxicity of B[a]P or BPD in intact cells. Cytotoxicity was assayed using the sulforhodamine B (SRB) method, as previously described. An assessment of acute (6 hour) cytotoxicity was chosen because concentrations of both PAH and galangin are highest in the media during shorter exposure times. Also, this time frame would allow for use of a higher, but still physiologically-achievable, concentration of galangin. Based on previous studies, some flavonols consumed in the diet may reach concentrations as high as 50 µM or more in the intestinal lumen (19). These same studies have shown that the bioavailability of flavonoids in general, however, is lower, limited both by poor transport (19) and by extensive metabolism (20).

Galangin significantly reduced the cytotoxicity of B[a]P in cells expressing either hCYP1A1 or hCYP1B1 (1.6-fold and 1.1-fold, respectively). We expected to see a higher fold protection, based on the results of the direct inhibition. Galangin also significantly reduced the cytotoxicity of BPD in both hCYP1A1 and hCYP1B1 cells (2.7-fold and 2.4-fold, respectively). Galangin appears to be more effective at inhibiting BPD toxicity as compared to B[a]P toxicity. This was also unexpected, given that BPD is already partially activated.

Next, we examined the effects of galangin on mutagenicity of B[a]P and BPD. Galangin effectively reduced the mutagenicity of B[a]P in both cell lines. There was a higher fold-reduction in cells expressing hCYP1A1, which does not parallel the direct inhibition data but agrees with the toxicity data. While galangin also effectively reduced mutagenicity of BPD in both cell lines, conversely, there was a higher fold-reduction in cells expressing hCYP1B1. These results suggest that the endpoints of cytotoxicity and
mutagenicity are isozyme specific and determined by different PAH metabolites that are formed from activation of the parent compound or further metabolism of the intermediate metabolite.

We investigated if the reduction in cytotoxicity and mutagenicity was due, not only to direct inhibition of CYP, but also to galangin affecting metabolism of B[a]P and BPD. Consumption profiles examining the metabolism of each compound showed galangin was blocking consumption of each PAH by hCYP1A1 and hCYP1B1. In the absence of galangin, we saw 70% consumption of B[a]P by hCYP1A1 cells and 73% by hCYP1B1 cells, over 4 hours. Galangin blocked/reduced that consumption to 9% and zero, respectively. We observed the same with BPD, where galangin reduced consumption from 81% to 16% in hCYP1A1 cells and from 49% to 7% in hCYP1B1 cells. Therefore, galangin’s direct inhibition of hCYP activity, as measured by the fluorescent substrate in intact cells, was predictive of its effects on consumption, cytotoxicity and mutagenicity of B[a]P and BPD.

Previous studies have shown that some flavonoids inhibit the formation of DNA adducts with B[a]P and aflatoxin B₁ (23, 24). Kim et al reported that when galangin was mixed with a mouse liver microsomal fraction, arylhydrocarbon hydroxylase was inhibited, leading to the reduced metabolic conversion of \( ^3 \text{H-B[a]P} \) in vitro (25). When calf thymus DNA was incubated with \( ^3 \text{H-B[a]P} \) in the presence of galangin, DNA-B[a]P adduct formation was shown to be reduced (25). We investigated the effect of galangin on protein adduct formation, and observed a reduction in \( ^3 \text{H-B[a]P} \) and a significant reduction in \( ^3 \text{H-BPD} \) protein adducts in the presence of galangin in hCYP1A1 and hCYP1B1 cells. We saw a 4.1-fold decrease and a 21.6-fold decrease in \( ^3 \text{H-B[a]P} \)
adducts in hCYP1A1 and hCYP1B1 cells, respectively. We observed an 11.1-fold and 10.2-fold reduction in \(^3\)H-BPD adducts in hCYP1A1 and hCYP1B1 cells, respectively.

The direct mechanism by which galangin inhibits DNA and protein adducts is unknown, but it has been suggested that the structure of galangin may produce this effect. The anti-clastogenic effect of flavonoids, including galangin, against the induction of micronuclei by B[a]P in polychromatic erythrocytes of mice was evaluated by Heo, et al. (26). The data revealed that in order to produce an anti-clastogenic effect, it was necessary for the flavonoid to have a 2,3-double bond and 3,5,7-hydroxyl groups. Galangin, having a 3-hydroxy group in the C-ring and 5,7-dihydroxy groups in the A-ring, supports this configuration. Galangin also has no hydroxyl in the B-ring.

Regarding the anti-mutagenicity of flavonoids, several important factors have been suggested (27, 28). First, a 4-keto substituent at C4 of a ring is required. Also, an unsaturated C2-C3 bond promotes the anti-mutagenic activity by making the compound more planar, limiting twisting of the B-ring, with reference to the C-ring. Galangin has both of these chemical properties (Figure 3). These chemical attributes of galangin may account for its potent enzyme inhibiting, metabolite modulating, and anti-mutagenic activities.

While much of the literature reports galangin to be an ideal CP agent, there have been conflicting reports that galangin causes mutations. In the Salmonella reversion assay, Brown et al. and MacGregor et al. found galangin to be mutagenic with or without metabolic activation (29, 30). Carver et al. reported that galangin induced mutations in the tk locus of CHO cells after metabolic activation of S9 mix (31). Other reports have shown that galangin does not exhibit any mutagenicity, with or without metabolic
activation (32, 33). Ironically, it is the chemical structure of galangin that supposedly contributes to its mutagenic properties. The absence of a hydroxyl group on the B-ring makes it a possible substrate for CYP. When metabolized by CYP, hydroxylation of the B-ring occurs, forming the metabolite kaempferol which is further metabolized to quercetin in a CYP-dependent reaction (34). Quercetin has been reported to be genotoxic, and potentially mutagenic, to V79 cells and this genotoxicity seems to depend on the production of reactive oxygen species (ROS) (34). Galangin, however, caused no mutagenicity in the V79MZ control cells, hCYP1A1, or hCYP1B1 cells used in these studies. Our studies support galangin as a promising CP agent.
References:


CHAPTER 4

Effects of Phenethyl Isothiocyanate (PE-ITC) and Genistein on hCYP1A1 and hCYP1B1 Activation of Benzo[a]pyrene (B[a]P) and Benzo[a]pyrene-7,8-dihydriodiol (BPD) to Toxic and Mutagenic Metabolites

Abstract:

V79MZ cells stably transfected to express hCYP1A1 or hCYP1B1 were employed to study whether direct inhibition of human cytochrome P450 (hCYP) activity, as measured by cleavage of the VIVID® BOMCC substrate to a fluorescent product in intact cells, was predictive of PE-ITC or genistein effects on the cytotoxicity and / or mutagenicity of B[a]P or BPD. We observed only 50% CYP inhibition by 30µM PE-ITC in cells expressing hCYP1B1, which was more potent than in cells expressing hCYP1A1, where we observed 33% inhibition. We observed 38% inhibition of CYP activity by 30µM genistein in hCYP1A1 cells. That inhibition was more potent in cells expressing hCYP1B1, where we observed 86% inhibition. Based on these data from the enzyme inhibition assay, we hypothesized that each CP agent would be better at inhibiting cytotoxicity and mutagenicity of B[a]P or BPD in cells expressing hCYP1B1 than in cells expressing hCYP1A1; however, this was not the case. As expected, we observed no reduction in B[a]P or BPD cytotoxicity in hCYP1A1 cells by PE-ITC or genistein. We observed a significant 2.3-fold reduction in B[a]P cytotoxicity by PE-ITC and 2.7-fold reduction in B[a]P cytotoxicity genistein in hCYP1B1 cells, which correlated with the inhibition assay. Unexpectedly, we did not observe any significant reductions in or BPD cytotoxicity in hCYP1B1 cells by either PE-ITC or genistein. We observed a significant
1.9-fold reduction in B[a]P mutagenicity by genistein in hCYP1B1 cells. Unexpectedly, there were no other significant reductions in B[a]P or BPD mutagenicity in hCYP1B1 cells by either CP agent; however, there was a significant 1.5-fold reduction in BPD mutagenicity by genistein in hCYP1A1 cells. Based on these results, we conclude that inhibition of CYP, as measured by cleavage of the VIVID® BOMCC substrate (O-dealkylation reaction), is not always predictive of CYP-mediated PAH metabolism (epoxidation reaction) when looking at the endpoints of cytotoxicity and mutagenicity.
**Introduction**

The mechanisms of prevention by chemopreventive (CP) agents remains incompletely understood. While indirect effects such as phase II enzyme induction have received much attention, there is also evidence for direct inhibitory effects on the metabolic activation of potent environmental carcinogens. Understanding how these agents affect cell signaling pathways that mediate processes such as apoptosis, cell cycle progression, and DNA repair are also important.

The cytochrome P450 (CYP) system is a major pathway through which the body metabolizes xenobiotics. Human CYP1A1 (hCYP1A1) and human CYP1B1 (hCYP1B1) are most active to metabolize benzo[a]pyrene (B[a]P). Human exposure to B[a]P is widespread and practically unavoidable. It is a ubiquitous environmental carcinogen found in tobacco smoke, charbroiled foods and incomplete combustion effluents. It is activated to its more reactive metabolite, B[a]P-7,8-diol (BPD), by Phase I enzymes, specifically, CYP1A1 and CYP1B1.

Gluconasturtiin is a glucosinolate that forms phenethyl isothiocyanate (PE-ITC) when hydrolyzed in a reaction involving myrosinase. PE-ITC is a constituent of cruciferous vegetables, including horseradish, cabbage, cauliflower, Brussels sprouts, radishes, and watercress (1). Isothiocyanates are known to be inhibitors of CYP (2, 3, 4). However, there have also been reports showing that ITCs can increase expression of Phase I enzymes. Many studies in bacterial and mammalian cells and laboratory animals, as well as in humans, show that ITCs could be involved in the induction of multistep carcinogenesis by acting as genotoxic agents (5, 6) or as co-carcinogens, enhancing the
activity of enzymes activating procarcinogens (7). It was also reported that gluconasturtiin and PE-ITC both have properties that can induce transformation in a Chinese hamster ovary (CHO) cell line (8). These conflicting results emphasize the importance of understanding how these agents modify cancer risks by various mechanisms.

Soybeans are a rich source of isoflavones. Genistein is one of the most abundant of the isoflavones in soybeans (9), often referred to as phytoestrogens because their chemical structures are similar to that of estrogen and they can bind, albeit weakly, to estrogen receptors. They act as partial agonists, and have weak estrogenic activity. Genistein has been shown to induce the expression of GSTs in human breast cells (10), thus conferring protection towards genotoxic carcinogens, which are GST substrates. Genistein was also shown to inhibit metastasis of human prostate cancer cells implanted in mice by inhibiting detachment of the cancer cells (11). This suggests that genistein might lower the incidence of tumor progression. In addition to its weak estrogenic properties, genistein also has antioxidant activity (12). Genistein has also been suggested to mediate its anticancer actions through other mechanisms such as inhibition of angiogenesis (13) and inhibition of tyrosine kinases (14), which play an important role in cell growth. Genistein has been shown to be a substrate for CYP1A1 and CYP1B1 (15). Reports have shown that genistein is metabolized in a manner similar to the isoflavone galangin, in which CYPs hydroxylate genistein at the 3’ position on the B ring to yield the metabolite, orobol (15).

We investigated whether inhibition of hCYP1A1 or hCYP1B1 activity, as measured by cleavage of the VIVID® BOMCC substrate to a fluorescent product in
intact cells, was predictive of PE-IT or genistein effects on the cytotoxicity and/or mutagenicity of B[a]P and BPD. We employed V79 Chinese hamster lung fibroblast cells stably transfected to express hCYP1A1 or hCYP1B1. This novel system allows us to study metabolism of PAH in the absence or presence of the CP agent under tightly controlled conditions, while maintaining cell architecture, enzyme compartmentalization, and biological responses.
**Experimental Procedures**

*Materials and Chemicals*

All chemicals used in these studies were of analytical grade and were obtained from Fisher (Atlanta, GA), Sigma (St. Louis), or Invitrogen (Carlsbad, CA). Advanced Dulbecco’s modified Eagle’s medium (Adv. DMEM) was purchased from Gibco/BRL (Grand Island, NY). B[a]P and BPD were purchased from Midwest Research Institute (Lexena, KS).

*Cell Lines and Culture*

Parental V79MZ Chinese hamster lung fibroblast cells were modified to express either hCYP1A1 or hCYP1B1, as previously described (16). Cells were grown and maintained in Advanced DMEM supplemented with 2% fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells were selected with 400 µg/ml G-418 and passaged every 2-3 days. Activities of hCYP were assayed at least once a week to maintain and verify stable expression levels.

The parent cells express no constitutive or inducible CYP activity (17). They do express an endogenous Pi-class hamster GST at low levels (18), but this GST is not active for the conjugation of reactive electrophiles of the PAH in these studies with glutathione (GSH) (19). Expression of the transfected CYP is stable and constitutive, rather than variable due to carcinogen induction. Also, expression of the enzymes is unhindered by background interference because each CYP is expressed against the same V79 cell background; therefore, each isozyme is comparable.
**CYP Fluorescent Enzyme Activity Assays**

VIVID® benzyloxymethyloxy-3-cyanocoumarin (BOMCC) substrates are blocked dyes that yield minimal fluorescence signal until cleaved at either of two potential cleavage sites, releasing a highly fluorescent product. This assay is performed in an intact cell system since both substrate and product are membrane-permeable. Cells expressing either hCYP1A1 or hCYP1B1 were plated at $1.8 \times 10^5$ in 2 ml Advanced DMEM (2% FBS) and allowed to attach and grow for 48 hours. At the time of the assay, 1 ml of serum-free media containing a 5µM concentration of BOMCC substrate was replaced on each plate and incubated at 37°C for 30 minutes. To determine the activity of the enzyme, the highly fluorescent product was then measured spectrofluorometrically in the medium at 409 nm excitation / 460 nm emission. Activities of hCYP1A1 and hCYP1B1 were measured using a known VIVID blue standard curve (0-100µM). Protein was determined by the Coomassie dye method (Bio-Rad, Hercules, CA). Final enzyme activity was determined by comparison to the standard curve and normalized to protein content on each plate and expressed as pmol / min / mg protein. It is important to note that hCYP1A1 has a higher specific activity with the BOMCC substrate than hCYP1B1.

In the direct enzyme inhibition studies, the assay was plated as above. At the time of the assay for the dose-dependent studies, 1 ml aliquots of media containing a range of concentrations of PE-ITC or genistein were replaced on the plates for a 30 minute pre-incubation at 37°C prior to adding the substrate. For the time-dependent studies, 1 ml of serum-free medium containing a fixed concentration (30µM PE-ITC or 30µM genistein) was replaced on each plate for pre-incubation times ranging from 0 minutes – 6 hours at
37°C prior to adding the substrate. Incubation for an additional 30 minutes at 37°C was allowed and enzyme activity was determined as described above.

**Cytotoxicity Assays**

Cytotoxicity was assayed using the sulforhodamine-B (SRB) method, as previously described (20). The principle of the assay is based on the ability of the protein dye (SRB) to bind protein basic amino acid residues of trichloroacetic acid-fixed cells. Cells were plated in Advanced DMEM (2% FBS) on 96 well plates at a density of 250 cells/well. This density has been optimized for a growth interval of up to 4 days. Cells were allowed to attach and grow for 16 – 24 hours prior to being dosed. At the time of dosing (for non-continuous exposure), media was removed and 150 µl of serum-free media was added to each well. A concentration calculated to yield 2µM PE-ITC or 30µM genistein in serum-free media (25 µl) was added for a 30 minute pre-incubation at 37°C prior to adding the PAH. It is important to note that we used 2µM PE-ITC in our cytotoxicity assays because higher concentrations proved toxic to the cells. An 8x concentration of B[a]P or BPD (25 µl) was added and incubated for 6 hours. At the end of the dosing period, medium was removed and 200 µl Adv DMEM (2% FBS) was added back to each well for the remainder of the 4 day growth interval. Following incubation for 4 days, cells were harvested by decanting the medium and fixing the cells in 100 µl cold 5% TCA for a minimum of 1 hr at 4°C. Cells were rinsed with a gentle stream of tap water, allowed to dry, and then stained for 10 minutes with 100 µl 0.4% SRB dye in 1% glacial acetic acid. Cells were then washed 4-5 times with 1% glacial acetic acid, dried, and the dye solubilized in 100 µl 10mM non-pH adjusted Tris-Base. Cell density was
determined by measuring absorbance with a microplate reader (Molecular Devices, Sunnyvale, CA) at 560 nm to quantitate protein as an indirect measure of cell number.

**hprt Mutagenicity Assays**

Cells were plated in Advanced DMEM (2% FBS) at a density of $5 \times 10^5$ on 100 mm plates and allowed to adhere to dishes for 16 – 24 hours prior to being dosed. At the time of dosing, media was removed and plates were rinsed twice with 2 ml serum-free media. Cells were pre-incubated with $2 \mu$M PE-ITC or $30 \mu$M genistein in serum-free media for 30 minutes at 37ºC prior to adding the PAH. It is important to note that we used $2 \mu$M PE-ITC in our mutagenicity assays because higher concentrations proved toxic to the cells. Cells were then exposed to 0.3µM B[a]P or 0.15µM BPD in DMSO (co-exposure with CP agent) for 6 hours, media was removed, and cells were rinsed twice with 2 ml Adv DMEM (2% FBS). Following incubation for an additional 3 hours with 2µM PE-ITC or 30µM genistein in 2% serum media, media was aspirated and 10 ml fresh serum-containing media was added to each plate and cells were allowed to recover for 72 hours. Cells were then subcultured at $5 \times 10^5$ cells / 100 mm plate and grown for 6 days, with one subculture at 3 days, to allow for phenotypic development. Cells were subcultured once more at the same density, and the next day 6-thioguanine (6-TG) was added at a concentration of 10µg 6-TG / ml medium in order to select for *hprt* mutants. Cells were maintained under 6-TG selection for 10 – 12 days, with two changes of serum media + 6-TG during that time, after which *hprt* mutant colonies were stained with 5mM methylene blue in methanol. Mutant colonies were scored manually and are expressed as colonies per million cells.
Statistical Methods

Descriptive statistics (median, mean, S.D., range) were summarized by groups and also graphically. Analysis of Variance (ANOVA) was performed for the analysis of experiments other than for cytotoxicity. Where applicable, Dunnett’s method was used for specific pairwise comparisons to control (e.g., no inhibitor, B[a]P only, etc.) Type I error. The homogeneity of variances assumption was checked with an O’Brien test and if violated, comparisons were performed using Kruskal-Wallis tests. Cytotoxicity data were analyzed using regression analysis, where concentration was modeled using restricted cubic splines with four knots based on available sample size and Harrell’s recommendations. The goal of this analysis was to get an appropriate test of the chemopreventive agent compared to no agent for each experiment, while appropriately accounting for survival vs. concentration relationships. Analyses were performed using SAS v9.2 (SAS Institute, Cary, NC) and Stata v10.1 (StataCorp, College Station, TX). A two-sided p-value<0.05 was considered statistically significant. A p-value between 0.05 and 0.1 was considered a trend.
Results

hCYP enzyme activity

The parental V79MZ control cells have no significant or inducible CYP activity. CYP enzyme activities in transfected cells were measured to ensure that expression levels were adequate and stable and that CYP activity was confirmed at background levels in our control cells. The CYP activity was greatly increased in cell lines transfected with hCYP1A1 (101-113 pmol / min / mg protein) or hCYP1B1 (8-10 pmol / min / mg protein), as compared to background levels in our control cells (0.16 pmol / min / mg protein) (Table1). Therefore, adequate CYP activity was obtained in transfected cells. CYP activity was measured after each passage of cells to insure expression of the transfected CYP was stable and constitutive.

Inhibition by PE-ITC

We observed a trend towards inhibition of hCYP1A1 (p=0.07) and hCYP1B1 (p=0.09) by 30µM PE-ITC, in a dose-dependent manner using an intact cell assay measuring cleavage of VIVID® BOMCC substrate (Figure 14a, 14b). Results for timed experiments showed 30µM PE-ITC inhibited hCYP1B1 by greater than 80% (p=0.002) at 6 hour pre-incubation time (Figure 15a, 15b).

Inhibition by Genistein

Genistein (30µM) inhibited hCYP1B1 by 86% (p<0.0001), compared to no inhibitor, in a dose-dependent manner, when measuring cleavage of the BOMCC substrate. There was a 38% trend towards reduction of hCYP1A1 by genistein (p=0.09)
(Figure 16a, 16b). Results for timed experiments were similar, where 30µM genistein inhibited hCYP1B1 by greater than 85% (p<0.0001). Inhibition was stable for up to six hour pre-incubation time (Figure 17a, 17b).

**Cytotoxicity of B[a]P and BPD +/- PE-ITC**

A dose-response of the cytotoxicity of the PAH in the absence or presence of PE-ITC was determined after a 30-minute pre-incubation with a range of PE-ITC concentrations followed by a 6 hour exposure to the PAH in the presence of PE-ITC. The results showed a significant (p<0.001) 2.3-fold reduction in B[a]P cytotoxicity by PE-ITC in hCYP1B1 cells. PE-ITC had no effect on B[a]P cytotoxicity in hCYP1A1 cells (Table 6). There was no effect of PE-ITC on BPD cytotoxicity in either cell line (Table 7). The V79MZ control cell line showed no cytotoxicity with PAH or PE-ITC (not shown).

**Cytotoxicity of B[a]P and BPD +/- Genistein**

A dose-response of the cytotoxicity of the PAH in the absence or presence of genistein was determined after a 30-minute pre-incubation with a range of genistein concentrations followed by a 6 hour exposure to the PAH in the presence of genistein. The results indicated a significant (p=0.05) 2.7-fold reduction of B[a]P cytotoxicity by genistein in hCYP1B1 cells (IC$_{50}$ = 0.73 ± 0.49 µM as compared to IC$_{50}$ = 2.0 ± 0.15 µM) (Table 8). There was no reduction of B[a]P cytotoxicity by genistein in hCYP1A1 cells (Table 8). Similarly, there was no reduction in BPD cytotoxicity in hCYP1A1 cells or hCYP1B1 cells. The V79MZ control cell line showed no cytotoxicity to PAH or genistein (not shown).
**Mutagenicity of B[a]P and BPD +/- PE-ITC**

Mutagenicity was measured by frequency of mutation at the *hprt* locus following exposure of cells to 0.3 µM B[a]P or 0.15 µM BPD in the absence or presence of PE-ITC for 6 hours. No reduction in B[a]P mutagenicity by PE-ITC was observed in hCYP1A1 cells or hCYP1B1 cells (Figure 18a, 18b). The V79MZ control cell line showed no mutagenicity with PAH or PE-ITC. The hCYP-transfected cells showed no mutagenicity with PE-ITC.

**Mutagenicity of B[a]P and BPD +/- Genistein**

Mutagenicity was measured by frequency of mutation at the *hprt* locus following exposure of cells to 0.3 µM B[a]P or 0.15 µM BPD in the absence or presence of genistein for 6 hours. There was a significant (p=0.01) 1.9-fold reduction in the mutagenicity of B[a]P in the presence of genistein in hCYP1B1 cells. Genistein had no effect on B[a]P mutagenicity in hCYP1A1 cells (Figure 19a, 19b). Genistein significantly (p=0.02) reduced BPD mutagenicity in hCYP1A1 cells by 1.5-fold, but had no effect on BDP mutagenicity in hCYP1B1 cells. The V79MZ control cell line showed no mutagenicity with PAH or genistein. The hCYP-transfected cells showed no mutagenicity with genistein.

*Summation tables on the effects of PE-ITC and genistein on CYP inhibition and B[a]P and BPD cytotoxicity and mutagenicity can be found in Chapter 5, Summary.*
Table 1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Genes Expressed</th>
<th>Human CYP Specific Activity (pmol / mg / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZ control</td>
<td>No transgenes</td>
<td>0.16 ± 0.2</td>
</tr>
<tr>
<td>V79MZh1A1</td>
<td>hCYP4501A1</td>
<td>101.2 ± 11.6</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>hCYP4501B1</td>
<td>7.9 ± 1.6</td>
</tr>
</tbody>
</table>

Table 1. Human Cytochrome P450 specific activities in parental and transfected cell lines. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are presented as the mean ± S.D. of at least 3 independent assays.
<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>B[a]P IC₅₀ (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.71 ± 0.12</td>
<td>None</td>
</tr>
<tr>
<td>V79MZh1A1 + PE-ITC</td>
<td>0.71 ± 0.18</td>
<td>None</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>0.73 ± 0.49</td>
<td>None</td>
</tr>
<tr>
<td>V79MZh1B1 + PE-ITC</td>
<td>1.7 ± 0.64</td>
<td>2.3*</td>
</tr>
</tbody>
</table>

**Table 6.** Cytotoxicity of B[a]P + 2µM PE-ITC in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in Experimental Procedures. Results are presented as the mean ± S.D. of 3 or more independent assays (p<0.001 for V79MZh1B1 vs V79MZh1B1 + PE-ITC).
Table 7

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>BPD IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + PE-ITC</td>
<td>0.04 ± 0.01</td>
<td>None</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>0.08 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1B1 + PE-ITC</td>
<td>0.08 ± 0.01</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 7. Cytotoxicity of BPD + 2µM PE-ITC in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in Experimental Procedures. Results are presented as the mean ± S.D. of 3 or more independent assays.
Table 8

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>B[a]P IC₅₀ (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.71 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + Genistein</td>
<td>0.47 ± 0.09</td>
<td>None</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>0.73 ± 0.49</td>
<td></td>
</tr>
<tr>
<td>V79MZh1B1 + Genistein</td>
<td>2.0 ± 0.15</td>
<td>2.7*</td>
</tr>
</tbody>
</table>

Table 8. Cytotoxicity of B[a]P + 20µM Genistein in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in *Experimental Procedures*. Results are presented as the mean ± S.D. of 3 or more independent assays (p=0.05 for V79MZ1B1 vs V79MZ1B1 + genistein).
Table 9.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>BPD IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.04 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + Genistein</td>
<td>0.03 ± 0.01</td>
<td>None</td>
</tr>
<tr>
<td>V79MZh1B1</td>
<td>0.08 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1B1 + Genistein</td>
<td>0.10 ± 0.02</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table 9.** Cytotoxicity of BPD + 20µM Genistein in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in *Experimental Procedures*. Results are presented as the mean ± S.D. of 3 or more independent assays.
Figure 14a. PE-ITC dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in *Experimental Procedures*. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); black bars represent V79MZ-h1A1.
Figure 14b. PE-ITC dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol/min/mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); gray bars represent V79MZh1B1.
Figure 15a. Stability of 30µM PE-ITC inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); black bars represent V79MZh1A1.
Figure 15b. Stability of 30µM PE-ITC inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol/min/mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); gray bars represent V79MZh1B1. p values are inhibitory concentrations compared to no inhibitor.
Figure 16a. Genistein dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); black bars represent V79MZh1A1.
**Figure 16b.** Genistein dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in *Experimental Procedures*. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); gray bars represent V79MZh1B1. p values are for comparison of activity in the presence of genistein to activity with no inhibitor.
Figure 17a. Stability of 30µM genistein inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); black bars represent V79MZh1A1.
Figure 17b. Stability of 30µM genistein inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); gray bars represent V79MZh1B1. p values are inhibitory concentrations compared to no inhibitor.
Figure 18a. Mutagenicity of 0.3μM B[a]P + 2μM PE-ITC in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprr mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the B[a]P exposure condition to the mutant colonies in the B[a]P + PE-ITC exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + PE-ITC.
Figure 18b. Mutagenicity of 0.15µM BPD + 2µM PE-ITC in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the BPD exposure condition to the mutant colonies in the BPD+ PE-ITC exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + PE-ITC.
Figure 19a. Mutagenicity of 0.3µM B[a]P + 30µM Genistein in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the B[a]P exposure condition to the mutant colonies in the B[a]P + genistein exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to B[a]P, light bars represent exposure to B[a]P + genistein. p values are B[a]P compared to B[a]P + genistein, within the same cell line.
Figure 19b. Mutagenicity of 0.15µM BPD + 30µM Genistein in V79MZ cells engineered to express hCYP1A1 or hCYP1B1 (6 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the BPD exposure condition to the mutant colonies in the BPD+ genistein exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + genistein. p values are BPD compared to BPD + genistein, within the same cell line.
In this current study, we have shown that PE-ITC and genistein both inhibit CYP1A1 and CYP1B1 similarly; however, their effects on the metabolism of B[a]P and BPD differ. Neither had an effect on cytotoxicity of B[a]P or BPD in hCYP1A1 cells; however, genistein significantly inhibited BPD mutagenicity in hCYP1A1 cells. Both agents significantly reduced B[a]P cytotoxicity in hCYP1B1 cells, however, each had a different effect on the cytotoxicity of BPD in hCYP1B1 cells, and on the mutagenicity of B[a]P and BPD in hCYP1B1 cells.

Phenethyl isothiocyanate (PE-ITC) is a constituent of cruciferous vegetables, such as cabbage, Brussels sprouts, and watercress (2). It is formed from the hydrolysis of its glucosinolate precursor, gluconasturtiin, a reaction involving myrosinase (21). Myrosinase is an enzyme that is physically separated from glucosinolates in plant cells but is released and activated when these cells are damaged, i.e., by chopping or chewing of the vegetable. Myrosinase activity has also been demonstrated in the gastrointestinal (GI) tract, where PE-ITC can undergo biotransformation by human gut microflora and is rapidly absorbed (22, 23). Following absorption, peak plasma concentrations of 0.943-2.27 µmol / l have been observed in humans (24). Plasma concentrations of PE-ITC have been shown to reach 9.2 µM in rats, after an oral dose of 10 µmol / kg of PE-ITC (25). These combined data show that micromolar concentrations of PE-ITC are achievable in vivo. Therefore, the concentration of 2 µM we chose for our studies is physiologically relevant.
Isothiocyanates are known to be effective inhibitors of CYP (2, 3, 4). It has been suggested that PE-ITC is a chemopreventive agent against lung cancer (2). Sticha et al, reported that PE-ITC inhibited B[a]P metabolism in lung microsomes isolated from mice (26). We also observed inhibition of B[a]P metabolism by PE-ITC, when measuring the endpoint of cytotoxicity, in hCYP1B1 cells. However, we did not see this effect in hCYP1A1 cells. PE-ITC had no effect on BPD cytotoxicity, and no effect on the mutagenicity of B[a]P and BPD.

PE-ITC seems to have differential effects on enzymes involved in carcinogen activation, depending on the substrate and the isozyme that is expressed. For example, treatment of mice with PE-ITC had no effect on CYP1A1 activity in lung extracts, with ethoxyresorufin-\(O\)-deethylase (EROD) as a substrate (27). However, in our studies, treatment with \(30\mu M\) PE-ITC trends towards reduction (\(p=0.07\)) of hCYP1A1 activity in intact cells when measuring cleavage of the VIVID® BOMCC substrate. Consistent with the weak enzyme inhibition observed, there was no effect by PE-ITC on cytotoxicity of B[a]P in hCYP1A1 cells. There was a significant 2.3-fold reduction in B[a]P cytotoxicity in hCYP1B1 cells (\(p<0.001\)). These results suggest that the BOMCC substrate and PAH are not metabolized similarly. Also, the endpoints of cytotoxicity and mutagenicity are isozyme specific and could be determined by different PAH metabolites that are formed from activation of the parent compound or further metabolism of the intermediate metabolite. CYP1A1 metabolism of B[a]P yields more phenols and quinones, which are more cytotoxic in nature (18). CYP1B1 metabolism of B[a]P yields more mutagenic metabolites (28). Given the minimal enzyme activity inhibition of hCYP1A1 by PE-ITC, this could be a possible explanation of why we see no effect on
cytotoxicity in hCYP1A1 cells. The cytotoxic burden is too high for PE-ITC to overcome. However, we see a significant inhibition in B[a]P toxicity in hCYP1B1 cells, where the cytotoxic burden is not as high. PE-ITC was unable to affect toxicity of BPD in hCYP1B1 cells, possibly because BPD is a better substrate and is partially activated.

While the trend towards inhibition of CYP by PE-ITC was a moderately larger hCYP1B1 than hCYP1A1 cells (50% vs 30%, respectively), it still had no effect on BPD toxicity or B[a]P mutagenicity in hCYP1B1 cells. These differential effects of PE-ITC on B[a]P and BPD mutagenicity in hCYP1B1 cells are not completely understood; however, it may be the difference in the type of reaction PE-ITC is affecting. Cleavage of the BOMCC substrate is an O-dealkylation reaction and metabolism of PAH is an epoxidation reaction. It is appears PE-ITC inhibits epoxidation less efficiently.

It is possible that there are other molecular targets through which PE-ITC could exert effects that could mask or offset any benefits from reduced activation. Induction of both cell cycle arrest and apoptosis are also known effects of PE-ITC. The cell cycle arrest effect of PE-ITC was first investigated in 1993 (29). PE-ITC (2 µM) caused the accumulation of HeLa cells at G2/M phase with inhibition of cell growth to 41-79% of the control (29). PE-ITC’s induction of apoptosis has been shown in both a p53-dependent and –independent manner in JB6 and PC-3 cells (30, 31). A 24 hour exposure of PC-3 cells to 10 µM PE-ITC resulted in a decrease in the levels of antiapoptotic proteins Bcl-2 (56% decrease) and Bcl-XL (44% decrease) (32). Similarly, activation of mitogen activated protein kinase pathways such as ERK, JNK, and p38 by PE-ITC were reported to be the possible mechanisms of growth arrest and apoptosis in PC-3, HL-60 and Jurkat cells (31, 33).
An induction of apoptosis by PE-ITC would cause an increase in toxicity. If PE-ITC was actually inhibiting the enzyme, an induction in apoptosis and increase in toxicity might cancel this effect out. This would give the impression that PE-ITC is not having an effect on toxicity, which is what we observed. This increase in apoptosis would lead to a decrease in mutagenicity because the cells with the highest burdens of mutations would stop growing and die. While the decrease in mutagenicity was not significant in either cell line, a chronic exposure time to PE-ITC may have yielded significant results. The acute exposure time in our studies was chosen because PAH may not be stable over long periods of time, and PE-ITC is known to be metabolized, which might be physiologically relevant to exposures in the GI tract or following non-dietary (nutraceutical) supplementation. It is important to note that higher concentrations of PE-ITC in our mutagenicity assay proved toxic to the cells. They rounded up and floated off the plate less than two hours after co-exposure to PAH and PE-ITC.

PE-ITC has also been shown to activate the MAPK pathway, possibly via an electrophile-mediated stress response (34). When the cells were co-exposed to PE-ITC and PAH, the activation of MAPKs could have occurred, serving as cellular stress sensors, eliciting a homeostatic response (35). This response might lead to increased GSH production. PE-ITC forms conjugates with GSH, in a GST-dependent reaction (36). If PE-ITC is conjugated to GSH in the cell, it is unable to inhibit CYP metabolism of PAH. Also, PE-ITC inhibition in the BOMCC assay was weak, especially in our hCYP1A1 cells; therefore, it is not surprising that it fails to block B[a]P or BPD activation, thereby reducing cytotoxicity or mutagenicity.
Genistein, a phytoestrogen derived from soy products, must be released from genistin in a hydrolysis reaction that can occur in the stomach (acid hydrolysis) and intestine (action of bacterial enzymes), in order to be biologically active. Genistein can also be further hydrolyzed to yield several other metabolites (37, 38). Clinically attainable concentrations in human plasma have been reported to be approximately 10 µM (39, 40, 41). However, given that genistein appears to be readily absorbed and metabolized in the GI tract, it is possible to attain higher concentrations in GI cells. Also, acute exposure in vitro is different from an in vivo chronic dietary exposure to soy isoflavones, which may result in low micromolar concentrations in serum. Continuous soy consumption throughout a life span may potentially result in accumulation of isoflavones in target tissues at levels exceeding those in serum. Therefore, for these studies, we worked with a higher (but non-toxic) concentration (30 µM) of genistein.

Genistein produced an effect that was similar to PE-ITC in our enzyme inhibition assay. A trend (p=0.09) towards inhibition of hCYP1A1 activity by 30µM genistein was less than 50%, with a more potent inhibition of hCYP1B1 activity at greater than 80% (p<0.001). However, there were differences in the effects of genistein on cytotoxicity and mutagenicity of B[a]P and BPD. Consistent with the enzyme inhibition assay, in hCYP1B1 cells, we observed a significant 2.7-fold and 1.9-fold reduction in B[a]P cytotoxicity and mutagenicity, respectively. However, there was no significant reduction of BPD cytotoxicity or mutagenicity by genistein in hCYP1B1 cells. This suggests that the presence of genistein could have modified B[a]P metabolism, where fewer (-)BPD metabolites are formed and more (+)BPD metabolites are formed. This shift towards the formation of less (-)BPD metabolites means a shift towards the formation of less (+)-anti-
BPDE formation. Given that (+)-anti-BPDE is the most mutagenic B[a]P metabolite, this would result in a reduction in mutagenicity.

We observed no effect of genistein on B[a]P cytotoxicity or mutagenicity in hCYP1A1 cells, also consistent with the enzyme inhibition data. There was no effect on BPD cytotoxicity in hCYP1A1 cells, but we observed a significant reduction in BPD mutagenicity by genistein in hCYP1A1 cells. There may have been a higher cytotoxic burden for genistein to overcome in hCYP1A1 cells, given that this isozyme yields more cytotoxic metabolites. The metabolites formed by hCYP1B1 are less toxic, and we observed a decrease in toxicity of B[a]P and BPD in hCYP1B1 cells.

Another possibility to consider is that, given that genistein is a substrate for CYP1A1 and CYP1B1 (15), genistein itself may be getting metabolized and the metabolites may be producing the effects. Roberts-Kirchhoff et al., showed that five different metabolites were observed when genistein was metabolized by rat CYPs in microsomes, and human liver microsomes produced different profiles, as well (15). One of the metabolites produced, orobol, appeared to have similar acitivity of genistein, which might include inhibition of angiogenesis or antioxidant properties. They also reported that evidence that CYP1A1 and CYP1B1 can hydroxylate genistein at the 3’ position on the B ring to yield orobol. This metabolism of genistein may affect metabolism of other CYP substrates, including BOMCC and PAHs.

PE-ITC and genistein inhibited hCYP1A1 and hCYP1B1 activity similarly, when measuring cleavage of the VIVID® BOMCC substrate in an intact cell enzyme assay. Despite this similarity, each agent gave differential effects on PAH metabolism. PE-ITC
significantly reduced B[a]P cytotoxicity in hCYP1B1 cells, while genistein significantly reduced mutagenicity of B[a]P in the same cell line. Genistein also significantly reduced BPD mutagenicity in hCYP1A1 cells; PE-ITC had no effect. The O-dealkylation reaction involved in the enzyme assay is different from the epoxidation reaction involved in PAH metabolism; therefore, the effects of PE-ITC or genistein on enzyme activity in the VIVID assay may not always predict the biological endpoints of cytotoxicity and mutagenicity. With that being said, one must consider the metabolites that are formed, resulting in toxicity and mutagenicity. The metabolic profile of hCYP1A1 yields more cytotoxic species, such as quinones and phenols (18), while hCYP1B1 yields more mutagenic metabolites, such as (+)-anti-BPDE (28). The CP agents might affect the metabolite profile, causing it to shift towards formation of more or less of either metabolite. This may affect the biological endpoints, as well. Also, the effects that these agents or their metabolites have on pathways in the cell, such as apoptosis, may also affect the endpoints of cytotoxicity and mutagenicity. Therefore, it is important to have a clear understanding of the mechanisms of these agents before employing them as chemopreventive.
References:


CHAPTER 5

SUMMARY

Cancer chemoprevention is a fast growing area of research focusing on the use of natural or synthetic substances to inhibit, delay, or reverse carcinogenesis (1). Fruits and vegetables are a major source of biologically active compounds called phytochemicals. It has been established in many epidemiological studies that populations that consume large quantities of fruits and vegetables in their diet lower their risk of developing cancer; therefore, the biologically active compounds found in these fruits and vegetables have been classified as chemopreventive (CP) agents. There are many CP agents being studied in the current literature, several of which we employed in these current studies. The few of particular interest here were resveratrol, galangin, phenethyl isothiocyanate (PE-ITC) and genistein.

Polycyclic aromatic hydrocarbons (PAH) are among the most widely studied carcinogens (2) that are ubiquitous in our environment and are likely causative agents for lung cancer (3). Benzo[a]pyrene (B[a]P) is perhaps the most extensively studied PAH in the literature. A main focus of studies in our lab has been on understanding enzymatic defense against the toxicity of carcinogens, with emphasis on understanding of the chemoprotective role of the glutathione-S-transferase superfamily of enzymes in mammalian systems. The studies here have taken a different perspective, with the main focus and goal being to understand how the aforementioned CP agents affect particularly cytochrome P450 (CYP) Phase I enzymes in their metabolism of carcinogens, and also glutathione-S-transferase (GST) Phase II enzymes.
Here, we proposed to 1) examine the direct effects (inhibition) of CP agents on hCYP1A1 or hCYP1B1 enzyme activity in intact cells, 2) examine the effects of CP agents on PAH activation by hCYP1A1 and hCYP1B1, and 3) analyze the mechanism of the differential inhibition of CYP activation of B[a]P versus BPD by CP agents. The ultimate goal of these studies was to develop an understanding of how the CP agents affect Phase I and Phase II enzymes in their metabolism of potent carcinogens such as B[a]P and BPD, and their metabolites. Agents that selectively inhibit Phase I enzymes, or preferentially activate Phase II more than Phase I enzymes would likely have the most promise in chemoprevention and in reducing carcinogenic DNA damage in humans. Tables 10-13 give a summary of the primary CP agents used in these studies, and their effects on CYP inhibition and on the metabolism of B[a]P and BPD.

In Chapters 2 and 3, we examined the effects of resveratrol and galangin on PAH metabolism. The biological activities for each agent are numerous. Resveratrol has been shown to be an anti-oxidant that inhibits COX action (4), to have anti-angiogenic properties (5) and to trigger apoptosis (4). Resveratrol is also a known inhibitor of Phase I enzymes (6). Chun et al. showed that resveratrol selectively inhibited CYP1A1 (7). Using human liver microsomes and EROD as a substrate, he observed that resveratrol showed mixed-type inhibition (competitive-noncompetitive). Resveratrol competed for the enzyme binding site, but it can still bind at a different site from the substrate (7). Galangin, like resveratrol, is a COX inhibitor (8). It is also anti-mutagenic (9), as well as anti-clastogenic (10). Its many other effects have been reviewed extensively (10, 11). Galangin is also a known inhibitor of CYP (12). Mixed inhibition by galangin was also
observed by Zhai et al., in microsomes containing CYP1A1 and CYP1A2, using MROD as a substrate. There was an increase in apparent $K_m$ and a decrease in $V_{max}$ (13).

In our studies, both CP agents proved to be effective inhibitors of CYP, as measured by the cleavage of the VIVID® BOMCC substrate. An interesting observation was the differential protection against BPD toxicity and mutagenicity by resveratrol and galangin. Both inhibited hCYP1B1 slightly more than hCYP1A1, and resveratrol stably inhibited both isozymes by 90% or greater in a dose-dependent manner. Based on these data, we hypothesized that there would be potent inhibition of PAH toxicity and mutagenicity by both agents at concentrations above 30µM resveratrol or 3µM galangin. What we observed was significant inhibition of B[a]P cytotoxicity in hCYP1A1 and hCYP1B1 cells, by both galangin and resveratrol. The inhibition of B[a]P mutagenicity was also similar between both agents. Unexpectedly, resveratrol did not inhibit BPD cytotoxicity in either cell line, or BPD mutagenicity in hCYP1A1 cells. While there was a trend in reduction of B[a]P mutagenicity by galangin ($p=0.07$) in hCYP1B1 cells, another unexpected observation was the insignificant reduction in B[a]P mutagenicity by both agents in hCYP1B1 cells, even though both agents moderately inhibited hCYP1B1 more than hCYP1A1.

Given that both agents were potent inhibitors of hCYP1A1 and hCYP1B1, the reasons for this differential protection by each agent is unclear. There are several factors to consider. First, there are differences in how the BOMCC substrate is metabolized compared to PAH metabolism. Cleavage of the BOMCC substrate is a CYP-mediated $O$-dealkylation reaction, whereas CYP catalyzes an epoxidation reaction when metabolizing PAH. Another factor is substrate specificity for the enzyme. For example, hCYP1B1 has
a lower specific activity towards the BOMCC substrate (7.9 ± 1.6 pmol/min/mg protein) compared to hCYP1A1 (101.2 ± 11.6 pmol/min/mg protein); however, it activates B[a]P at a higher rate than hCYP1A1 (14). Therefore, the effects of each CP agent on CYP activity, when measuring cleavage of the BOMCC substrate, may not always predict the CP agent’s effects on cytotoxicity and / or mutagenicity of B[a]P or BPD.

The binding of the CP agent to the enzyme may cause a shift in the metabolic profile. The mixed-type inhibition by resveratrol as reported by Chun et al. (7) and the mixed-type inhibition by galangin as reported by Zhai et al. (13) suggest that this allosteric binding may cause a conformational change in the enzyme, altering the metabolism of the PAH. That may be what we are seeing with resveratrol and the lack of effect it has on BPD toxicity and mutagenicity. Based on our consumption data, we know that BPD is being metabolized in the presence of resveratrol. Resveratrol has no effect on that consumption in hCYP1A1 cells. A conformational change in the enzyme by resveratrol may shift the metabolite formation of BPD. hCYP1A1 yields more cytotoxic species, such as quinones and phenols (14), while hCYP1B1 yields more mutagenic metabolites, such as (+)-anti-BPDE (15). Resveratrol might cause a shift towards formation of more or less of either metabolite. This may affect the biological endpoints, as well.

It is possible that BPD is a better substrate than resveratrol. Therefore, it is able to be further metabolized to the (+)-anti-BPDE, thus resveratrol has no effect on cytotoxicity. Galangin, on the other hand, appears to be a better substrate than B[a]P or BPD. To determine the metabolism of each PAH in the absence or presence of each agent, consumption profiles were completed for B[a]P (6 hours) and BPD (2 hours).
What we observed was that resveratrol appears to slow, but not block consumption of B[a]P and BPD in hCYP1A1 and hCYP1B1 cells. These results give reason as to why we see no effect on BPD biological endpoints. It may also suggest that the slower metabolism of B[a]P in the presence of resveratrol is enough to inhibit cytotoxicity and mutagenicity of the parent compound, given that B[a]P must be activated by CYP before it becomes carcinogenic (16).

Galangin appears to inhibit metabolism of both compounds by hCYP1A1 and hCYP1B1. Galangin also provides a significant 91% (11.1-fold) and 90% (10.2-fold) reduction of covalent B[a]P-protein adducts in hCYP1A1 and hCYP1B1 cells, respectively. This observation complemented the finding by Kim et al, who showed that DNA-B[a]P adduct formation was reduced in calf thymus DNA that was incubated with $^3$H-B[a]P in the presence of galangin (17). The protection against PAH mutagenicity by galangin was predictive of protection against protein adducts by galangin. We observed reductions in $^3$H-BPD protein adducts, and a trend towards reduction in $^3$H-B[a]P protein adducts.

Isothiocyanates are known to be inhibitors of CYP (18, 19, 20). However, in Chapter 4 we observed that PE-ITC is a weak inhibitor of hCYP1A1, with BOMCC substrate. Genistein also proved to be a weak inhibitors of hCYP1A1, with BOMCC substrate. Consistent with this observation, neither had an effect on toxicity of B[a]P or BPD in hCYP1A1 cells. Both were more potent inhibitors of hCYP1B1, with the BOMCC substrate and each significantly reduced B[a]P toxicity in hCYP1B1 cells. However, neither had an effect on BPD toxicity in cells expressing hCYP1B1. PE-ITC had no effect on B[a]P or BPD mutagenicity in hCYP1A1 or hCYP1B1 cells. The lack
of a significant effect by PE-ITC on PAH cytotoxicity or mutagenicity in hCYP1A1 cells is not surprising, given that it only moderately inhibited the isozyme. It was also a moderate inhibitor of hCYP1B1; therefore, a significant reduction in B[a]P cytotoxicity was not expected. It is possible that PE-ITC reduced B[a]P toxicity in hCYP1B1 cells because PAH metabolites in these cells are less cytotoxic; however, the BPD toxicity may have been too much of a burden for PE-ITC to overcome. The PAH metabolites of hCYP1A1 are more cytotoxic; therefore, PE-ITC had no effect on toxicity in those cells. PE-ITC had no effect on the endpoint of mutagenicity in hCYP1A1 cells, either.

Genistein had no effect on B[a]P toxicity or mutagenicity in hCYP1A1 cells. This is not surprising given that it was such a weak inhibitor of BOMCC substrate in these cells. However, genistein significantly reduced BPD mutagenicity in hCYP1A1 cells. It is possible that there is less of a mutagenic burden in hCYP1A1 cells, due to less formation of mutagenic PAH metabolites by hCYP1A1. Genistein significantly reduced B[a]P toxicity and mutagenicity, and BPD toxicity in hCYP1B1 cells. This was predicted, based on the enzyme inhibition data. It seems the effects of genistein on PAH toxicity and mutagenicity are isozyme-specific.

PE-ITC and genistein may not be acting as simple inhibitors, but may be exerting their effects in other ways. Induction of both cell cycle arrest and apoptosis are known effects of PE-ITC (21). Also, PE-ITC forms conjugates with GSH in the cell, catalyzed by GST (22). Conjugation to GSH makes PE-ITC unavailable to inhibit CYP-mediated metabolism of PAH. Genistein is a substrate of CYP1A1 and CYP1B1 (23). The metabolic profile of genistein may affect PAH metabolic distribution. Any of genistein’s
metabolites may shift the metabolite distribution of BPD to form more of the (+)-anti BPDE.

All things considered, the main point to take from these studies is that BOMCC substrate cleavage and PAH metabolism are not equal. BOMCC substrate and PAH are not metabolized via the same mechanism. BOMCC is metabolized by CYP via an O-dealkylation reaction. B[a]P is oxidized in a monooxygenation reaction to form multiple electrophiles, including (+)B[a]P-7,8-oxide. This species can be further metabolized to form (-)B[a]P 7, 8-dihydrodiol, catalyzed by epoxide hydrolase. This diol species is then metabolized by CYP in an epoxidation reaction to form (+)-anti-BPDE. A CP agent may be an effective inhibitor of the BOMCC substrate cleavage in an O-dealkylation reaction, but have no effect on CYP-catalyzed monooxygenation or epoxidation of PAH. Also, substrate specificity is critical in determining how carcinogen or CP agent may interact metabolically. They may interact with different residues of the CYP enzyme and bind different sites of the enzyme to form distinct bound enzyme-substrate complexes.

Next, while much effort is taken to control for such things as expression of CYP, exposure time and concentrations of PAH and CP agents, there may be other factors that are harder to ascertain, for example, 1) the dominant metabolites formed from PAH metabolism ± CP agent, 2) direct binding of CP agents to the PAH, and 3) effects on cell signaling, apoptosis and repair pathways. Examining each point separately, first, CYP activation of B[a]P yields both (+) and (-) BPD enantiomers. If (-) BPD is the dominant metabolite formed, it can be further metabolized to (+)-anti-BPDE, the most mutagenic of the metabolites. The presence of CP agents may shift metabolism profile of B[a]P to
yield more (+)-anti-BPDE. This may explain why resveratrol did not reduce BPD toxicity.

Last, a review of the literature shows that these agents affect other parts of the cell besides directly inhibiting CYP. Resveratrol can trigger apoptosis (24). Galangin has potent anti-oxidant properties (25). PE-ITC affects cell signalling pathways (26, 27) and directly affects Nrf2-Keap1 complex (28). Genistein has been shown to inhibit detachment of cancer cells in mice (29). All of these actions of CP agents and more, that we are unable to control for in our system, could be indirectly affecting PAH metabolism.

These are factors that should be considered, as well, if these studies were to be extrapolated into an animal model. In addition, in an animal model, disposition of carcinogens is dependent upon the balance between activation and detoxification of carcinogenic compounds, as well as the rate of elimination via excretion. Other Phase I enzymes and their inducibility by PAHs become a factor in animal models and in humans. We looked at each CP agent separately, but they are usually found as mixtures in the environment or in a diet. The amounts consumed, whether exposure is dietary vs supplemental, and the environmental factors (smoker vs non-smoker) would all need to be considered.

In Appendix 1, we investigated direct inhibition of GST by CP agents in vitro using 1-chloro-2,4-dinitrobenzene (CDNB) substrate. Purified enzyme stocks (hGSTM-1 and hGSTA-1) were employed to determine if any CP agents were potent inhibitors of GST. We observed zero to moderate inhibition of each isozyme by the CP agents, but only at supraphysiological concentrations (100µM – 1mM). hGST-M1 was inhibited no
more than 21% by any of the agents and hGST-A1 less than 30%, with the exception of PE-ITC, which inhibited hGST-A1 by 51%. PE-ITC could form conjugates with glutathione (GSH) in the cell, and these conjugates may act as inhibitors themselves, through feedback (product) inhibition. Isothiocyanates have been shown to form GSH conjugates, especially when GST is present; therefore, we assayed the isothiocyanates after a 10 minute pre-incubation with GST and GSH, prior to adding the substrate (Table 16). We observed greater inhibition of hGST-M1 following the 10 minute pre-incubation; however, none of the agents inhibited hGST-A1 more following the 10 minute pre-incubation period, except sulforaphane. GST inhibition by these agents would likely be due to intracellular accumulation of the agent, cellular GSH depletion, or conjugate feedback inhibition. Although we did not observe strong inhibition of GST-M1 or GST-A1 in a solution assay, this does not rule out inhibition in intact cells, where accumulation of conjugates may rapidly reach steady-state. However, our results suggest that ITC conjugates do not block GST conjugation of CDNB.

In Appendix 2, we looked at the effects that S-nitrosoglutathione (GSNO) had on CYP-dependent metabolism of BPD. It has been demonstrated that NO inhibited CYP-dependent reactions when microsomal preparations were exposed to NO (30). Inhibition of CYP by NO has also been demonstrated in V79 Chinese hamster cells genetically engineered to stably express rat or human CYP (31). Our studies showed that GSNO effectively inhibited both hCYP1A1 and hCYP1B1 in a dose-dependent manner (greater than 80% for both). However, it did not reduce the cytotoxicity or mutagenicity of BPD in hCYP1A1 cells.
Collectively, what we have shown is that there is a need for broad-based toxicological studies on any potential new chemopreventive agent. The effects observed when measuring one endpoint are not always predictive of the effects on other biological endpoints such as cytotoxicity and mutagenicity. Additionally, the effects in an \textit{in vitro} system may not always extrapolate to an \textit{in vivo} system. There are many factors to consider. The unexpected harmful outcomes (e.g. lung cancer increase) found in a large-scale clinical trial of β-carotene as a cancer chemopreventive agent in smokers and in those exposed to asbestos offer excellent examples (32).
Table 10

<table>
<thead>
<tr>
<th>Resveratrol</th>
<th>hCYP1A1</th>
<th>hCYP1B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIVID® BOMCC Inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (30µM)</td>
<td>90%</td>
<td>94%</td>
</tr>
<tr>
<td>Time (6 hour pre-incubation)</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>Cytotoxicity – 6 hour exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD</td>
<td>↑→</td>
<td>↑→</td>
</tr>
<tr>
<td>Mutagenicity – 6 hour exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Consumption (HPLC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P (5 min vs 6 hour)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD (5 min vs 2 hour)</td>
<td>→</td>
<td>↓</td>
</tr>
<tr>
<td>Protein Adducts – 6 hour exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD</td>
<td>↓</td>
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</table>

**Table 10.** A summation table on the effects of resveratrol on CYP inhibition and B[a]P and BPD cytotoxicity, mutagenicity, PAH consumption and total protein adduct formation. Down arrow indicates “reduction”; side arrow indicates “no effect”; up arrow indicates “sensitization”; black arrow indicates significance; clear arrow indicates no significance.
Table 11

<table>
<thead>
<tr>
<th>Galangin</th>
<th>hCYP1A1</th>
<th>hCYP1B1</th>
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<tbody>
<tr>
<td><strong>VIVID® BOMCC Inhibition</strong></td>
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</tr>
<tr>
<td>Dose (3µM)</td>
<td>49%</td>
<td>99%</td>
</tr>
<tr>
<td>Time (30 min pre-incubation)</td>
<td>38%</td>
<td>100%</td>
</tr>
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<td><strong>Cytotoxicity – 6 hour exposure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Mutagenicity – 6 hour exposure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B[a]P</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Consumption (HPLC)</strong></td>
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</tr>
<tr>
<td>B[a]P (5 min vs 6 hour)</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD (5 min vs 2 hour)</td>
<td>↓</td>
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</tr>
<tr>
<td><strong>Protein Adducts – 6 hour exposure</strong></td>
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</tr>
<tr>
<td>B[a]P</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BPD</td>
<td>↓</td>
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</table>

Table 11. A summation table on the effects of galangin on CYP inhibition and B[a]P and BPD cytotoxicity, mutagenicity, PAH consumption and total protein adduct formation. Down arrow indicates “reduction”; side arrow indicates “no effect”; up arrow indicates “sensitization”; black arrow indicates significance; clear arrow indicates no significance.
<table>
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<tr>
<th>PE-ITC</th>
<th>hCYP1A1</th>
<th>hCYP1B1</th>
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<tr>
<td>B[a]P</td>
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<td>BPD</td>
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<td>Mutagenicity – 6 hour exposure</td>
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<tr>
<td>B[a]P</td>
<td></td>
<td></td>
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<tr>
<td>BPD</td>
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</tbody>
</table>

**Table 12.** A summation table on the effects of PE-ITC on CYP inhibition and B[a]P and BPD cytotoxicity and mutagenicity. Down arrow indicates “reduction”; side arrow indicates “no effect”; up arrow indicates “sensitization”; black arrow indicates significance; clear arrow indicates no significance.
Table 13

<table>
<thead>
<tr>
<th>Genistein</th>
<th>hCYP1A1</th>
<th>hCYP1B1</th>
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<tr>
<td><strong>VIVID® BOMCC Inhibition</strong></td>
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</tr>
<tr>
<td>Dose (30µM)</td>
<td>38%</td>
<td>86%</td>
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<td>Time (6 hour pre-incubation)</td>
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<td>88%</td>
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</thead>
<tbody>
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<td>B[a]P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPD</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Mutagenicity – 6 hour exposure</strong></th>
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</thead>
<tbody>
<tr>
<td>B[a]P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 13.** A summation table on the effects of genistein on CYP inhibition and B[a]P and BPD cytotoxicity and mutagenicity. Down arrow indicates “reduction”; side arrow indicates “no effect”; up arrow indicates “sensitization”; black arrow indicates significance; clear arrow indicates no significance.
References:


APPENDIX 1

Effects of CP Agents on Purified Human Glutathione S-Transferase Mu-1 (hGST-M1) and Human Glutathione S-Transferase Alpha-1 (hGST-A1) Enzymes

Introduction

Enzymes of the glutathione S-transferase (GST) superfamily catalyze nucleophilic attack by the sulfhydryl group of glutathione (GSH) on substrates that contain an electrophilic molecule. The conjugation of these compounds with GSH inactivates their electrophilic centers and renders them more water soluble and more readily excreted from the cell by the transmembrane multidrug resistance-associated protein (MRP) (1). This is an important defense mechanism for the cell.

It has been suggested that chemopreventive agents exert their potential through two major actions: 1) their inhibition of Phase I enzymes leading to decreased formation of the carcinogenic derivatives of xenobiotics, and/or 2) their induction of Phase II enzymes leading to increased excretion of the metabolites. However, it is possible that some CP agents may also inhibit GSTs, and therefore limit their protective effects. Indeed, formation of the GSH-ITC conjugate is catalyzed by GSTs, and the conjugate causes product inhibition if it accumulates to high levels, which might occur in cells, since the conjugate is not membrane permeable. Thus, we expected the ITCs, in particular, to inhibit GST activity. We investigated direct inhibition of GST by CP agents in vitro using 1-chloro-2,4-dinitrobenzene (CDNB) substrate. Purified enzyme stocks (hGST-M1 and hGST-A1) were employed to determine if the CP agents we studied were
potent inhibitors of GST. We observed a moderate inhibition of each isozyme by the CP agents, but only at supraphysiological concentrations (100µM – 1mM).
Experimental Procedures

Purified GST and GST Enzyme Assay

Purified enzyme stocks (hGST-M1 and hGSTA-1) were previously prepared in the lab by GSH affinity chromatography of lysates of *E. coli* that express each GST from a prokaryotic expression vector, as described previously (2). Purity was verified by migration as a single band at the appropriate size by SDS-PAGE analysis. The GST assay is a modification of the method described by Habig (3). Briefly, 10ul of purified enzyme (~ 16 mU GSTM-1 GSTA-1) was assayed at room temperature in a solution of 0.1mM K$_2$PO$_4$, pH 6.5 and 1mM of reduced glutathione (GSH). CP agents (10ul) were added at various concentrations (0.1µM – 1mM), prior to adding the substrate. The isothiocyanates were also assayed after a 10 minute pre-incubation with GSH and GST, prior to adding the substrate, to allow for GSH-ITC conjugate formation. The reaction was initiated by addition of 1-chloro-2,4-dinitrobenzene (CDNB) in anethanolic solution over a range of concentrations from 100µM to 1mM (final concentration). The data presented is for 1mM CDNB. The change in absorbance was monitored at 340 nm for 90 seconds (six intervals) and activity, corrected for nonenzymatic reagent blank, was calculated using the ΔA/minute and extinction coefficient, and expressed as nmol/min/mg protein. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard.
<table>
<thead>
<tr>
<th>Dose Condition</th>
<th>hGSTµ Specific Activity (No inhibitor)</th>
<th>hGSTµ Specific Activity (with inhibitor - 100µM)</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average GST</td>
<td>Average GST</td>
<td></td>
</tr>
<tr>
<td>Allyl ITC</td>
<td>738 (nmol / min)</td>
<td>805 (nmol / min)</td>
<td>None</td>
</tr>
<tr>
<td>Benzyl ITC</td>
<td>943 (nmol / min)</td>
<td>764 (nmol / min)</td>
<td>21%</td>
</tr>
<tr>
<td>Phenethyl ITC</td>
<td>841 (nmol / min)</td>
<td>779 (nmol / min)</td>
<td>7%</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>815 (nmol / min)</td>
<td>878 (nmol / min)</td>
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<tr>
<td>Resveratrol</td>
<td>880 (nmol / min)</td>
<td>796 (nmol / min)</td>
<td>10%</td>
</tr>
<tr>
<td>Quercetin</td>
<td>610 (nmol / min)</td>
<td>949 (nmol / min)</td>
<td>None</td>
</tr>
<tr>
<td>CAPE</td>
<td>862 (nmol / min)</td>
<td>684 (nmol / min)</td>
<td>21%</td>
</tr>
<tr>
<td>Curcumin</td>
<td>688 (nmol / min)</td>
<td>572 (nmol / min)</td>
<td>17%</td>
</tr>
<tr>
<td>Genistein</td>
<td>910 (nmol / min)</td>
<td>806 (nmol / min)</td>
<td>11%</td>
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<td>Daidzein</td>
<td>715 (nmol / min)</td>
<td>597 (nmol / min)</td>
<td>17%</td>
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<tr>
<td>Equol</td>
<td>780 (nmol / min)</td>
<td>675 (nmol / min)</td>
<td>13%</td>
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<tr>
<td>Alpha Tocopherol</td>
<td>791 (nmol / min)</td>
<td>649 (nmol / min)</td>
<td>18%</td>
</tr>
</tbody>
</table>

**Table 14. Inhibition of Human Glutathione-S-transferase Mu by Chemopreventive Agents.** The GST enzyme assay was performed as described in *Experimental Procedures*. The average GST is a measure of specific activity towards 1-chloro-2,4-dinitrobenzene (CDNB). Results are presented as the mean of 3 or more independent assays.
Table 15. Inhibition of Human Glutathione-S-transferase Alpha by Chemopreventive Agents. The GST enzyme assay was performed as described in Experimental Procedures. The average GST is a measure of specific activity towards 1-chloro-2,4-dinitrobenzene (CDNB). Results are presented as the mean of 3 or more independent assays.
<table>
<thead>
<tr>
<th>Dose Condition (nmol / min)</th>
<th>Average GST Specific Activity (No inhibitor) (nmol / min)</th>
<th>Average GST Specific Activity (with inhibitor - 100µM) (nmol / min)</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hGSTµ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allyl ITC</td>
<td>677</td>
<td>491</td>
<td>27%</td>
</tr>
<tr>
<td>Benzyl ITC</td>
<td>946</td>
<td>677</td>
<td>28%</td>
</tr>
<tr>
<td>Phenethyl ITC</td>
<td>955</td>
<td>754</td>
<td>21%</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>770</td>
<td>657</td>
<td>15%</td>
</tr>
<tr>
<td><strong>hGSTα</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allyl ITC</td>
<td>1103</td>
<td>1115</td>
<td>None</td>
</tr>
<tr>
<td>Benzyl ITC</td>
<td>1025</td>
<td>756</td>
<td>26%</td>
</tr>
<tr>
<td>Phenethyl ITC</td>
<td>1093</td>
<td>687</td>
<td>37%</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>1062</td>
<td>864</td>
<td>19%</td>
</tr>
</tbody>
</table>

**Table 16. Inhibition of Human Glutathione-S-transferase by Isothiocyanates Following a 10 minute pre-incubation.** The GST enzyme assay was performed as described in *Experimental Procedures*. The average GST is a measure of specific activity towards 1-chloro-2,4-dinitrobenzene (CDNB). Results are presented as the mean of 3 or more independent assays.
Results and Discussion

There are seven classes of cytosolic GST recognized in mammalian species, including GST Pi, Mu, and Alpha. The Pi and Mu class GSTs have a high activity for BPDE conjugation, and Alpha class GST can efficiently conjugate the potent carcinogens dibenzo[a,l]pyrene (DBP) and (−)-anti-DBP-11,12-diol-13,14-epoxide ((−)-anti-DBPDE) (4). In rodent models, a considerable body of data indicates that enhancement of GST expression is a key element in the reduction by CP agents of chemical-induced DNA damage and carcinogenesis. For example, the ITC sulforaphane has been shown to induce GSTs and other protective enzymes in rat models (5). Also, curcumin was shown to increase the levels of several mouse GST isozymes in hepatic tissue (6).

We investigated direct inhibition by CP agents of GST in vitro using purified enzyme stocks (hGST-M1 and hGST-A1) and 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. One of the major actions of CP agents is their induction of Phase II enzymes, including GST, leading to increase conjugation and excretion of metabolites (9). However, it is possible that some CP agents may also inhibit GSTs, and therefore limit their protective effects. In particular, the ITCs are known to be conjugated by GSTs, and may thus act as competitive or product inhibitors. We wanted to determine if any of the CP agents were potent inhibitors of GST. What we observed was zero to moderate inhibition of each isozyme by the CP agents, but only at supraphysiological concentrations (100µM) (Tables 14, 15). hGST-M1 was inhibited no more than 21% by any of the agents and hGST-A1 less than 30%, with the exception of PE-ITC, which inhibited hGST-A1 by 51%. 
The CP agent could work as a direct, albeit weak, inhibitor of the enzyme. The agent could also form conjugates with glutathione (GSH) in the cell, and these conjugates may act as feedback inhibitors themselves, through product inhibition. ITC’s have been shown to form these conjugates; therefore, we assayed the isothiocyanates after a 10 minute pre-incubation with GSH, prior to adding the substrate (Table 16). We observed more inhibition of hGST-M1 by each of the ITCs following a 10 minute pre-incubation, but none inhibited the enzyme more than 28%. However, none of the ITCs inhibited hGST-A1 more following the 10 minute pre-incubation period, except sulforaphane. Inhibition of hGST-A1 by any of the ITCs after the 10 minute pre-incubation period was no greater than 37%.

There are several factors that could determine if an agent would act as an inhibitor of GST. First, the isozyme that is expressed is important. The CP agents in these studies inhibited hGST-A1 more potently than hGST-M1. Second, the CP agent itself may be a substrate for the enzyme, leading to increased inhibition. That may be the case for phenethyl-ITC and hGST-A1. Next, the amount of GSH in the cell could influence inhibition. ITCs form conjugates with GSH, and these conjugates could feedback inhibit the enzyme. Finally, the concentration of the CP agent is important. We see here, even at concentrations of 100µM, the enzymes are moderately inhibited. However, given a supplemental dose of the CP agent, peak plasma level concentrations might reach 1mM or upwards. This could also have an effect on the balance between depletion and re-synthesis of GSH. Based on the data presented here, these CP agents don’t appear to be strong inhibitors, even at relatively high concentrations (100 µM).
References:


APPENDIX 2

Effects of S-nitrosoglutathione (GSNO) on hCYP1A1 and hCYP1B1 Activity and on hCYP1A1 Activation of Benzo[a]pyrene-7,8-dihydrodiol (BPD) to Toxic and Mutagenic Metabolites

Introduction

Endogenously derived nitric oxide \( (NO)^2 \) plays important bioregulatory roles in a number of physiological processes, such as the control of blood pressure, neurotransmission, platelet aggregation, and the cytostatic and cytotoxic action of macrophage cells (1, 2). Since NO binds to the catalytic heme moiety of hemoproteins such as CYP, hemoproteins may represent a primary target of NO within cells.

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (B[a]P) and its metabolite benzo[a]pyrene-7,8-diol (BPD) are environmental pollutants that have been implicated as potent lung carcinogens. Human exposure to these compounds is unavoidable. BPD can be further metabolized by CYP to form \((+)-anti\)-B[a]P-7,8-diol-9,10-epoxide \([(+)\text{-anti-}BPDE]\), which is the most carcinogenic B[a]P metabolite (3) and is highly reactive towards nucleophilic sites in DNA and other macromolecular structures in the cell, possibly leading to cancer.

It has been demonstrated that NO inhibits CYP-dependent reactions when microsomal preparations were exposed to NO (4). Inhibition of CYP by NO has also been demonstrated in V79 Chinese hamster cells genetically engineered to stably express rat or human CYP (5). Here, we investigated if S-nitrosoglutathione (GSNO), an NO
donor, was a possible inhibitor of CYP-dependent activation of BPD. If so, this might suggest that it could be used as a possible chemopreventive (CP) agent.

We employed V79 Chinese hamster lung fibroblast cells that have been stably transfected to express hCYP1A1 and hCYP1B1 isozymes. This novel system allows us to study metabolism of PAH in the absence and presence of GSNO under tightly controlled conditions, while maintaining cell architecture, enzyme compartmentalization, and biological responses. We wanted to determine whether inhibition of hCYP1A1 or hCYP1B1 activity, as measured by cleavage of the fluorescent VIVID BOMCC substrate in intact cells, was predictive of GSNO’s effects on the biological endpoints of cytotoxicity and / or mutagenicity of BPD.
Experimental Procedures

Materials and Chemicals

All chemicals used in these studies were of analytical grade and were obtained from Fisher (Atlanta, GA), Sigma (St. Louis), or Invitrogen (Carlsbad, CA). Advanced Dulbecco’s modified Eagle’s medium (Adv. DMEM) was purchased from Gibco/BRL (Grand Island, NY). GSNO was obtained from Dr. Bruce King’s laboratory. B[a]P and BPD were purchased from Midwest Research Institute (Lexena, KS).

Cell Lines and Culture

Parental V79MZ Chinese hamster lung fibroblast cells were modified to express either hCYP1A1 or hCYP1B1, as previously described (6). Cells were grown and maintained in Advanced DMEM supplemented with 2% fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells were selected with 400 µg/ml G-418 and passaged every 2-3 days. CYP activity was measured after each passage of cells to insure expression of the transfected CYP was stable and constitutive.

The parent cells express no constitutive or inducible CYP activity (7). They do express an endogenous Pi-class hamster GST at low levels (8), but this GST is not active for the conjugation of reactive electrophiles of the PAH in these studies with glutathione (GSH) (9). Expression of the transfected CYP is stable and constitutive, rather than variable due to carcinogen induction. Also, expression of the enzymes in unhindered by background interference because each CYP is expressed against the same V79 cell background; therefore, each isozyme is comparable.
**CYP Fluorescent Enzyme Activity Assays**

VIVID® benzyloxymethyloxy-3-cyanocoumarin (BOMCC) substrates are blocked dyes that yield minimal fluorescence signal until cleaved at either of two potential cleavage sites, releasing a highly fluorescent product. This assay is performed in an intact cell system, as both substrate and product are membrane-permeable. Cells expressing either hCYP1A1 or hCYP1B1 were plated on 6-well plates at $1.8 \times 10^5$ in 2 ml Advanced DMEM (2% FBS) and allowed to attach and grow for 48 hours. At the time of the assay, 1 ml of serum-free media containing a 5µM concentration of BOMCC substrate was replaced on each plate and incubated at 37°C for 30 minutes. To determine the activity of the enzyme, the highly fluorescent product was then measured spectrofluorometrically in the medium at 409 nm excitation / 460 nm emission. Activities of hCYP1A1 and hCYP1B1 were measured using a known VIVID blue standard curve (0-100µM). Protein was determined by the Coomassie dye method (Bio-Rad, Hercules, CA). Final enzyme activity was determined by comparison to the standard curve and normalized to protein content on each plate and expressed as pmol / min / mg protein. It is important to note that hCYP1A1 has a higher specific activity with the BOMCC substrate than hCYP1B1.

In the direct enzyme inhibition studies, the assay was plated as above. At the time of the assay for the dose-dependent studies, 1 ml of media containing a range of concentrations of GSNO was replaced on the plates for a 30 minute pre-incubation at 37°C prior to adding the substrate. For the time-dependent studies, 1 ml of serum-free medium containing a fixed concentration GSNO (30µM) was replaced on each plate for pre-incubation times ranging from 0 minutes – 3 hours at 37°C prior to adding the
substrate. Incubation for an additional 30 minutes at 37°C was allowed and enzyme activity was determined as described above.

**Cytotoxicity Assays**

Cytotoxicity was assayed using the sulforhodamine-B (SRB) method, as previously described (10). The principle of the assay is based on the ability of the protein dye (SRB) to bind protein basic amino acid residues of trichloroacetic acid-fixed cells. Cells were plated in Advanced DMEM (2% FBS) on 96 well plates at a density of 250 cells / well. This density has been optimized for a growth interval of up to 4 days. Cells were allowed to attach and grow for 16 – 24 hours prior to being dosed. At the time of dosing (for non-continuous exposure), media was removed and 150 µl of serum-free media was added to each well. A concentration calculated to yield 1mM GSNO in serum-free media (25 µl) was added for a 30 minute pre-incubation at 37°C prior to adding the PAH. An 8x concentration of BPD (25 µl) was added and incubated for 3 hours. At the end of the exposure period, medium was removed and 200 µl Adv DMEM (2% FBS) was added back to each well for the remainder of the 4 day growth interval. Following incubation for 4 days, cells were harvested by decanting the medium and fixing the cells in 100 µl cold 5% TCA for a minimum of 1 hr at 4°C. Cells were rinsed with a gentle stream of tap water, allowed to dry, and then stained for 10 minutes with 100 µl 0.4% SRB dye in 1% glacial acetic acid. Cells were then washed 4-5 times with 1% glacial acetic acid, dried, and the dye solubilized in 100 µl 10mM non-pH adjusted Tris-Base. Cell density was determined by measuring absorbance with a microplate reader (Molecular Devices, Sunnyvale, CA) at 560 nm to quantitate protein as an indirect measure of cell number.
**hprt Mutagenicity Assays**

Cells were plated in Advanced DMEM (2% FBS) at a density of 5 x 10^5 cells per 100 mm plates and allowed to adhere for 16 – 24 hours prior to being dosed. At the time of dosing, media was removed and plates were rinsed twice with 2 ml serum-free media. Cells were pre-incubated with 1mM GSNO in serum-free media for 30 minutes at 37°C prior to adding the PAH. Cells were then exposed to 0.15µM BPD in DMSO (co-exposure with CP agent) for 3 hours, media was removed, and cells were rinsed twice with 2 ml Adv DMEM (2% FBS). Following incubation for an additional 3 hours with 1mM GSNO in 2% serum media, 10 ml fresh serum-containing media was added to each plate and cells were allowed to recover for 72 hours. Cells were then subcultured at 5 x 10^5 cells / 100 mm plate and grown for 6 days, with one subculture at 3 days, to allow for phenotypic development. Cells were subcultured once more at the same density, and the next day 6-thioguanine (6-TG) was added at a concentration of 10µg 6-TG / ml medium in order to select for hprt mutants. Cells were maintained under 6-TG selection for 10 – 12 days, with two changes of serum media + 6-TG during that time, after which mutant hprt mutant colonies were stained with 5mM methylene blue in methanol. Mutant colonies were scored manually and are expressed as colonies per million cells.
### Table 17

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>BPD IC\textsubscript{50} (µM)</th>
<th>Fold Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V79MZh1A1</td>
<td>0.1 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>V79MZh1A1 + GSNO</td>
<td>0.1 ± 0.01</td>
<td>None</td>
</tr>
</tbody>
</table>

**Table 17.** Cytotoxicity of BPD ± GSNO in V79MZ cells engineered to express hCYP1A1 (3 hour exposure). Cytotoxicity was assayed by the sulforhodamine dye method as described in *Experimental Procedures*. Results are presented as the mean ± S.D. of 3 or more independent assays.
Figure 20a. GSNO dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in *Experimental Procedures*. Results are expressed as pmol/min/mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); black bars represent V79MZh1A1.
Figure 20b. GSNO dose-dependent inhibition of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); gray bars represent V79MZh1B1
Figure 21a. Stability of GSNO inhibition over time of human Cytochrome P450 activity in V79MZ cells engineered to express hCYP1A1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S. D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); black bars represent V79MZh1A1.
Figure 21b. Stability of GSNO inhibition over time of Cytochrome P450 activity of V79MZ cells engineered to express hCYP1B1. The CYP fluorescent enzyme activity assay was performed as described in Experimental Procedures. Results are expressed as pmol / min / mg protein. Results are presented as the mean ± S.D. of 3 independent assays. Clear bars represent V79MZ (no transgenes); gray bars represent V79MZh1B1.
Figure 22. Mutagenicity of BPD + GSNO in V79MZ cells engineered to express hCYP1A1 (3 hour exposure). The hprt mutagenicity assay was performed as described in Experimental Procedures. Fold-protection is the ratio of mutant colony numbers in the BPD exposure condition to the mutant colonies in the BPD+ GSNO exposure condition. Results are presented as the mean ± S.D. of 3 independent assays. Black bars represent exposure to BPD, light bars represent exposure to BPD + GSNO.
Results and Discussion

Here, we investigated if inhibition of hCYP1A1 or hCYP1B1 activity, as measured by cleavage of the fluorescent VIVID BOMCC substrate in intact cells, was predictive of GSNO’s effects on the biological endpoints of cytotoxicity and/or mutagenicity of BPD. GSNO (30µM) effectively inhibited hCYP1A1 by 88% and hCYP1B1 by 84% in a dose-dependent manner when measuring cleavage of VIVID® BOMCC substrate (Figure 20). Results for timed experiments show greater than a 65% inhibition of hCYP1A1 and hCYP1B1 by 30µM GSNO at 1 hour, but that activity was not maintained over 3 hours. Activity returned and was 40% and 35% in hCYP1A1 and hCYP1B1 cells, respectively (Figure 21).

Next, we wanted to determine if this direct inhibition of CYP’s cleavage of the BOMCC substrate was predictive of GSNO’s effects on the toxicity of BPD. A dose-response of the cytotoxicity of BPD in the absence or presence of 30µM GSNO was determined after a 30-minute pre-incubation with GSNO followed by a 3 hour exposure to the BPD in the presence of GSNO. GSNO did not protect against cytotoxicity of exposure to BPD for 3 hours in hCYP1A1 cells (Table 17). These studies were not done in cells expressing hCYP1B1. The V79MZ control cell line showed no cytotoxicity to BPD or GSNO (not shown). The hCYP1A1 cells showed no cytotoxicity to GSNO (not shown).

Finally, we determined if mutagenicity of BPD, in the absence or presence of 1mM GSNO, was reduced. Mutagenicity was measured by frequency of mutation at the \textit{hprt} locus following exposure of cells to 0.15µM BPD in the absence or presence of
1mM GSNO for 3 hours. GSNO had no effect on the mutagenicity of BPD in hCYP1A1 cells (Figure 22). These studies were not done in cells expressing hCYP1B1. The V79MZ control cell line showed no mutagenicity to BPD or GSNO. The hCYP1A1 transfected cells showed no mutagenicity to GSNO.

This data shows that, while GSNO effectively inhibited CYP activity in a dose-dependent manner when measuring cleavage of the BOMCC substrate, it does not inhibit cytotoxicity or mutagenicity of BPD. These studies did not examine GSNO’s effects on B[a]P toxicity or mutagenicity, nor did we look at the effect of GSNO on B[a]P or BPD toxicity or mutagenicity in hCYP1B1 lines. It is possible that we might have seen a different outcome with that CYP isozyme, or when looking at B[a]P as a substrate. The potent inhibition of each CYP isozyme by GSNO supports the finding of previous studies (4, 5); however, GSNO’s inhibition of the BOMCC substrate is not predictive of its effects on PAH toxicity or mutagenicity. GSNO effectively inhibits the O-dealkylation reaction occurring by cleavage of the BOMCC substrate, but is ineffective at inhibiting the epoxidation reaction that occurs though CYP-mediated metabolism of BPD.
References:


### Table 18

<table>
<thead>
<tr>
<th>CP Agents</th>
<th>Cytotoxicity in Parent V79 Cell Line</th>
<th>IC₅₀ (µM) @ Cont. Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MZ</td>
<td>1A1</td>
</tr>
<tr>
<td>Allyl ITC</td>
<td>6.0 ± 1.52</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Benzyl ITC</td>
<td>2.0 ± 0.20</td>
<td>2.0 ± 0.14</td>
</tr>
<tr>
<td>Phenethyl ITC</td>
<td>3.0 ± 0.17</td>
<td>3.0 ± 0.34</td>
</tr>
<tr>
<td>Sulforaphane</td>
<td>2.0 ± 0.9</td>
<td>2.0 ± 0.24</td>
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<td>Genistein</td>
<td>5.0 ± 0.86</td>
<td>5.0 ± 1.13</td>
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<td>&gt;100</td>
<td>94.0 ± 2.91</td>
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<tr>
<td>Equol</td>
<td>70.0 ± 2.23</td>
<td>55.0 ± 8.27</td>
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<tr>
<td>Quercetin</td>
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<td>&gt;100</td>
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<td>Resveratrol</td>
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<td>Galangin</td>
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<td>1.0 ± 0.20</td>
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<tr>
<td>Curcumin</td>
<td>2.0 ± 0.12</td>
<td>2.0 ± 0.09</td>
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<tr>
<td>Alpha Tocopherol</td>
<td>13.1 ± 0.21</td>
<td>13.0 ± 0.03</td>
</tr>
</tbody>
</table>

**Table 18.** Cytotoxicity of CP Agents in Parental V79 Cell Line at Continual Exposure. IC₅₀ expressed as µM. Results are presented as the mean + S.D. of at least three independent assays.