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Title: DNA BINDING AND BEYOND: AN INVESTIGATION OF PROTEINS INVOLVED IN PAH-INDUCED CARCINOGENESIS

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William M. Baird

Exposure to polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (B[*a*]P) has been determined to be a risk factor for various forms of human cancer. PAH DNA adducts have been shown to cause mutations, but carcinogenesis is also accompanied by alterations in gene expression. Inhibiting individual cytochrome P450s could clarify the interaction of P450s and other enzymes in the activation of polycyclic aromatic hydrocarbons to DNA binding intermediates. Phosphorodiamidate morpholino oligomers (PMOs), a class of antisense agents were targeted against cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1). No significant inhibition of enzyme activity or expression was observed with any PMO used as measured by ethoxyresorufin-*O*-deethylase (EROD) activity and immunoblots. It was demonstrated that BPDE may react with PMOs in vitro, and PMOs may be segregated in lysosomes, blocking their efficacy. Nonspecific effects by the PMO on CYP1A1 activity were observed. These observations indicate multiple confounding effects in the use of PMOs for this purpose. Many of the genes regulated by histone deacetylases are involved in proliferation, cell function, and differentiation, and HDAC inhibitors are of great interest in cancer research. To probe epigenetic regulation of CYP1A1, MCF-7

cells were treated with two HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA). SAHA and TSA increased EROD activity and in RT-PCR. SAHA and TSA reduced B[*a*]P induced CYP1A1 and CYP1B1 mRNA levels. B[*a*]P DNA binding was not significantly altered by SAHA or TSA treatment. To assay global protein expression changes after treatment with PAH, MCF-7 cells were treated with B[*a*]P, DB[*a,l*]P, coal tar extract (SRM 1597) and diesel exhaust extract (SRM 1975), Proteins were separated by two-dimensional electrophoresis, and analyzed using PDQuest. Spots of interest were excised and identified by matrix assisted laser desorption/ionization time of flight time of flight mass spectroscopy. Alterations in expression of heat shock proteins, cytoskeletal proteins, DNA associated proteins, and glycolytic and mitochondrial proteins were observed. Universally increased expression was observed for tubulin alpha and myosin light chain alkali, cyclophilin B, heterogeneous nuclear riboprotein B1, and alpha enolase. Additional proteins exhibiting change in expression included histone H2A.1, heat shock protein 70-2, galectin-3, nucleoside diphosphate kinase, ATP synthase, and electron transfer flavoprotein.

DNA BINDING AND BEYOND: AN INVESTIGATION OF PROTEINS INVOLVED IN PAH-INDUCED CARCINOGESESIS

By

Louisa Ada Hooven

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APPROVED:

Redacted for privacy

Major Professor, representing Biochemistry and Biophysics

Redacted for privacy

Head of Biochemistry and Biophysics

Redacted for privacy

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Louisa Ada Hooven, Author

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CONTRIBUTION OF AUTHORS

William M. Baird[1,2] has provided guidance and advice for all the work in this thesis as well as support from funding from the National Cancer Institute.

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[1] Department of Biochemistry and Biophysics,
[2]Department of Environmental and Molecular Toxicology
Oregon State University, Corvallis, Oregon
[3]Institute for Cancer Prevention, American Health Foundation Cancer Center,
Valhalla, New York
[4]Toxicology and Molecular Biology Branch,
National Institute of Ocupational Safety and Health, CDC, Morgantown, WV

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ABBREVIATIONS

[^{33}P]ATP .....adenosine-5'-O-(3-[^{33}P]-triphosphate

1A1 .....cytochrome P450 1A1

1B1 .....cytochrome P450 1B1

1DE.....1 dimensional electrophoresis

2DE.....2 dimensional electrophoresis

AHR.....aryl hydrocarbon receptor

AIDS.....acquired immune deficiency syndrome

ANOVA.....analysis of variance

AP-1 .....activator protein-1

APS .....ammonium persulfate

ARNT.....aryl hydrocarbon nuclear transporter

ATP.....adenosine triphosphate

B[*a*]P .....benzo[*a*]pyrene

BPDE .....benzo[a]pyrene diol epoxide

CHAPS.....3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate

CYP.....cytochrome P450

CYP1.....cytochrome P450 family 1

CYP1A1 .....cytochrome P450 1A1

CYP1B1.....cytochrome P450 1B1

DB[*a,l*]P .....dibenzo[*a,l*]pyrene

DHFR.....dihydrofolate reductase

DMBA.....dimethylbenzanthracene

DMSO.....dimethylsulfoxide

EDTA.....ethylenediamine tetraacetate acid

EPEI.....ethoxylated polyethylenimine

ER alpha.....Estrogen receptor alpha

EROD.....ethoxyresorufin *O*-deethylation

FCS.....fetal calf serum

GAPDH.....glyceraldehyde-3-phosphate dehydrogenase

GDI.....guanine nucleotide dissociation inhibitor

GDP.....guanosine diphosphate

GST.....glutathione S transferase

H2A.1.....histone 2A.1

HAT.....histone acetyl transferase

HDAC.....histone deacetylase

HEP G2.....cell line derived from human hepatoma

HEPES.....N-2-hydroxyethylpiperazine-*N*'-2-ethane-solfonic acid

HIF alpha.....hypoxia-inducible factor alpha

hnRNA.....heteronuclear RNA

hnRNP B1.....heterogeneous ribonucleoprotein B1

HPLC.....high performance liquid chromatography

HSP 27.....heat shock protein 27

HSP 70.....heat shock protein 70

IARC.....International Agency for Research on Cancer

IEF.....isoelectric focusing

IPG.....immobilized pH gradient

MALDI TOF/TOF.....matrix assisted laser desorption ionization time of flight/time of flight mass spectroscopy

MC.....methylchrysene

MCF-7.....human mammary carcinoma derived cell line

NF-κB.....nuclear factor-kappa B

NMR.....nuclear magnetic resonance

NMU.....nitrosomethyl urea

NPDK.....nucleoside diphosphate kinase

NRE.....negative response element

PAH.....polycyclic aromatic hydrocarbon

PBS.....phosphate buffered saline

PBS-T.....phosphate buffered saline with Tween-20

pI.....isolelectric focusing point

PMO.....phosphorodiamidate morpholino oligomers

ROS.....reactive oxygen species

RT-PCR.....reverse transcriptase polymerase chain reaction

SAHA.....suberoylanilide hydroxamic acid

SDS-PAGE.....sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA.....small interfering RNA

SRM .....standard reference mixture

TCDD 2,3,7,8,-tetrachlorodibenzo-p-dioxin

TEMED .....tetramethylethylenediamine

TPA.....12-*O*-tetradecanoylphorbol-13-acetate

Tris .....tris(hydroxymethyl)aminomethane

Trx-1 .....thioredoxin 1

TSA .....trichostatin A

Tween-20.....polyolyethylene-sorbitan monolaurate

UTR .....untranslated region

V79.....Chinese hamster V79 cell line

WT .....wildtype

XRE .....xenobiotic responsive elemenent

Dedicated

To the memory of my father

Dr. Edward Frank Hooven

who earned his own Ph.D. from Oregon State University

June, 1971

DNA BINDING AND BEYOND: AN INVESTIGATION OF PROTEINS INVOLVED IN PAH-INDUCED CARCINOGESESIS

INTRODUCTION

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are large, aromatic, planar compounds formed by a variety of combustion processes. The ability of PAH-containing mixtures to induce human cancer has been observed since 1775, when the British surgeon Sir Percival Pott demonstrated a correlation between the exposure of chimney sweeps to soot and the incidence of scrotal cancer [1]. PAH occur naturally as a result of forest fires, and in coal, peat, crude oil and shale oils [2]. High occupational exposure to PAHs occurs in workers involved in aluminum production, iron and steel foundries, fossil fuel processing, wood impregnation, roofing, road paving, and other industries [3]. Additionally, workers exposed to diesel exhaust are exposed to high levels of PAH. Such elevated exposures are associated with elevated risks of lung, skin, and bladder cancer [4]. Many compounds characterized as PAH are found in tobacco smoke condensate, although for non-smokers, the principal route of exposure is through diet [5]. The environmental ubiquity of PAH and their negative association with human health adds urgency to the ongoing study of these compounds.

Modern research on PAH-induced carcinogenesis began with the isolation of benzo[*a*]pyrene (B[*a*]P) from coal tar in 1930, and the subsequent demonstration that B[*a*]P initiates tumors when repeatedly painted on mouse skin. Later it was observed that B[*a*]P is metabolized into diol epoxides which may form covalent adducts with DNA. [1] In the case of lung cancer, patterns of G→T transversion hotspots could be observed which correlated with preferential B[*a*]P binding sites in the p53 gene. Mutations in p53 are found in approximately 60% of human lung cancers. These findings are considered to constitute a mutational "fingerprint" of PAH carcinogenesis [6].

Many individual PAH have been tested for tumorigenicity. It was observed that PAH with bay regions (Figure 1-1) were likely to be potent carcinogens, and those with fjord regions even more potent. However, no structure-activity relationship could consistently account for the carcinogenicity of PAH [7]. This work is further

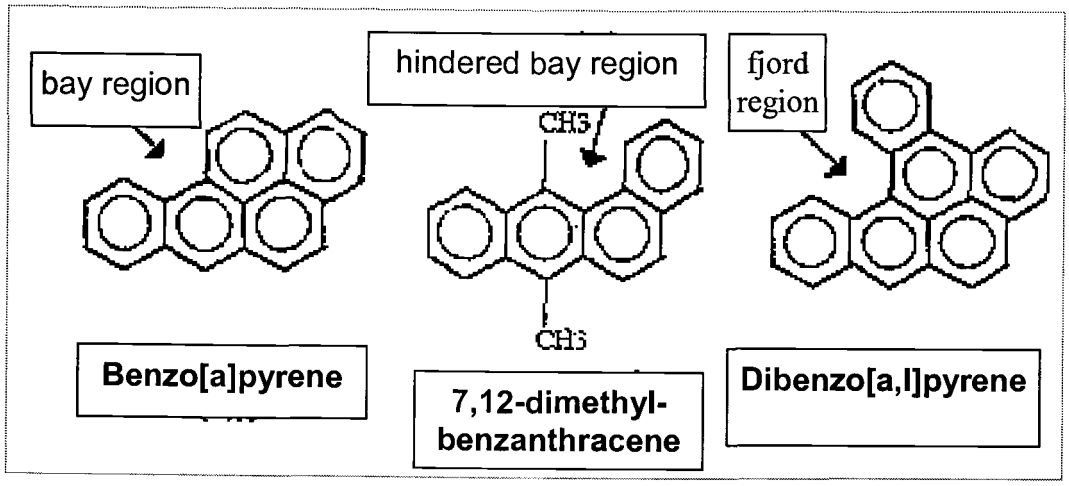


Figure 1-1. Structural features of PAH thought to contribute to carcinogenicity.

Benzo[*a*]pyrene (B[*a*]P), a known carcinogen, contains a bay region. The diol epoxide metabolite of B[*a*]P which binds to DNA contains an epoxide group next to the bay region. Methyl groups in 7,12-dimethyl-benzanthracene (DMBA) hinder this structural feature further, as does the extra benzene ring in dibenzo[*a,l*]pyrene. (DB[*a,l*]P) The compounds are arranged in order of their carcinogenicity, with DB[*a,l*]P being the most potent.

complicated by the fact that PAH occur in complex mixtures in the environment [8] (Figure 1-2). Such mixtures may contain hundreds of PAH compounds, many of which have exhibited no carcinogenic potential. However, two or more substances within a mixture may compete for receptors or metabolizing enzymes, and such synergistic action may result in cocarcinogenic or chemopreventive effects [9-11].

Inconsistencies remain in the study of PAH induced carcinogenesis. Variations in PAH-DNA binding do not always parallel observed tumor levels. For example, two of the four diol epoxide metabolites of B[*a*]P [(+)-*anti*]B[*a*]P-7R,8S-diol-9-S,10R-epoxide and [(+)-*syn*]B[*a*]P-7R,8S-diol-9-S,10R-epoxide (Figure 1-3) bind to DNA and are mutagenic in V79 cells or the Ames test. However, only the (+)-*anti* metabolite induces epidermal tumors when repeatedly applied to mouse skin. [12] In our own lab, we have observed that relative to B[*a*]P, a PAH mixture derived from coal tar forms few DNA adducts, yet is more tumorigenic in mouse skin [13]. This is a trend which may be observed in other mixtures [14] (Figure 1-4). These idiosyncrasies point to factors contributing to PAH-induced carcinogenesis beyond DNA binding ability.

Molecular mechanisms of PAH metabolism

Cytochromes P450 are a large family of enzymes involved in drug, xenobiotic, and steroid metabolism. Cytochrome P450 1A1 and 1B1 (CYP1A1 and CYP1B1) are substrate inducible, allowing the cell to respond to varying exposures to the PAH which these enzymes metabolize. CYP1A1 and CYP1B1 are induced via the aryl hydrocarbon receptor (AHR), which binds planar PAH, such as B[*a*]P, more readily than compounds distorted from planarity, such as DB[*a,l*]P. This cytosolic receptor, after dimerizing with the aryl hydrocarbon nuclear translocator (ARNT), enters the nucleus and interacts with genes containing a xenobiotic response element (XRE), resulting in induced or upregulated proteins involved in xenobiotic detoxification [15] (Figure 1-5).

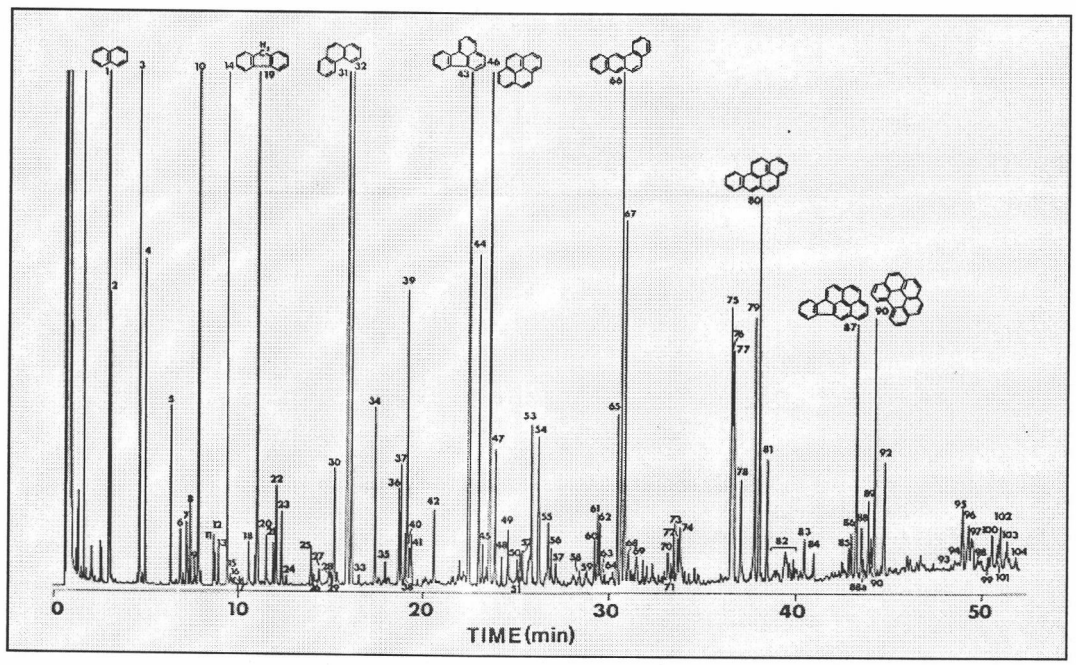
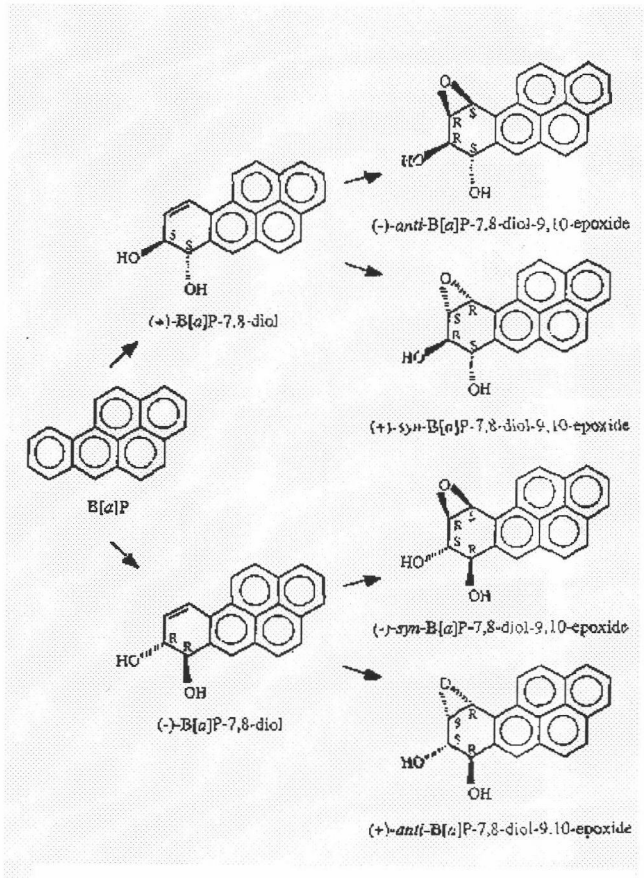


Figure 1.2. Capillary column gas chromatography analysis of SRM 1597. Analysis of coal tar extract (standard reference mixture 1597) illustrates that environmental PAH mixtures are composed of many compounds. Source: National Institute of Standards and Technology.



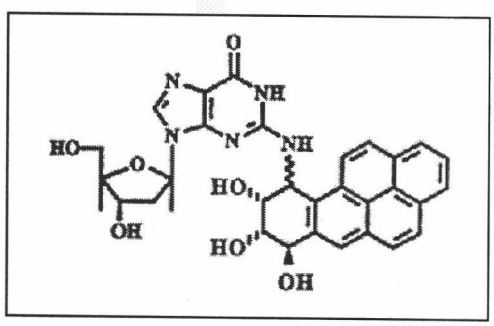


Figure 1-3
Top: Metabolism of B[*a*]P to diol epoxides.
The BPDE isomers containing the epoxide oxygen and 7-hydroxyl on the same face of the molecule are called *syn*; opposite face, *anti*. The syn and anti pairs are enantiomers. The [(+)-*anti*]B[*a*]P-7R,8S-diol-9-S,10R-epoxide is the most tumorigenic. The (+)-*syn* metabolite also binds to DNA.

Left: The bay-region epoxide may open in a cis or trans fashion to adduct to DNA bases.

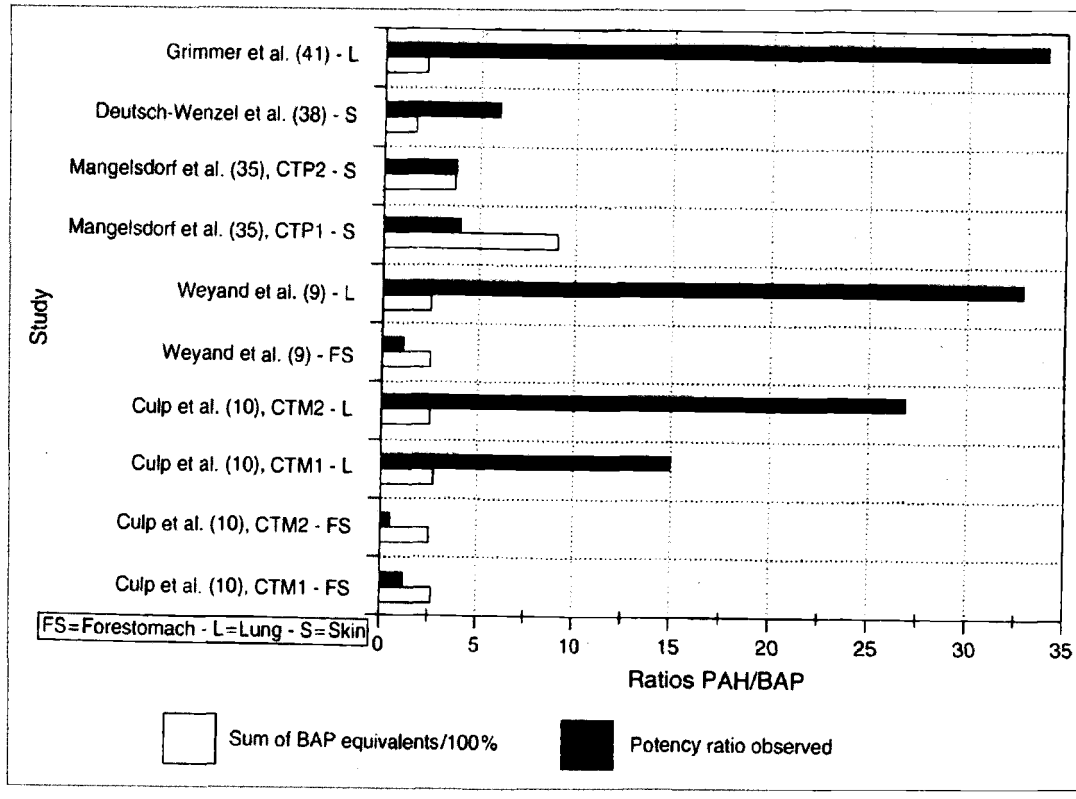


Figure 1-4. Comparison of carcinogenic potency of environmental PAH mixtures compared to estimated potency in terms of B[*a*]P equivalents.
Multiple studies in mice underscore the difficulty in estimating carcinogenic potency of environmental mixtures. The mixtures in these studies, from top to bottom included diesel automobile exhaust condensate, flue gas condensate, coal tar product 2, coal tar product 1, manufactured gas plant residue in lung, manufactured gas plant residue in forestomach, coal tar mixture 2 and coal tar mixture 1 in lung, and coal tar mixture 2 and coal tar mixture 1 in forestomach.
Source: K. Schneider et al, J. Appl. Toxicol. 22, 73-83 2002. The numbers following the authors of studies cited above relate to references from the Schneider paper.

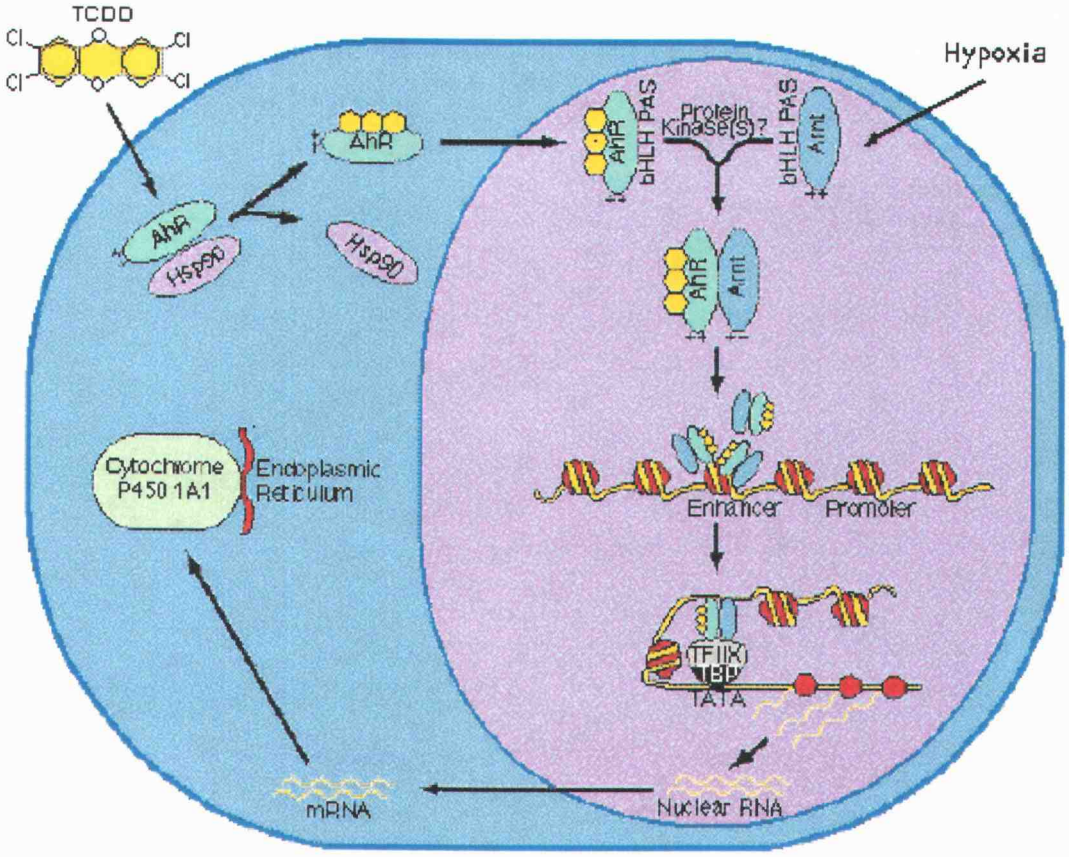


Figure 1-5. Induction of CYP1A1 by ligands of the aryl hydrocarbon receptor (AhR)

Planar, hydrophobic ligands bind to the AhR, which travels to the nucleus and dimerizes with the aryl hydrocarbon nuclear translocator (Arnt). This dimer binds to xenobiotic response elements upstream of genes such as CYP1A1, causing their transcription. In this diagram, TCDD is shown as a ligand. When PAH are the ligand, they are metabolized by CYP1A1. In this way, PAH induce their own metabolism.

Source: J.P. Whitlock Jr. laboratory web page, Department of Molecular Pharmacology, Stanford University, Stanford, California
http://www.stanford.edu/group/whitlock/research.html

CYP1A1 and CYP1B1 are monooxygenases which convert inert, lipophylic PAH to reactive electrophiles so they may be conjugated and excreted from the cell. It is in this process of detoxification that the PAH diol expoxides cabable of binding DNA may arise (Figure 1-6). P450s first add an epoxide group to the PAH, which is converted to a diol by epoxide hydrolase. This PAH metabolite may be further metabolized by P450s to form a diol epoxide. CYP1A1 and CYP1B1 have broad and overlapping specificity but vary in their efficiency at metabolizing specific compounds [16].

Two pairs of enantiomeric diol epoxides may result from this reaction, although ultimate DNA adducts observed appear to indicate high stereoselectivity. These metabolites may covalently bind via a *cis* or *trans* addition of the exocyclic amino groups of purines to diol epoxides (Figure 1-3, left). The ability of adducts to bind to DNA and persist to form mutations may depend upon the conformation of the adduct within the DNA sequence and the ability of repair enzymes to identify the lesion, which may be intercalated within the DNA helix [17].

While CYP1A1 and CYP1B1 are principal players, many enzymes are known to be crucial in the pathway to tumorigenesis. Many conjugating enzymes contribute to detoxification of PAH, including GSH transferase, UDP glucuronosyl transferase, epoxide hydrolase, and methyl transferase. To a lesser degree, PAH are activated by prostaglandin synthase, lipoxygenase, or one-electron oxidation [18]. PAH are known as complete carcinogens, because they can induce mouse skin tumors. However, such induction requires repeated, prolonged exposure, indicating that PAH treatment is required not just for initiation, but for promotion of tumors [12]. Promotion may be thought of as the transformation of a cell to a dormant cancer cell, and subsequent clonal expansion of that cell to a group of tumor cells. The molecular mechanisms of these processes have not been well elucidated [19]. The pathways involved in PAH metabolism overlap with several others. CYP1A1 and 1B1 are essential to estrogen metabolism [20], and ARNT also participates in cellular response to hypoxia as

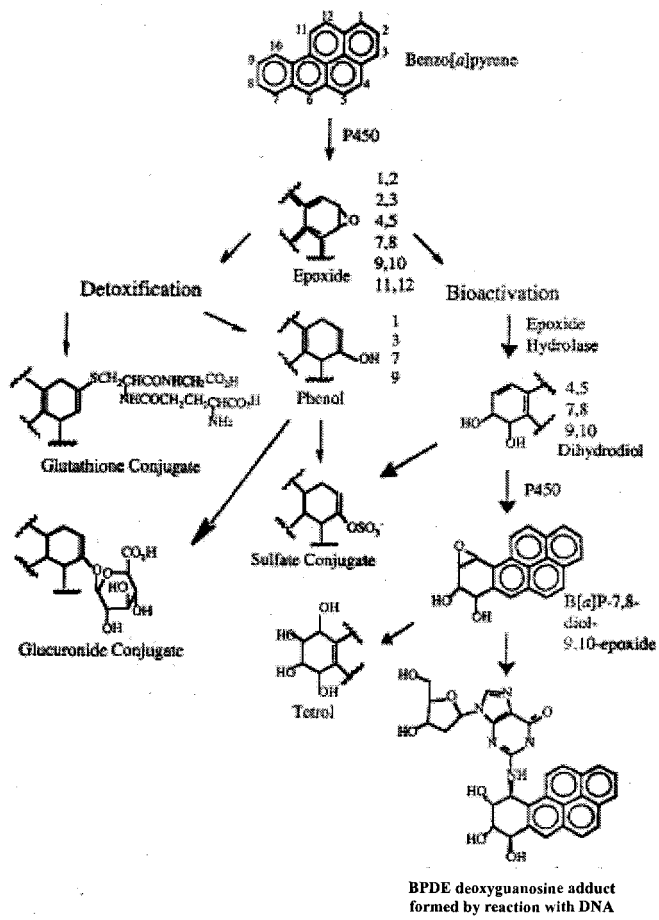


Figure 1-6. Activation of PAH to DNA binding species.
Cytochrome P450s add an epoxide group to hydrophobic PAH. This species may be conjugated and detoxified, or further bioactivated by epoxide hydrolase and additional epoxidation by P450s. Bay or fjord region diol epoxides have the potential to bind to DNA.

HIFalpha [21] and the AHR exhibits crosstalk with the estrogen receptor [22]. The role of PAH in carcinogenesis is far greater than the ability of these compounds to bind to DNA. The correlation of polymorphisms in selected genes and epidemiology of human cancers reveals that multifactorial relationships between enzymes contribute to cancer susceptibility. GSTm1, epoxide hydrolase, CYP1A1 and other genetic polymorphisms may together influence genotoxicity [20,23]. Small sequence differences in 1A1 or 1B1 can influence activity towards specific PAH ligands [16]. Lack of expression or overexpression of a given gene product can profoundly influence tumor promotion [24]. The complement of proteins induced or inhibited by a specific PAH compound or mixturehelp to determine its ability to cause cancer.

While the binding of PAH to DNA may be essential for tumorigenesis, it is not sufficient, and many questions remain in chemical carcinogenesis. The methods for analyzing DNA binding ability of PAH are well established. Stereospecificity of adduction may be observed, and NMR and computer modeling illustrate how lesions may influence DNA replication and repair. Yet how can DNA binding occur without subsequent tumorigenesis, as in the case of some compounds? What protein expression changes must occur beyond those involved in PAH metabolism for tumors to emerge? What are the mechanisms of induction, inhibition, or regulation of these proteins? What are the functions of individual proteins within this milieu?

Methods of study of PAH metabolism

Because the incidence of skin cancer in workers in the coal gasification industry was high [12], the first focus of PAH study was dermal application. After it was discovered that coal tar, a by-product of coal gasification, repeatedly applied to rabbit ears produced tumors, mice were developed as a model, because of their convenience. Single or multiple applications of the compound in question may be applied, followed by repeated applications of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This has become a standard technique for screening potential carcinogens [12]. While useful as an initial screen for human carcinogens, this technique suffers from

several drawbacks. The activity and specificity of metabolizing enzymes may vary greatly between species, possibly accounting for widely varying DNA binding and tumor levels, and making it difficult to compare data to human biology [25]. The repeated application of the tumor promoter TPA may make it difficult to distinguish between compounds which are initiators, initiators/promoters, and compounds which are simply promoters [26].

In vitro studies using synthesized PAH and PAH metabolites have yielded much information regarding the ability of individual metabolites to bind DNA, as well as the stereochemistry and conformation of these adducts. The ability of these lesions to impede the progress of polymerases explains how they may result in mutations. Individual metabolizing enzymes have been overexpressed in baculovirus/insect cell, yeast, *Salmonella*, and other systems [16,27-29] to compare their ability to metabolize specific compounds . These methods are highly focused on individual compounds and enzymes, and offer important mechanistic detail. However, these techniques do little to explain additional effects PAH may have on the cells that contribute to tumorigenesis.

Cell culture models are very useful when intact enzyme systems, rather than enzymes isolated in yeast or insect cells, can be observed. Chemical inhibitors of cytochrome P450s have often been used to characterize the function of these enzymes. However, many of these reagents have overlapping specificity, and it is unknown what non-specific effects they may have. Such inhibitors may have different activity for P450s from various species [30]. The ability to isolate the individual function of metabolizing enzymes within an intact enzyme system is dependent on the development of specific inhibitors.

Knockout mice have been developed for CYP1A1, CYP1B1, and other xenobiotic metabolizing enzymes. These mice validate the importance of metabolizing enzymes in tumor formation, but unexpected paradoxes point to the need for more study. For

example, in CYP1B1 knockout mice, dimethylbenzanthracene (DMBA)-induced tumors were reduced, compared to wild type, except in the lung, where they were increased [24]. Additionally, rodents express a broader spectrum of cytochrome P450s than do humans [31]. An AHR knock-in mouse has been developed by first knocking out the mouse AHR and replacing it with the human receptor. This animal model has been tested for its responsiveness to tetrachlorodibenzodioxin (TCDD), which binds tightly to the AHR, resulting in CYP1A1 and CYP1B1 upregulation, as well as many other effects. Mouse populations containing the human AHR were compared to those expressing the murine AHR, and while CYP1A1 and CYP1B1 responded as expected, TCDD-induced teratogenicity was greatly reduced [32], again demonstrating significant differences between murine and human xenobiotic responses.

Correlating cancer occurrence in PAH-exposed humans with DNA adduct levels and genotype has revealed that variant enzymes may play an important role in cancer susceptibility. Such efforts have identified variations in CYP1A1 and glutathione S-transferases, and other enzymes in vulnerable individuals. While such studies most accurately pinpoint dangerous exposure conditions, a major goal of toxicology is to predict and prevent cancer. Several decades may pass between exposure and tumorigenesis, by which time many additional individuals may have been exposed. Additionally, these data are able to measure phylogenetic differences in populations, as well as genetic alterations in tumors, but not the mechanism of progression from initial DNA damage to cancer.

High throughput methods, including DNA arrays, protein arrays, and proteomics, allow for the simultaneous measurement in changes of expression or effect. These methods increase the possibility of discovering new markers and pathways as well as therapeutic targets. DNA arrays measure changes in mRNA levels, and the number of publications in this area is increasing exponentially. Technological data analysis improvements continue to increase the usefulness of this technique. However, the changes observed in mRNA copies do not always parallel those observed in protein

levels. Protein arrays use antibodies or other means to probe for multiple known proteins, but do not find changes in unknown proteins. A principal technique in proteomics is the separation of proteins by two-dimensional electrophoresis and subsequent analysis by mass spectrometry. This offers the theoretical advantage of monitoring changes in all proteins in a given sample. Recent technological advances have increased the reproducibility of this method, and continuing software developments allow for improved statistical analysis and database searching.

Approaches used in this work

The research of chemical carcinogenesis by PAH has a long and fruitful history, yet many questions remain. To address some of these questions, three approaches were used in this work. To keep this work relevant to human biology, all of this work used MCF-7 cells, which are derived from human breast carcinoma, and are known to metabolically activate PAH.

In the first approach, morpholino antisense technology was used to attempt to block CYP1A1 and CYP1B1 protein synthesis. By subtracting one cytochrome P450 within an intact population of xenobiotic metabolizing enzymes, the function of this enzyme within the system would be revealed. In the second approach, the possibility that CYP1A1 is under epigenetic control was explored by treating cells with histone deacetylase inhibitors. In the third project, two-dimensional electrophoresis and mass spectroscopy were used to explore an expanded spectrum of protein expression after cells were treated with individual PAH and SRMs. This work seeks to examine the ability of xenobiotic metabolizing enzymes as a system, and how PAH or PAH mixtures influence the population and levels of these enzymes.

Antisense inhibition of cytochrome P450s

Phosphorodiamidate morpholino oligomers (PMOs) are believed to duplex with complementary target RNA and sterically block the ribosome from producing protein. This method is highly effective in zebrafish embryos, and has been in use for several

decades in cell culture and animal models with varying success. Phosphorothioate antisense oligos targeted against CYP1A1 have been shown to significantly reduce CYP1A1 activity in rats [33], and have shown some effect in Hepa-1 cells [34]. The action of PMOs is reported to be very precise, with less nonspecific effects than phosphorothioates, and active over a greater concentration range [35]. In Chapter Two the ability of PMOs to inhibit CYP1A1 and CYP1B1 in human MCF-7 cells was examined. This inhibition technique, if successful, could clarify the role of CYP1A1 and CYP1B1 not only in PAH metabolism, but in the metabolism of other toxins. Additionally, antisense oligos could be applied to other enzymes involved in xenobiotic metabolism. Inhibition of CYP1A1 by PMOs was at best minimal, and unexpected non-specific effects were observed. The possibility that PMOs are sequestered in the cell or interact with B[*a*]P, which is used to induce CYP1A1/1B1 is examined. For antisense to be useful in cell culture, the trafficking of these molecules once they have been delivered to the cell must be understood.

Upregulation of CYP1A1 by histone deacetylase inhibitors

Suberoylanilide hydroxamic acid (SAHA) is a hybrid polar compound which has been shown to suppress the proliferation of cancer cells in culture, and reduce tumors in animal models, and is the subject of ongoing clinical trials as a chemotherapeutic agent. SAHA, like trichostatin A (TCA) acts as a histone deacetylase (HDAC) inibitor. HDAC inhibitors block the remodeling and tight packing of chromatin, and increase the expression of approximately two percent of genes.

Because of its chemotherapeutic and chemopreventive properties, SAHA was initially tested in our lab for its ability to modulate B[*a*]P DNA adduct levels. No significant effect on DNA binding was observed. However, SAHA increased CYP1A1 levels and activity as shown by immunoblotting, RT-PCR, and EROD assays. This work was repeated using TSA, which is a structurally related, but more potent HDAC inhibitor. TSA increases CYP1A1 levels and activity to an even greater degree, but has no

significant effect on B[*a*]P DNA adducts levels. Interestingly, TSA also reduced CYP1A1 and CYP1B1 mRNA levels induced by B[*a*]P.

Examination of PAH-related protein expression changes using two-dimensional electrophoresis

A great deal of research has been performed examining the role of individual proteins in xenobiotic metabolism. To build on this work, DNA arrays are beginning to be used to examine the spectrum of genes involved in metabolism. Initial work has been published with data obtained from rats and mice treated with B[*a*]P. We wished to compare actual protein levels, and to use MCF-7 cells, which would allow comparison with previous work in our lab. Two-dimensional electrophoresis (2DE) is a common approach for proteomic analysis. Complex protein mixtures are first separated by isoelectric focusing point (pI) and further separated by molecular weight using SDS-PAGE. The resolved proteins are excised from the gel and analyzed by mass spectroscopy. We have undertaken a proteomic analysis of MCF-7 cells treated with B[*a*]P, DB[*a,l*]P, and standardized extracts from coal tar and diesel exhaust. Our results have shown alterations in protein expression of heat shock proteins, cytoskeletal proteins, DNA associated proteins, and glycolytic and mitochondrial proteins.

Scientific research owes much of its success to its ability to dissect complex structures and systems into manageable, analyzable subunits. It is difficult to understand or even recognize information that may be lost in this process [36]. DNA adduct formation may work in concert with non-genotoxic pathways in inducing tumors [37]. Combinatorial or synergistic effects may be involved both in the activity of enzymes induced by a specific compound [20], as well as the compounds within a given mixture [9,11]. The importance of differences in populations of enzymes has been demonstrated by comparing polymorphisms in metabolizing and detoxifying enzymes to human PAH exposures and cancer epidemiology. Questions remain in the study of PAH regarding co-carcinogens, such as benzo[*e*]pyrene [38] and benzo[*g,h,I*] perylene [39], highly mutagenic compounds, such as fluoranthene, which show low

carcinogenicity [40], and carcinogenic PAH, which show low DNA binding as our lab has seen with coal tar mixture [41]. Why doesn't the apparent genotoxicity of PAH or PAH mixtures always correlate with rodent tumorigenicity? What are the synergistic interactions of PAH within mixtures that cause them to be more or less carcinogenic than predicted? An additional layer of complexity may be involved in PAH induced carcinogenesis, and an overarching question presents itself. What mechanisms aside from the well known CYP metabolism and adduction to DNA of PAH contribute to carcinogenesis? In this work, we have sought to identify some of the additional factors and proteins that may be involved in this process immediately after PAH exposure.

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EFFECT OF MORPHOLINO ANTISENSE OLIGOMERS ON CYTOCHROME P450 1A1 AND 1B1 ACTIVITY AND POLYCYCLIC AROMATIC HYDROCARBON DNA ADDUCTS IN MCF-7 CELLS

Louisa Ada Hooven

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W. M. Baird, L. A. Hooven, B. Mahadevan, A. Luch, A. Seidel, and P.L. Iversen

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ABSTRACT

The aim of this study was to develop a highly specific method to inhibit CYP1A1 and CYP1B1 activity. Inhibiting the activity of an individual cytochrome P450 enzyme could clarify the interaction of P450s and other enzymes in the activation of polycyclic aromatic hydrocarbons to DNA binding intermediates. Existing cytochrome P450 inhibitors often lack specificity for a single P450 enzyme. Phosphorodiamidate morpholino oligomers (PMOs) are a class of antisense agents that are designed to bind tightly and specifically to RNA and thus alter hnRNA processing and inhibit mRNA translation. Three different PMOs were targeted against CYP1A1 and three were targeted against CYP1B1. Three control PMO oligomers were also used. Several methods were attempted for loading the PMOs into cells. Use of a cationic reagent, ethoxylated polyethylenimine (EPEI) was found to be the best technique to load the PMOs into the human mammary carcinoma MCF-7 cell line, which were then treated with benzo[*a*]pyrene (B[*a*]P) to induce CYP1A1 activity. Catalytic activity was measured using ethoxyresorufin-*O*-deethylase (EROD) assays of intact cells or microsomes prepared from treated cells. No significant inhibition of enzyme activity was observed with any PMO used. Protein levels in immunoblots and DNA adduct measurements exhibited little change when treated with PMOs. It was demonstrated that BPDE may react with PMOs in vitro, indicating that B[*a*]P may interact with PMOs in cells, potentially inhibiting their ability to bind to RNA. Fluorescent microscopy revealed that while PMOs entered the cells, they may be segregated in lysosomes, blocking their efficacy. Additionally, EROD activity appears to be elevated in cells treated with B[*a*]P and scrambled control PMO, compared to B[*a*]P alone. These observations point to multiple confounding effects in the use of PMOs in this experimental model.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are a large group of ubiquitous environmental contaminants formed by the incomplete combustion of carbon compounds and are found in sources such as automobile exhaust, tobacco smoke and coal tar. The correlation between human cancer and exposure to PAH has been studied since 1775, when Sir Percival Pott noted increased levels of scrotal cancer in chimney sweeps exposed to soot. Later animal studies confirmed the association between sources of PAH and cancer [42]. Contemporary studies observe the relationship between cancer rates and polymorphisms in carcinogen metabolizing enzymes as well as specific mutational fingerprints left by PAH [43,44].

Many individual PAH have been isolated or synthesized to compare structural features with carcinogenic activity. Many of the most potent carcinogenic PAH contain 4 to 6 aromatic rings and contain a bay or fjord region although this is not an absolute determination of carcinogenicity [7]. Structural models of various PAH bound to DNA have revealed how these bulky DNA lesions might interact with the DNA helix and interfere with DNA replication, repair and translation [45-47]. Studying individual PAH has answered many metabolic and structural questions of how PAH metabolites bind to DNA and initiate mutations.

PAH are initially metabolized by cytochrome P450 enzymes, which are a large group of enzymes involved in the metabolism of drugs, steroid hormones, and xenobiotic compounds such as PAH. The CYP1 family of these enzymes acts as monooxygenases, transforming lipophilic PAH into oxygen substituted reactive species which may then be conjugated by other enzymes (Figure 2-1). These charged and water soluble conjugates may then be excreted. Alternatively, CYP1 enzymes may further oxidize the PAH to a diol epoxide which may then react with the exocyclic amino group of purine bases to form covalent adducts which can result in miscoding to form a mutation when the DNA is replicated [7,48]. CYP enzymes vary in

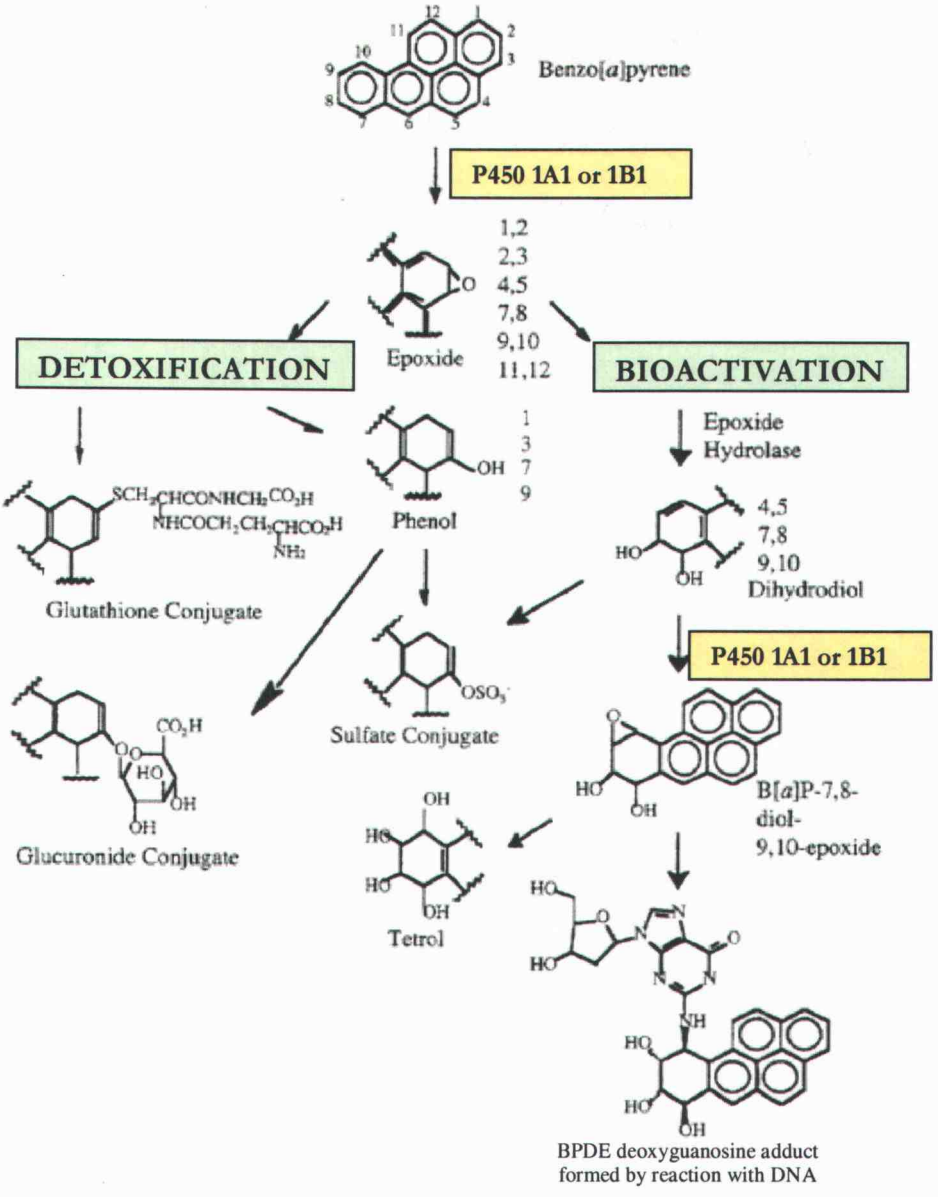


Figure 2-1. Metabolism of polycyclic aromatic hydrocarbons. Hydrophobic PAH may be metabolized to polar compounds which are then conjugated and excreted. Alternatively, PAH may be further bioactivated to diol epoxides which may then bind to the exocyclic amines of DNA. Cytochrome P450 1A1 and 1B1 are important in both detoxification and bioactivation.

their expression and substrate specificity. CYP1A1 has been implicated in the activation of many carcinogens and has been well characterized. CYP1B1, a more recent discovery, appears to be important in the metabolism of dibenzo[*a,l*]pyrene, a very potent carcinogen [49,50].

CYP1A1 is barely detectable until it is induced by a ligand of the aryl hydrocarbon receptor (AhR), typically a planar, lipophilic compound such as B[*a*]P or TCDD. The liganded AhR travels to the nucleus, where it dimerizes with the Ah receptor nuclear translocator (Arnt) to generate a DNA-binding transcription factor. The dimer binds to xenobiotic responsive elements (XREs), which induce the transcription of many genes [15]. The nucleotide core of this sequence is GCGTG and is important for AhR/Arnt binding, with flanking sequences contributing to transcriptional activation [51]. CYP1B1 is expressed constitutively in low levels, but may also be induced via the AhR [52].

P450s comprise a superfamily of proteins involved in the metabolism of many endogenous and exogenous compounds. Because variation in P450 activity may alter the fate of drugs, it has long been a concern of pharmacology. Likewise, variation in CYP expression or activity may influence the outcome of PAH exposure. Two steps of PAH metabolism involve CYP1A1 and CYP1B1 (Figure 2-1). The substrate specificities of these two enzymes are overlapping but distinct. For example, when V79 cells expressing only CYP1A1 are exposed to the potent carcinogen DB[*a*,l]P, more polar DNA adduct products are observed than those in DB[*a,l*]P-treated cells expressing only CYP1B1 [53]. Differential expression of these two enzymes may result in varying adduct patterns, which in turn may influence the health outcome of those exposed to PAH.

The development of nearly all cancers are accompanied by changes in gene expression as compared to normal cells. The human cytochrome P450 family is highly polymorphic and evidence is accumulating that these variations may alter susceptibility

to cancer. A high inducibility phenotype of CYP1A1 may be associated with elevated incidences of lung and breast cancer [54-56]. Certain polymorphisms of CYP1B1 exhibit a significant association with increased lung and breast cancer incidences [57]. While conflicting data have also been observed, polymorphisms that influence the activity of carcinogen metabolizing P450s likely impact human cancer rates. CYP1A1 variants have been shown to differentially metabolize benzo[*a*]pyrene and dibenzo[*a,l*]pyrene – a change in a single amino acid may alter substrate specificity [58]. Understanding the interaction of such polymorphisms with environmental carcinogens will assist in the prediction of those at high risk of cancer.

Chemical inducers and inhibitors are available for both CYP1A1 and 1B1. However, many of these reagents have shown non-specific effects. For example, 7,8 - benzoflavone, a known inhibitor of several cytochrome P450s, has also been observed to have effects on the cell cycle [59]. Such inhibitors may have overlapping effects on other P450s than the intended target [60]. Some of these compounds may act on the AhR pathway, rather than directly on a specific protein. Knockout mice have been developed for a number of carcinogen metabolizing enzymes [24,61,62]. In a study by Buters et al, 7.5% of CYP1B1 knockout mice treated with the carcinogenic PAH dimethylbenzanthracene (DMBA) developed lymphomas, wheareas 70% of WT mice developed lymphomas, underscoring the role of P450s in activating carcinogens [63]. Because mice are an important animal model in understanding carcinogenesis, it is imperative to understand the specific action of murine P450s. However, orthologous cytochrome P450s in mice are not identical to those in humans [31], and of course human knockouts cannot be developed.

Non-human cell lines expressing single human P450s have been useful in understanding human metabolism of PAH [14]. For example, a number of P450s have been expressed in *Salmonella* and *E. coli.* [64]. However, examining a single enzyme may be misleading. In the CYP1B1 knockout study cited above, the knockout mice developed more lung adenomas than WT mice, likely due to the induction of CYP1A1

by DMBA [63]. This indicates that the ratio of P450 enzymes may be important to the amount of final DNA adducts which are carcinogenic. To more accurately understand carcinogenesis by PAH in humans, human cell lines expressing a normal spectrum of P450s are useful. MCF-7 cells, a line of human mammary carcinoma cells, express a number of cytochrome P450 enzymes in addition to 1A1 and 1B1. Inhibiting either CYP1A1 or CYP1B1 in these cells will aid in separating the roles of these enzymes in the activation of PAH in human cells.

Antisense technology offers a highly specific means of inhibiting protein synthesis. An oligomer is designed which is complementary to the mRNA of the protein of interest. Older antisense designs, such as phosphorothioates utilized backbones which required RNAse activity to degrade the mRNA. Such designs were limited in their efficacy, degraded quickly, were difficult to load into cells, and exhibited nonspecific effects [35,65,66]. Because of their solubility, phosphorodiamidate morpholino oligomers (PMOs) (Figure 2-2, inset) are easier to load into cells than other antisense designs, are resistant to enzymatic degradation, and act by blocking the ribosome from binding to a specific mRNA transcript [67] (Figure 2-2), rather than through an RNase mechanism. PMOs also bind more tightly to RNA than do older backbone designs [35,66]. These features purportedly allow for more predictable targeting and specific action toward a given protein. PMOs have been very successfully utilized in blocking protein synthesis when injected into zebrafish embryos, in some cell studies, and are being tested in a number of clinical trials in humans [68-73].

To determine how PAH DNA adducts are affected when the activity of CYP1A1 or CYP1B1 is reduced, we attempted to develop the use of phosphorodiamidate morpholino antisense oligomers (PMOs) to selectively inhibit these enzymes in MCF-7 cells. Our first aim was to optimize a method for loading PMOs into these cells. Secondly, we attempted to inhibit cytochrome P4501A1 using this method and measure the affect on PAH-DNA adducts. Finally, we repeated some of these assays

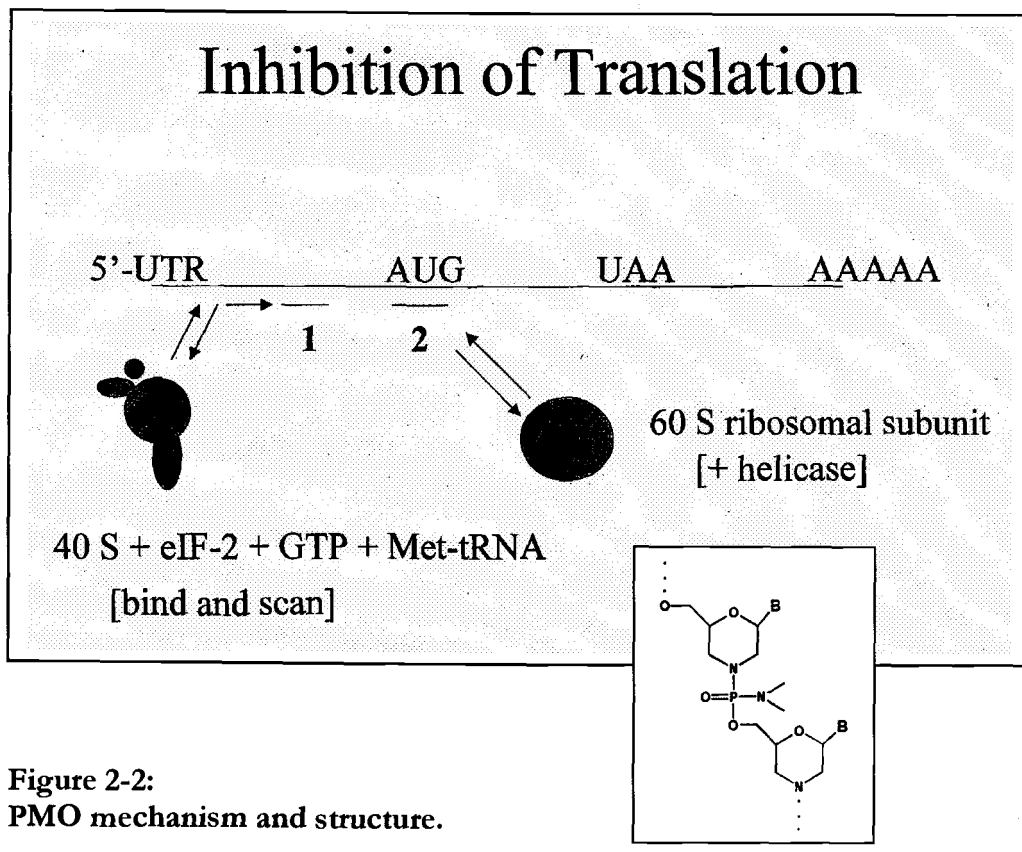


Figure 2-2:
PMO mechanism and structure.

Figure 2-2, top. Inhibition of translation by phosphorodiamidate morpholino oligomers. Phosphorodiamidate morpholino oligomers are believed to bind tightly and specifically to hnRNA, with an ability to invade secondary structure. The antisense oligomer may then interfere with splicing or ribosome machinery, preventing processing and/or translation to protein. PMOs targeted near the start site, as indicated by the number 2 in the figure, have been found to inhibit protein translation more effectively than oligomers directed to other sites in the untranslated region, such as the oligomer indicated by the number 1 in the figure. *Figure courtesy of Pat Iversen, AVI Biopharma, Corvallis, Oregon*

Figure 2-2, inset. Backbone design of phosphorodiamidate morpholino oligomers. The ribose sugar of DNA is replaced with a morpholine moiety, and a phosphorodiamidate joining replaces the phosphodiester linkage.

using PMOs against cytochrome P450 1B1. Several approaches were used in an attempt to explain the lack of inhibition of CYP1A1 or CYP1B1 by PMOs.

MATERIALS AND METHODS

Antisense oligomers

Morpholino antisense oligomers were a generous gift from AVI Biopharma, or purchased from Gene Tools, both of Corvallis, Oregon. The sequences of these oligos are shown 5' to 3', below.

Cytochrome P450 1A1 (CYP1A1)

1A1-AUG21:

GGAAAAGCATGATCAGTGTAC

1A1-AUG25:

CCGACATGGAGATTGGGAAAAGCAT

A fluorescein labeled version of this sequence was used for loading studies.

1A1-SA18:

GTGTAGGGATCTTGGAGG

Cytochrome P450 1B1 (CYP1B1)

1B1-AUGSA18-1:

GCTGGTGCCCATGCTGGG

1B1-AUGSA18-2:

CTGAGGCTGGTGCCCATG

1B1-AUGSA25:

GGCTGAGGCTGGTGCCCATGCTGCG

Control PMOs

1A1-SCRAM20:

GAGAGTCACTCAACTAAGAG

1B1-SCRAM18:

TGACTCGCCGGACGGGTC

GT-Standard

CCTCTTACCTCAGTTACAATTTATA

Cell culture

MCF-7 cells originated from human mammary carcinoma tissue [74] and were provided to the Baird lab by the Purdue University Cell Culture Laboratory. MCF-7 cells were grown in 1:1 F12 Nutrient Mixture and Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY), with 10% fetal bovine serum (Integren, Purchase, NY), 15 mM HEPES buffer, 14.3 mM $NaHCO_3$, 200 units/ml penicillin, 200 μg/ml streptomycin, and 25 μg/ml ampicillin. The cells were grown in T75 cm^2 flasks and and maintained at 37°C with 5% CO_2. The cells were split 1:4 when they covered the surface area of the bottom of the flask.

Loading antisense oligomers into MCF-7 cells

Scrape Loading of PMOs

MCF-7 cells were grown until they covered the surface of 6 well culture plates. The medium was replaced with 1 ml of medium containing various concentrations of PMO targeted to CYP1A1. A cell scraper (Sarstedt Ltd, London, UK) was used to gently lift the cells from the surface of the plate. The cells were then removed to a fresh plate.

Passive Loading of PMOs

MCF-7 cells were grown in 24 well plates in the presence of various concentrations of PMOs. When the cells covered the surface of the plate, they were treated with B[*a*]P and later examined for EROD activity.

Syringe Loading of PMOs

Syringe loading is adapted from a protocol provided by AVI BioPharma [67]. MCF-7 cells in T75cm^2 flasks were first trypsinized and the cells centrifuged to form a cell pellet. The cells were then resuspended in medium and counted using a haemocytometer or Coulter counter, and 15 ml vials of 2 million cells each were aliquotted and allowed to rest for 20 minutes. They were then pelleted again and suspended in 1 ml of complete medium containing the appropriate PMO in concentrations varying between 5 and 75 μM. The cells and PMO were passed through a 25 7/8 G syringe 8 times (four in, four out), and then plated in 96 or 24 well plates, or 25 cm^2 flasks.

Loading of PMOs using EPEI

EPEI (ethoxylated polyethylenimine), generously provided by Gene Tools, Corvallis, Oregon, is a weakly basic delivery reagent which binds to the charged backbone of DNA primers which are designed to be complementary to the uncharged morpholino oligo [75]. This complex containing the antisense oligomer, complementary DNA, and EPEI can then enter the cell. The following method was adapted from a protocol obtained from Gene Tools. MCF-7 cells were grown until they covered approximately 80% of the surface area of the flask, and refed with complete medium. 24 hours later the medium was removed and replaced with serum-free medium containing 1.4 μM morpholino (AVI Biopharma, Corvallis, Oregon), 1.0 μM DNA partially complementary to the morpholino, and 0.56 μM EPEI. Three hours later the medium was removed and replaced with complete medium.

Cell treatment with PAH

MCF-7 cells were treated with 1 μg/ml B[*a*]P in DMSO, 0.01 μg/ml DB[*a,l*]P in DMSO, or DMSO alone (vehicle control). B[*a*]P and DB[*a,l*]P were obtained from Chemsyn Science Laboratories (Lenexa, KS). In the case of syringe loading, the cells

were treated six hours after loading. When loaded with EPEI, cells were treated with PAH one hour after replacement with complete medium.

Cell harvest

Medium was aspirated from MCF-7 cells in T75 cm^2 flasks, which were then rinsed with 5 mls trypsin-versene. (0.05% Trypsin, 0.14 NaCl, 3mM KCl, 0.1 M Na_2HPO_4, 1.5mM KH_2PO_4, and 0.5 mM EDTA) The cells were then incubated with another 5 mls of trypsin-versene until they became detached from the flask. 5 mls of F/D medium with 10% fetal calf serum was then added to the flask. The cells were then decanted into a 50 ml centrifuge tube, and the flask rinsed with 10 mls PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4), which was added to the centrifuge tube. The cells were centrifuged at 2,000 rpm (478xg) in a bench top IEC HNSII centrifuge (International Equipment Co, Vernon Hills, IL). The supernatant was aspirated, and the pellet resuspended in 10 mls PBS, and centrifuged. The PBS was removed, and the pellet was again resuspended in PBS. After centrifugation, the resulting pellet was stored at -80°C.

Measurement of EROD activity in intact MCF-7 cells

The fluorescent measurement of the conversion of ethoxyresorufin to resorufin is a common way to assay CYP1A1 activity [76,77]. 250,000 MCF-7 cells were plated in each well of a 24 well plate after syringe loading or before EPEI loading of PMO. After treatment in at least triplicate with B[*a*]P or DMSO, cells were incubated for 24 hours, and rinsed twice with medium. The medium was then replaced with phenol red free medium containing 5 μM ERES and 1.5 μM salicylamide. The fluorescence was then measured in a SpectraMax Gemini fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA) at intervals over 60 minutes, with an excitation wavelength of 530 nm and emission at 585 nm.

Microsome isolation

Cell pellets were homogenized in a steel homogenizer in homogenization buffer (0.25 M KPO4, 0.15 M KCl, 10 mM EDTA, and 0.25 mM phenylmethylsulfonylfluoride (PMSF), and centrifuged at 9,000 rpm (15,000xg) for 20 minutes at 4°C in a Sorvall RC-5B Superspeed Refrigerated Centrifuge (Sorvall, Albertville, MN). The supernatant was centrifuged at 58,000 rpm (100,000xg) for 90 minutes at 4°C in a Beckman TL-100 Ultracentrifuge (Beckman, St. Louis, MO), and the pellet homogenized in microsome dilution buffer [78]. (0.1M KPO_4, 20% glycerol, 10 mM EDTA, 0.1 mM DTT, and 0.25 mM PMSF).

Protein quantification

Total protein concentration of microsomal samples was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used to create a standard curve ranging from 10 μg to 50 μg protein in 50 μl water. 3 to 10 μl of sample in 50 μl water were compared to this sample curve to obtain protein concentration. 1 ml of 50:1 BCA assay reagent A/BCA reagent B were added to the standards and the samples, which were then vortexed. After incubation for 30 minutes at 37°C, the absorbance of the standards and samples were measured at 562 nm on a Beckman DU-650 Spectrophotometer (Beckman Instruments Inc., St. Louis, MO).

Microsomal EROD assay

2 μl of 10 mM NADPH was added to 50 μg of microsomes in 0.1 M Tris-HCl and 1 μM ethoxyresorufin in 96 well black plates (Greiner Labortechnik, Frickenhausen, Germany), and emission measured at 585 nm with excitement at 530 nm in a SpectraMax Gemini fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA) every 30 seconds for ten minutes. The samples were performed in at least triplicate. A resorufin standard curve from 25 to 100 pM was used to determine relative fluorescent units (RFUs) per picomole resorufin, which was then used to calculate picomoles resorufin per minute per mg microsomes [79].

SDS-PAGE and immunoblotting

A 7½% 1.5 mm sodium dodecyl sulfate (SDS) polyacrylamide separating gel was used to resolve 50 μg of microsomal proteins into bands. Separating gels were cast by using 7.5 percent by weight acrylamide, 2 % N,N'-bis-methylene acrylamide (bis-acrylamide) (BioRad, Hercules, CA), 0.375 M Tris-base, pH 8.8, 0.05% tetramethylethylenediamine (TEMED) (BioRad, Hercules, CA), 0.1% SDS, and 0.03% ammonium persulfate (APS). The gel was allowed to polymerize for at least 45 minutes. The stacking gel was 3% by weight acrylamide, 0.8% bis-acrylamide, 0.18 M Tris-base, 0.12% TEMED, 0.14% SDS, and 0.03% APS, which was allowed to polymerize 30 minutes. Biorad precast ReadyGels (Biorad, Hercules, CA) were also used. 50 μg of microsomal protein in up to 70 μl of sample buffer (0.625 M Tris-base (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue) were loaded into each well. 10 μg of microsomes from V79 cells overexpressing CYP1A1 or CYP1B1 were used as positive controls. The samples were denatured by boiling for 3 minutes. 12 μl of Kaleidoscope molecular weight markers (BioRad, Hercules, CA) was also loaded in at least one well. The gels were run at 60V for approximately 10 hours at 4°C in a Thermo EC EC120 Vertical MiniGel System (Thermo EC, Holbrook, NY).

The proteins were transferred to a nitrocellulose membrane using a BioRad Mini Transblot Cell (BioRad, Heculues, CA) at 30V overnight at 4°C, and blocked with 1:3 NapSURE Blocker (Geno Technology, St. Louis, MO) in PBS-T (0.3% w/v Tween-20 in PBS) for at least one hour. The membrane was washed 5 minutes for 3 times with 1:7 Nap-SURE/PBS-T, and incubated with the primary antibody for two hours in 1:7 NapSURE/PBS-T. Human CYP1A1 was detected using a 1:3000 rabbit polyclonal antibody provided by Dr. F. P. Guengerich, Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville TN. Human CYP1B1 was detected with a 1:1000 rabbit polyclonal antibody provided by Dr. C. Marcus, Department of Pharmacology and Toxicology, University of New Mexico,

Albuquerque, NM). The blots were incubated with peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) (1:20,000 or 1:30,000) in 1:7 NapSURE/PBS-T for 30 minutes. The membrane was again washed for 5 minutes 3 times with 1:7 Nap-SURE/PBS-T. CYP1A1 and CYP1B1 were detected using Amersham 1:1 Enhanced Chemuluminescence Western Blotting Detection reagents 1 and 2 (Amersham Pharmacia Biotech, Buckinghamshire, England). Kodak Biomax Light Film (Eastman Kodak, Rochester NY) was exposed to the blots and developed using an X-omat apparatus. Blots were sometimes incubated with Western Reprobe (Genotechnology, St. Louis, MO) for 30 minutes, to strip blots for reprobing.

Isolation of DNA

Frozen cell pellets were homogenized in 0.75 ml EDTA/SDS buffer (10 mM Tris, 1 mM Na_2EDTA, 1% SDS, pH 8) in a glass homogenizer, and removed to a 15 ml conical centrifuge tube. The homogenizer was rinsed with an additional 0.75 ml of EDTA/SDS buffer which was added to the centrifuge tube. 30 µl of RNase mixture (50 units per ml RNase T1and 25µg/ml RNase A (both Boehringer Mannheim, Indianapolis, IN) were added to the mixture, which was then incubated for 1 to 2 hours at 37°C with shaking. 75µl proteinase K (500 µg/ml proteinase K [Sigma, St. Louis, MO]) was incubated with the sample for an additional 1 to 2 hours with shaking. The sample was decanted into a 15 ml conical phase lock centrifuge tube (Eppendorf, Westbury NY), and 1.5 ml of equilibrated phenol (USB Corp., Cleveland, OH) added. The tube was shaken for 5 minutes, and centrifuged for 6 minutes at 3000 rpm on a bench top IEC HNSII centrifuge (International Equipment Co, Vernon Hills, IL). 0.75 ml of equilibrated phenol and 0.75 ml of 24:1 chloroform:isoamyl alcohol were added, and the sample was shaken for 5 minutes. The sample was centrifuged for 6 minutes at 3000 rpm, and 1.5 ml 24:1 chloroform:isoamyl alcohol were added, and the sample shaken for 5 minutes. The sample was again centrifuged 6 minutes at 3000 rpm. The layer above the gel lock was transferred to a clean 15 ml conical centrifuge tube. 150 µl 5M NaCl and 3 ml ice cold 100% ethanol were added to precipitate the DNA. The precipitated DNA was removed by using a glass hook,

rinsed in 70% ethanol, and redissolved in 1 ml sterile double distilled water. The DNA was quantified by spectrophotometry at 260 nm using a Beckman DU-650 Spectrophotometer (Beckman Instruments Inc., St. Louis, MO).

^{33}P Postlabeling of adducted DNA

10 μg DNA in water was evaporated to 5 μl using a Speed Vac (Savant Instruments Inc., Farmingdale, NY) and digested with 0.6 U of nuclease P1 (Sigma Chemical Co., St. Louis, MO) and 350 mU prostatic acid phosphatase (Sigma Chemical Co., St. Louis, MO), and 1 μl of nuclease P1 buffer (0.125 M sodium acetate, 30 mM $ZnCl_2$, pH 5.2). The digested DNA was again reduced to 5 μl using a Speed-Vac, and labeled with 50 μCi [γ-^{33}P]ATP using 18 U T4 polynucleotide kinase (USB Corp., Cleveland, OH), and 2 μl kinase buffer (0.5 M Tris, 8 mM spermidine, 100 mM MgCl2, 100 mM dithiothreitol (DTT), pH 9.6) at 37°C for 1 hour. The resulting labeled adducted dinucleotides were digested to mononucleotides using 15 mU Type VII *Crotalus atrox* venom phosphodiesterase (Sigma Chemical Co., St. Louis, MO).

Isolation of adducted mononucleotides

Waters C_{18} Sep Pak columns (Millipore Corp., Milford, MA) were used to separate the adducted mononucleotide from other digestion products. The columns were first conditioned by running 10 ml HPLC grade methanol, 10 ml ddH_20, and 10 ml Sep Pak loading buffer (0.5 M KH_2PO_4, pH 6.0) using a 10 ml syringe. The digested and labeled DNA was passed slowly through the syringe in 10 ml Sep Pak loading buffer. The sample was passed through the column a second time, and the column washed three times with 10 ml ddH_20, then ddH_20 with 5% basic methanol (5% ammonium hydroxide in HPLC grade methanol). The labeled adducted nucleotides were then eluted by passing 2 ml of basic methanol through the syringe. 2 10 μl aliquots of the product were counted in a Tricarb Liquid Scintillation Counter (Packard Instruments Co., Meriden, CT) with Flo-V scintillation fluid (Packard Instruments Co., Meriden, CT).

HPLC separation of DNA adducts

An aliquot of each sample measuring $1x10^6$ dpm or less was reduced to approximately 10 µl using a Speed-Vac. The sample was resuspended in 100 µl of 1:1 buffer A and B. Buffer A for B[*a*]P and DB[*a,l*]P samples was filtered 0.1 M NH_4H_2PO4, pH 5.5. Buffer B for B[*a*]P samples was 100% HPLC grade methanol, and for DB[*a,l*]P samples 10% acetonitrile and 90% HPLC grade methanol.

Reverse phase HPLC separation of DNA adducts was carried out using a Beckman ultrasphere ODS 5 µm 4.6 mm x 250 mm C_{18} column using Beckman 110B solvent delivery modules, system organizer, and injection module (Beckman Instruments Inc., St. Louis, MO), and detected on a Radiomatic Flo-one Beta radiochromatography detector (Packard Instruments Co., Meriden, CT). B[*a*]P Adducts were eluted using a gradient of 44%-60% solvent B over 40 minutes, 60-80% solvent B over 10 minutes, isocratic elution at 80% solvent B over 10 minutes, and 80-44% solvent B over 5 minutes. DB[*a,l*]P adducts were eluted using a gradient of 1 ml/minute 44% solvent B for 50 minutes, 55%-80% solvent B over 10 minutes, and 80%-44% solvent B over 10 minutes.

Fluorescence measurement of BPDE interaction with PMO

BPDE in tetrahydrofuran was added to solutions of PBS or PMO in PBS to final concentrations of 5 µM BPDE and 1 µM PMO, 1 µM PMO, and 5 µM BPDE. The solutions were incubated for one hour at 37° and the fluorescence spectra measured on an SLM 8000 with the excitation held at 398 nm and emission held at 342 nm.

Measurement of fluorescence in medium/cell lysate

MCF-7 cells were plated in 24 well cell culture plates. After the cells had grown to cover the plate surface, the medium was replaced with serum-free medium containing 1.4 µM morpholino (AVI Biopharma, Corvallis, Oregon), 1.0 µM DNA partially complementary to the morpholino, and 0.56 µM EPEI. An aliquot of the PMO-

containing medium was retained for later analysis. Three hours later the medium was removed and replaced with complete medium. An aliquot of the removed medium was retained. One hour later an aliquot of lysed cells were reserved. The remaining cells were treated with B[*a*]P or DMSO. One hour later, an aliquot of medium from cells treated with PMO was reserved. Aliquots of medium from the remaining cells were retained at 4, 9, 18, and 24 hours after B[*a*]P treatment, as well as an aliquot of lysed cells from 24 hours after treatment. The volumes of these samples were adjusted so that the volume of lysed cells from one well was equal to the volume of medium from one well. The fluorescence of these samples was compared at excitation of 495 and emission of 522.

Cell fixing, staining, and microscopy

The initial visualization of fluorescently labeled PMO was perfomed on an Olympus Nikon Diaphot 300 inverted fluorescent microscope (Figures 2-3 and 2-5). MCF-7 cells were syringe loaded with PMO and then cultured in a 6 well cell culture plate, or loaded with PMO/DNA/EPEI and allowed to incubate before imaging.

In later imaging (Figures 2-9 & 2-13) MCF-7 cells were plated on 12 mm round glass coverslips in 24 well culture plates. The cells were loaded with fluorescein-tagged antisense oligos (Gene Tools, Philomath, OR) and treated with DMSO or B[*a*]P. The cells were later washed 3 times with 1 ml PBS each well, and then fixed with 1 ml each well 3% formaldehyde in PBS for 30 minutes at room temperature. The cells were again washed with PBS, and stained with 1 μg/ml Hoechst stain (Molecular Probes, Eugene, OR) in PBS for ten minutes at room temperature to visualize nuclei. The Hoechst/PBS mixture was replaced with PBS, and the coverslip removed, blotted lightly, and mounted on a microscope slide using 4 μl Prolong Antifade Reagent (Molecular Probes Inc., Eugene, OR). To view lysosomes, 75 nM LysoTracker Red Probe (Molecular Probes Inc., Eugene, OR) was incubated with the cells for one hour at 37°C before fixing and Hoechst staining. A Zeiss Axiovert S100 TV fluorescent microscope, with filter sets from Chroma Technology. Filter set 31000, excitation 360,

emission 460 was used to visualize Hoechst nuclear staining, and filter set 41001, excitation 480, emission 535 was used to visualize the fluorescein labeled PMO

RESULTS

Initial comparison of methods for loading PMOs into MCF-7 cells

The delivery of antisense oligomers to their target is an ongoing challenge in the antisense arena. Several methods have been demonstrated to be useful in assisting the entry of these molecules into cultured cells, but must be optimized for a given cell line and the question being addressed. These methods may be divided into three main categories. Passive loading is achieved by growing the cells in medium containing the PMO. Physical methods temporarily perturb membrane integrity, allowing PMOs to pass from surrounding medium into the cytosol. Delivery may also be mediated by a number of commercially available reagents.

Passive loading was attempted in MCF-7 cells with a range of concentrations of PMOs against the CYP1A1 start site. None of these concentrations had a significant effect on initial intact cell EROD assays, indicating no effect on CYP1A1 levels. Scrape loading was also attempted. In this method, cells are scraped from the plate surface in the presence of medium containing the PMO. PMOs are hypothesized to enter the cytosol due to transient opening of cell pores [67]. The MCF-7 cells adhered to each other and were released from the plate surface in sheets when scraped. These sheets did not readhere when transferred to a new plate. None of these loading methods were found to be appropriate for this study.

Syringe loading is another physical method, in which cells in suspension with the PMO are passed several times through a syringe needle. With this method, no adverse affects to the cells were observed. A fluorescently labeled PMO was syringe loaded into MCF-7 cells. Initial flow cytometry measurements indicated that nearly 40% of cells appeared to contain fluorescence. This was confirmed by fluorescent microscopy,

which indicated that 47% of the cells were noticeably fluorescent, indicating that they had been loaded with PMOs (Figure 2-3).

To measure the effect on protein activity, various concentrations of two PMOs targeted against CYP1A1 were syringe loaded into MCF-7 cells and subjected to an intact cell EROD assay (Figure 2-4). An 18mer PMO targeted against a splice acceptor site (1A1-SA18), reduced EROD activity by approximately 30% compared to untreated control when loaded at concentrations of 50-75 μM. Experimental variation exceeded the difference between samples. A 21mer targeted toward the start site (1A1-AUG21) also exhibited no significant change. A control PMO was not provided for this experiment, but was obtained for subsequent work.

Syringe loading required extended cell handling times and large concentrations of PMO, while only a fraction of the cells needed could be processed at a time. These considerations kept experiments small, yielding insufficient amounts of material for extracting microsomes for immunoblots or ERODs. Moreover, because the end goal of this work was to examine the effects on PAH/DNA binding, which itself can vary significantly, greater inhibition would be necessary to observe an effect in DNA adducts.

Loading PMOs into MCF-7 cells using EPEI

At this time a specialized delivery reagent, ethoxylated polyethylenimine (EPEI) became available from Gene Tools, LLC (Philomath, Oregon). The antisense oligo is paired with a partially complementary DNA oligo, and EPEI, which is partially ionized in culture media, interacts with the charged DNA backbone. The positively charged EPEI/antisense/DNA complex is thought to be endocytosed, and becomes increasingly ionized within the acidic endosome/lysosome, thereby permeating the compartmental membrane and releasing PMO into the cytosol [75].

A fluorescently labeled PMO was obtained and loaded into MCF-7 cells. Fluorescent micrographs document the loading of PMOs using this method (Figure 2-5). The

Figure 2-3. Entry of fluorescein labeled PMOs into MCF-7 cells using syringe loading. 2 million MCF-7 cells were passed through a syringe in the presence of 75 μM PMO. The cells were then plated in a six well plate. Cells were examined under visible light (top) and fluorescence (bottom). Approximately 47% of the cells display fluorescence.

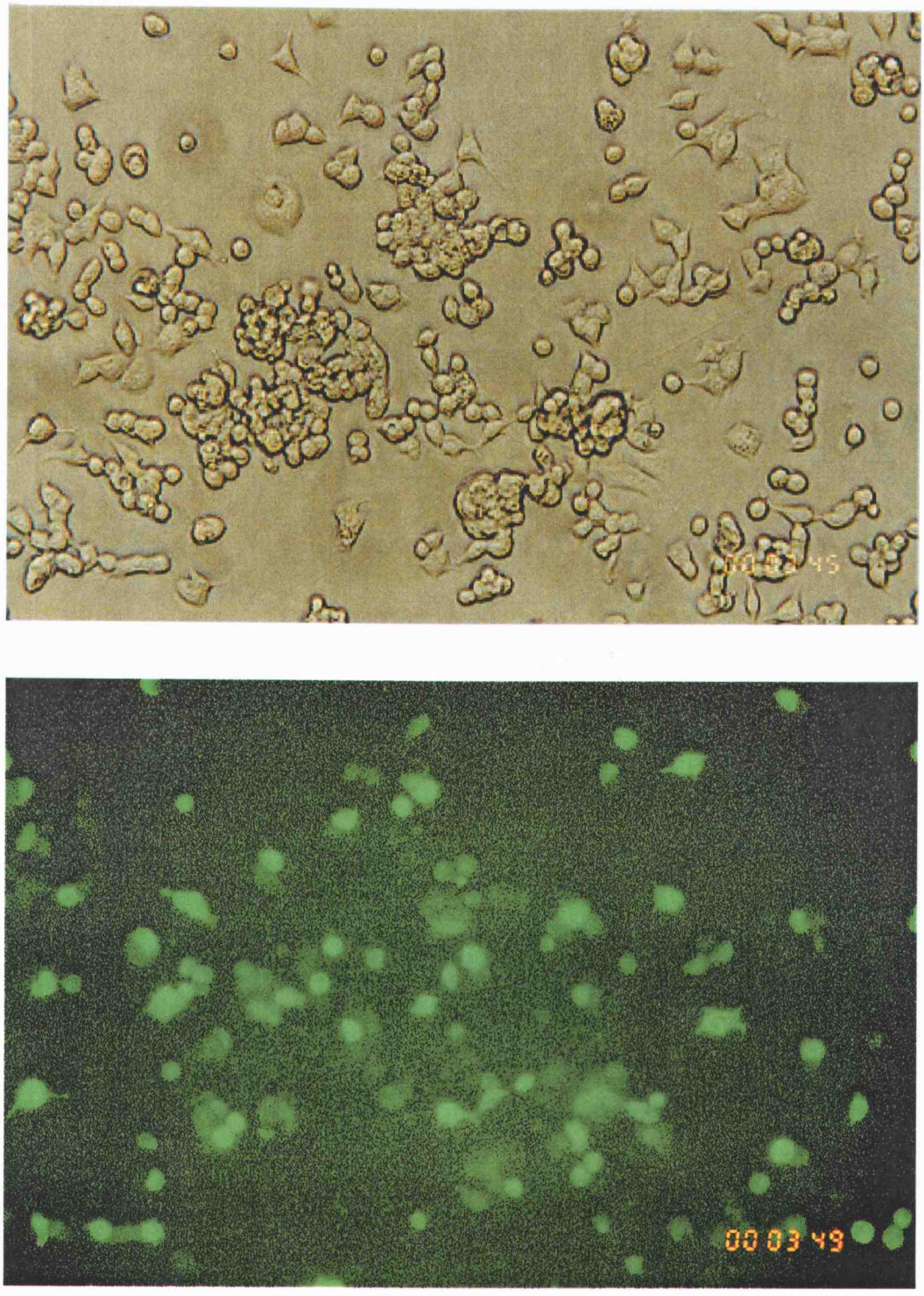


Figure 2-3. Entry of fluorescein labeled PMOs into MCF-7 cells using syringe loading

Figure 2-4. EROD activity of intact MCF-7 cells syringe loaded with PMOs targeted against CYP1A1. MCF-7 cells were syringe loaded with 0, 25 μM, 50 μM, and 75 μM of two PMOs against CYP1A1 and treated with B[*a*]P to induce CYP1A1 activity. Green bars represent EROD activity of cells treated with 1A1-SA18. White bars represent EROD activity of cells treated with 1A1-AUG21. This assay was repeated 4 times. Error bars indicate standard deviation. Comparing these values using ANOVA analysis gave a p-value of 0.38, indicating a lack of significant differences between these values.

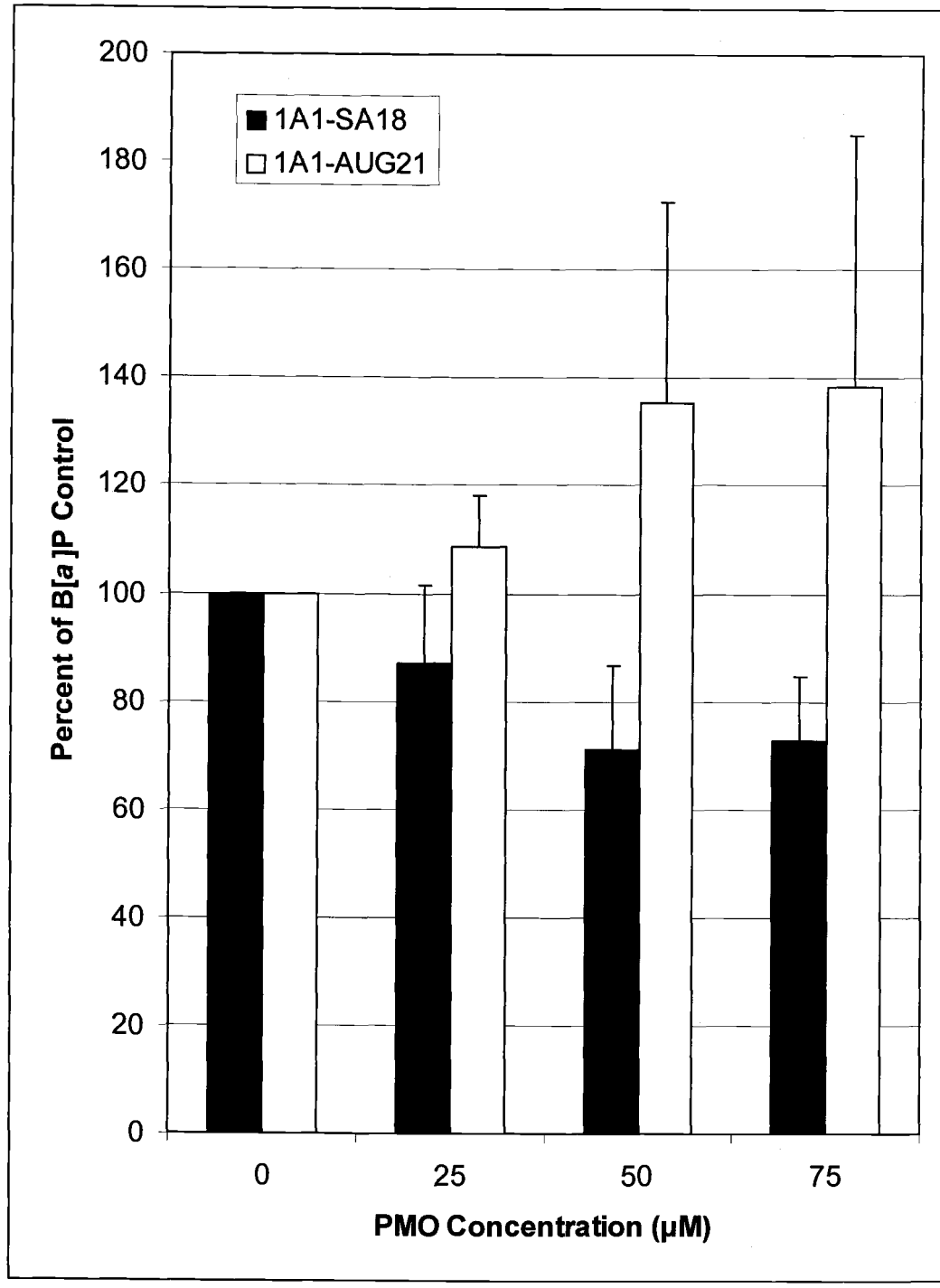


Figure 2-4. EROD activity of MCF-7 cells syringe loaded with PMOs targeted against CYP1A1

Figure 2-5. Ethoxylated polyethylenimine (EPEI) loading of PMO into MCF-7 cells. A fluorescently labeled oligo was paired with partially complementary DNA and combined with EPEI in serum-free media. MCF-7 cells were then covered with this medium and incubated for 3 hours. A fluorescent microscope was used to observe the cells 16 hours later. A majority of the cells were fluorescent, indicating entry of the PMO into cells.

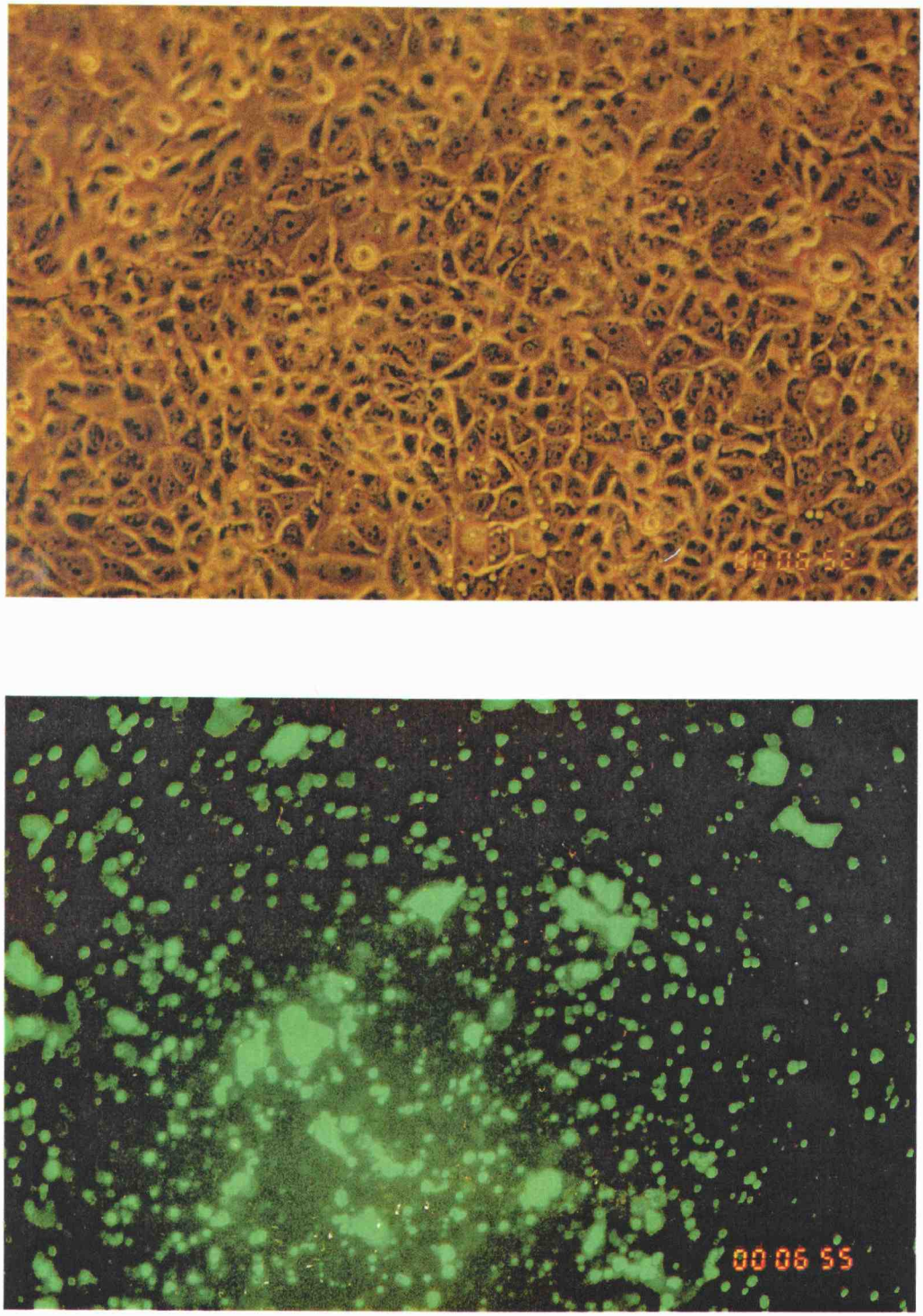


Figure 2-5. Ethoxylated polyethylenimine (EPEI) loading of PMO into MCF-7 cells

majority of cells displayed fluorescence, indicating that a large percentage of cells have been loaded with antisense oligo. When observed under visible light, no difference in appearance was observed between loaded and unloaded cells and no cell toxicity was observed.

To ascertain whether decreased enzyme activity corresponded with this apparently efficient loading method, intact cell ERODs were performed. The cells were loaded with PMOs against CYP1A1 and later treated with B[*a*]P to induce CYP1A1. The level of EROD activity after B[*a*]P treatment has been observed to peak after approximately 12 hours and remain elevated well beyond 24 hours. The precise timecourse of availability of PMO to target after loading is unknown. Therefore a number of trials were made to discover the interval between PMO loading and B[*a*]P treatment that would maximally inhibit EROD activity. To probe the effect of cell density, additional experiments were performed to optimize the time between cell plating and PMO loading. In the resulting protocol, cells were plated 12 hours before being treated with EPEI/DNA/PMO, and treated with B[*a*]P one hour after the treatment medium was removed. EROD activity was measured 24 hours later.

Using this protocol, MCF-7 cells were loaded with two PMOs against CYP1A1 as well as a scrambled PMO control. Compared to either B[*a*]P alone or scrambled control PMO (1A1-SCRAM20), neither a PMO targeted to the start site of CYP1A1 (1A1-AUG21) or to a splice acceptor site (1A1-SA18) had statistically significant effects on EROD activity (Figure 2-6 left). To confirm these results, the treatment was repeated, and microsomes were isolated from harvested cells. A microsomal EROD assay yielded very similar results: neither PMO targeted against CYP1A1 inhibited activity to a significant extent compared to control (Figure 2-6 right).

EROD activity principally represents the catalytic activity of CYP1A1. However, CYP1B1 contributes a small fraction of this activity. To examine the effect of PMOs on CYP1A1 protein levels alone the microsomes were subjected to SDS-PAGE and

immunoblotting. Compared to DMSO, B[*a*]P induced CYP1A1 as expected. No significant change in CYP1A1 was noted in cells treated with PMOs targeted against CYP1A1 before B[*a*]P (Figure 2-7 top). Cells treated with two PMOs spanning the start site and a splice acceptor site of CYP1B1 also showed no reduction in protein levels (Figure 2-7 bottom), indicating that lack of inhibition was not unique to CYP1A1.

Despite the lack of inhibition recorded in EROD assays and immunoblots, DNA binding assays were performed. 1A1-AUG21 and 1A1-SA18 were compared to 1A1-SCRAM20 in both B[*a*]P and DB[*a,l*]P treated cells. DNA adducts in cells loaded with 1A1-AUG21 did not vary significantly from those treated with B[*a*]P alone. Cells loaded with 1A1-SA18 displayed increased DNA binding over cells treated with B[*a*]P alone. However, cells loaded with control oligo 1A1-SCRAM20 also yielded increased adduct levels (Figure 2-8 A). Cells treated with antisense oligos and DB[*a,l*]P showed no significant difference in DNA binding between samples and controls (Figure 2-8 B). This was not unexpected since CYP1B1 is believed to be principally responsible for activating DB[*a,l*]P [50].

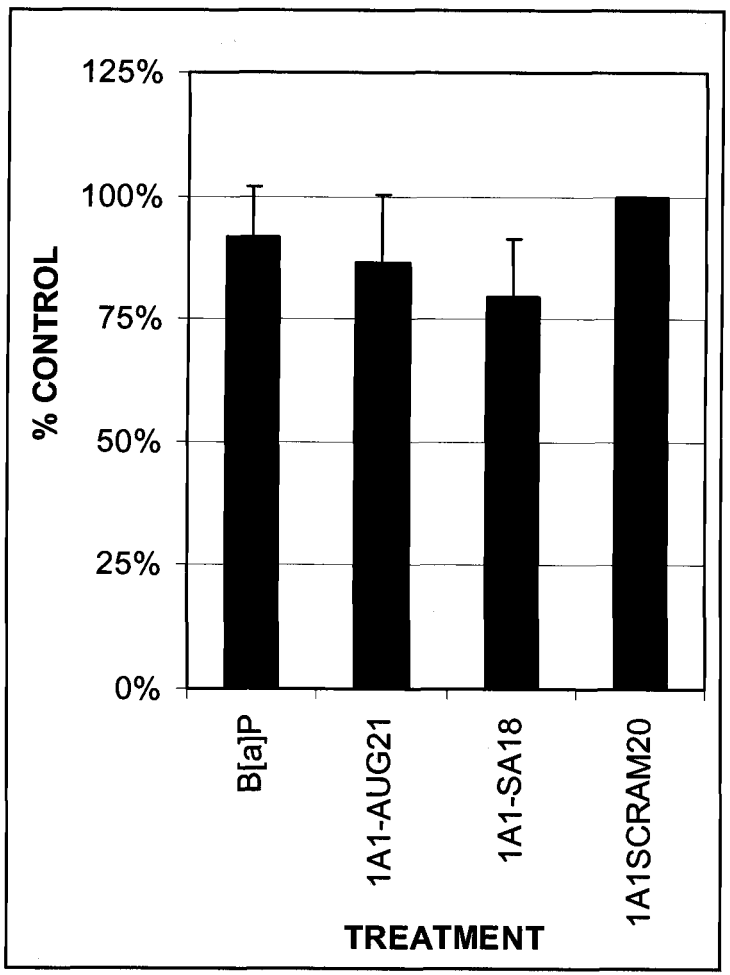
Use of 25mer PMOs

PMOs 25 units in length have been demonstrated to increase inhibition substantially compared to shorter PMOs in cell free systems. The PMOs in the studies above were 18 to 21 units long. To test whether longer PMOs would increase inhibition, 25mer PMOs were obtained targeting CYP1A1 and CYP1B1. An additional PMO was created against CYP1A1 with a fluorescein tag and loading of cells using EPEI was again assayed. The Zeiss Axiovert S100 TV fluorescent microscope used in this assay exhibited greater resolution than the Olympus Nikon Diaphot 300 inverted fluorescent microscope used in previous experiments, and it was possible to observe that 100% of the cells were loaded with antisense (Figure 2-9). The fluorescence was visible most intensely in the nuclei with diffuse as well as punctate signal in the cytosol.

Figure 2-6. Effect of PMOs targeted against CYP1A1 and CYP1B1 on intact cell and microsomal EROD activity.

Left: EROD activity of intact MCF-7 cells treated with B[*a*]P alone, or B[*a*]P and PMOs antisense to CYP1A1, or a scrambled, control PMO. The activity of the sample treated with 1A1SCRAM20 was used as a control. Samples treated with B[*a*]P, B[*a*]P plus 1A1-AUG21, or B[*a*]P plus 1A1-SA18 are expressed as a percent of control. This assay was repeated 4 times Error bars represent standard deviation. ANOVA analysis gave a p-value of 0.89, indicating that none of these treatments had a significant effect on EROD activity.

Right: Microsomal EROD activity of MCF-7 cells treated with B[*a*]P alone, or B[*a*]P amd PMOs antisense to CYP1A1, or a scrambled, control PMO. Values are expressed as picomole resorufin per minute per milligram microsomal protein. This assay was repeated 3 times. Error bars represent standard deviation. ANOVA analysis gave a p-value of 0.42, indicating that none of these samples showed a significant effect in EROD activity.



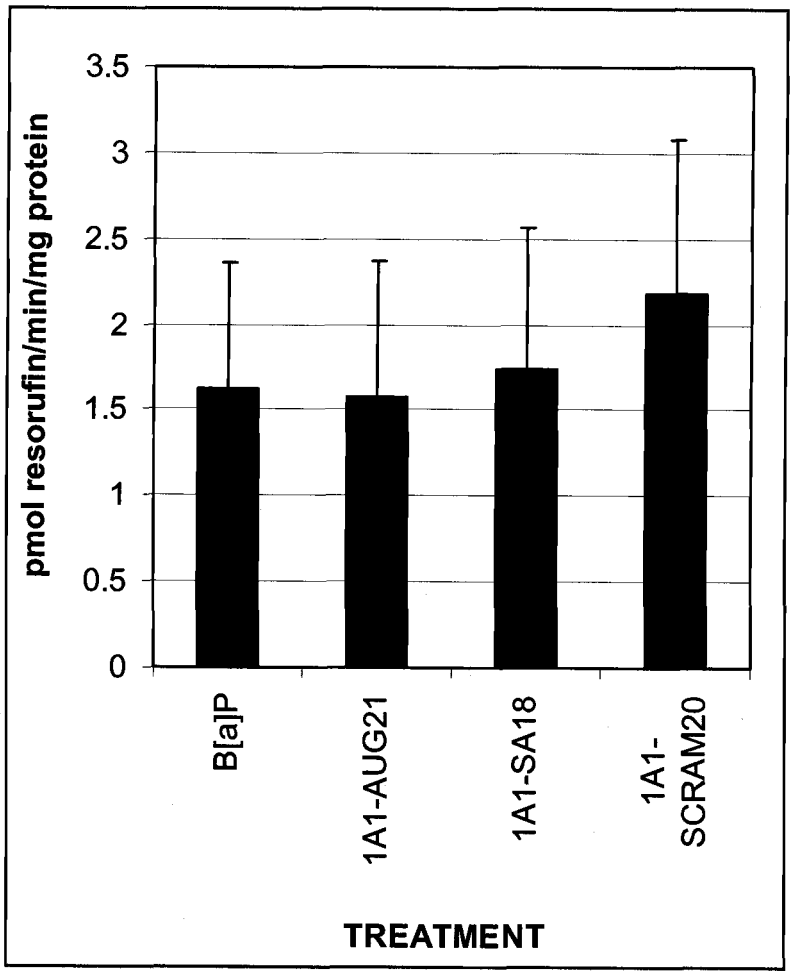


Figure 2-6. Effect of PMOs targeted against CYP1A1 and CYP1B1 on intact cell and microsomal EROD activity.

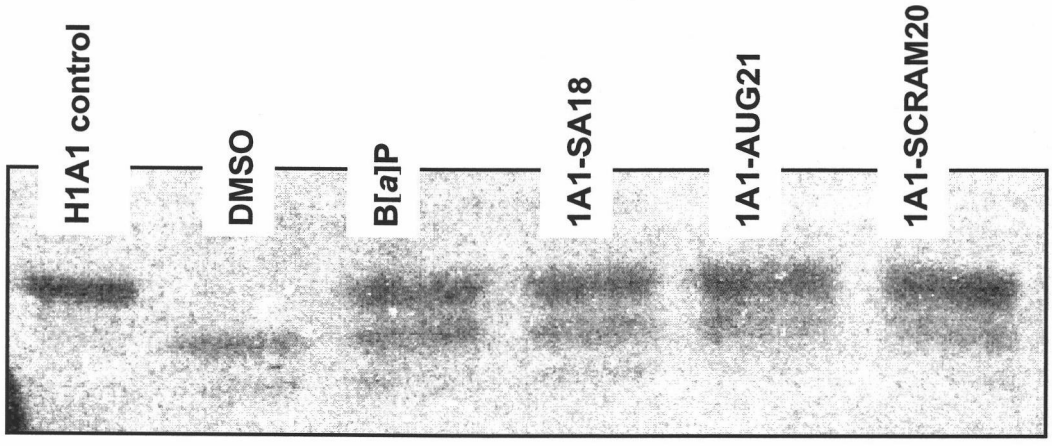


Figure 2-7 top. Immunoblot for CYP1A1 in MCF-7 cells EPEI-loaded with PMOs targeted to CYP1A1 mRNA.
Positive control, labeled H1A1 control, is microsomes isolated from V79 cells overexpressing human CYP1A1 [13]. Cells were treated with DMSO, B[*a*]P alone, or PMOs plus B[*a*]P. Little, if any change in CYP1A1 protein levels can be observed in cells treated with 1A1-SA18 or 1A1-AUG21 AUG PMOs compared to 1A1-SCRAM20.

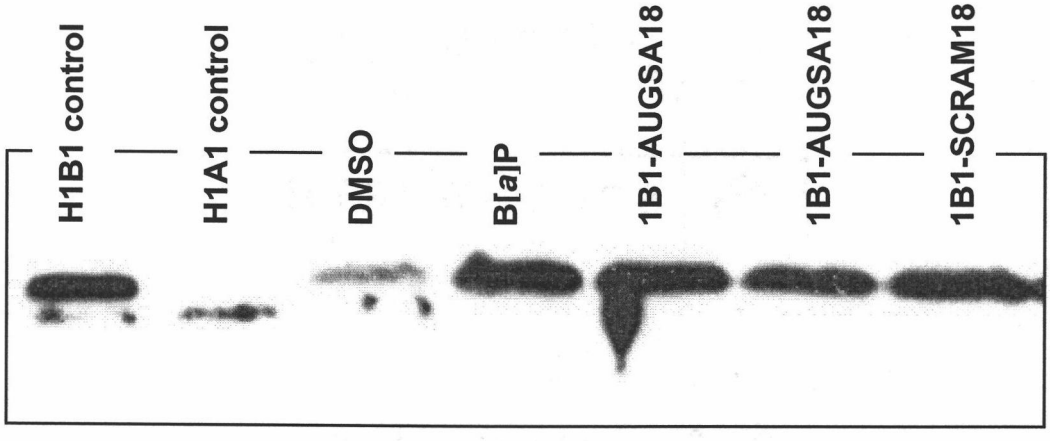
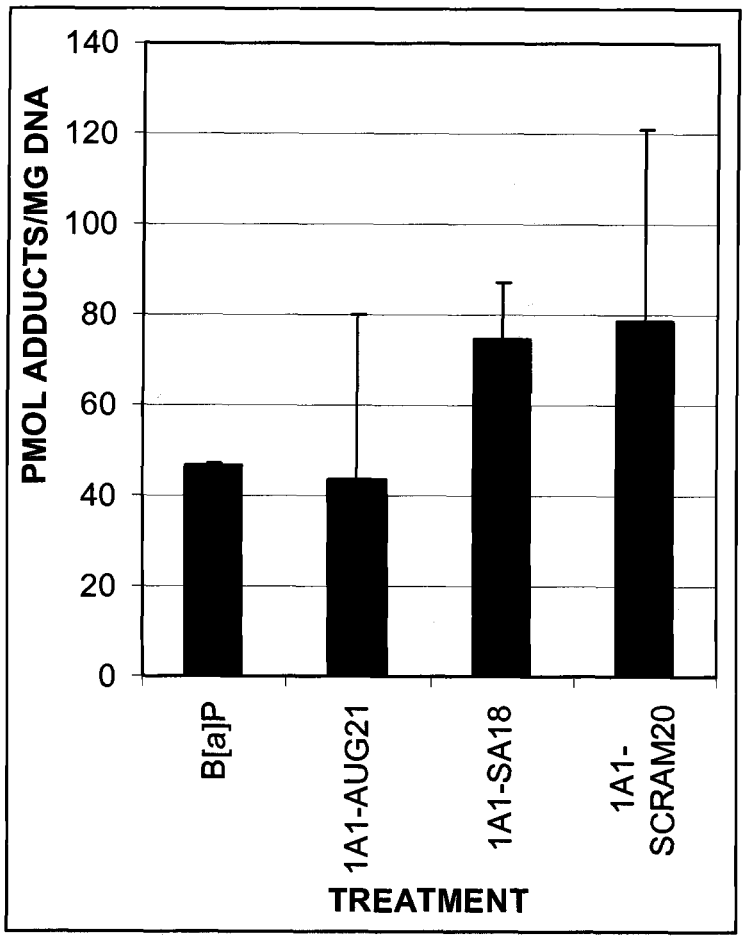


Figure 2-7 bottom: Immunoblot to detect CYP1B1 in MCF-7 cells loaded with PMOs targeted against CYP1B1 using EPEI.
Control (H1B1 control) is microsomes isolated from V79 cells overexpressing CYP1B1 [13]. Microsomes from V79 cells expressing CYP1A1 are also shown to distinguish band of interest. Cells treated with vehicle only (DMSO) display a detectable level of CYP1B1, while those treated with B[*a*]P show higher levels. Little if any difference in CYP1B1 protein expression can be observed in cells treated with 1B1-AUGSA18 or 1B1-AUGSA18-2 compared to 1B1-SCRAM18.

Figure 2-8: B[*a*]P and DB[*a,l*]P DNA binding in cells treated with PMOs targeted against CYP1A1.

Figure 2-8 left: B[*a*]P DNA adducts. MCF-7 cells were treated PMOs targeting CYP1A1 and with B[*a*]P. DNA was isolated from these samples, and assayed for DNA. Adducts in antisense treated samples appear to be reduced compared to scramble treated cells, but not when compared to B[*a*]P alone. Samples are compared to a B[*a*]P DNA adduct standard for quantification. Three trials were performed. Error bars represent standard deviation. ANOVA analysis gave a p-value of 0.5, indicating that treatments had no significant effects.

Figure 2-8 right: DB[*a,l*]P adducts. MCF-7 were treated with PMOs targeted against CYP1A1 and DB[*a,l*]P. After 24 hours, cells were harvested and examined for DNA binding. Samples are compared to a B[*a*]P DNA adduct standard for quantification. Three trials were performed. Error bars represent standard deviation. ANOVA analysis gave a p-value of 0.77. None of the treatments had a significant effect on DB[*a,l*]P DNA binding.



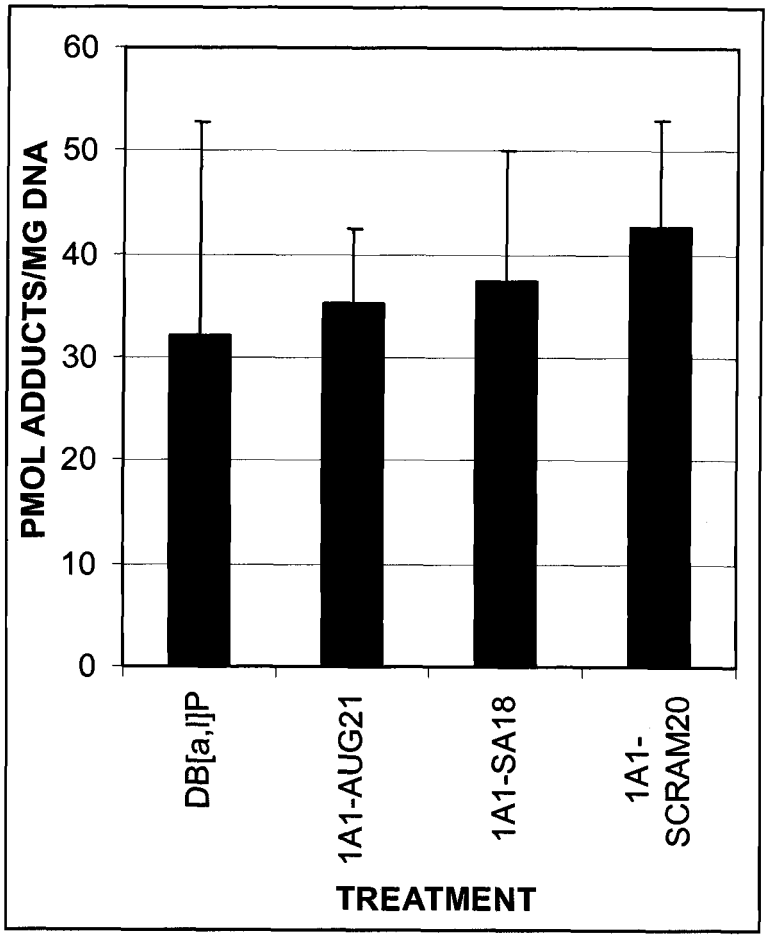


Figure 2-8: B[*a*]P and DB[*a,l*]P DNA binding in cells treated with PMOs targeted against CYP1A1.

Intact cells were then loaded with the 25mer PMO against CYP1A1 and assayed for EROD activity. Compared to a scrambled standard (GT-standard) or B[*a*]P alone, no statistically significant differences were observed in cells loaded with a PMO against CYP1A1 (1A1-AUG25) or CYP1B1 (1B1-AUGSA25) (Figure 2-10 left). To confirm these results, microsomes were isolated from treated cells and assayed for EROD activity. No inhibition was observed by either PMO when compared to B[*a*]P (Figure 2-10 right). Immunoblots also revealed little if any variation between control and treated samples (Figure 2-11).

Summary of attempts to inhibit CYP1A1 and CYP1B1 using PMOs

Several variables were analyzed in the attempt to inhibit CYP1A1 and CYP1B1. Varying the loading method had no significant effect on the degree of inhibition observed although antisense was observed to have entered the cells. Many additional factors in the treatment protocol, such as cell density, and the time between antisense loading and the induction of CYP1A1 and CYP1B1 with B[*a*]P, were systematically altered in an attempt to optimize inhibition to no avail. Finally, increasing the PMO length did not decrease enzyme activity or protein levels any further. The paucity of inhibition in this study is consistent with that found by Heidi Einolf [34], who attempted to inhibit CYP1A1 in the mouse hepatoma lines Hepa-1 and BP'C4 using phosphorothioate oligonucleotides. Up to 44.5% inhibition of the appearance of water soluble B[*a*]P metabolites was achieved. However, attaining this level of inhibition required the fastidious control of multiple variables, yet the results were found to vary considerably and the research was discontinued [34].

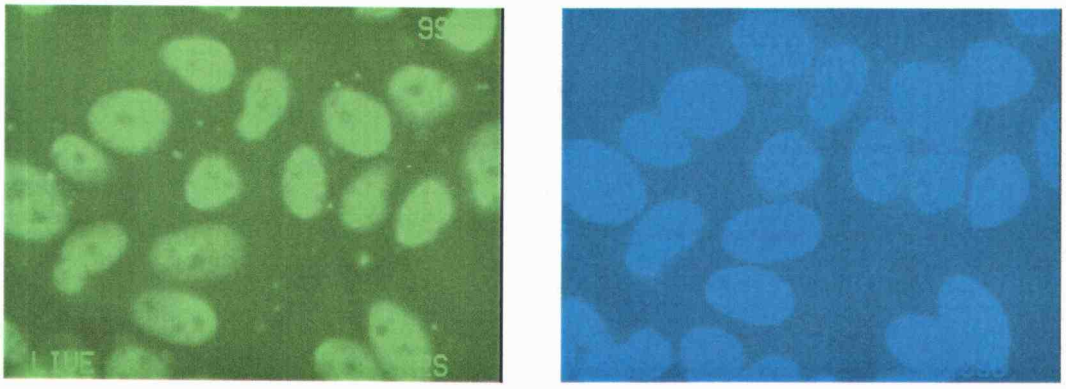


Figure 2-9. Entry of PMOs into MCF-7 cells loaded with EPEI. Left panel shows fluorescein-tagged PMOs in nucleus, with some points of fluorescence visible in the cytoplasm. Right panel shows cells labeled with Hoechst, which accumulates in the nucleus. 100% of cells contain PMO.

Figure 2-10. Intact cell and microsomal EROD activity of MCF-7 cells loaded with 25mer PMOs against CYP1A1.

Left: Intact cells assayed for EROD activity after loading with 25mer PMOs and treated with B[a]P. GT-STANDARD was used as a control. The values of other samples are expressed as percent of control. The assay was repeated 3 times. Error bars represent standard deviation. ANOVA gave a p-value of 0.33

Right: Microsomal protein from samples treated with B[a]P or B[a]P plus two 25 mer PMOs against CYP1A1 were assayed for EROD activity. The assay was repeated twice. Error bars represent standard deviation. ANOVA analysis gave a p-value of 0.35.

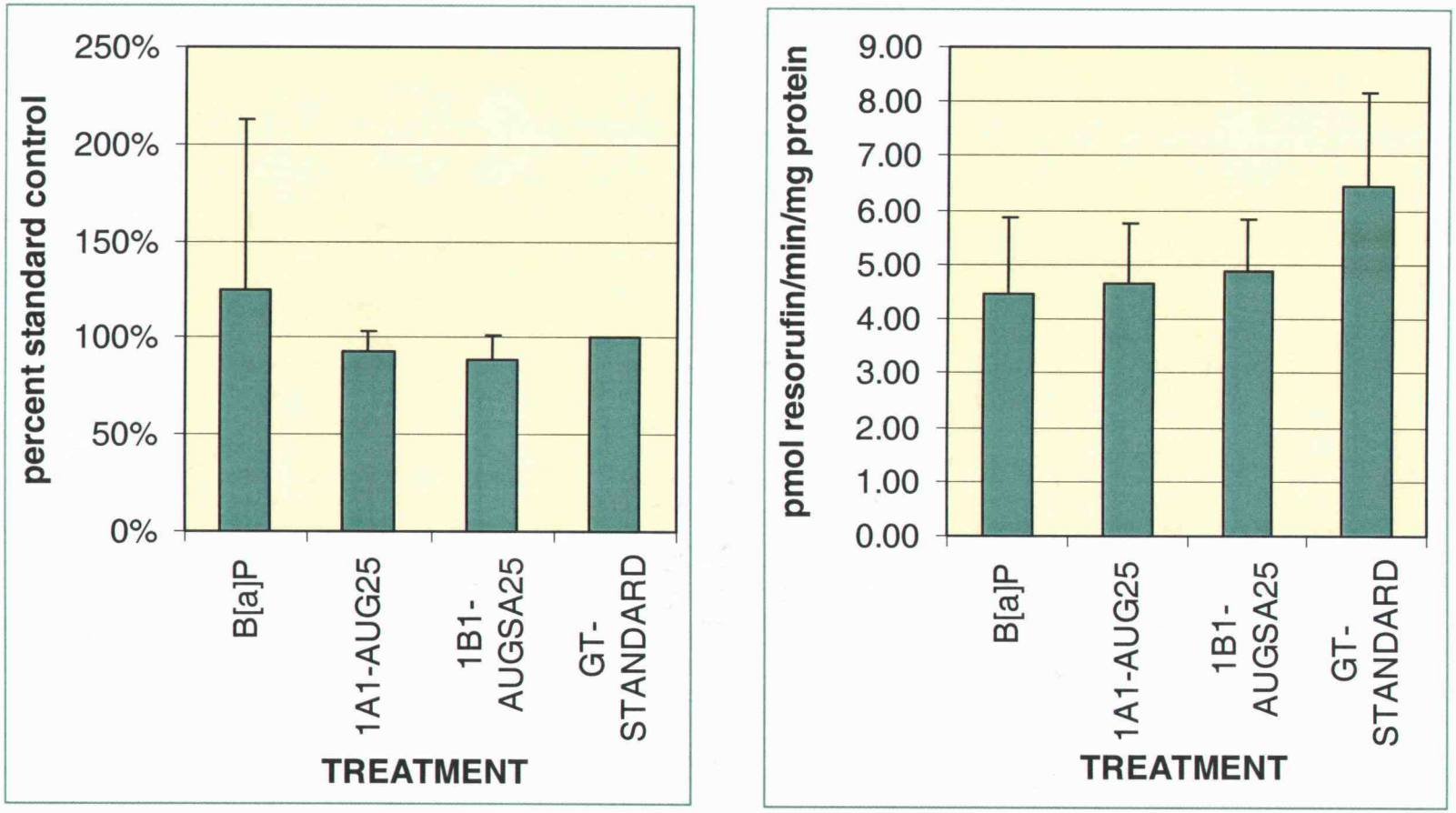


Figure 2-10. Intact cell and microsomal EROD activity of MCF-7 cells loaded with 25mer PMOs against CYP1A1.

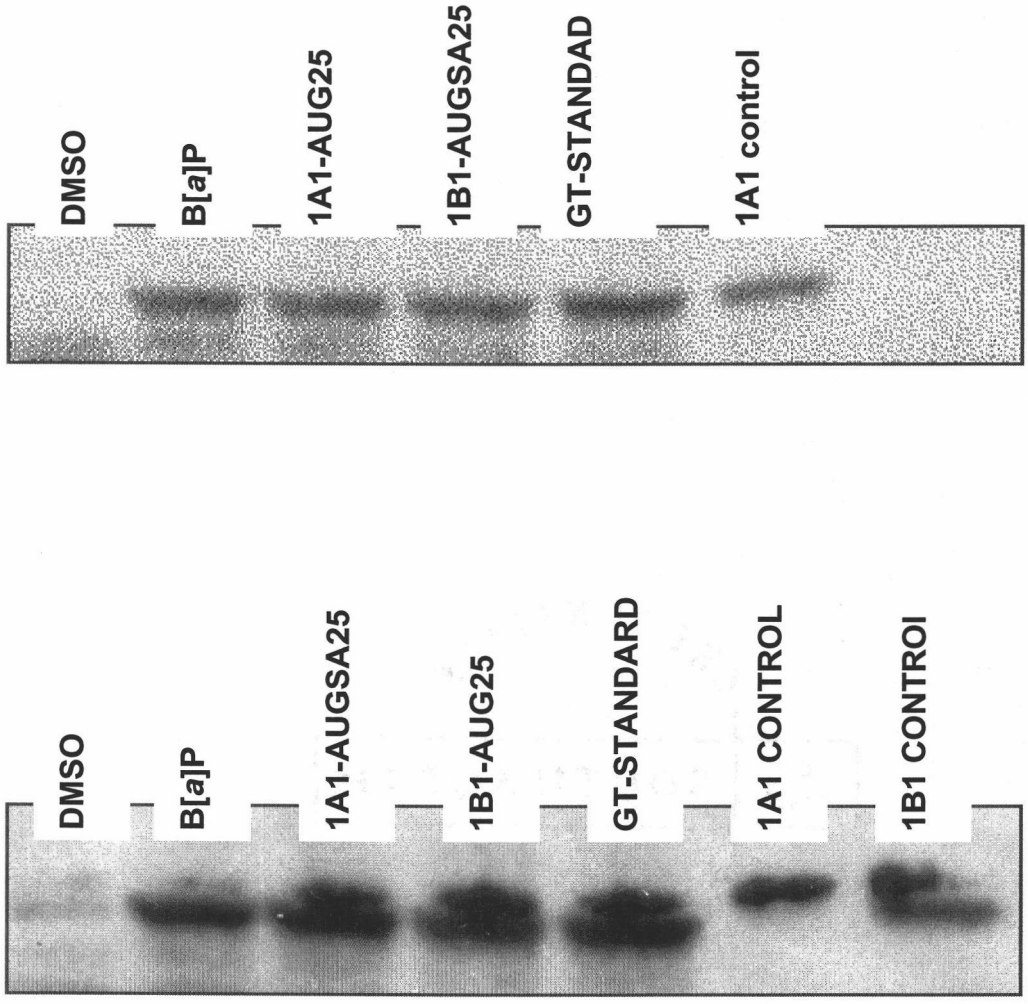


Figure 2-11. Immunoblot of microsomal proteins isolated from MCF-7 cells loaded with 25mer PMOs against CYP1A1 and CYP1B1. The samples were treated with DMSO, B[*a*]P, or PMO plus B[*a*]P. The top panel shows an immunoblot in which the proteins are probed with an antibody against human CYP1A1. Bottom panel shows proteins probed with an antibody against human CYP1B1.

Evaluating the efficacy of antisense experiments requires multiple controls. My work compared B[*a*]P treated samples to samples treated with B[*a*]P plus targeted antisense oligomers. Most of my assays also included an antisense oligo of similar nucleotide content but scrambled sequence. Such non-specific antisense oligomers should have no effect on B[*a*]P induced EROD activity. However, substantial variation was observed between these two positive controls. The difference between positive controls prevents quantification of enzyme activity inhibition in these studies, as the values of samples treated with PMOs designed as inhibitors generally fall in between these two values.

Closer examination of loading

Although fluorescence could be observed in the cells (Figure 2-10), it was uncertain what quantity of PMO this represented. To estimate this, cells were treated with fluorescein tagged PMO, and the fluorescence tracked over time. An aliquot of the medium used to treat the cells was retained, as well as medium removed after treatment. Aliquots of the medium replaced over the cells were collected at intervals up to 24 hours, and cells were harvested 1 hour and 24 hours after treatment and lysed. The fluorescence in these samples was measured and compared.

The medium removed from cells after treatment contains substantially less fluorescence than is found in medium with PMO/DNA/EPEI used to treat the cells (Figure 2-12). Fluorescence approximately corresponding to this difference is found in cells lysed one hour after treatment. However, cells lysed 24 hours after treatment contain a fraction of this fluorescence, and medium removed from cells at intervals after treatment exhibited nearly as much fluorescence as the original cell lysate. Calculating from this data reveals that approximately 7.7 million PMO molecules remain in each cell after 24 hours.

Greater magnification of cells loaded with a fluorescently tagged PMO indicated that fluorescence congregated both in the nucleus, and both diffusely and in punctate areas

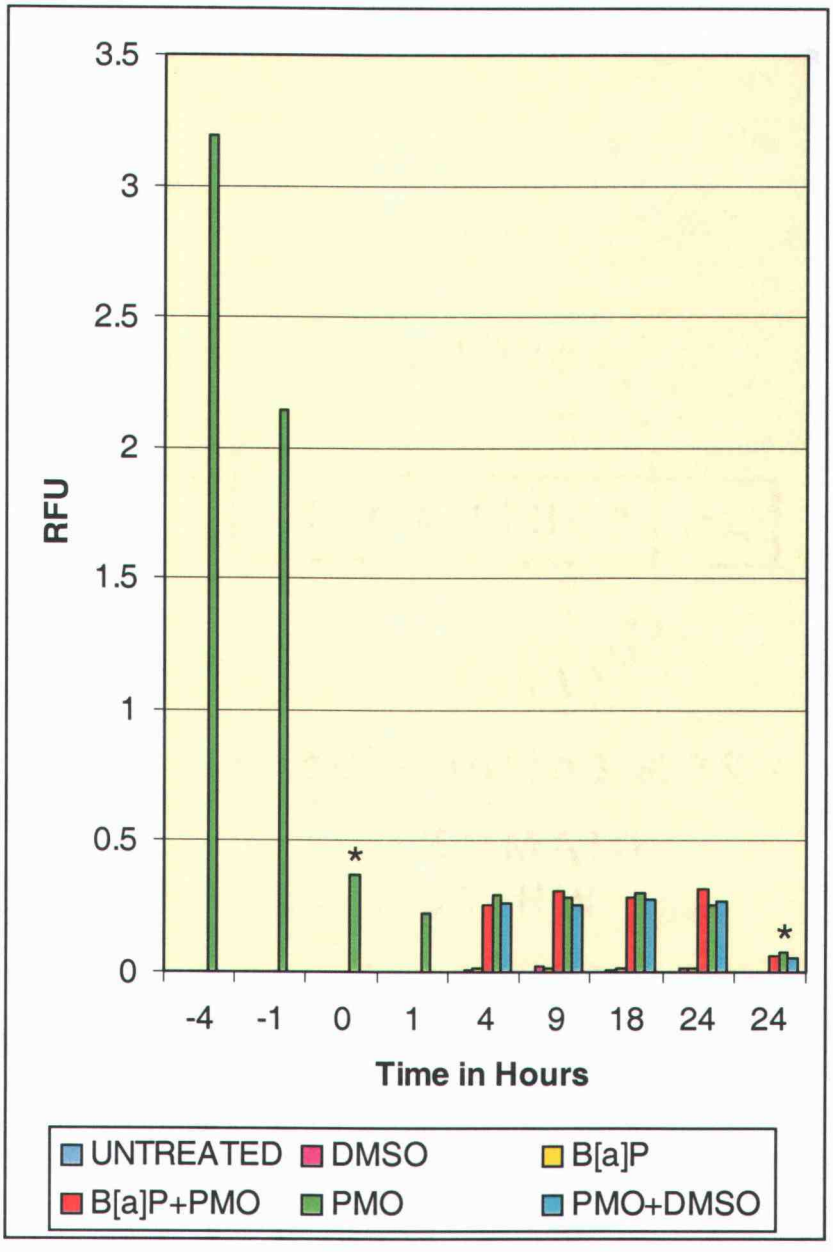


Figure 2-12. Fluorescence measured in media and cell lysate from MCF-7 cells treated with fluorescein tagged PMO/DNA/EPEI. Cells were loaded with PMO complex and sampled over a 24 hour period. An asterisk (*) represents cell lysate samples. The remaining columns represent aliquots of media reserved before or at various intervals after treatment.

in the cytosol. To determine the nature of these cytosolic points of fluorescence, cells were treated with LysoTracker Red Probe (Molecular Probes Inc., Eugene, OR). This stain indicated the position of lysosomes in the cells. Many of these points correlated with the fluorescence associated with the PMO (Figure 2-13). The sequestration of antisense oligonucleotides with various backbone designs into subcellular vesicular structures has been a barrier to their efficacy. Apparently the unique backbone design of PMOs does not prevent this phenomenon.

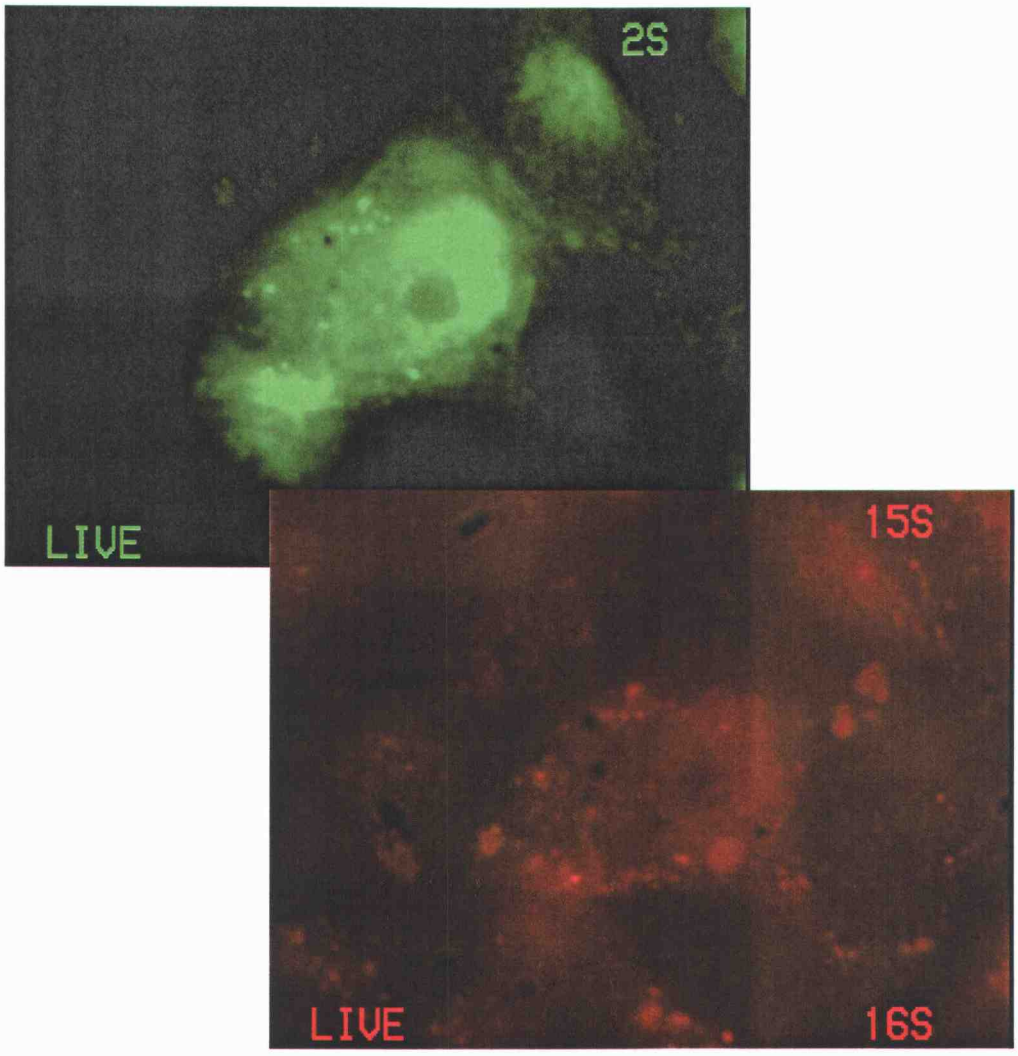
Possible interference of B[*a*]P with PMOs

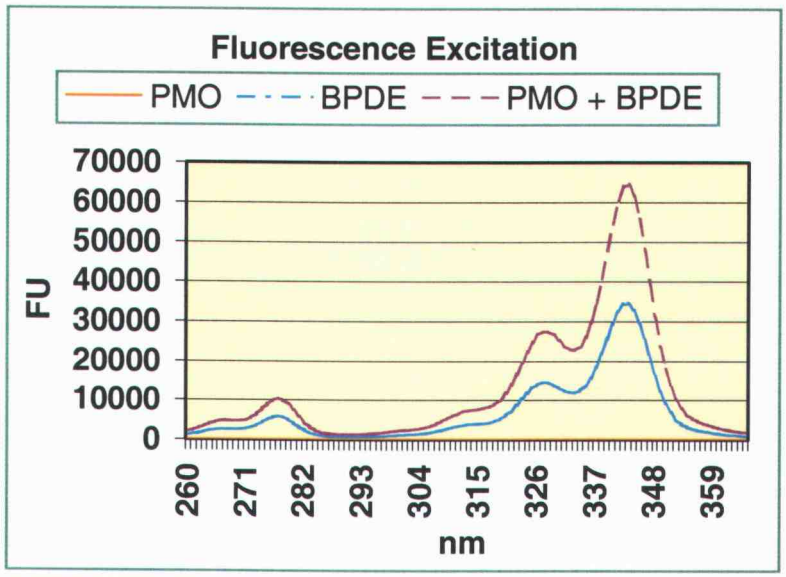
B[*a*]P is metabolized to [(+)-*anti*]B[*a*]P-7R,8S-diol-9-S,10R-epoxide (BPDE), which may then adduct to DNA. Because PMOs are DNA analogs, their ability to interact with BPDE was examined. BPDE, which fluoresces at 360-500 nm when covalently attached to DNA [80], was reacted with a PMO (Figure 2-14). The PMO exhibited no fluorescence at the wavelengths observed, while BPDE exhibited maximal excitation at 342 nm and emission at 398 nm. BPDE is unstable and reacts with water to form highly fluorescent tetrols, and this spectrum accounts for such background. When PMO was reacted with BPDE, these peaks increased in intensity, indicating that BPDE may react with PMOs in a similar manner to DNA. Such a reaction might hinder the ability of a PMO to fully duplex with its RNA target.

DISCUSSION

Cytochrome P450 1A1 and 1B1 are essential in determining the fate of many xenobiotic compounds. In turn, many of these compounds induce or inhibit enzymes involved in their own metabolism. While much has been characterized, an indefinite number of additional enzymes are involved in the metabolism of exogenous compounds. While some of these enzymes have been expressed in mammalian cells, bacteria, or yeast to gain an understanding of their individual functions, it is more difficult to specifically and completely subtract them so as to elucidate their role in

Figure 2-13. PMOs colocalize with lysosomes. Fluorescein-tagged PMOs (top panel) form punctate fluorescence in the cytosol. When stained with Lysotracker Red, many of these points can be observed to colocalize.





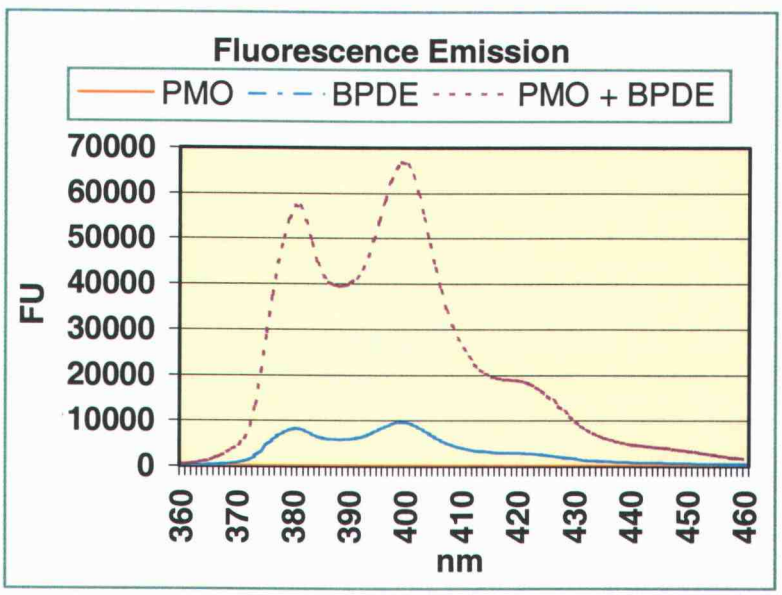


Figure 2-14. Fluorescent spectra of PMO, BPDE, and PMO + BPDE. BPDE was added to PMO. The fluorescence spectra were measured at excitation at 398 nm and emission at 342 nm.

complex multi-enzyme human systems. The creation of specific, reliable inhibitors for cytochrome P450 1A1 and 1B1 remains a worthwhile goal.

Inhibiting the production of proteins from mRNA using antisense persists as an elegantly simple and seductive concept, despite many setbacks. It originated at least a quarter of a century ago [81,82] and its proponents have never wavered in their enthusiasm. A handful of companies worldwide tout this technology, and have met with some success, particularly in "knock-down" of proteins in zebrafish embryos with morpholinos. Only one antisense compound has made it through clinical trials to therapeutic application. However, the compound, Vitravene by Isis of Carlsbad CA, designed to combat cytomegalovirus retinitis in AIDS patients, is used only when more effective, first-defense treatments have failed. It is delivered by direct injection to the eye. Delivery in organisms remains problematic and is a major stumbling block in bringing antisense to market as effective therapeutic compounds [83].

Success has been mixed as well in cell culture. Considering the length of time antisense technology has been available, relatively few accounts of successful applications in cell culture have been reported. Improving PMO delivery and efficacy is an ongoing effort [75]. However, it is unknown how many unsuccessful studies have been undertaken in cell culture using antisense. In our study, although antisense was delivered to the cells, insufficient inhibition was observed to continue with meaningful DNA binding experiments. Many explanations are possible for this result, as well as the general lack of efficacy of antisense.

When MCF-7 cells are treated with B[*a*]P, CYP1A1 activity rises steadily, nearing maximum levels at 12 hours, remaining elevated at 24 hours and beyond. CYP1A1 protein has an instability index of 40.85, which classifies it as unstable. (ExPASy web site) CYP1A1 mRNA has a half life of 2.4 hours in human HepG2 cells [84]. This elongated expression time despite rapid protein and mRNA turnover may quickly

exhaust PMOs targeted to CYP1A1 if they remain bound tightly to mRNA while new copies are continually produced.

Possibly, B[*a*]P binds to the PMO, and interferes with its ability to duplex with its targeted mRNA, as suggested in Figure 2-13. More generally, while a single morpholino/RNA duplex has been show to possess a higher melting temperature than that of other oligo/RNA duplexes [85], this has not been universally demonstrated. The six membered morpholine ring that makes up the backbone of PMOs surely differs in flexibility from the five carbon ribose ring of RNA. This difference may limit PMOs from completely conforming to intricate three dimensional RNA structures. The structure of RNA varies around the translational start site and is known to be important in translation regulation [86]. It would be interesting to compare melting curves of DNA/RNA and PMO/RNA to ascertain whether an elevated melting temperature were consistent over a variety of sequences. In addition it would be informative to attempt to crystallize antisense oligomers with their RNA targets, to verify the ability of synthetic oligonucleotides to conform to RNA structure.

It is possible that PMOs become trapped in cellular compartments such as endosomes/lysosomes, and are prevented from reaching their intended target, as evidenced by the fluorescence micrographs in Figure 2-12. This sequestration has been observed by others with other antisense designs [87] and mechanisms of nucleic acid transport in cells are being elucidated [88], implying that trafficking of endogenous oligonucleotides already occurs in cells. The fluorescein tag used in our loading studies adds a charge to the PMO, and a caveat to our results, as the charge may positively influence loading (observations by AVI Biopharma). It is unclear in our work, as in any antisense study, how many oligomers reach their RNA target.

PMOs are reported to work by competing with the ribosome for the start site or splice site of RNA. This simple mechanism discounts additional regulatory mechanisms that may be present in protein synthesis. If the protein synthesis complex is unable to bind

to the start site, it may continue scanning the RNA for an alternative start site, in a process known as leaky scanning, or an internal initial site may be standard in to a given protein's expression [86]. Proteins exist which may regulate transcription by interacting with sequences within the 5' UTR. These proteins are often upregulated in response to physiological stimuli, and could conceivably hinder binding of PMOs. Regulation of translation may also be mediated by 3' UTR sequences and the poly(A) tail of RNA. Further understanding of these mechanisms may shed light on their possible interference with antisense action.

While a few possible mechanisms for lack of inhibition by PMOs in cell culture have been mentioned here, many more may exist. Why then, do morpholinos inhibit proteins involved in zebrafish development so efficiently [69]? Is it because PMOs are delivered by microinjection, instead of by endocytosis? It would be informative to microinject MCF-7 cells with PMOs, and compare compartmentalization with cells loaded with EPEI. However, this would not be a practical approach for analyzing effects on PAH metabolism due to the large number of cells required for PAH measurement.

PMOs are reported to bind tightly and specifically to target RNA, and non-specific effects have not been reported. Yet in the present study, not only was no significant inhibition observed with targeted PMOs, but treatment with scrambled control PMOs appeared to if anything, slightly increase EROD activity. To isolate this effect, MCF-7 cells could be syringe-loaded with PMO to avoid any effects of EPEI, not treated with B[*a*]P, and assayed for EROD activity. CYP1A1 induction following xenobiotic treatment is sometimes, although not consistently, associated with P-glycoprotein and multidrug resistance enzymes involved in drug efflux [89]. Further exploration of the effect of PMO on EROD activity in these studies may reveal clues about the fate of PMOs within the cell. Given the general lack of efficacy in antisense in cell culture, this approach was not pursued.

CYP1A1 and CYP1B1 are intimately involved in the transformation of PAH to mutagenic DNA binding species. The development of specific inhibitors for these compounds would expedite many lines of research. The chemical inhibitors which do exist often have overlapping activity for multiple P450s, although a few show some potential for distinguishing between P450s 1A1 and 1B1 [30,60]. These inhibitors may cause non-specific effects as well. CYP1A1 and CYP1B1 knockout mice have yielded interesting and sometimes paradoxical results, but a large volume of work has not yet resulted from these recently developed models [24,62,63,90]. While in many ways murine carcinogenesis positively compares to human carcinogenesis, in other ways it does not [58,91]. This must be considered when applying these results to human carcinogenesis.

Since this work was completed, a new method for inhibition of CYP1A1 and CYP1B1 has become available. Small interfering RNA (siRNA) are 21-22 nucleotide double stranded RNAs which recognize and facilitate the destruction of complementary RNAs. Methods have been refined which allow these constructs to be used in mammalian cells [92,93]. Specific kits have become available for using siRNA to inhibit both CYP1A1 and CYP1B1 (SuperArray Bioscience Corporation). The makers assert that at least 70% inhibition can be achieved. This method has already been used to silence AhR and Arnt in MCF-7 and HepG2 cells, resulting in reductions in CYP1A1 levels and activity and effects on cell cycle progression [94]. However, the application of new approaches such as siRNA must be looked forward to with very cautious optimism. Antisense technology was met with similar enthusiasm, and while this enthusiasm may be merited for knocking down protein expression in zebra fish embryos, its application is limited in other settings. The concept of a general method of specific inhibition is seductive, but must be greeted critically.

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SUBEROYLANILIDE HYDROXAMIC ACID AND TRICHOSTATIN A INDUCE CYP1A1 AND CYP1B1 BUT DECREASE B[*a*]P-INDUCED EXPRESSION WITH NO EFFECT ON DNA BINDING

Louisa Ada Hooven[1], Brinda Mahadevan[2], Channa Keshava[4], Dhimant Desai[3], Shantu Amin[3], Ainsley Weston[4], and William M. Baird[1,5]

[1] Department of Biochemistry and Biophysics,

[2]Department of Environmental and Molecular Toxicology

Oregon State University, Corvallis, Oregon

[3]Institute for Cancer Prevention, American Health Foundation Cancer Center, Valhalla, New York

[4]Toxicology and Molecular Biology Branch, National Institute of Occupational Safety and Health, CDC, Morgantown, WV

[5] To whom correspondence should be addressed

Email: William.Baird@ orst.edu

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ABSTRACT

Exposure to polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (B[*a*]P) have been determined to be a risk factor for various forms of human cancer. B[*a*]P binds to the aryl hydrocarbon receptor (AHR), which acts as a transcription factor for cytochrome P450 1A1 (CYP1A1) and other xenobiotic metabolizing enzymes. The metabolites of B[*a*]P formed by CYP1A1 may bind to DNA, and if the DNA interaction products are not repaired, may cause mutations. The expression of CYP1A1 may be influenced by interaction of ligand with AHR, or alternative pathways which are less well understood. Histone deacetylases (HDACs) participate in the remodeling of chromatin, affecting the expression of fewer than 2% of genes. HDAC inhibitors act to reverse this epigenetically controlled suppression. Because many of the genes regulated in this manner are involved in proliferation, cell function, and differentiation, HDAC inhibitors are of great interest in cancer research. In this work, MCF-7 cells were treated with two HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA). When CYP1A1 activity, as measured by ethoxyresorufin-*O*-deethylase (EROD) activity, was examined in intact cells, SAHA and TSA increased CYP1A1 activity to a greater extent than B[*a*]P. When examined in microsomes extracted from treated cells, this effect was smaller. To further examine this effect, we examined the level of expression of CYP1A1 and CYP1B1 in cells exposed to various concentrations of SAHA, TSA, and B[*a*]P by RT-PCR. At some concentrations, SAHA and TSA increased CYP1A1 and CYP1B1 mRNA levels. When cells were co-treated with SAHA and B[*a*]P or TSA and B[*a*]P, CYP1A1 and CYP1B1 mRNA levels were reduced. Despite this result, when B[*a*]P DNA binding was examined in cells co-treated with SAHA or TSA, no significant difference in DNA adducts was observed.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) result from the combustion of organic matter and are widely dispersed in the environment. The association between exposure to PAH and human cancer has been recognized for centuries, and a large body of research exists which examines this relationship [1,2]. Elevated exposures to PAH correspond with increased risk of lung, skin, and bladder cancer, and may contribute to breast cancer incidence [3,4]. The metabolism of PAH, which bestows mutagenic properties on these compounds, has been studied extensively [5]. Yet many lines of inquiry remain open, including the variable expression and interaction of PAH metabolizing enzymes.

Benzo[*a*]pyrene (B[*a*]P) is a particularly well-studied carcinogenic PAH which is found in coal tar, tobacco smoke, and other environmental mixtures. B[*a*]P binds to the aryl hydrocarbon receptor (AHR), inducing cytochrome P450 1A1 (CYP1A1) as well as other detoxifying enzymes. In the course of epoxidating B[*a*]P, reactive electrophiles may be formed which are resistant to further enzymatic processing but rather bind to the exocyclic amines of purine bases [5]. These bulky DNA lesions may result in mutations, and B[*a*]P is thought to be responsible for the particular p53 mutations found in the lung tissues of smokers [6]. Approximately 10% of the human population exhibits highly inducible CYP1A1 activity, which in combination with variation in other xenobiotic metabolizing enzymes, may contribute to cancer susceptibility [7]. Amounts of B[*a*]P adducts formed have been observed to be different in groups with polymorphisms in CYP1A1 and other enzymes [8]. Tumors may have distinct P450 profiles, altering their ability to metabolize chemotherapeutic compounds [9].

Because CYP1A1 plays a key role in xenobiotic transformation and carcinogenesis as well as hormone metabolism, gene regulation of this protein has been studied extensively. Inducers of CYP1A1 may interact with the AHR, which dimerizes with the aryl hydrocarbon nuclear translocator (ARNT), and is transported to the nucleus

[10]. This dimer associates with xenobiotic responsive elements (XREs), at least seven of which are upstream of the human CYP1A1 gene [11]. B[*a*]P causes a robust and prolonged increase in CYP1A1 protein levels and activity through this pathway. Less well understood examples of CYP1A1 induction may involve the retinoic acid receptor or protein tyrosine kinase activation [12]. Negative regulatory elements (NRE) appear to modulate CYP1A1 transcriptional activity [13].

While variation in genetic sequence is important to understand cancer risk and treatment, epigenetic activation or repression of genes may also contribute to human cancer. The pattern of lysine acetylation of the amino terminal tails of histones are determined by histone deacetylases (HDAC) and histone acetyl transferases (HAT). Together with other histone modifications and DNA methylation, these patterns contribute to an epigenetic code recognized by proteins involved in regulation of gene expression [14]. These modifications induce the open euchromatin formation, allowing transcription of epigenetically controlled genes. HDACs are important components of epigenetic regulation, and are recruited to DNA by protein complexes. Their deacetylase action allows chromatin to form higher order closed heterochromatin structure, preventing transcription [15]. A number of compounds reverse HDAC activity, affecting fewer than 2% of expressed genes. Because these changes in expression are associated with altered growth, arrest, differentiation, or apoptosis of cells in culture, and the inhibition of tumor growth in animal models, several HDAC inhibitors have entered clinical trials. The local dynamic remodeling of chromatin and nucleosomal DNA packaging is key to regulation of genes affecting proper cell function, differentiation, and proliferation. Inappropriate expression of some of these genes has been associated with cancer, indicating that further study of epigenetic regulation holds great promise [16,17].

Trichostatin A (TSA), a linear hydroxamic acid, was isolated from *Streptomyces hygroscopicus* and identified as a fungal antibiotic [18]. TSA was later observed to be a potent, specific, and reversible HDAC inhibitor [19]. Suberoylanilide hydroxamic acid

(SAHA), a structurally similar compound, shows strong anti-proliferative effects but low toxicity *in vivo* [20]. The antitumor activity of SAHA has been demonstrated in several animal models, and has shown promise in phase I clinical trials [21]. The accumulation of acetylated histones in peripheral mononuclear cells and tumor cells in patients correlated with SAHA concentrations. In phase II clinical trials, SAHA patients with prior therapy-resistant cutaneous T cell lymphomas have shown positive responses [14]. SAHA has also been tested as a chemopreventive agent. When continuously fed to rats in the diet, SAHA has been shown to reduce the development of N-methylnitrosurea (NMU) induced mammary and lung tumors [22,23]. Due to these chemotherapeutic and chemopreventive properties, in this work SAHA and TSA were examined for their ability to influence B[*a*]P DNA adducts. Like NMU, B[*a*]P causes DNA mutations which initiate carcinogenesis.

Transcriptional repression by epigenetic mechanisms has been demonstrated in a wide variety of tumor types. When genes involved in tumor suppression, cell cycle, DNA repair, invasion and metastasis are inactivated, tumor cells may be given a growth advantage. The reexpression of these genes can alter sensitivity to existing cancer therapies or suppress cell growth [17]. While reinactivating the expression of some genes may be useful in preventing or treating cancer, the unexpected induction of other genes may have unintended clinical consequences. In this work, SAHA and TSA have been found to substantially increase EROD activity, which measures CYP1A1 activity, in intact cells. Microsomal EROD assays and RT-PCR analysis also revealed elevated CYP1A1 activity, and CYP1A1 and CYP1B1 expression after treatment with these two compounds. Interestingly, both SAHA and TSA reduced the induction of CYP1A1 and CYP1B1 by B[*a*]P as measured by RT-PCR. Paradoxically, no difference in DNA binding was noted when MCF-7 cells were co-treated with B[*a*]P and the HDAC inhibitors SAHA and TSA.

MATERIALS AND METHODS

Cell culture and treatment

MCF-7 cells originated from human mammary carcinoma tissue [24], and were provided to the Baird lab by the Purdue University Cell Culture Laboratory. MCF-7 cells were grown in 1:1 F12 Nutrient Mixture and Dulbecco's Modified Eagle Medium (F/D) (Gibco BRL, Grand Island, NY) , with 10 percent fetal bovine serum (Integren, Purchase, NY), 15 mM HEPES buffer, 14.3 mM $NaHCO_3$, 200 units/ml penicillin, 200 μg/ml streptomycin, and 25 μg/ml ampicillin. The cells were grown in T75 cm^2 flasks and maintained at 37°C with 5% CO_2. The cells were split 1:4 when they covered the surface area of the bottom of the flask.

Cells were treated with 0.01, 0.10, 1.0, 2.0, or 4.0 μM B[*a*]P, SAHA, TSA or an equal volume of dimethylsulfoxide (DMSO) as vehicle control). B[*a*]P and DB[*a,l*]P were obtained from Chemsyn Science Laboratories (Lenexa, KS). SAHA was prepared as previously described. TSA was obtained from Dr. Roderick Dashwood and Mindy Myzak of the Linus Pauling Institute, Oregon State University, or purchased from Biomol (Plymouth Meeting PA). The cells were harvested or treated after 12 or 24 hours.

Cell harvest

Medium was aspirated from MCF-7 cells in 75 cm^2 flasks, which were then rinsed with 5 mls trypsin-versene. (0.05 % trypsin, 0.14 NaCl, 3mM KCl, 0.1 M Na_2HPO_4, 1.5mM KH_2PO_4, and 0.5 mM EDTA) The cells were then incubated with another 5 mls of trypsin-versene until they became detached from the flask. Five mls of F/D media with 10% fetal calf serum was added to the flask, and the cells decanted into a 50 ml centrifuge tube, and the flask rinsed with 10 mls PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4), which was added to the centrifuge tube. The cells were centrifuged at 2,000 rpm (478xg) in a bench top IEC HNSII centrifuge (International Equipment Co, Vernon Hills, IL). The supernatant was aspirated, and

the pellet resuspended in 10 mls PBS, and centrifuged. The PBS was removed, and the pellet was again resuspended in PBS. After centrifugation, the resulting pellet was stored at -80°C.

Measurement of EROD activity in intact MCF-7 cells

250,000 MCF-7 cells were plated in each well of a 24 well culture plate. After treatment in at least triplicate with B[*a*]P, SAHA, TSA, or DMSO, cells were incubated for 12 or 24 hours, and rinsed twice with media. The media was then replaced with phenol red free media containing 5 μM ethoxyresorufin (ERES) and 1.5 μM salicylamide. The fluorescence was then measured in a SpectraMax Gemini fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA) at intervals over 60 minutes, with an excitation wavelength of 530 nm and emission at 585 nm [25].

Microsome isolation

Cell pellets were homogenized in a steel homogenizer in homogenization buffer (0.25 M KPO_4, 0.15 M KCl, 10 mM EDTA, and 0.25 mM phenylmethylsulfonylfluoride (PMSF), and centrifuged at 9,000 rpm (15,000xg) for 20 minutes at 4°C in a Sorvall RC-5B Superspeed Refrigerated Centrifuge (Sorvall, Albertville, MN). The supernatant was centrifuged at 58,000 rpm (100,000xg) for 90 minutes at 4°C in a Beckman TL-100 Ultracentrifuge (Beckman, St. Louis, MO), and the pellet homogenized in microsome dilution buffer [26] (0.1M KPO_4, 20% glycerol, 10 mM EDTA, 0.1 mM DTT, and 0.25 mM PMSF) [26].

Protein quantification

Total protein concentration of microsomal samples was determined by the bicinchonic acid (BCA) protein assay (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used to create a standard curve ranging from 10 μg to 50 μg protein in 50 μl water. 3 to 10 μl of sample in 50 μl water were compared to this sample curve to obtain protein concentration. 1 ml of 50:1 BCA assay reagent A/BCA reagent B was added

to the standards and the samples, which were then vortexed. After incubation for 30 minutes at 37°C, the absorbance of the standards and samples were measured at 562 nm on a Beckman DU-650 Spectrophotometer (Beckman Instruments Inc., St. Louis, MO).

Microsomal EROD assay

2 µl of 10 mM NADPH was added to 50 µg of microsomes in 0.1 M Tris-HCl and 1 µM ERES in 96 well black plates (Greiner Labortechnik, Frickenhausen, Germany), and emission measured at 585 nm with excitement at 530 nm in a SpectraMax Gemini fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA) every 30 seconds for ten minutes. The samples were performed in at least triplicate. A resorufin standard curve from 25 to 100 pM was used to determine relative fluorescent units (RFUs) per picomole resorufin, which was then used to calculate picomoles resorufin per minute per mg microsomal protein [27].

SDS-PAGE and immunoblotting

50 µg of microsomal protein in sample buffer (0.625 M Tris-base (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue) was loaded into each well of a 7 ½% 1.5 mm sodium dodecyl sulfate (SDS) polyacrylamide precast Criterion gel (Biorad, Hercules, CA). 10 µg of microsomes from V79 cells overexpressing CYP1A1 was used as positive control. The samples were denatured by boiling for 3 minutes. 10 µl of dual color Precision Plus Protein Standards (BioRad, Hercules, CA) ran with the samples. The gels were run at 200V for 65 minutes in a Criterion Cell (BioRad, Hercules, CA).

The proteins were transferred to a Trans-blot transfer membrane (BioRad, Hercules CA) using a Criterion Blotter (BioRad, Heculues, CA) at 100V for 30 minutes at 4°C. The membrane was blocked with 1:3 NapSURE Blocker (Geno Technology, St. Louis, MO) in PBS-T (0.3% w/v Tween-20 in PBS) for at least one hour, washed 3 times for

5 minutes with 1:7 Nap-SURE/PBS-T, and incubated with the primary antibody for two hours in 1:7 NapSURE/PBS-T. Human CYP1A1 was detected using a 1:3000 rabbit polyclonal antibody provided by Dr. F. P. Guengerich, Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville TN. The blots were incubated with peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) (1:20,000 or 1:30,000) in 1:7 NapSURE/PBS-T for 30 minutes. The membrane was again washed 3 times for 5 minutes with 1:7 Nap-SURE/PBS-T. CYP1A1 was detected using Amersham 1:1 Enhanced Chemiluminescence Western Blotting Detection reagents 1 and 2 (Amersham Pharmacia Biotech, Buckinghamshire, England). Kodak Biomax Light Film (Eastman Kodak, Rochester NY) was exposed to the blots and developed using a model M6B RP X-omat Processor (Eastman Kodak, Rochester NY).

Isolation of DNA

Frozen cell pellets were homogenized in 1.5 ml EDTA/SDS buffer (10 mM Tris, 1 mM Na_2EDTA, 1% SDS, pH 8) in a glass homogenizer. 30 µl of RNase mixture (50 units per ml RNase T1 and 25µg/ml RNase A [both Boeheringer Mannheim, Indianapolis, IN]) were added to the mixture, which was then incubated for 1 to 2 hours at 37°C with shaking. 75 µl proteinase K (500 µg/ml proteinase K [Sigma, St. Louis, MO]) was incubated with the sample for an additional 1 to 2 hours with shaking. The sample was decanted into a 15 ml light phase lock centrifuge tube (Eppendorf, Westbury NY), and 1.5 ml of equilibrated phenol (USB Corp., Cleveland, OH) added. The tube was shaken for 5 minutes, and centrifuged for 6 minutes at 3000 rpm on a bench top IEC HNSII centrifuge (International Equipment Co, Vernon Hills, IL). 0.75 ml of equilibrated phenol and 0.75 ml of 24:1 chloroform:isoamyl alcohol were added, and the sample was shaken for 5 minutes. The sample was centrifuged for 6 minutes at 3000 rpm, and 1.5 ml 24:1 chloroform:isoamyl alcohol were added, and the sample shaken for 5 minutes. The sample was again centrifuged 6 minutes at 3000 rpm. The layer above the gel lock was transferred to a clean 15 ml conical centrifuge tube. 150 µl 5M NaCl and 3 ml ice cold 100% ethanol were added

to precipitate the DNA. The precipitated DNA was removed by using a glass hook, rinsed in 70% ethanol, and redissolved in 1 ml sterile double distilled water. The DNA was quantified by spectrophotometry at 260 nm using a Beckman DU-650 Spectrophotometer (Beckman Instruments Inc., St. Louis, MO). One tenth volume 5M NaCl and twice volume 100% ethanol were used to reprecipitate 10 μg DNA. After centrifugation, the supernatant was removed and the pellet rinsed with 70% ethanol. The pellet was dried and resuspended in 5 μl of sterile water.

^{33}P Postlabeling of adducted DNA

The DNA was digested with 0.6 U of nuclease P1 (Sigma Chemical Co., St. Louis, MO) and 350 mU prostatic acid phosphatase (Sigma Chemical Co., St. Louis, MO) in nuclease P1 buffer (0.125 M sodium acetate, 30 mM $ZnCl_2$, pH 5.2). The digested DNA was again reduced to 5 μl using a Speed-Vac (Savant Instruments Inc., Farmingdale, NY), and labeled with 50 μCi [γ-^{33}P]ATP using 18 U T4 polynucleotide kinase (USB Corp., Cleveland, OH), and 2 μl kinase buffer (0.5 M Tris, 8 mM spermidine, 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), pH 9.6) at 37°C for 1 hour. The resulting labeled adducted dinucleotides were digested to mononucleotides using 15 mU Type VII *Crotalus atrox* venom phosphodiesterase (Sigma Chemical Co., St. Louis, MO). Unused [γ-^{33}P]ATP was hydrolyzed with 100 mU potato apyrase (Sigma Chemical Co., St. Louisa, MO) at 37° for 1 hour.

Isolation of adducted mononucleotides

Waters C_{18} Sep Pak columns (Millipore Corp., Milford, MA) were used to separate the adducted mononucleotide from other digestion products. The columns were first conditioned by running 10 ml HPLC grade methanol, 10 ml double distilled water (ddH_20) , and 10 ml Sep Pak loading buffer (0.5 M KH_2PO_4, pH 6.0) using a 10 ml syringe. The digested and labeled DNA was passed slowly through the syringe in 10 ml Sep Pak loading buffer. The sample was passed through the column a second time, and the column washed three times with 10 ml ddH_2O, then ddH_2O with 5% basic methanol (5% ammonia hydroxide in HPLC grade methanol). The labeled adducted

nucleotides were then eluted by passing 2 ml of basic methanol through the syringe. 2 10 µl aliquots of the product were counted in a Tricarb Liquid Scintillation Counter (Packard Instruments Co., Meriden, CT) with Flo-V scintillation fluid (Packard Instruments Co., Meriden, CT).

HPLC separation of DNA adducts

An aliquot of each sample measuring $1x10^6$ dpm or less was reduced to approximately 10 µl using a Speed-Vac. The sample was resuspended in 100 µl of 1:1 buffer A and B. Buffer A for B[*a*]P and DB[*a,l*]P samples was filtered 0.1M $NH_4H_2PO_4$, pH 5.5. Buffer B for B[*a*]P samples was 100% HPLC grade methanol, and for DB[*a,l*]P samples 10% acetonitrile and 90% HPLC grade methanol.

Reverse phase HPLC separation of DNA adducts in the first experiment was carried out using a Beckman ultrasphere ODS 5 µm 4.6 mm x 250 mm C_{18} column using Beckman 110B solvent delivery modules, system organizer, and injection module (Beckman Instruments Inc., St. Louis, MO), and detected on a Radiomatic Flo-one Beta radiochromatography detector (Packard Instruments Co., Meriden, CT). B[*a*]P DNA adducts were eluted using a gradient of 44%-60% solvent B over 40 minutes, 60-80% solvent B over 10 minutes, isocratic elution at 80% solvent B over 10 minutes, and 80-44% solvent B over 5 minutes. In the second adduct experiment, a Varian 420 Auto Sampler (Varian Analytical Instruments, Walnut Creek, CA) introduced samples to a Waters Symmetry 4.6 mm x 250 mm C_{18} column on a Varian Pro Star 210 HPLC instrument. The labeled adducted nucleotides were eluted using a gradient of 44% solvent B for five minutes, 55% for 5 minutes, 60% for 20 minutes, 90% for five minutes, and 100% for five mintues. Peaks were detected with a 400 µl dry cell on a β-RAM® Model 3 (IN/US Systems, Inc., Tampa FL) radioactive detector [28].

MTT assay

35,000 MCF-7 cells were plated in each well of a 96-well culture plate. The cells were incubated for 18 hours. The medium was removed and replaced with medium

containing 4 μM B[*a*]P, 0.01, 0.10, and 2.0 μM TSA and SAHA in 100 μl. DMSO and untreated cells were used as controls. After 24 hours, 10 μl MTT reagent (ATCC, Manassa, VA) was added to each well. After 4 hours, purple precipitate became visible and 100 μl detergent reagent was added. The plate was shielded from light, and left at room temperature overnight. The absorbance at 570 nm was compared for six wells of each sample in a SpectraMax Gemini fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA) [29].

Quantitative real-time polymerase chain reaction (RT-PCR)

Relative quantitation of RNA was performed by RT-PCR using Taq-Man™ technology (Applied Biosystems). The cDNA was synthesized from total RNA using Advantage RT-PCR kit (Clontech). The cDNA was diluted 1:10 and 2 μl cDNA was used as a template to perform real-time PCR in a 50 μl reaction mixture containing 2X Taq-Man Universal PCR Master Mix and 20X Assays-on-Demand Gene Expression Primers and Probes for both *CYP1A1* (Hs00153120_m1/X02612), *CYP1B1* (Hs00164383_m1/U03688) and *GAPDH* (Hs99999905_m1/NM002046) (Applied Biosystems/GenBank). This two step PCR reaction was performed on an ABI 7700 Sequence Detection System. Each sample was assayed in duplicate and the Cycle Threshold (C_T) values were normalized to the housekeeping gene (*GAPDH*) and the fold change was calculated using $2^{-\Delta\Delta C}{}_T$ method [30].

RESULTS

SAHA and TSA induce EROD activity in intact MCF-7 cells

MCF-7 cells were first treated with the HDAC inhibitor SAHA. When intact cells were assayed at 12 hours, as little as 0.2 μM SAHA displayed increased EROD activity (Figure 3-1). 2.0 μM SAHA showed approximately the same activity as 4 μM B[*a*]P, and 4 μM SAHA showed approximately twice as much activity as 4 μM B[*a*]P. At 24 hours, less activity was noted in the samples treated with 0.2 μM and 1.0 μM SAHA than at 12 hours. However, in cells treated with 2.0 μM SAHA, EROD activity was

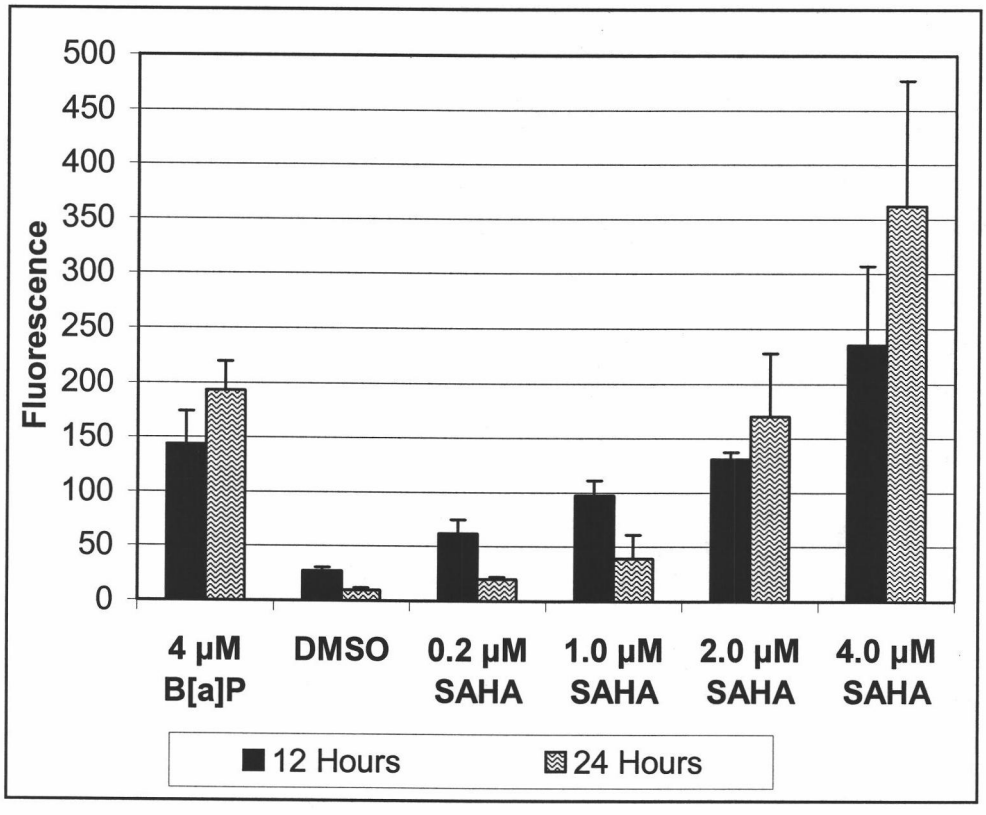

Figure 3-1. Induction of EROD activity by SAHA. MCF-7 cells in 24 well plates were treated with 4 µM B[*a*]P, an equivalent volume of DMSO, or the indicated concentrations of SAHA. After 12 or 24 hours, the media was removed and replaced with phenol red free medium containing 5 µM ERES and 1.5 µM salicylamide. Fluorescence emission was assayed at 585 nm with an excitation at 530 nm, at intervals for 60 minutes. Numbers represent relative fluorescence units. Triplicate wells were measured in each plate. Data shown is the average of two plates. Error bars indicate standard deviation.

nearly as high as cells treated with 4.0 μM B[*a*]P, and the sample treated with 4.0 μM SAHA showed nearly twice the activity of that treated with 4 μM B[*a*]P. A similar trend was noted when cells were treated with both SAHA and TSA. for 24 hours. At 0.2 μM TSA showed a marked increase in EROD activity, while 1.0 μM TSA showed activity nearly that of 4 μM B[*a*]P (Figure 3-2).

Relative to B[a]P, SAHA and TSA induced EROD activity is lower in microsomes than in intact cells

When microsomal samples from cells treated with 0.2 μM SAHA were examined for EROD activity, they were not distinguishable from DMSO. 1.0 μM SAHA however, showed slight EROD activity, but less than that induced by 1.0 μM B[*a*]P. 2.0 μM SAHA showed slightly increased EROD activity, but only 22% of that of 2.0 μM B[*a*]P. 4.0 μM SAHA treated samples yielded 40% of the EROD activity of those treated by 4 μM B[*a*]P. This varies considerably from the intact cell EROD data, where 2.0 μM SAHA activity was nearly that of B[*a*]P (Figure 3-3). TSA behaves in a similar manner and relative to B[*a*]P, shows greatly decreased EROD activity in microsomal EROD assays compared to intact cells at 24 hours (Figure 3-4). At the highest concentration, 2 μM, TSA treated samples gave 14% of that of B[*a*]P treated samples. CYP1A1 protein levels in samples treated with SAHA as observed in immunoblots were a small fraction of that of B[*a*]P (Figure 3-5).

SAHA and TSA increase CYP1A1 and CYP1B1 expression as measured by RT-PCR

RNA was isolated from MCF-7 cells treated with SAHA, TSA, and B[a]P and subjected to RT-PCR (Figure 3-6). At 0.01 μM, SAHA and TSA produced a 0.77 fold change in CYP1A1 expression compared to 1.85 by B[*a*]P. 0.10 μM SAHA and TSA induced CYP1A1 expression 2.86 and 0.81 fold, respectively, compared to 3.69 for B[*a*]P. At 1.0 μM, B[*a*]P increased CYP1A1 expression 25.24 fold, while SAHA and TSA increased expression 0.98 and 3.10 fold. CYP1B1 expression was also increased. 0.01 μM concentrations increased expression 1.36, 1.10, and 1.29 fold, while 0.10 μM

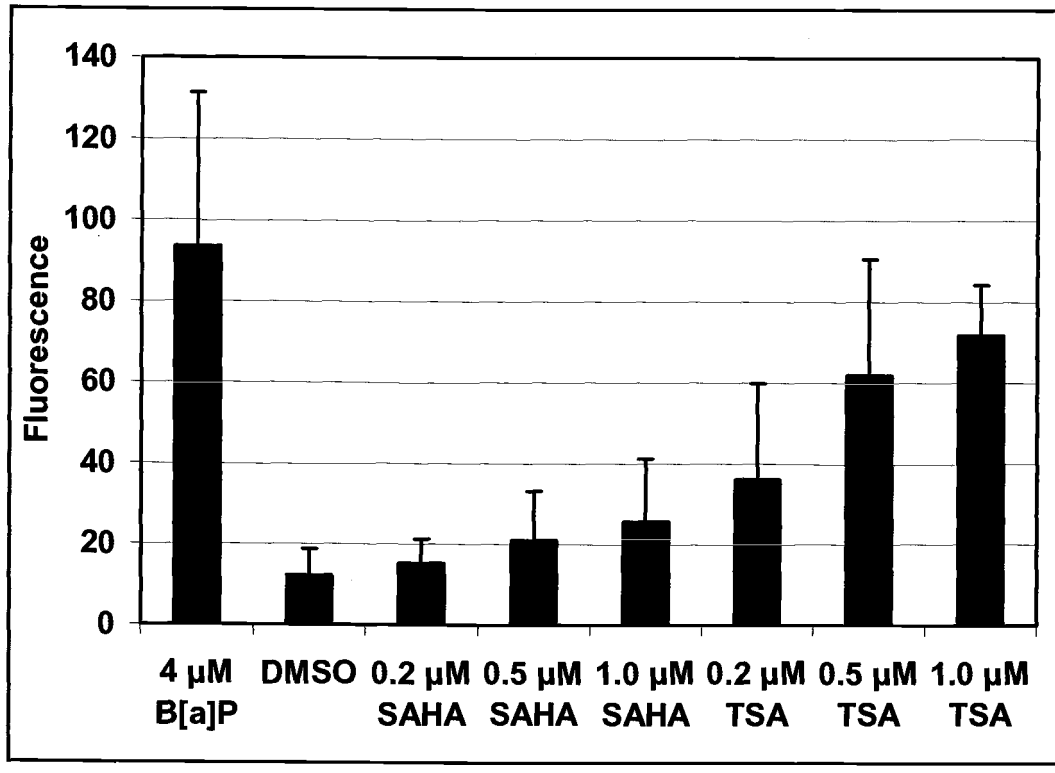

Figure 3-2: Increase in EROD activity in intact MCF-7 cells after treatment with SAHA and TSA. MCF-7 cells in 24 well plates were treated with 4 µM B[*a*]P, an equivalent volume of DMSO, or the indicated concentrations of SAHA or TSA. After 24 hours, the medium was removed and replaced with phenol red free medium containing 5 µM ERES and 1.5 µM salicylamide. Fluorescence emission was assayed at 585 nm with an excitation at 530 nm at intervals for 60 minutes. Numbers are in relative fluorescence units. Triplicate wells were measured in each plate. Data shown is the average of three plates. Error bars represent standard deviation.

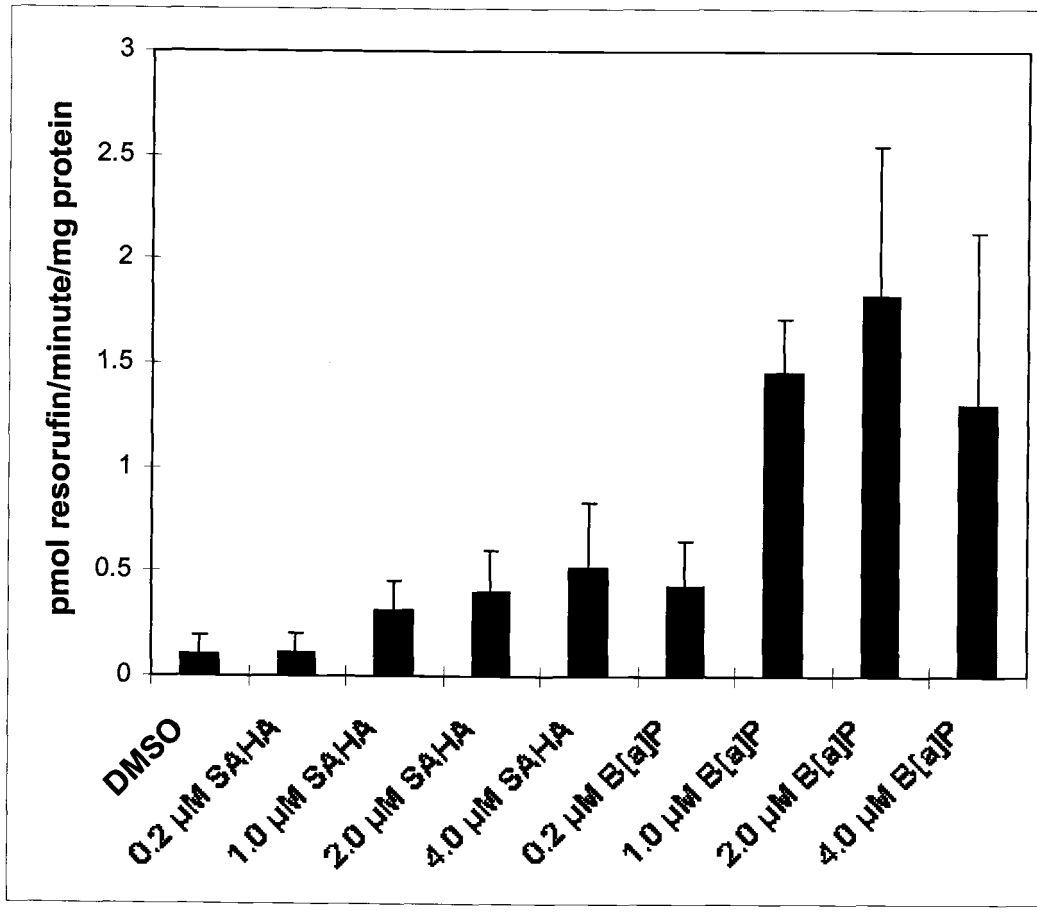

Figure 3-3: Comparison of microsomal EROD activity of MCF-7 cells treated with B[*a*]P and SAHA. MCF-7 cells were harvested 24 hours after treatment with varying concentrations of SAHA, B[*a*]P, or an equivalent volume of DMSO. Microsomes were harvested from the cell pellets and assayed for EROD activity. Results are the average of three separate experiments, with error bars showing standard deviation.

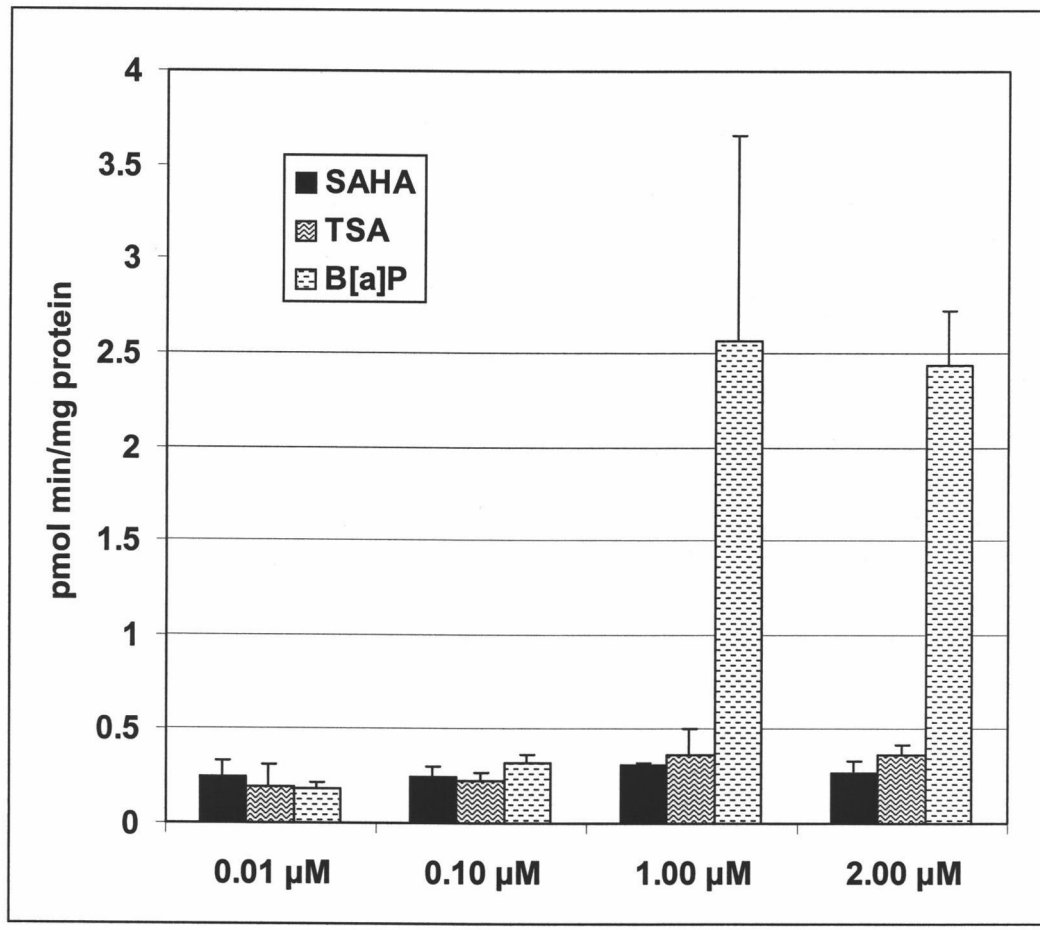

Figure 3-4: Comparison of microsomal EROD activity of MCF-7 cells treated with SAHA, TSA, and B[*a*]P. MCF-7 cells were treated with varying concentrations of SAHA, TSA, and B[*a*]P, and harvested after 24 hours. Microsomes were isolated from the cell pellet and assayed for EROD activity. Results shown are the average of two independent experiments, with error bars displaying standard deviation.

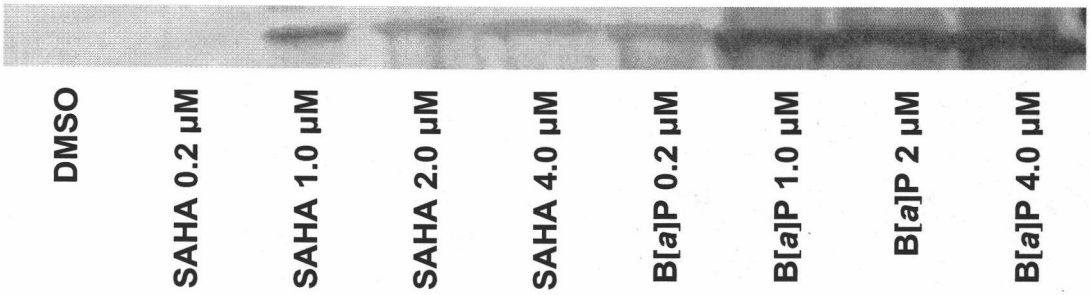

Figure 3-5. Treatment with SAHA elevates CYP1A1 protein levels in MCF-7 cells. Cells were treated with varying concentrations of SAHA or B[*a*]P, and harvested 24 hours later. Microsomes were isolated from the cell pellet. 50 µg of protein from each sample was subjected to SDS-PAGE and immunoblotting, and probed with an antibody against CYP1A1.

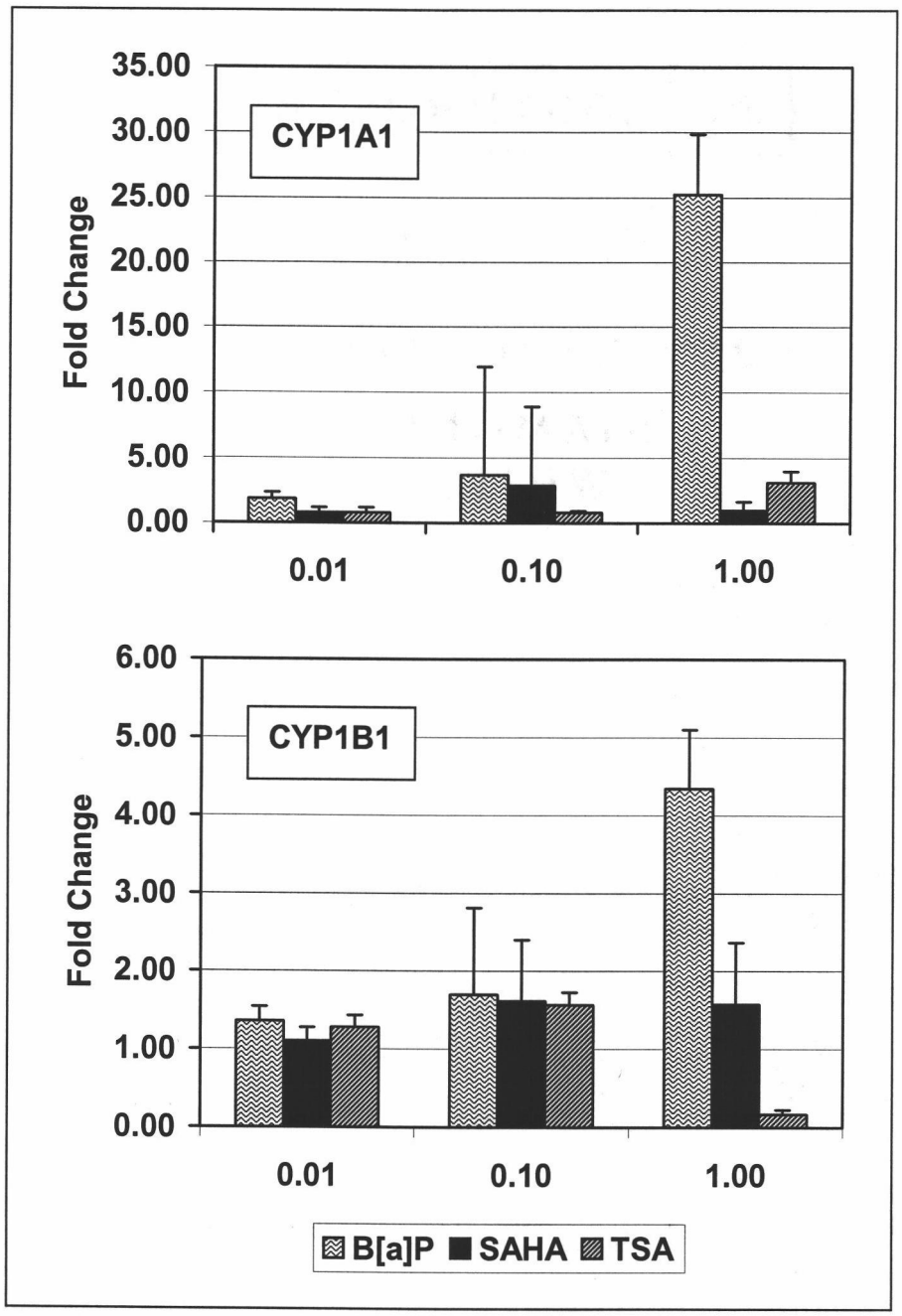

Figure 3-6. RT-PCR of samples treated with B[*a*]P, SAHA, and TSA. MCF-7 cells were treated as indicated and harvested after 24 hours. RNA was isolated from the cell pellet immediately after cell harvest, and subjected to RT-PCR. Top graph indicates fold change in expression of CYP1A1 after treatment with 0.01 μM, 0.10 μM, and 1.00 μM B[*a*]P, SAHA, and TSA. Bottom graph indicates fold change in expression of CYP1B1 after treatment with 0.01 μM, 0.10 μM, and 1.00 μM B[*a*]P, SAHA, and TSA.

treatments increased expression 1.70, 1.62, and 1.57 fold, and 1.0 μM increased expression 4.35, 1.58, and 0.17 fold for B[*a*]P, SAHA, and TSA, respectively.

SAHA and TSA reduce B[a]P induced expression of CYP1A1 and CYP1B1

RT-PCR of samples treated with 1.0 μM B[a]P, or 1.0 μM B[a]P plus 1.0 μM SAHA or TSA showed that B[*a*]P induced expression of CYP1A1 is reduced by approximately 68% when co-treated with TSA. B[*a*]P induced expression of CYP1B1 was reduced by approximately 46% when co-treated with SAHA, and 77% when co-treated with TSA (Figure 3-7).

When MCF-7 cells are co-treated with B[a]P and SAHA or TSA, no difference is noted in DNA binding

EROD activity principally measures CYP1A1 activity. Because CYP1A1 is involved in the metabolism of B[*a*]P, it was hypothesized that treatment with SAHA or TSA, which increase EROD activity, might also influence adduct levels. However, no statistical difference was noted between cells treated with 4 μM B[*a*]P, or cotreated with B[*a*]P and 1 or 2 μM SAHA (Figure 3-8). When DNA binding experiments were repeated to include 2 μM TSA, the DNA binding was not statistically different from that of B[*a*]P treatment alone (Figure 3-9). The concentrations of SAHA and TSA used in these experiments were not cytotoxic, as shown by MTT assay (Figure 3-10). The SAHA values were identical to those of DMSO.

DISCUSSION

In the present study, SAHA and TSA were found to induce EROD activity in intact MCF-7 cells. The ability of SAHA to induce EROD activity exceeded that of B[*a*]P. However, this effect was diminished in microsomal EROD assays. CYP1A1 and CYP1B1 mRNA were increased by SAHA and TSA treatment, Work by others [31] has also shown an increase in CYP1A1 mRNA in MCF-7 cells after TSA treatment. TSA has been shown to derepress CYP1A1 induction in human fibroblasts, allowing

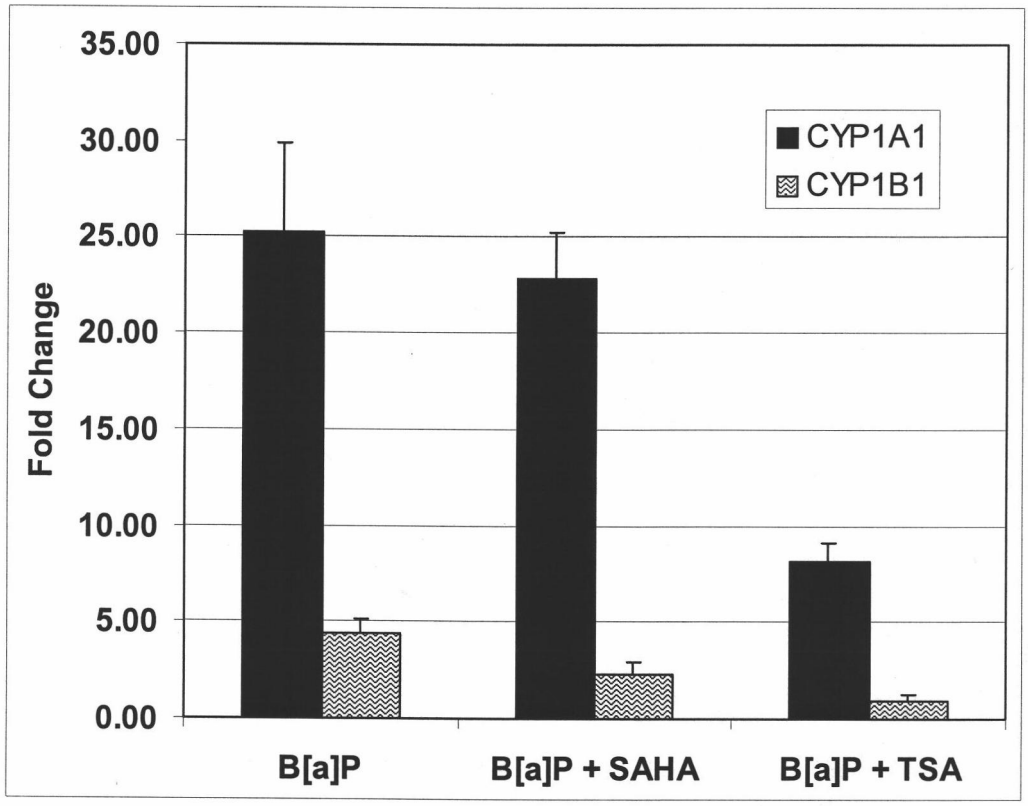

Figure 3-7. CYP1A1 and CYP1B1 expression after co-treatment with B[*a*]P and SAHA or TSA. MCF-7 cells were treated with 1.0 μM B[*a*]P, or 1.0 μM B[*a*]P and 1.0 μM SAHA or 1.0 μM TSA. The cells were harvested after 24 hours and probed for CYP1A1 and CYP1B1 expression by RT-PCR. Black bars represent CYP1A1 expression, while hatched bars represent CYP1B1.

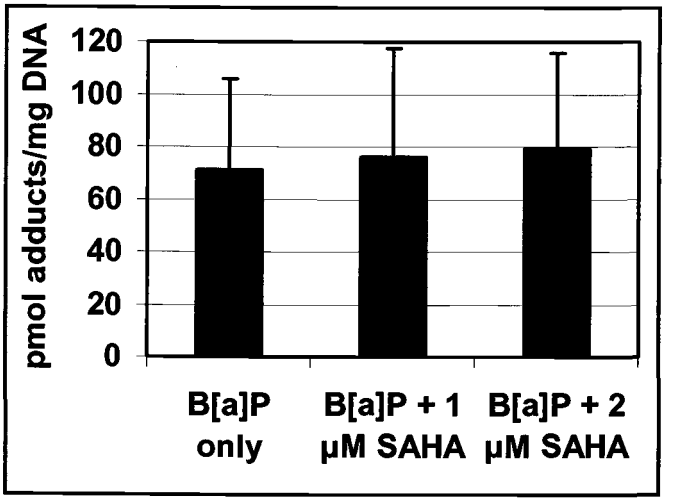

Figure 3-8: B[*a*]P DNA binding in MCF-7 cells co-treated with SAHA. MCF-7 cells were treated with 4 µM B[a]P or 4 µM B[*a*]P plus 1 or 2 µM SAHA. The cells were harvested after 24 hours and DNA was isolated from the cell pellets. The DNA was isolated, postlabeled and assayed for DNA binding using HPLC. Bars represent the average of three separate experiments, with error bars representing standard deviation.

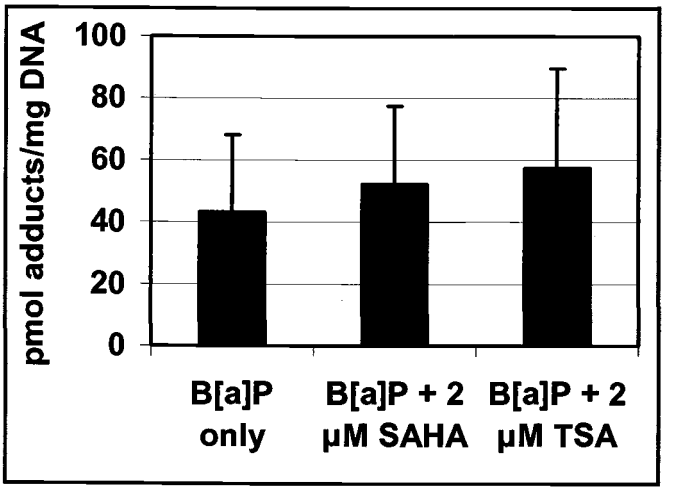

Figure 3-9: B[*a*]P DNA binding in MCF-7 cells co-treated with SAHA or TSA. MCF-7 cells were treated with 4 µM B[*a*]P, or 4 µM B[*a*]P plus 2 µM SAHA or 2 µM TSA. The cells were harvested after 24 hours. DNA was isolated, postlabeled and assayed for DNA binding using HPLC. Bars represent the average of three separate experiments, with error bars representing standard deviation.

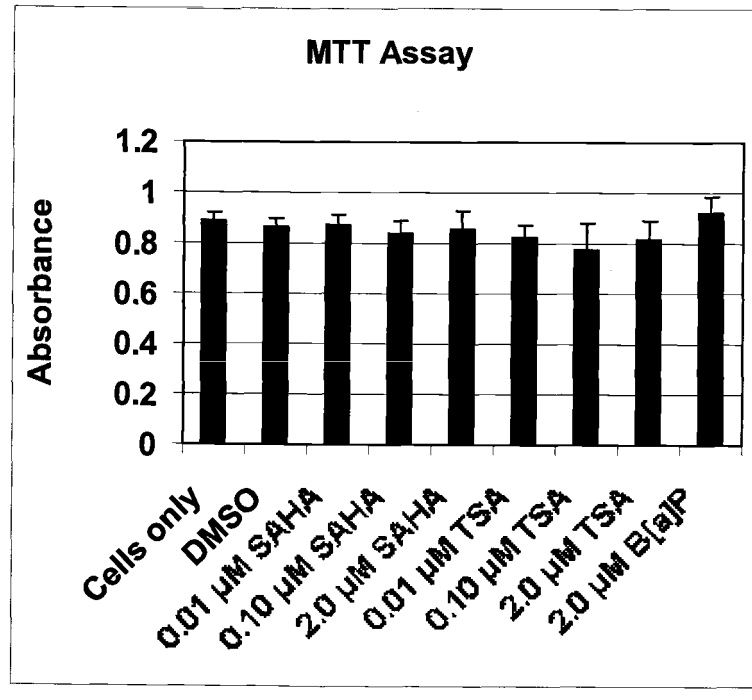

Figure 3-10: MTT assay of MCF-7 cells treated with SAHA, TSA, and B[*a*]P. MCF-7 cells in a 96 well plate were treated with varying concentrations of SAHA, TSA, and B[*a*]P. After 24 hours, MTT reagent was added to each well. After four hours, purple precipitate became visible and detergent reagent was added. After incubating overnight, the absorbance at 570 nm was compared. Six wells of each sample were examined. Error bars represent standard deviation.

expression in the presence of TCDD. Interestingly, that study, which assayed RNA blots, did not reveal TCDD independent induction of CYP1A1 [32].

In our work, a substantial difference was noted between induction of EROD activity by SAHA and TSA in intact cells, and microsomal EROD measurements and mRNA levels, relative to B[*a*]P. This may be due to inherent properties of these assays. The half life of CYP1A1 mRNA has been shown to be 2.4 hours in HepG2 cells [33], and CYP1A1 is known to be unstable. EROD measurements using intact cells may avoid issues of protein and mRNA degradation and more directly measure CYP1A1 activity. P450 induction by B[*a*]P acts through the AHR while SAHA and TSA act as HDAC inhibitors. The rise and fall of mRNA and protein levels and enzyme activity induced by AHR ligands may follow a different temporal pattern than that induced by HDAC inhibitors.

Activity of the murine AHR promoter has been shown to be increased by the HDAC inhibitors *n*-butyrate and TSA [34], indicating that expression of AHR is under epigenetic influence. While it is well established that AHR and ARNT contribute to the regulation of CYP1A1, and it has been demonstrated that in the presence of ligand, increased AHR and ARNT gene expression influence the inducibility of CYP1A1 [35], increased AHR expression does not by itself induce or increase CYP1A1. In intact cultured rat hepatocytes, TSA has been shown to increase the ability of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to induce CYP1A1 [36]. In our study, SAHA and TSA increased CYP1A1 activity and expression in the absence of a known ligand. These results, and those of others [31] suggest that CYP1A1 as well as AHR are epigenetically regulated in MCF-7 cells.

SAHA has been shown to upregulate thioredoxin-binding protein 2, and down regulate thioredoxin (Trx) [37]. Thioredoxin-1 (Trx-1) has been shown to regulate the constitutive expression of CYP1B1, and the inducible expression of CYP1A1 and CYP1B1 [38]. Trx-1 is one of a family of small redox proteins and has been shown to

be important in many human diseases, including cancer. While the induction of CYP1A1 and CYP1B1 may increase oxidative stress [39], Trx-1 acts as a reducing co-factor [40] and an antioxidant [41]. Trx may influence the DNA binding and transactivating activity of redox sensitive transcription factors including NF-κB, p53, AP-1, the glucocorticoid receptor, and AHR/ARNT [38]. The effect of SAHA and TSA on CYP1A1 and CYP1B1 may be mediated through Trx.

MCF-7 cells are estrogen responsive [42]. HDAC inhibitors have been show to differentially affect proliferation in cells that are estrogen receptor positive and estrogen receptor negative [43]. Estrogen receptor alpha (ER alpha) is the major form of estrogen receptor in breast cancer. However, loss of ER alpha is associated with aggressive cancer [44]. TSA has also been shown to induce reexpression of ER alpha in MCF-7 cells [45]. CYP1A1 and CYP1B1 are essential to estrogen metabolism, and there is evidence of cross talk between ER alpha and AHR regulated responses, including CYP1A1 inducibility. It is possible that ER alpha and CYP1A1 share common epigenetic regulatory mechanisms

To examine the effect of the elevated CYP1A1 activity we observed, we examined the ability of SAHA and TSA to influence B[*a*]P DNA binding. However, no meaningful difference in DNA adducts from the control was observed. Interestingly, when cells were co-treated with B[*a*]P and SAHA or TSA, CYP1A1 and CYP1B1 expression as measured by RT-PCR was reduced. CYP1A1 and CYP1B1 are pivotal enzymes in a network of pathways. Their induction *via* the AHR is accompanied by that of many other enzymes. It is possible that not only CYP1A1, but other detoxifying enzymes are influenced by HDAC inhibitors. Further research is necessary to understand how these two pathways of induction interact.

The role of genetics in cancer has long been recognized. Recent developments have highlighted the role of epigenetic misregulation in tumor onset and progression [46]. As these mechanisms are further elucidated, new strategies for the development of

chemotherapeutic treatments will be considered. HDAC inhibitors represent a diverse class of antineoplastic agents. They are of great interest because of their ability to suppress the growth of tumor cells *in vitro*, and reduce the growth of tumors in animal models. SAHA alone has been found to perturb cell cycle proteins [16,47], downregulate survival signaling pathways [48], disrupt the cellular redox state [49], and exhibit anti-inflammatory properties [50]. Our work is the first to show that CYP1A1 activity is increased when MCF-7 cells are treated with SAHA or TSA. We have also shown that SAHA and TSA reduce the expression of CYP1A1 and CYP1B1 induced by B[*a*]P. Because SAHA and other HDAC inhibitors are being investigated for their usefulness in preventing and treating cancer, it is critical that this effect be further examined. TSA is biotransformed rapidly by rat hepatocytes, likely involving cytochrome P450s [51]. If HDAC inhibitors used for clinical trials are transformed in the same manner, additional induction of P450s could expedite this metabolism, decreasing the amount of drug available. HDAC inhibitors appear to be promising chemotherapeutic agents, but further investigations of their roles both in cancer biology and carcinogenesis are warranted.

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Proteomic Analysis of MCF-7 Cells Treated with the Carcinogens Benzo[*a*]pyrene, Dibenzo[*a,l*]pyrene, Coal Tar Extract, and Diesel Exhaust Extract

Louisa Ada Hooven and William M. Baird[1]

Oregon State University, Corvallis, Oregon

[1]To whom correspondence should be addressed

Email: William.baird@orst.edu

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental contaminants which result from the combustion of organic materials. The association between exposure to these compounds and incidence of cancer in humans has been a subject of intense research. Benzo[*a*]pyrene (B[*a*]P) and dibenzo[*a,l*]pyrene (DB[*a,l*]P) are carcinogenic PAH, but in the environment occur in complex mixtures of compounds. While the other members of such mixtures may not be carcinogenic individually, they synergistically contribute to initiation and promotion of cancer. Mutations linked to PAH DNA adducts have been implicated in the carcinogenicity of these compounds, but the initiation and progression of cancer is also accompanied by multiple alterations in gene expression. We have used a proteomic approach to analyze protein expression changes induced in MCF-7 cells by B[*a*]P, DB[*a,l*]P, coal tar extract (SRM 1597) and diesel exhaust extract (SRM 1975). Samples from these cells were sequentially extracted into two fractions based on protein solubility and further separated using two-dimensional electrophoresis. The gels were analyzed using PDQuest. Spots of interest were excised and identified by MALDI-TOF-TOF. These results reveal both similarities and differences in the expression patterns of these compounds. Our results have shown alterations in protein expression of heat shock proteins, cytoskeletal proteins, DNA associated proteins, and glycolytic and mitochondrial proteins. The proteins that were universally increased in expression were tubulin alpha and myosin light chain alkali, cyclophilin B, heterogeneous ribonucleoprotein B1, a protein involved in access to telomerase and mRNA maturation, and alpha enolase. Additional proteins with altered expression include histone H2A.1, heat shock protein 70-2, galectin-3, nucleoside diphosphate kinase, ATP synthase, and electron transfer flavoprotein.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are a large group of ubiquitous environmental contaminants formed by the incomplete combustion of carbon compounds. Humans are routinely exposed to these compounds, which are found in sources such as automobile exhaust, tobacco smoke and coal tar. The correlation between human cancer and exposure to PAH has been studied since 1775, when Sir Percival Pott noted increased levels of scrotal cancer in chimney sweeps exposed to soot. Later animal studies have confirmed and detailed the association between exposure to sources of PAH and cancer [1]. Contemporary studies observe the relationship between cancer rates and polymorphisms in carcinogen metabolizing enzymes, as well as specific mutational fingerprints believed to be the result of the interaction of PAH with DNA [2,3].

Specific structural features of PAH are thought to contribute to carcinogenic activity. Many of the most potent carcinogenic PAH contain 4 to 6 aromatic rings and contain a bay or fjord region although this is not an absolute determination of carcinogenicity [4]. Structural models of various PAH bound to DNA have revealed how these bulky DNA lesions interact with the DNA helix and interfere with DNA replication, repair and translation [5-7]. Studying individual PAH has answered many metabolic and structural questions of how PAH metabolites bind to DNA and initiate mutations.

PAH are initially metabolized by cytochrome P450 enzymes. CYP1A1 has been implicated in the activation of many carcinogens and has been well characterized. CYP1B1, a more recent discovery, appears to be important in the metabolism of dibenzo[*a,l*]pyrene, a very potent carcinogen [8,9]. The charged and water soluble PAH metabolites formed by these enzymes may be excreted, or CYP1 enzymes may further oxidize the PAH. The resulting diol epoxides have been demonstrated to react with the exocyclic amino group of purine bases to form covalent adducts and result in mutational miscoding when the DNA is replicated [4,10].

A strong association exists between PAH DNA adducts and cancer incidence. However, evidence also exists which implies that additional processes mediated by PAH contribute to carcinogenesis. Some PAH have been found to bind readily to DNA, but are not carcinogenic. For example, the DNA binding of dibenzo[*a,l*]anthracene is greater than that of its carcinogenic isomer, dibenzo[*a,h*] anthracene, but is non-carcinogenic [11]. Benzo[*g,h,i*]perylene has been shown to bind to DNA in mouse skin, but demonstrates little or no carcinogenicity [12]. When the ability of B[*a*]P metabolites to cause tumors in mice is compared to the mutagenicity in V79 cells or the Ames test, a discrepancy is observed. The *anti* isomer of B[*a*]P 7,8-dihydrodiol-9,10 epoxide is mutagenic as measured in the Ames test, and is tumorigenic. However, the *syn* isomer of the same compound is also highly mutagenic, but not tumorigenic [13]. In general, bacterial mutagenicity data are poorly coordinated with cancer data [14]. These examples are representative of a body of evidence indicating that the ability of PAH to form DNA adducts is only one contributing factor to their carcinogenicity [15].

A major difficulty in studying PAH is their occurrence in complex mixtures. These mixtures may contain hundreds of compounds. The carcinogenic potential of the most abundant species in these mixtures is often well established. Summing these potentials is a method of calculating the risk of exposure to PAH mixtures. Unfortunately, the actual carcinogenicity, which is dependent on the route of exposure, often does not equal, and sometimes far exceeds calculated carcinogenicity [16]. When individual PAH are used as co-treatments, cocarcinogenic or anticarcinogenic effects have been observed. For example, when B[*a*]P is co-treated with dibenzo[*a,e*]pyrene, benzo[*g,h,i*]perylene, benzo[*e*]pyrene or fluoranthene [17,18] an increase in B[*a*]P DNA adducts is observed. However, benzo[*e*]pyrene is an anticarcinogen when co-treated with 7,12-dimethylbenz(*a*)anthracene (DMBA) [19]. These effects are thought to be the result of differential induction or competition for metabolizing enzymes.

CYP1A1 and CYP1B1 are induced when ligands such as B[*a*]P or TCDD interact with the aryl hydrocarbon receptor (AHR). This receptor pairs with the aryl hydrocarbon nuclear receptor translocator (ARNT) and travels to the nucleus, where it interacts with xenobiotic response elements (XRE) to regulate the expression of many genes [20]. Multiple downstream effects are induced through this pathway, which has exhibited crosstalk with hypoxia, estrogen receptor, and multiple signal transduction pathways [21]. Several of the enzymes induced *via* the AHR in addition to CYP1A1 and CYP1B1 help determine the fate of PAH. The balance of expression and activities of epoxide hydrolase (EH) and glutathione S-transferase (GSTM1) and other conjugating enzymes help determine whether PAH will form DNA binding metabolites or be excreted from the cell [22].

The products of PAH metabolism also influence gene expression. Hydroxy metabolites, epoxides, and quinones formed from PAH can be further metabolized to reactive oxidative species (ROS). The resulting perturbation in redox balance can induce genes responsive to the antioxidant response element (ARE), including enzymes associated with glutathione biosynthesis, redox proteins, and drug-metabolizing enzymes [23]. Many compounds which interact with the AHR also interact with the ARE. ROS can also contribute DNA damage, and both DNA adducts and ROS may explain the sister chromatid exchanges and chromosomal breakage that are evident after PAH treatment [24].

The multitude of gene expression changes following PAH treatment determine the metabolism of PAH to DNA damaging species. This damage is thought of as the initiation stage of carcinogenesis. Many of the same genes and effects are implicated in tumor promotion and further stages of carcinogenesis. Because a large portion of these effects are mediated by the AHR, the affinity of PAH with the AHR has been investigated as a more accurate measure of carcinogenic potency [14]. However, when one or several gene expression changes are compared to carcinogenicity, the

relationship is less clear. This indicates that focusing on selected changes in expression does not sufficiently describe the initiation/promotion processes induced by PAH.

Using developing technologies, several research groups have addressed this challenging problem of simultaneously observing global expression changes following treatment with PAHs. Rats and mice treated with B[*a*]P have been examined for altered gene expression using DNA microarrays [25,26]. While yielding some interesting results, the studies are limited in scope by the size of microarray [26] or the immense task of analyzing the data, and principally reported increases in P450s and GST. Studies using microarray technology [27] and RAGE, rapid analysis of gene expression [28] have looked at the effects of benzo[*a*]pyrene diol epoxide on gene expression in cells. The latter study, from the MacLeod lab, focused on the upregulation of DNA repair, cell cycle, and transcription factor genes. These high throughput methods, which analyse the levels of gene transcripts in the cell, are extremely powerful, although challenging to implement and interpret [29].

Instead of looking at variation in mRNA transcripts, proteomics employs a variety of technologies to study the protein complement of a given sample. Two dimensional electrophoresis (2DE) is a common approach for proteomic analysis. Complex protein mixtures are first separated by isoelectric focusing point (pI) and further separated by molecular weight using SDS-PAGE. The resolved proteins are excised from the gel and analyzed by mass spectroscopy. The application of 2DE has identified new potential biomarkers in cancer, and further elucidated the roles of existing cancer effectors [30,31]. In order to investigate the multiple effects of PAH on gene expression simultaneously, we have undertaken a proteomic analysis of MCF-7 cells treated with B[*a*]P, DB[*a,l*]P, and standardized extracts from coal tar and diesel exhaust.

In this work we have treated MCF-7 cells with B[*a*]P and DB[*a,l*]P, and DMSO. After dividing the protein samples by solubility, two dimensional gels were produced. The spots were detected and analyzed, and spots which differed significantly from the DMSO control were excised and analyzed by mass spectrometry. This process was repeated with extracts from two environmental PAH mixtures derived from coal tar and diesel exhaust. These mixtures were obtained from the National Institute of Standards and Technology. Our results have shown alterations in protein expression of heat shock proteins, cytoskeletal proteins, DNA associated proteins, and glycolytic and mitochondrial proteins.

METHODS

Cell culture

MCF-7 cells originated from human mammary carcinoma tissue [32], and were provided to the Baird lab by the Purdue University Cell Culture Laboratory. MCF-7 cells were grown in 1:1 F12 Nutrient Mixture and Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY), with 10% fetal bovine serum (Integren, Purchase, NY), 15 mM HEPES buffer, 14.3 mM $NaHCO_3$, 200 units/ml penicillin, 200 μg/ml streptomycin, and 25 μg/ml ampicillin. The cells were grown in T75 cm^2 flasks and maintained at 37°C with 5% CO_2. The cells were split 1:4 when they covered the surface area of the bottom of the flask.

Cell treatment with PAH

B[*a*]P and DB[*a,l*]P were obtained from Chemsyn Science Laboratories (Lenexa, KS). Standard reference material extracts of coal tar (SRM 1597) and diesel exhaust (SRM 1975) in toluene were obtained from the National Institute of Standards and Technology (Gaithersberg, MD). The toluene in which the mixtures were dissolved was evaporated under nitrogen and replaced with DMSO. MCF-7 cells were grown in 20 mls of F/D with 10% FBS, and treated with 1 μg/ml B[*a*]P, 0.01 μg/ml DB[*a,l*]P, 20 μg/ml SRM 1597, or 20 μg/ml SRM 1975 in DMSO, or DMSO alone (vehicle

control). The SRM 1597 dose contains 0.2 μg/ml B[*a*]P. The cells were exposed to these treatments for 24 hours.

Cell harvest

Medium was aspirated from two 75 cm^2 flasks of MCF-7 cells, which were then rinsed with 5 mls trypsin-versene. (0.05% Trypsin, 0.14 NaCl, 3mM KCl, 0.1 M Na_2HPO_4, 1.5 mM KH_2PO_4, and 0.5 mM EDTA) The cells were then incubated with another 5 mls of trypsin-versene until they became detached from the flask. Five mls of F/D medium with 10% fetal calf serum was then added to the flask. The cells were then decanted into a 50 ml centrifuge tube, and the flask rinsed with 10 mls PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4), which was added to the centrifuge tube. The cells were centrifuged at 2,000 rpm (478xg) in a bench top IEC HNSII centrifuge (International Equipment Co, Vernon Hills, IL). The supernatant was aspirated, and the pellet resuspended in 10 mls PBS, and centrifuged. The PBS was removed, and the pellet was again resuspended in PBS. After centrifugation and aspiration of supernatant, the resulting pellet was stored at -80°C.

Sample preparation and fractionation

8.5 M Urea was prepared by dissolving in water with gentle heating and addition of 5 g deionizing resin Bio-Rex 501-X8 (BioRad, Hercules CA) per liter, and filtering through a Whatman No. 1 paper. Multiple surfactant solution was prepared using this solution to a final concentration of 5 M urea, 2 M thiourea, 2 mM tributylphosphine, 2% CHAPS, 2% SB 3-10, 0.2% 3-10 carrier ampholytes (BioRad, Hercules CA), and 0.0002% Bromophenol blue. 200 μl 40 mM filtered tris with 0.1 μl endonuclease (Sigma, St. Louis MO) was added to cell pellet. The sample was sonicated for 5 seconds at level 4 on a Sonic Dismembrator (Fisher Scientific), and incubated at room temperature for 20 minutes. The sample was removed to a 1.5 ml centrifuge tube, and centrifuged at 15,000 g for 10 minutes. The supernatant was reserved as the "tris fraction" and immediately stored at -80°. The pellet was twice rinsed by vortexing in 500 μl 40 mM tris and centrifuged at 15,000 g for 10 minutes. The supernatant was

removed, and 200 µl "multiple surfactant solution" was added to the pellet. The pellet was sonicated at level 4 for 5 seconds, and centrifuged at 15,000 g for 10 minutes. The supernatant was reserved as "Multiple Surfactant fraction" and stored at –80°.

Protein quantification

5 µl of the tris samples, and 25 µl of the multiple surfactant samples were quantified in duplicate using the RCDC Protein Assay (BioRad, Hercules, CA) using the microfuge tube assay protocol. 125 µl of RC reagent I was added to each sample, which was vortexed and incubated for 1 minute. 125 µl Reagent II was added to each tube, which was vortexed, and centrifuged at 15,000 g for 5 minutes to precipitate the protein. The supernatant was aspirated, and the process repeated with 125 µl of RC reagent I and 40 µl of RC reagent II. The samples were inverted to drain. 127 µl Reagent A' was added to each tube, which was vortexed to redissolve protein. The samples were held at room temperature for 5 minutes before vortexing again and adding 1 ml of reagent B. After vortexing, the samples were incubated at room temperature for 15 minutes and the absorbance read at 750 nm. Pre-diluted bovine serum albumin protein assay standards were used as standards. (Pierce, Rockford, IL)

IPG strip rehydration and isoelectric focusing

150 µg of protein was brought to a volume of 185 µl in multiple surfactant solution. 11 cm pH 3-10 nonlinear IPG strips (BioRad, Hercules, CA) were rehydrated overnight with this sample solution at room temperature in disposable rehydration trays. The strips were covered in mineral oil to prevent dehydration. The strips were loaded in a Protean IEF isoelectric focusing cell (BioRad, Hercules CA) and covered in mineral oil. Hydrated electrode wicks were mounted over the electrodes. The strips were conditioned at 250 V for 30 minutes at 20°C to remove excess salts. The voltage was then ramped to 8,000 V over 2 ½ hours, and maintained at 8,000 V for an additional 3.5 hours. After focusing the voltage was held at 500 V until the strips were removed to a fresh rehydration tray and immediately stored at –80°C.

Equilibration and SDS-PAGE

SDS-PAGE Equilibration base buffer was composed of 6 M Urea, 0.375 M tris pH 8.8, 2% SDS, and 20% glycerol. 2% dithiothreitol w/v was added to one portion, and 2.5% w/v of iodoacetamide was added to another portion. Four ml of the SDS-PAGE equilibration buffer with DTT was added to each strip, and the strips were shaken for 15 minutes. The buffer was removed and replaced with 4 ml of SDS-PAGE equlibration buffer with iodoacetamide, and shaken for 15 minutes. The strips were dipped several times in SDS-PAGE running buffer, composed of 25 mM tris, 192 mM glycine, and 0.1% sodium dodecyl sulfate. The strips were mounted over an 8-16% Criterion pre-cast IPG + 1 acrylamide gel and covered in ReadyPrep overlay agarose (BioRad, Hercules, CA). 3 μl of Precision Plus unstained molecular weight standards (BioRad) was loaded in the small well of the gel. 50 V was applied to the gels for 30 minutes, and then 200 V for 65 minutes at 4°C. The gels were shaken gently in 75 mls gel fixative (10% methanol, 7% acetic acid) for 45 minutes at room temperature, rinsed with ultrapure water (Millipore, Bedford MA) and placed in 75 ml Sypro Ruby gel stain (BioRad, Hercules, CA). After shaking gently overnight, the stain was replaced with gel fixative and shaken for 45 minutes. The gels were rinsed and stored in deionized water at 4°C until they were imaged.

Imaging and analysis

Gels were imaged using a Model 1000 VersaDoc imaging system (BioRad, Hercules, CA). Multiple exposures were recorded directly in PDQuest format. The gels were stored at 4°C in ZipLoc® bags in a small volume of 0.005% sodium azide. A matchset was created of triplicate gels of each sample. Automated spot detection and matching was initially used, with additional manual refinement. Analysis sets were created of spots that varied quantitatively greater than 1.5 fold or less than –1.5 fold from the DMSO control. Additional sets were formed of spots found to vary in expression according to a Student's t-test, with a significance level of 90%. The intersection of these sets formed a spot set which was further reduced by elimination of low intensity spots, spots near the edges of gels, and artifactual spots.

Spot cutting and digesting

Spots were manually excised with a #11 Xacto blade, using an ultraviolet light box to illuminate the Sypro Ruby fluorescent stain. The spots were placed in 500 µl 100 mM ammonium bicarbonate, and vortexed for 10 minutes. The solution was replaced with 500 µl acetonitrile, and vortexed for 10 minutes. This process was repeated, and the gel pieces evaporated to dryness in a SpeedVac. The gel pieces were rehydrated for 45 minutes in 50µl ice cold trypsin solution composed of 20 µg sequencing grade trypsin (Promega, Madison WI) in 760 µl 100mM ammonium bicarbonate. The trypsin solution was removed, replaced with 50µl ammonium bicarbonate, and the mixture incubated overnight at 37°C. The digestion liquid was collected. 50 µl 0.1% trifluoroacetic acid in ammonium bicarbonate was added to the gel pieces and the mixture vortexed for 15 minutes. This rinse step was repeated. The digestion liquid and rinse solutions were pooled and dehydrated by SpeedVac, then rehydrated in 5 µl 0.1% TFA in water. ZipTips (Millipore, Bedford MA) were used, following the manufacturers instructions, to desalt the samples in preparation for mass spectrometry.

Mass spectrometry

The spot digests were mixed in a 1:2 ratio of a-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA. Molecular mass analysis was performed by matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry using an Applied Biosystems ABI4700 TOF/TOF mass spectrometer (Applied Biosystems Incorporated, Framingham, MA) with an accelerating voltage of 20 kV. Data were always acquired in the MALDI reflector mode using internal calibration standards of the following monoisotopic molecular masses: desArg^1 bradykinin, 904.47; angiotensin I, 1296.69; Glu^1-fibrinopeptide B, 1570.68, ACTH (clip1-17), 2093.09; ACTH (clip 18-39), 2465.20; ACTH (clip 7-38), 3657.93 Da as supplied by Applied Biosystems. Data in the collision induced decay (CID) mode were calibrated using fragment ions from Glu^1-fibrinopeptide B. CID MS/MS spectra were generated by acceleration of

precursor ions at 8 kV and selection with a timed gate with a window of ± 5 Da. Gas pressure (air) in the CID chamber was set at 1 μTorr. Fragment ions were further accelerated by 14 kV prior to entry into the reflector. Data collection and analysis were performed using the Data Explorer software package also supplied by Applied Biosystems (Framingham, MA).

RESULTS

MCF-7 cells were treated with DMSO, B[*a*]P, DB[*a,l*]P, coal tar extract, and diesel exhaust extract. 24 hours later the cells were harvested. The samples were divided into two fractions by solubility. The first fraction was proteins soluble in 40 mM tris. The second fraction consisted of proteins soluble in a multiple surfactant solution. After 2DE, and imaging, the gels were analyzed in four sets. The first two sets consisted of the tris and multiple surfactant solution fractions of DMSO, B[*a*]P, and DB[*a,l*]P treated samples. These spot numbers have the prefix BT and BU, respectively. The second two sets consisted of the tris and multiple surfactant solution fractions of DMSO, coal tar extract, and diesel exhaust extract, and these spots numbers have the prefix MT and MU.

A matchset was created of triplicate gels of each sample. Automated spot detection and matching was initially used. Approximately 1,500 spots were detectable in each gel, and the software parameters were modified so that low intensity spots were neglected and approximately 700 spots were used in analysis. The matching was further refined manually with particular attention to the 300 most intense spots. The mean correlation of variance between these sets was 42.6. Analysis sets were created of spots that varied quantitatively greater than 1.5 fold or less than –1.5 fold from the DMSO control. Additional sets were formed of spots found to vary in expression according to a Student's t-test, with a significance level of 90%. The intersection of these sets formed sets composed of an average of 118 spots (Figure 4-1 through 4-4). The number of spots was further reduced by eliminating low intensity and artifactual

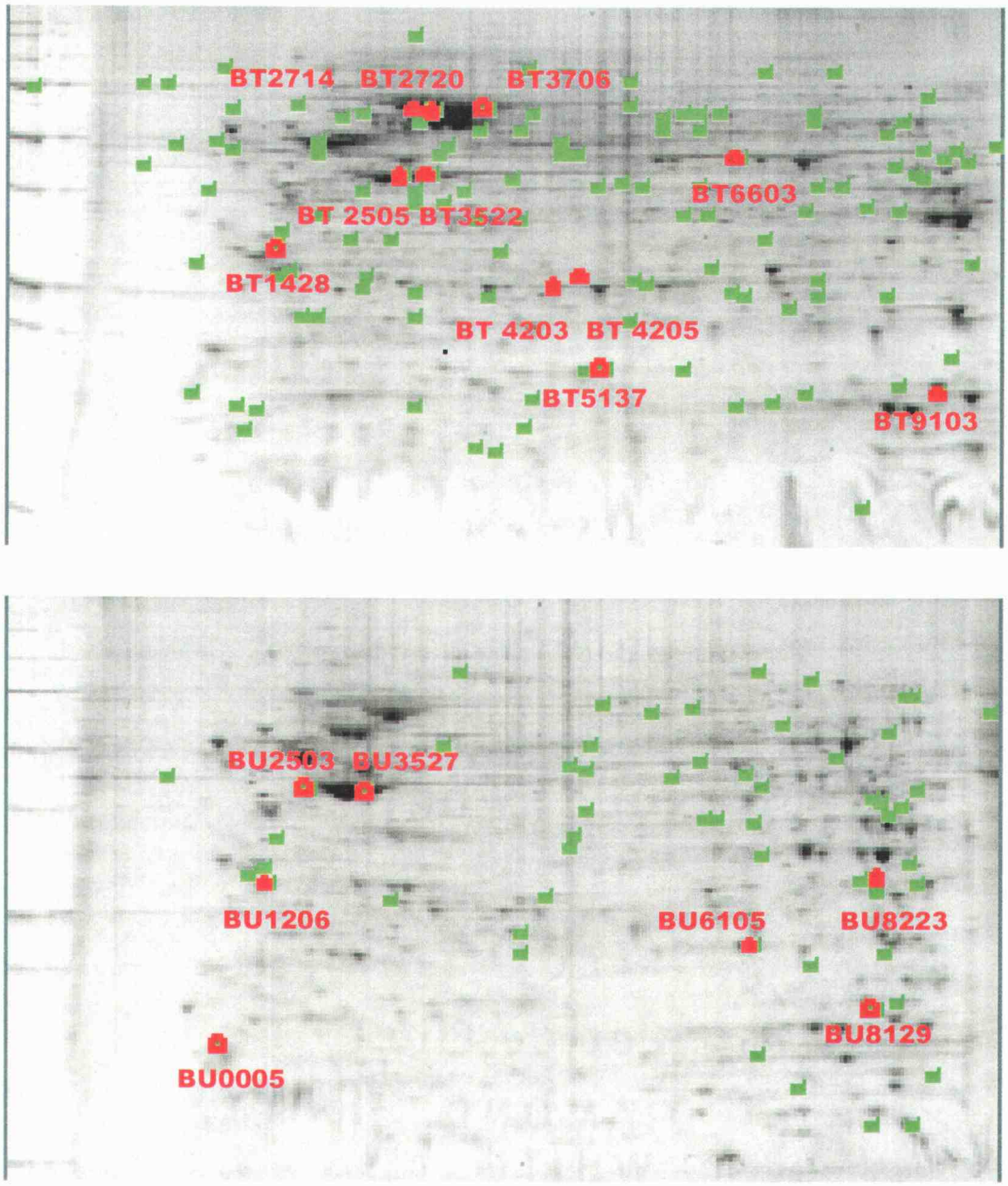

Figure 4-1. Representative raw 2DE gel images from the tris fraction (top) and multiple surfactant fraction (bottom) of the DMSO/B[*a*]P/DB[*a,l*]P matchsets.

Spots marked with green are spots selected using statistical and quantitative analysis sets in PDQuest. After eliminating artifactual and low intensity spots, spots of interest were excised from gels and submitted to MALDI-TOF-TOF. The spots marked in red are those that were identified by mass spectroscopy. They are listed in Table 4-1. Proteins from the tris gel set are prefixed with BT, and those from the multiple surfactant fraction BU when referred to in the text and tables.

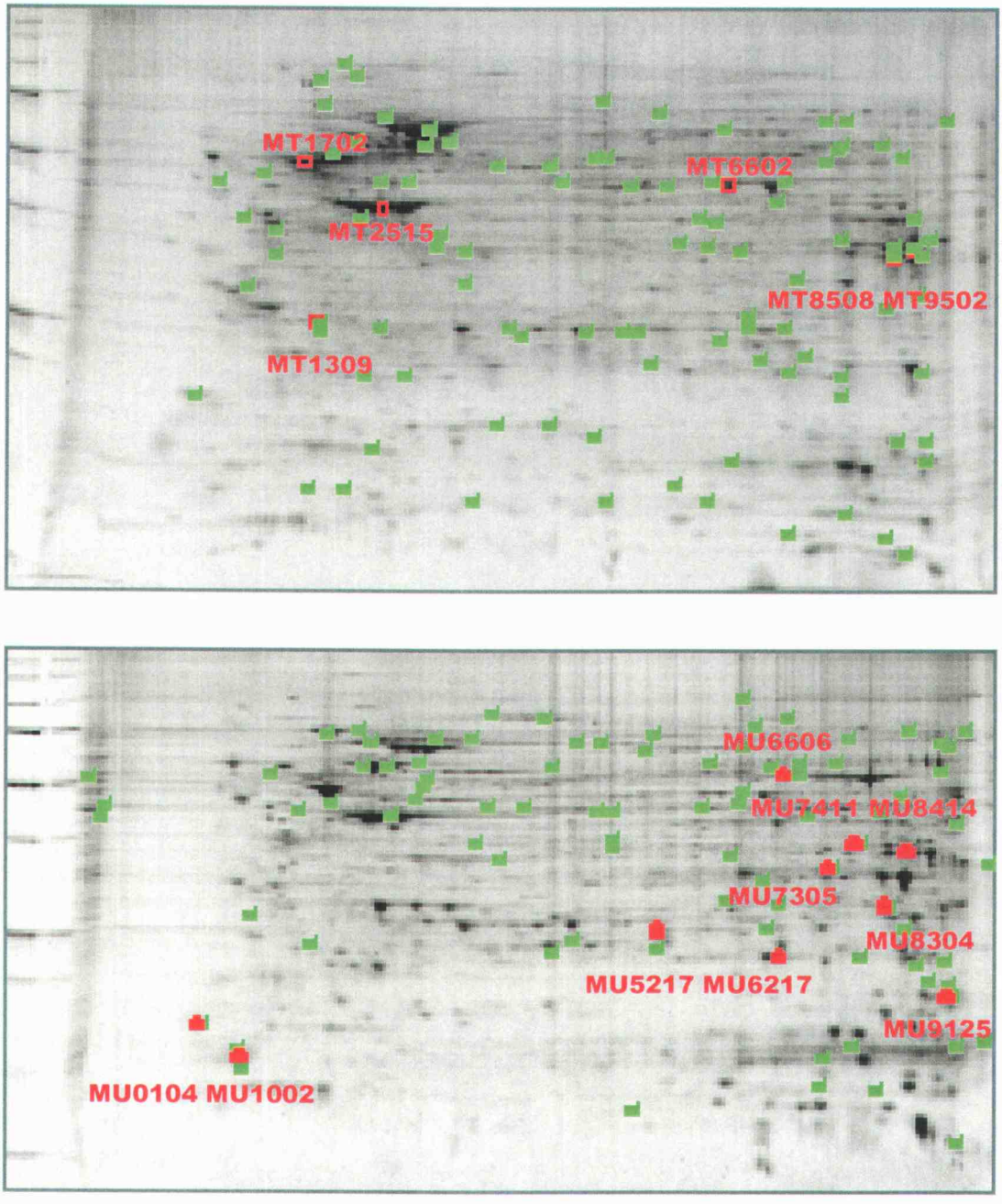

Figure 4-2. Representative raw 2DE gel images from the tris fraction (top) and multiple surfactant fraction (bottom) of the DMSO/coal tar extract/diesel exhaust extract matchsets.

Spots marked with green are spots selected using statistical and quantitative analysis sets in PDQuest. After eliminating artifactual and low intensity spots, spots of interest were excised from gels and submitted to MALDI-TOF-TOF. The spots marked in red are those that were identified by mass spectroscopy and are listed in Table 4-2. Proteins from the tris gel set are prefixed with MT, and those from the multiple surfactant fraction MU when referenced in text and tables.

spots resulting from dust. A number of spots that were found to be increased or decreased in treated samples were selected, excised, and submitted to mass spectrometry. The proteins that were identified are marked on Figures 4-1 and 4-2 and listed in Tables 4-1 (B[*a*]P/DB[*a,l*]P) and 4-2 (coal tar and diesel exhaust).

To clarify these results, the resulting proteins were grouped according to functional association. A number of spots were identified as cytoskeletal proteins, including keratin and actin. While these proteins are sometimes found as artifacts in 2DE, the spots we identified varied statistically from the DMSO control when analyzed in three separate gels (Figure 4-3 and 4-4). One spot identified as actin gamma increased 8 fold in DB[*a,l*]P compared to DMSO. Tubulin alpha and beta chains and myosin were increased in all samples, while cofilin expression was reduced in B[*a*]P and DB[*a,l*]P samples (Figure 4-5). Four proteins associated with signal transduction were also identified. Nucleoside diphosphate kinase expression was increased in all samples, while galectin-3 was increased in all samples but diesel exhaust. Rho GDP dissociation inhibitor increased 1.67 fold in coal tar and calmodulin increased 2.14 fold in coal tar (Figure 4-6). Five heat shock proteins (HSP) showed altered expression. HSP 70-2 was increased greater than 3 fold in B[*a*]P and DB[*a,l*]P samples. HSP 70 increased in DB[*a,l*]P and coal tar. The expression of HSP 27 varied between adjacent spots, increasing in response to DB[*a,l*]P in one spot, while being reduced in response to coal tar in another. Cyclophilin B was increased in all samples (Figure 4-7). Three spots were identified as heterogeneous ribonucleoprotein B1 (hnRNP B1), two in multiple surfactant fractions and one in the tris fraction, indicating that multiple forms or modifications of this protein may exist. The first spot was increased 2.40 and 4.16 fold in B[*a*]P and DB[*a,l*]P, while the second showed increased expression in coal tar (3.14) and diesel exhaust samples (3.03 fold). The third spot increased in intensity in response to diesel exhaust (2.64 fold). DHFR was elevated in B[a]P treated samples, and histone H2A.1 was elevated in DB[*a,l*]P and diesel exhaust samples (Figure 4-8). Seven spots correlated with proteins involved in glyclolysis and the electron transport chain. Alpha enolase increased significantly in all samples in one spot, and increased in

Table 4-1. Identified proteins in DMSO/B[*a*]P/DB[*a,l*]P gels.
Three separate experiments were performed in which MCF-7 cells were treated with DMSO, B[*a*]P, or DB[*a,l*]P. The resulting samples were fractionated by solubility. 2DE gels were generated from these samples. After imaging and analysis, spots of interest were excised, digested, and submitted to MALDI-TOF-TOF. Spots with a prefix of BT are spots from the fraction soluble in 40 mM Tris. Spots with a prefix of BU are spots from the fraction soluble in multiple surfactant solution. Fold change represents the average ratio of spot intensity of the sample compared to the average correlating spot in DMSO samples.

TABLE 4-1 Identified proteins in DMSO/B[*a*]P/DB[*a,l*]P gels			Fold change	
spot	**protein**	**accession number(s)**	**B[*a*]P**	**DB[*a,l*]P**
BT-1428	Actin gamma	ATHUB	2.89	1.56
BT-2505	Actin gamma	ATHUB	1.24	2.02
BT-2712	Tubulin alpha	AAH04949	3.13	2.24
BT-2714	Keratin, type II cytoskelatal 1 (Cytokeratin 1)	K2C1_HUMAN	1.85	2.29
BT-2720	AF134726 NID -- Hsp 70-2	AAD21815	3.41	4.26
BT-3522	Actin gamma	ATHUB	2.30	2.34
BT-3706	Heat-shock 70kd protein fragment	1HJOA	0.76	1.83
BT-4205	Heat shock protein 27	HHHU27	1.21	1.76
BT-4203	Heat shock protein 27	HHHU27	0.63	0.84
BT-5137	Nucleoside-diphosphate kinase	A33386	1.43	3.38
BT-6603	Alpha enolase	ENOA_HUMAN	2.15	2.00
BU-0005	Myosin light chain alkali, non-muscle isoform	MLEN_HUMAN	2.25	2.16
BU-1206	Keratin, type II cytoskelatal 1 (Cytokeratin 1)	K2C1_HUMAN	1.53	1.29
BU-2503	Keratin 19, type I cytoskeletal	KRHU9	1.97	1.20
BU-3527	Actin gamma	ATHUB	4.91	8.06
BU-6105	Dihydrofolate reductase	DYR_HUMAN	2.12	0.63
BU-7004	Histone H2A.1	HSHUA1	1.26	2.71
BU-8129	Cofilin	SI2632	0.49	0.64
BU-8223	Galectin-3 carbohydrate recognition domain	1A3K	2.27	2.76
BU-8418	Heterogeneous nuclear ribonucleoprotein B1	B34504	2.40	4.16

Table 4-2. Identified proteins in DMSO/coal tar extract/diesel exhaust extract gels.

Three separate experiments were performed in which MCF-7 cells were treated with DMSO, standardized coal tar extract, or standardized diesel exhaust extract. The resulting samples were fractionated by solubility. 2-DE gels were generated from these samples. After imaging and analysis, spots of interest were excised, digested, and submitted to MALDI-TOF-TOF. Spots with a prefix of MT are spots from the fraction soluble in 40 mM Tris. Spots with a prefix of MU are spots from the fraction soluble in multiple surfactant solution. Fold change represents the average ratio of spot intensity of the sample compared to the average correlating spot in DMSO samples.

TABLE 4-2 Identified proteins in DMSO/coal tar/diesel exhaust gels			Fold change	
spot	protein	accession number(s)	Coal Tar	Diesel
MT-1309	Rho gdp dissociation inhibitor alpha, chain E	1CCOE	1.67	1.39
MT-1702	Tubulin beta chain	UBHU5B	2.33	1.22
MT-2515	Actin gamma	ATHUB	2.79	0.54
MT-6602	Alpha enolase	ENOA_HUMAN	1.50	1.77
MT-8404	Heterogeneous nuclear ribonucleoprotein B1	B34504	0.91	2.64
MT-8508	Glyceraldehyde-3-phosphate dehydrogenase	DEHUG3	2.02	0.86
MT-9502	Keratin, type II cytoskeletal 1	K2C1_HUMAN	0.30	1.08
MU-0104	Calmodulin	MCHU	2.14	1.27
MU-1002	H+ transporting two-sector ATPase	S22348	0.98	1.11
MU-5217	Homo sapiens ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	AAH00649	1.66	1.31
MU-6217	Dihydrofolate reductase	DYR_HUMAN	1.28	1.28
MU-6606	H+ transporting two-sector ATPase	PWHUA	1.19	2.94
MU-7305	Voltage-dependent anion channel	Q9BWK8	0.79	0.38
MU-7411	Keratin, type II cytoskelatal 1 (Cytokeratin 1)	K2C1_HUMAN	2.02	1.43
MU-8304	Electron transfer flavoprotein	1EFVB	2.16	1.89
MU-8414	Heterogeneous nuclear ribonucleoprotein B1 - human	B34504	2.46	3.03
MU-9125	Peptidylprolyl isomerase	CSHUB	3.14	3.04

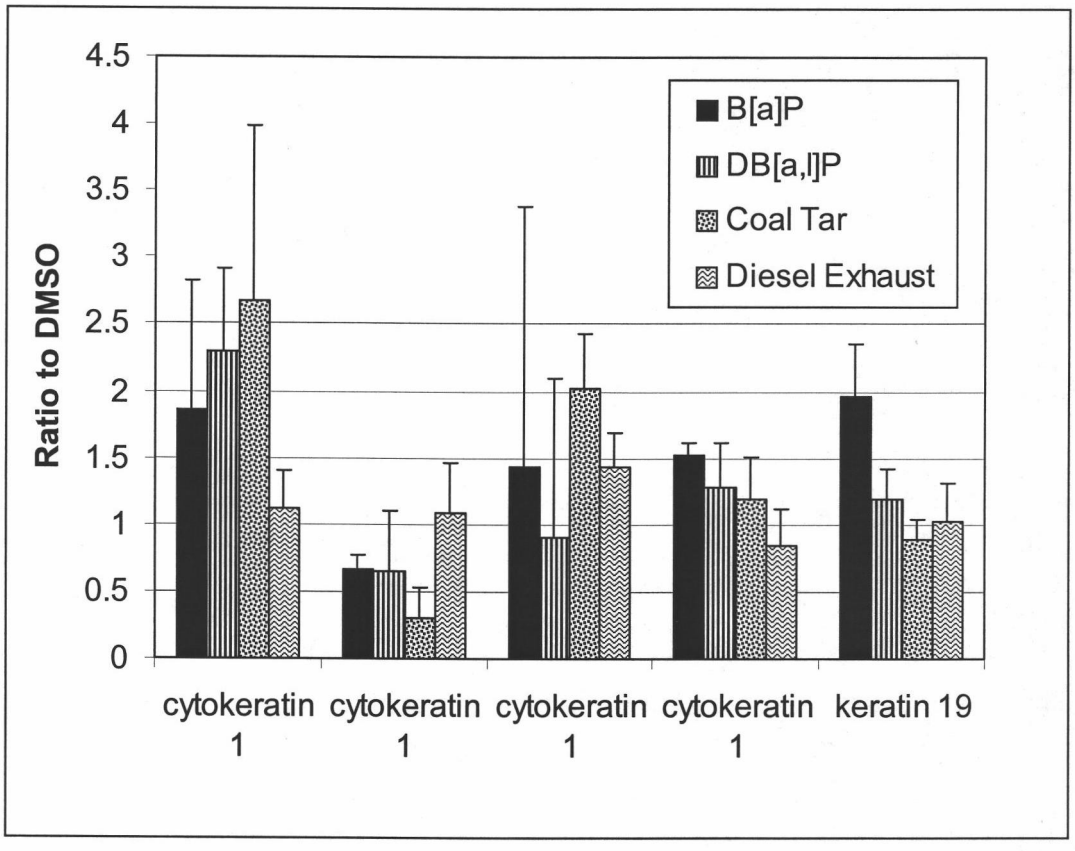

Figure 4-3 Changes in spot intensity of proteins identified as keratin. From right to left, the spot numbers correlating to the matchset the spots were identified in are BT-2714, MT-9502, MU-7411, BU-1206, and BU-2503. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

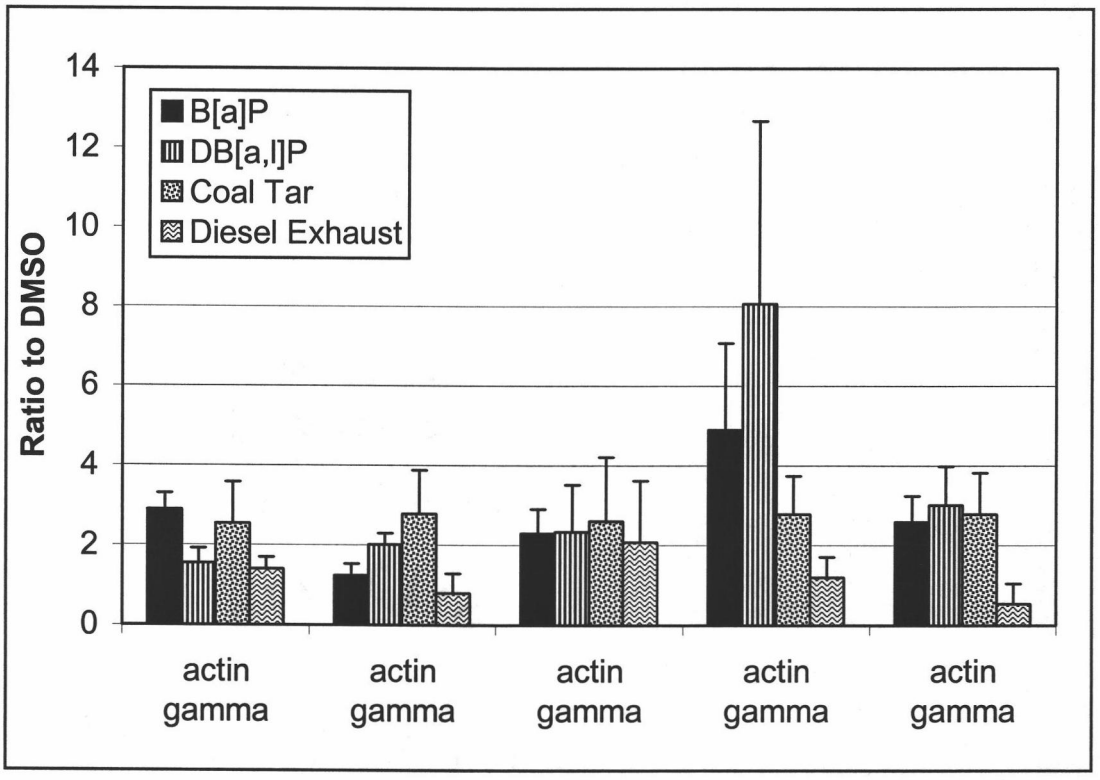

Figure 4.4. Changes in spot intensity of proteins identified as actin.
From right to left, the spot numbers correlating to the matchset the spots were identified in are BT-1428, BT-2505, BT-3522, BU-3527, and MT-2515. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

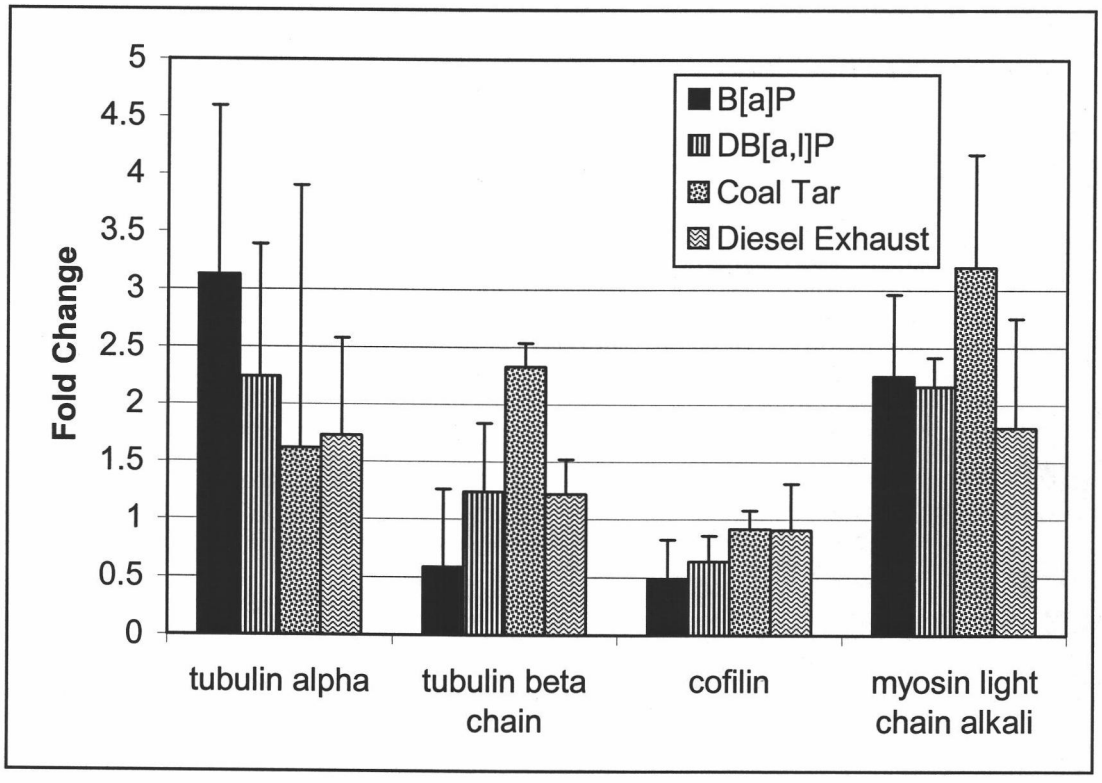

Figure 4.5 Changes in spot intensity of cytoskeletal proteins.
From right to left, the spot numbers correlating to the matchset the spots were identified in are BT-2712, MT-1702, BU-8129, and BU-0005. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

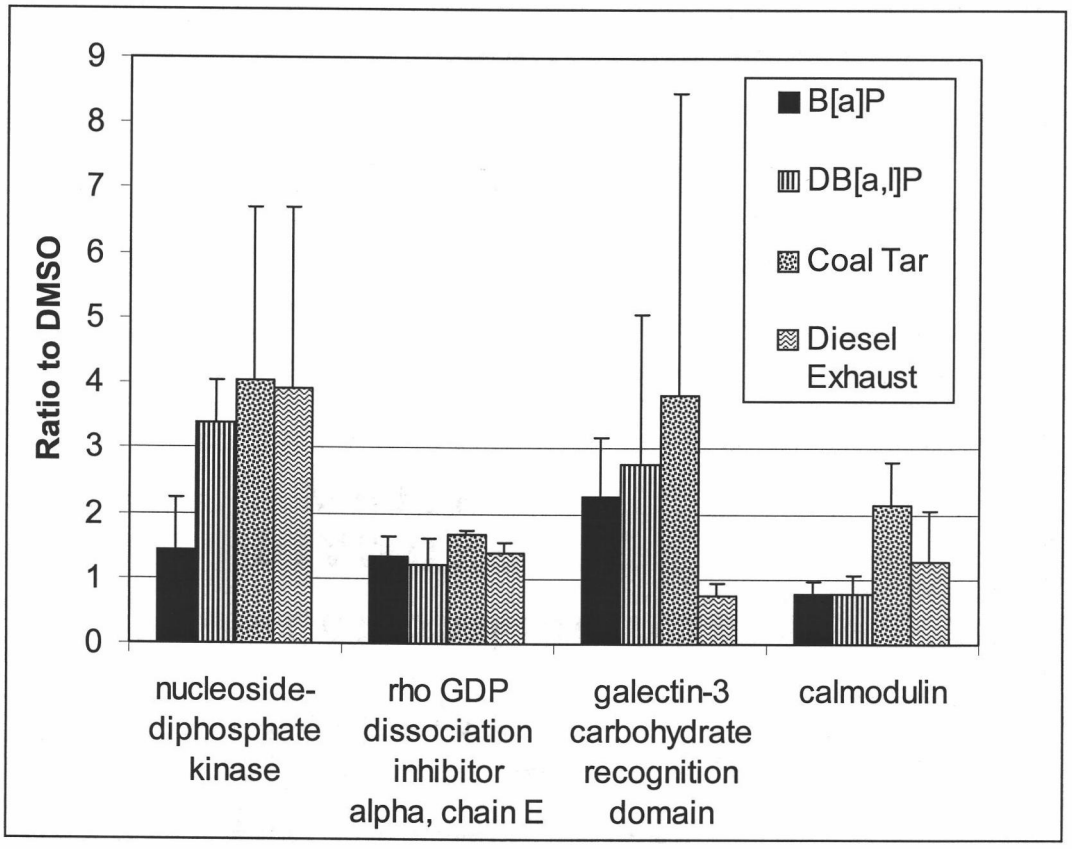

Figure 4.6 Changes in spot intensity of signal transduction proteins.
From right to left, the spot numbers correlating to the matchset the spots were identified in are BT-5313, MT-1309, BU-8223, and MU-0104. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

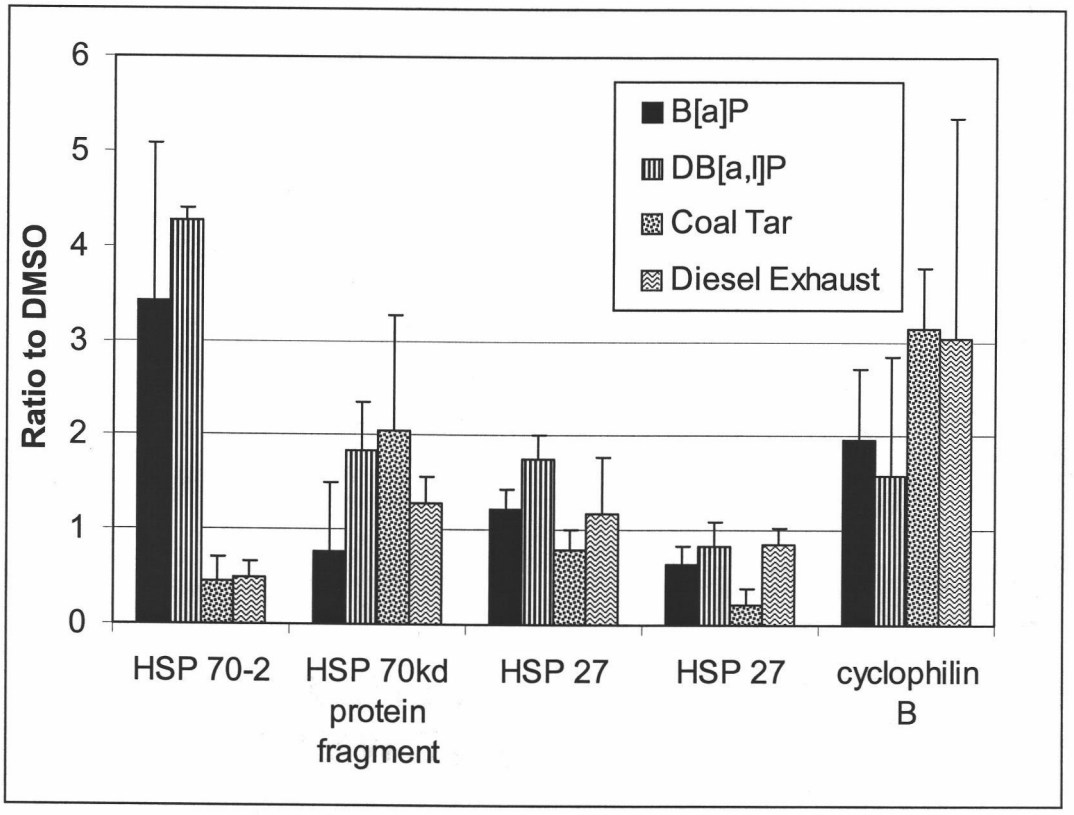

Figure 4.7 Changes in spot intensity of heat shock/chaperone proteins.
From right to left, the spot numbers correlating to the matchset the spots were identified in are BT-2720, BT-3706, BT-4203, BT-4205, and MU-9125. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

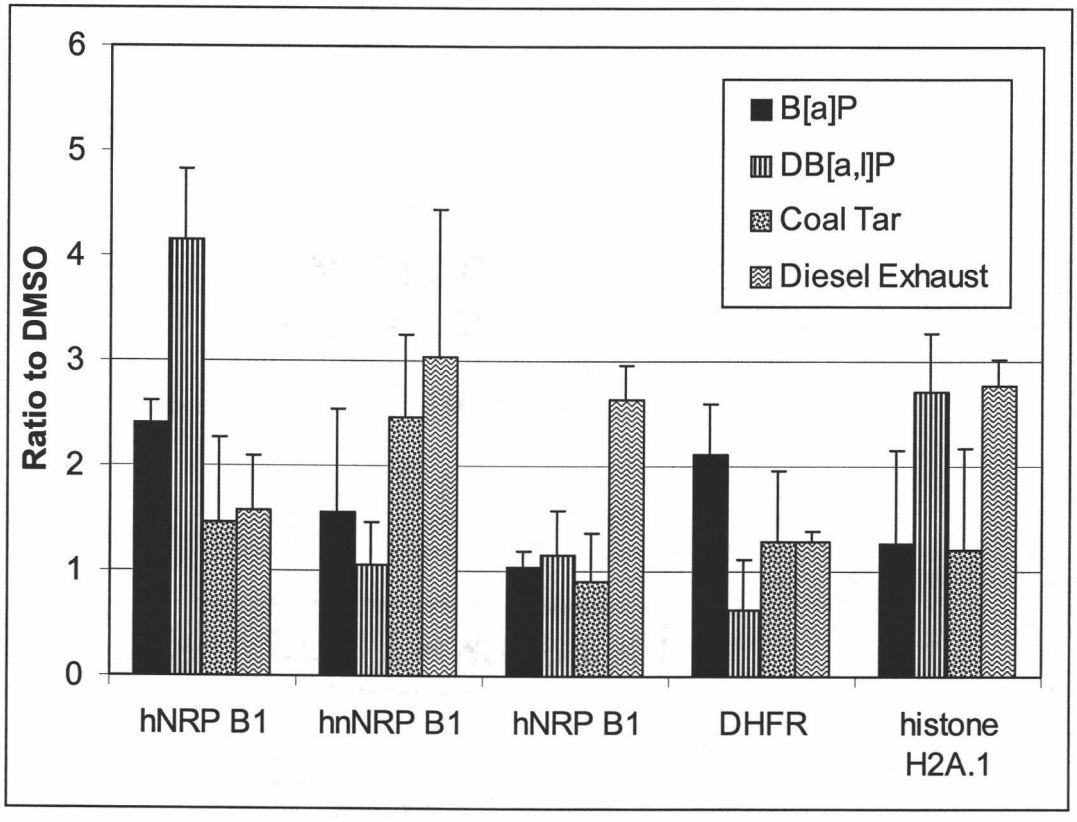

Figure 4.8 Changes in spot intensity of DNA associated proteins.
From right to left, the spot numbers correlating to the matchset the spots were identified in are BU-8418, MU-8414, MT-8404, BU-6105, and BU-7004. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

coal tar and diesel exhaust samples in another spot. GAPDP1 increased in expression in B[*a*]P, DB[*a,l*]P, and coal tar samples (Figure 4-9). Proteins associated with the electron transport chain including ATP synthase, cytochrome *c* reductase, voltage dependent anion channel, and electron transfer flavoprotein were principally increased overall (Figure 4-10).

Expression patterns were compared between B[*a*]P, DB[*a,l*]P, coal tar, and diesel exhaust. The proteins that were universally increased in expression were tubulin alpha and myosin light chain alkali, cyclophilin B, hnRNP B1, and alpha enolase. Additional proteins that exhibited altered expression included H2A.1, HSP 70-2, galectin-3, nucleoside diphosphate kinase, ATP synthase, and electron transfer flavoprotein. The ratio of these changes to the DMSO control may be found in Tables 4.1 and 4.2.

DISCUSSION

An immense body of literature has accumulated over many decades describing the effects of PAH in carcinogenesis. This work ranges from structural details of PAH-DNA binding, elucidation of PAH metabolism and characterization of the enzymes involved, to epidemiological studies relating cancer incidences to exposure and population differences. Most research is restricted to a single aspect of PAH induced carcinogenesis. Carcinogenesis in general is not completely understood, and reviews are published periodically discussing various models for this complex process [13,33,34]. In order to investigate the multiple effects of PAH on gene expression simultaneously, we have undertaken a proteomic analysis of MCF-7 cells treated with B[*a*]P, DB[*a,l*]P, and standardized extracts from coal tar and diesel exhaust. Our results have shown alterations in protein expression of heat shock proteins, cytoskeletal proteins, DNA associated proteins, and glycolytic and mitochondrial proteins.

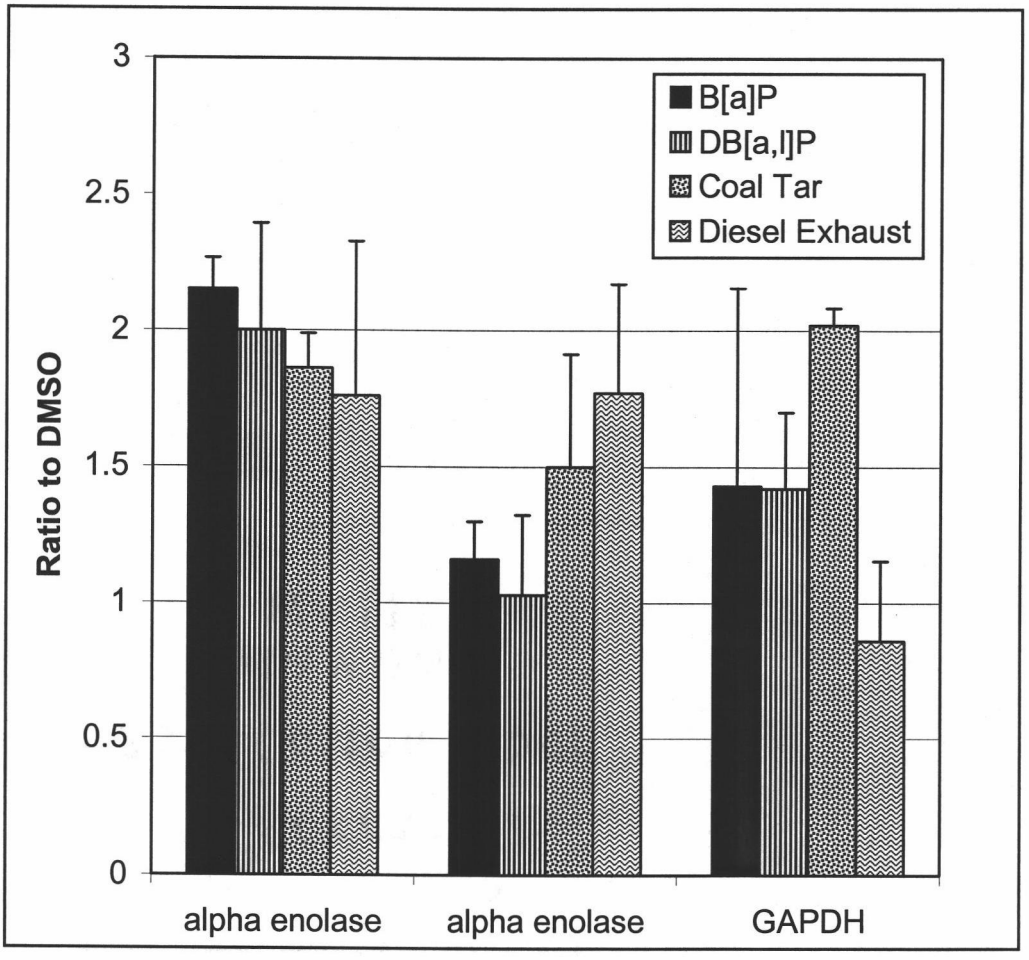

Figure 4.9. Changes in spot intensity of glycolytic proteins.
From right to left, the spot numbers correlating to the matchset the spots were identified in are BT-6603, MT-6602, and MT-8508. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

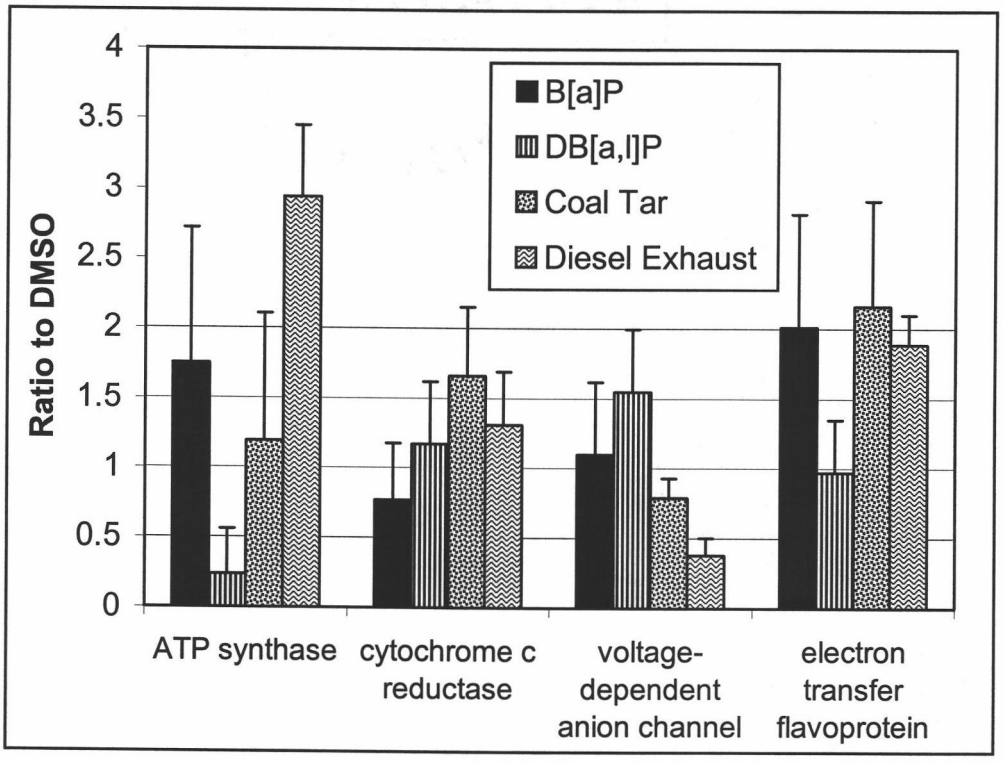

Figure 4.10 Changes in spot intensity of proteins associated with mitochondria.

From right to left, the spot numbers correlating to the matchset the spots were identified in are MU-6606, MU-5217, MU-7305, and MU-8304. At least one of the samples for each spot increased or decreased significantly compared to DMSO control sample as measured by a Student's t-test with a confidence level of at least 90%. Error bars indicate standard deviation.

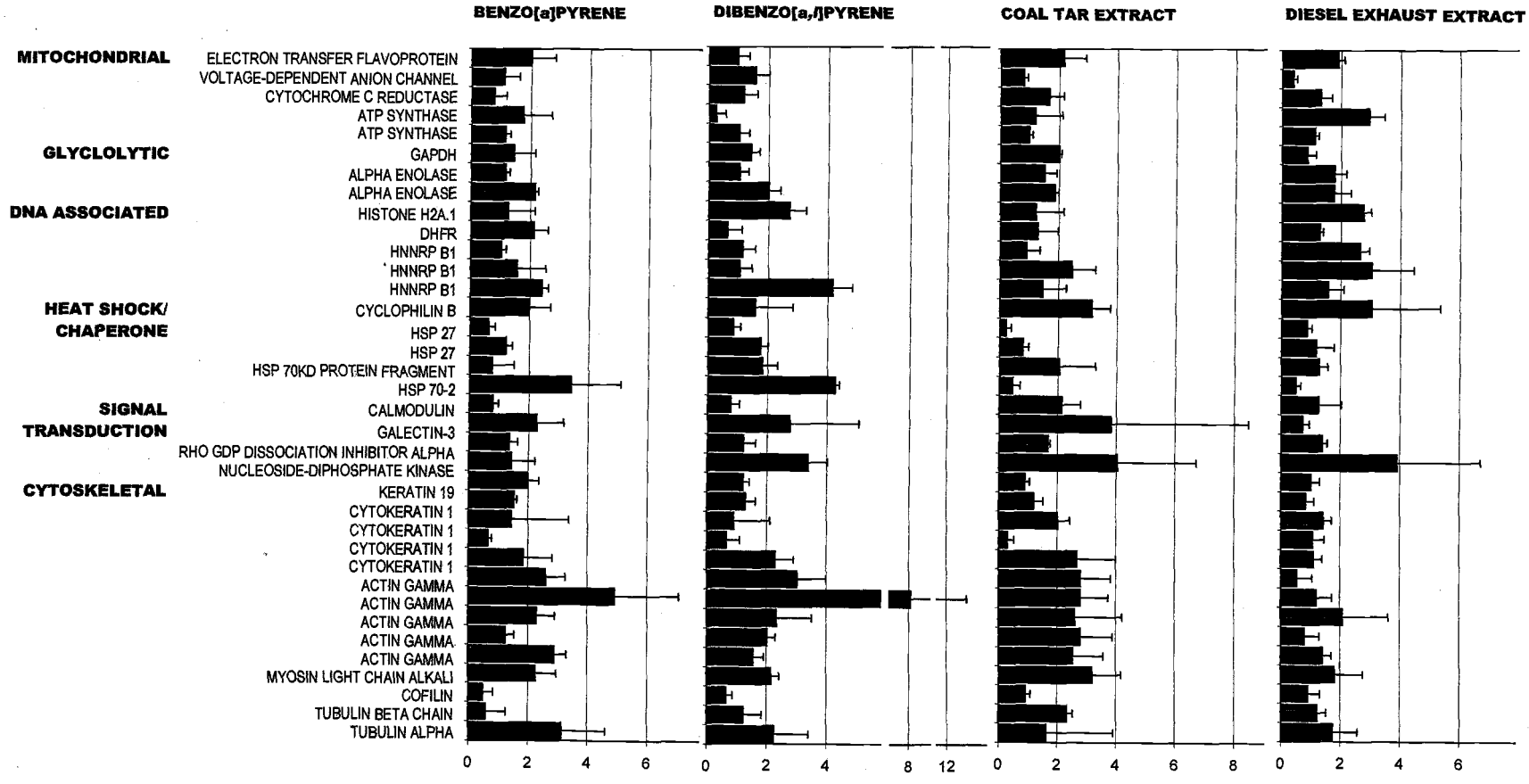

Figure 4-11. Comparison of proteins identified in B[*a*]P, DB[*a,l*]P, coal tar extract, and diesel exhaust extract.
Bars represent ratio to DMSO. Proteins are grouped according to functional association. In some cases, more than one spot was identified as a given protein.

Other research groups, using DNA arrays, have treated rodents and cells with B[*a*]P and Methylchrysene (MC) [26,35]. These efforts suffer from some limitations. Proteins which are already known to be involved in xenobiotic metabolism are chosen as members of the DNA array chip. The results reported in both these papers focus on well known P450s and GSTs. This limits opportunities to discover pathways not previously implicated in carcinogenesis. A limitation of DNA array work is that only RNA transcripts are measured, instead of the final protein product. The regulation of RNA translation is not completely understood, and likely varies with each transcript.

2DE analysis of proteomic changes of course also has its own limitations. Small proteins, large proteins, and low abundance proteins are difficult to resolve. So while this method asks a broader question than do DNA array approaches, it measures the tip of the iceberg. For example, CYP1A1 and CYP1B1, which are known to be expressed in response to B[*a*]P and other PAH, are not seen in our proteomic analysis. Others have compared the ability of 2DE and 1DE to detect these proteins in microsomes. 2DE was much less efficient at resolving these proteins than 1DE [36]. In our work, we have not enriched these proteins by isolating microsomes, but rather have fractionated our samples by solubility. Thus it is no surprise that we did not detect P450s. We have, however, identified a number of proteins that change in expression in response to PAH treatment. Grouping these proteins by function reveals interesting trends.

Cytoskeletal proteins

The expression of cytoskeletal proteins contributes to cellular organization and differentiation, and their disregulation is associated with tumor metastasis. Cytoskeletal proteins help to maintain cell shape and enable intracellular transport. Cytoskeletal reorganization also occurs during apoptosis. Intermediate filaments, such as keratins, are thought to act as signal and/or energy transducers between the extracellular matrix and cell nucleus and may contribute to the regulation of gene expression. The inappropriate expression of keratins has been observed in the mouse skin tumor

promotion model [37-39], and in human cancers [40]. B[*a*]P has been shown to alter keratin expression in rabbit bladder epithelium [41]. Perturbation of the actin/myosin cytoskeleton correlates with higher proliferation rates and uncontrolled movement. [42]. Cofilin participates in actin filament turnover and cytoskeletal reorganization [43]. Microtubules are composed of alpha and beta tubulin, and participate in multiple cellular functions and have become a target of cancer chemotherapy [44]. In our work, the expression of various forms of keratin, actin, myosin, and tubulin, are altered, and principally upregulated, depending upon the treatment. Cofilin levels are reduced in cells treated with B[*a*]P and DB[*a,l*]P. Changes in cytoskeletal proteins are the molecular basis for morphological changes. The cellular complement of structural proteins is under scrutiny as a biomarker of cancer [45]. The alterations seen in this research could be the result of cells apoptosing as a result of interactions with PAH metabolites [46], or indicative of changes in cell phenotype very early in cellular transformation. Further study of how PAHs induce these changes and how they affect associated cellular processes is warranted.

Signal transduction proteins

The components of the cytoskeleton are dynamic, and interact with many proteins. Small GTPases such as Rho contribute to the regulation of the microfilament network, which in turn affects cell-cell adhesion and malignant transformation [47]. Rho guanine nucleotide dissociation inhibitor alpha (Rho GDI), was particularly elevated by coal tar in our studies. Rho GDPases have been observed to be overexpressed in human tumors [47], and Rho GDI acts as a negative regulator of Rho GTPase. Rho GDI has been shown to modulate estrogen receptor transcriptional regulation by this action [48].

Nucleoside diphosphate kinases (NDPK) were originally designated as housekeeping enzymes required for the synthesis of nucleoside triphosphates. Additional activities of these enzymes affects many cellular processes including cell growth and differentiation [49]. Nm23, an NPDK, can act as both a GTPase-activating and a guanine nucleotide

exchange factor for Rad, another small GTPase protein of unknown function. Paradoxically, while we saw a significant increase in expression of Nm23 in all samples, in other work it is associated with reduced tumor metastasis [50].

Rho and Rad are believed to participate in signal transduction similarly to Ras. The Ras gene has long been known to be susceptible to B[*a*]P induced mutations, and to be activated by B[*a*]P. Calmodulin, a ubiquitous Ca^{2+} binding protein, participates in the phosphorylation/dephosphorylation cycle of proteins, participating in the regulation of cellular metabolism, cytoskeletal organization, cell proliferation, and apoptosis. Ca^{2+} levels participate in the cycling of Ras [51], and have been found to be increased after B[*a*]P treatment [52]. Calmodulin levels were found to be increased by coal tar extract in our studies, and may contribute to the actions of differentially expressed signaling proteins we have observed.

Galectins are carbohydrate binding proteins which also modulate cell adhesion and regulate cell growth. Galectin-3 was found to be upregulated in all our samples except those treated with diesel exhaust extract. Galectin-3 has been found to translocate to the mitochondria following apoptotic stimuli, and inhibit cytochrome *c* release. This explains the role of galectin-3 in inhibiting apoptosis [53].

The differential expression of proteins from multiple signaling pathways by PAH is observed. These changes could be the indirect result of DNA damage, the downstream effects of PAH metabolites, or direct interaction of PAH with receptors involved in these pathways. These possibilities could be further explored by systematically examining the effects of PAH on proteins further upstream in these signaling pathways.

DNA associated proteins

Dihrodrofolate reductase (DHFR), which was significantly upregulated in B[a]P treated samples, has long been of interest in cancer research. Inhibiting DHFR with

methotrexate reduces the rate of DNA synthesis. An amplification independent increase in DHFR has been noted in carcinogen treated cells by others [54]. The increase in DHFR is perhaps in response to increased need for DNA synthesis and cell replication. Expression in DB[*a,l*]P treated cells is reduced, and only slightly elevated in samples treated with complex mixtures.

The synthesis of of histones is coupled to DNA synthesis. The complement of histones and histone subtypes has been shown to vary between tissues, cell types, and carcinomas [55]. In fetal tissues, the ratio of H2A-1 to H2A-2 is greater than in adult tissue, but cannot be correlated to cell growth rate. The expression of the H2A.1 gene has been shown to be under the control of the transcription factor E2F, which is important in the expression of genes important to DNA synthesis and cell cycle progression [56]. Histone H2A.1 was particularly increased in cells treated with DB[*a,l*]P and diesel exhaust.

Heterogeneous nuclear ribonucleoprotein (hnRNP) levels were increased in all samples, particularly in B[*a*]P and DB[*a,l*]P treated cells. This protein is found to be elevated in cancers and cell lines derived from cancers [57,58]. HnRNP is an RNA-binding protein required for mRNA maturation. HnRNP is also implicated in promoting telomerase access to telomeres by interacting with telomeres. Telomeres are repetitive elements at the ends of human chromosomes which are normally shortened with each cell division. Reactivation of telomerase is essential for carcinogenesis.

Studies in MCF-7 cells treated with hydrocarbon carcinogens has revealed that cells do not arrest in G1 phase, but accumulate in S phase with damaged DNA [59]. The increase in levels of proteins associated with DNA synthesis observed in our work may be the result of this phenomenon. This inappropriate response to DNA damage may help to explain the carcinogenicity of PAH, but further study is needed to understand the mechanism behind this response.

Chaperones

Heat shock proteins (HSP) function as molecular chaperones in response to cellular stress. Their expression level can determine the fate of the cell in the face of potentially lethal conditions. HSP 27 and HSP 70 inhibit apoptosis, and may participate in carcinogenesis [60]. The overexpression of these proteins can increase the tumorigenic potential of tumor cells. Two spots in our work were identified as HSP 70-2, or an HSP 70 fragment. The first was found to be elevated in B[*a*]P and DB[*a,l*]P samples, and reduced in samples treated with PAH mixtures. The second was increased in DB[*a,l*]P and coal tar. HSP 70 has been found to be responsive to a wide variety of xenobiotics, and may provide a useful marker for toxicant exposure [61]. HSP 27 was identified in two adjacent spots, indicating that protein modifications affecting charge may be differentially affected by treatment. In the first, levels were significantly increased in samples treated with B[*a*]P, and in the second spot, all samples showed decreased levels. The levels of another chaperone protein were universally increased in our studies. A peptidyl-prolyl isomerase, cyclophilin B, acts as a cochaperone with HSP 40 in the endoplasmic reticulum (ER). These proteins are members of a large ER complex associated with incompletely folded immunoglobulin heavy chains. This complex can bind to unfolded proteins in the ER, rather than assembling onto substrate proteins [62]. While differential expression of chaperones is not unexpected in response to xenobiotic treatment, because of the influence of these proteins on apoptotic outcome, they are worth examination. The function of cyclophilin B in PAH-mediated carcinogeneis in particular is not immediately evident.

Mitochondrial proteins

In solid tumors, elevated expression of subunits of the mitochondrial electron chain may reflect a mitochondrial response to perturbation in cellular energy requirements. Such alterations may be a general characteristic of cancer cells [63]. In our work we have noted various levels of expression changes in ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1, mitochondrial ATP synthase, voltage dependent

anion channel, and electron transfer flavoprotein chain E. B[*a*]P is associated with mitochondrial dysfunction [64]. The ratio of BPDE adducts in mitochondrial DNA has previously been shown to be 40 to 90 times greater than that in nuclear DNA [65,66], and most cancer cells contain mitochondrial DNA mutations [67]. These mutations can initiate a cascade of events leading to persistent oxidative stress [68]. Interestingly, while B[*a*]P has been shown to stimulate nuclear DNA synthesis, mitochondrial DNA synthesis was inhibited [69]. Our results and those cited here point to multiple effects of PAH on mitochondria.

Glycolytic proteins

The ability of cancer cells to overproduce lactic acid was observed by Otto Warburg in London more than 70 years ago. The Warburg effect is one of the most universal effects observed in solid tumors [70]. Increased levels of two proteins involved in glycolysis, Alpha enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were observed in our work. In addition to being a glycolytic enzyme, alpha enolase is a multifunctional enzyme that may serve as a cell surface plasminogen receptor, and has recently been found to play a role in autoimmune disorders. It also serves as a heat-shock protein and binds cytoskeletal and chromatin structures [71]. GAPDH, often thought of as a "housekeeping gene" is found to be upregulated in several forms of cancer [72,73]. GAPDH has been implicated in DNA replication and repair, nuclear RNA export, membrane fusion, microtubule bundling, and phosphotransferase activity [74]. GAPDH transcription and activity has been found to be elevated in response to TCDD [75]. Increased expression of glycolytic enzymes in tumors has recently been shown to be modulated by the transcription factors c-MYC and Hypoxia-inducible factor -1 (HIF-1) [70]. HIF-1 interacts with ARNT, which is also the transcription factor partner for AHR.

Differences in expression between MCF-7 cells treated with individual PAH compounds and complex environmental mixtures containing PAH

Our work has revealed altered expression of a number of proteins in response to treatment by B[*a*]P, DB[*a,l*]P, and standard reference material extracts of coal tar and diesel exhaust. Many of these proteins have not previously associated with PAH induced carcinogenesis. However, many have been identified as being elevated in tumors. The existing expression analyses of tumors using DNA arrays and proteomics exceeds that examining initial carcinogenesis, and is informative about later stages of cancer. To understand the initiating events of carcinogenesis in a human system, our work used the human breast carcinoma cell line MCF-7. Expression patterns were compared between B[*a*]P, DB[*a,l*]P, coal tar, and diesel exhaust. The proteins that were universally increased in expression were tubulin alpha and myosin light chain alkali, cyclophilin B, hnRNP B1, and alpha enolase. Additional prominently altered proteins include H2A.1, HSP 70-2, galectin-3, nucleoside diphosphate kinase, ATP synthase, and electron transfer flavoprotein. Our proteomic analysis attempted to assay expression changes in a very large population of proteins. Our findings should be followed with more focused proteomic studies of mitochondrial, nuclear, or other cellular protein fractions.

Benzo[*a*]pyrene and dibenzo[*a,l*]pyrene are known carcinogens, but only laboratory animals are routinely exposed to such pure compounds. However, it is useful to compare them to mixtures in order to simplify the understanding of PAH induced carcinogenesis and metabolic mechanisms. The other components of environmental PAH mixtures may also be singly tested for genotoxicity, tumorigenicity, and metabolism in animals, cells, and other models. These results may or may not be useful, because different models may yield disparate answers or may not agree with human epidemiological studies. Coal tar has been examined many times in the laboratory for tumorigenicity, yet only individuals who are subjected to prolonged industrial exposure to coal tar may suffer increased incidences of cancer. Coal tar is a component of treatments for dandruff and psoriasis, but it is not established that it is

carcinogenic in this context. Diesel exhaust has demonstrated an ability to induce cancer in the lab, but its actual ability to cause cancer in humans remains under investigation. It is evident that the effects of PAH are very complex and multidimensional.

This work sought to look beyond DNA damage and tumorigenesis and observe subtle changes in protein expression. Each of these compounds bore some similarities in induced expression changes to each other, as well as differences. B[*a*]P has been intensely studied for its role in carcinogenesis, and serves as a baseline comparison for the expression changes induced by PAH. Interestingly, while DB[*a,l*]P was applied to cells in a hundredth the concentration of B[*a*]P, the difference in expression in some proteins is much larger. DB[*a,l*]P, is a weaker agonist of the AHR pathway than B[*a*]P, and so must be inducing these changes through a different pathway or pathways. Some of these changes are observed in the other treatments as well. Coal tar appears to in particular affect cytoskeletal proteins. Diesel exhaust yields perhaps the most interesting spectrum of expression changes, in particular in proteins that have been classified here as mitochondrial, glycolytic, and DNA associated. These expression changes beg many questions. How would these studies in human breast cancer cells compare to cells from other tissues or animal models? How might they affect the health of humans and other organisms environmentally exposed to PAH mixtures? Are these transient expression changes, or are the cells permanently altered? This work demonstrates that the study of the effects of PAH on human health is far from complete.

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CONCLUSION

The development of cancer is conceptualized as occurring in phases, beginning with initation by mutagenesis. If mutations occur in critical genes involved in growth control, a cell may be transformed, progressing towards malignancy. Cancer initiation is itself a complex process, and may be broken down into several phases. The process begins with the metabolism of the carcinogen, which can then adduct to DNA. The resulting bulky DNA lesions are repaired, or interfere with polymerase fidelity and are fixed as mutations. The metabolizing enzymes present at the time of exposure determine the fate of the carcinogen. Carcinogens and their metabolites can induce changes in enzyme levels. These changes may influence PAH metabolism, or may contribute to subsequent stages of carcinogenesis. The work described in this thesis attempts to further characterize alterations in protein expression by polycyclic aromatic hydrocarbons (PAH), a class of carcinogens, as well as enzymatic metabolism of PAH.

The association between exposure to PAH and human cancer has been recognized for centuries, and a large body of research exists which examines this relationship. [1,2] Benzo[*a*]pyrene (B[*a*]P) was among the first carcinogenic PAHs isolated, and was found to be a complete carcinogen when painted repeatedly on mouse skin. Later it was observed that B[*a*]P is metabolized into diol epoxides which may form covalent adducts with DNA. Elevated exposures to PAH correspond with increased risk of lung, skin, and bladder cancer, and may contribute to breast cancer incidence [3,4]. In the case of lung cancer, patterns of G→T transversion hotspots could be observed which correlated with preferential PAH binding sites in the p53 gene. Mutations in p53 are found in approximately 60% of human lung cancers [5]. These findings were thought to constitute a mutational "fingerprint" of PAH carcinogenesis, and agree with the multi-hit model of carcinogenesis.

Specific structural features of PAH are thought to be important to their carcinogenic activity. Many of the most potent carcinogenic PAH contain 4 to 6 aromatic rings and contain a bay or fjord region although exceptions have been observed [6]. Structural

models of various PAH bound to DNA have revealed how these bulky DNA lesions interact with the DNA helix and interfere with DNA replication, repair and transcription [7-9]. PAH adducts may also interfere with nucleosome formation and structure, affecting the organization of DNA [10].

Cytochrome P450s are a large family of enzymes which are responsible for the metabolism of xenobiotics, steroid hormones, and other compounds. Cytochrome P450 1A1 (CYP1A1) has been implicated in the activation of many carcinogens and has been well characterized. CYP1B1, a more recent discovery, appears to be important in the metabolism of dibenzo[*a,l*]pyrene (DB[*a,l*]P), a very potent carcinogen [11,12]. The highly reactive PAH metabolites formed by these enzymes can be excreted from the cell, or CYP1 enzymes may further oxidize the PAH. The resulting diol epoxides have been demonstrated to react with the exocyclic amino group of purine bases to form covalent adducts and result in mutational miscoding when the DNA is replicated [6,13].

Many additional enzymes are known to be involved in the pathway to tumorigenesis. Conjugating enzymes contribute to detoxification of PAH, including GSH transferase, UDP glucuronyl transferase, epoxide hydrolase, and methyl transferase. To a lesser degree, PAH may also be activated by prostaglandin synthase, lipoxygenase, one electron oxidation, and other pathways. Aside from diol epoxides, additional products of PAH metabolism also influence gene expression. Hydroxy metabolites, epoxides, and quinones formed from PAH can be further metabolized to reactive oxidative species (ROS) [14]. ROS can also contribute to DNA damage, and also induce genes *via* the antioxidant response element (ARE), including enzymes associated with glutathione biosynthesis, redox proteins, and drug-metabolizing enzymes [15]. Many PAH-induced effects are mediated by the AhR, and the affinity of PAH with the AhR has been investigated as a more accurate measure of carcinogenic potency [16]. However, when one or several gene expression changes are compared to carcinogenicity, the relationship is less clear.

Some PAH have been found to bind readily to DNA, but are not carcinogenic. For example, Benzo[*g,h,i*]perylene has been shown to bind to DNA in mouse skin, but demonstrates little or no carcinogenicity [17]. The DNA binding of dibenzo [*a,c*]anthracene is greater than its carcinogenic isomer, dibenzo[*a,h*] anthracene, but is non-carcinogenic [18]. The *anti* and *syn* isomers of B[*a*]P 7,8-dihydrodiol-9,10 epoxide are both mutagenic as measured in the Ames test, but only one is tumorigenic [1]. In general, bacterial mutagenicity data are poorly coordinated with cancer data. [16]. These examples are representative of a body of evidence indicating that the ability of PAH to form DNA adducts is only one contributing factor to their carcinogenicity.

In the Baird lab, we have observed that relative to B[*a*]P, a PAH mixture derived from coal tar forms few DNA adducts, yet is more tumorigenic in mouse skin. This is a trend which may be observed in other mixtures [19]. Environmental PAH mixtures may contain hundreds of compounds. The carcinogenic potential of the most abundant species in these mixtures is often well established. Summing these potentials or using complex statistical models are methods of calculating the risk of exposure to PAH mixtures. Unfortunately, the actual carcinogenicity often does not equal, and sometimes far exceeds calculated carcinogenicity [20]. When individual PAH are used as co-treatments, cocarcinogenic or anticarcinogenic effects have been observed. For example, when B[*a*]P is co-treated with dibenzo[*a,e*]pyrene, benzo[*g,h,i*]perylene, benzo[*e*]pyrene or fluoranthene [21,22], an increase in B[*a*]P DNA adducts is observed. However, benzo[*e*]pyrene is an anticarcinogen when co-treated with 7,12-dimethylbenz(*a*)anthracene (DMBA) [23]. These effects are thought to be the result of differential induction or competition for metabolizing enzymes.

PAH are known as complete carcinogens, because they can induce mouse skin tumors. However, such induction requires repeated, prolonged exposure of low doses or subcutaneous application of high doses, indicating that PAH treatment is required not just for initiation, but promotion of tumors. Promotion may be thought of as the

transformation of a cell to a dormant cancer cell, and subsequent clonal expansion of that cell. The ability of B[*a*]P and other PAH to act singly or in concert as initiators and promoters depends on multiple variables such as the species or organ being studied and the treatment protocol [1]. While multiple studies have examined these relationships, understanding on a molecular level is still in its infancy. It is likely that initiation and promotion interact synergistically. Oncogenes and tumor suppressor genes have been identified in developed cancers, as have mutations which are commonly observed after PAH exposure in murine models [24].

While the binding of PAH to DNA may be essential for tumorigenesis, it is not sufficient, and many questions remain in chemical carcinogenesis. The methods for analyzing PAH-DNA binding are well established. However, in some cases, DNA binding occurs without subsequent tumorigenesis, and sometimes carcinogens act with little observable DNA binding. There is still much to learn about PAH-induced carcinogenesis, and carcinogenesis in general. The continuing development of microarray and proteomic technologies will contribute greatly to understanding the timing and full extent of changes in gene expression during the process of cancer induction. Several approaches have been used in this work to examine the effects of PAH which in turn may modulate their ability to initiate cancer.

In Chapter Two, I attempted to develop a method for inhibiting CYP1A1 and CYP1B1 in order to further examine their roles in activating PAH to metabolites involved in DNA binding. In Chapter Three, I examined the effect of a histone deacetylase inhibitor in epigenetic regulation of CYP1A1 and CYP1B1, and the effect on DNA adducts. In Chapter Four, I compare the protein expression changes caused by treatment with B[*a*]P, DB[*a,l*]P, coal tar extract, and diesel exhaust extract.

Effect of morpholino antisense inhibitors on CYP1A1, CYP1B1, and B[*a*]P DNA adducts

CYP1A1 and CYP1B1 are intimately involved in the transformation of PAH to mutagenic DNA binding species. The development of specific inhibitors for these enzymes would expedite many lines of research. The chemical inhibitors which do exist often exhibit nonspecific or overlapping activity for multiple P450s [25,26]. CYP1A1 and CYP1B1 knockout mice have yielded interesting and sometimes paradoxical results, but a large volume of work has not yet resulted from these recently developed models [27-30]. Expression systems expressing single human P450s have been useful in understanding metabolism of PAH [31]. However, examining an isolated enzyme may be misleading. The ratio of P450 enzymes may be important to the amount of final DNA adducts which are carcinogenic. To more accurately understand carcinogenesis by PAH in humans, human cell lines expressing a normal spectrum of P450s are useful. MCF-7 cells, a line of human mammary carcinoma cells, express a number of cytochrome P450 enzymes in addition to 1A1 and 1B1. Inhibiting either CYP1A1 or CYP1B1 in these cells will aid in separating the roles of these enzymes in the activation of PAH in human cells.

I attempted to develop the use of phosphorodiamidate morpholino antisense oligomers (PMOs) to selectively inhibit P450s in MCF-7 cells. A method for loading PMOs into these cells was successfully developed. However, the ability of this method to inhibit CYP1A1 and CYP1B1 was insufficient to gain additional understanding of PAH metabolism. A non-specific effect of PMOs on EROD activity and observations about the fate of PMOs after treatment led us to conclude that multiple confounding affects made this method unsuitable in this model.

Antisense persists as an elegantly simple and seductive concept, despite many setbacks. It originated at least a quarter of a century ago, [32,33] and has met with some success, particularly in "knock-down" of proteins in zebrafish embryos with PMOs. However, delivery in cell culture or other organisms remains problematic [34]. Since this work

was completed, a new method for inhibition of CYP1A1 and CYP1B1 has become available. Small interfering RNA (siRNA) are 21 to 22 nucleotide double stranded RNAs which recognize and facilitate the destruction of complementary RNAs. Methods have been refined which allow these constructs to be used in mammalian cells [35,36]. However, the application of new approaches such as siRNA must be looked forward to with very cautious optimism, considering the buoyant enthusiasm antisense technology has engendered. The concept of a universally applicable method of specific inhibition is alluring, but must be approached critically.

The HDAC inhibitors SAHA and TSA increase CYP1A1 levels and activity

The local dynamic remodeling of chromatin and nucleosomal DNA packaging is key to regulation of genes affecting proper cell function, differentiation, and proliferation. Inappropriate expression of some of these genes have been associated with cancer. The pattern of lysine acetylation of the amino terminal tails of histones are determined by histone deacetylases (HDAC) and histone acetyl transferases (HAT). Together with other histone modifications and DNA methylation, these patterns contribute to an epigenetic code recognized by proteins involved in regulation of gene expression [37]. These modifications induce the open euchromatin formation, allowing transcription of epigenetically controlled genes. The deacetylase action of HDACs allows chromatin to form higher order closed heterochromatin structure, preventing transcription [38]. Transcriptional repression by epigenetic mechanisms has been demonstrated in a wide variety of tumor types. When genes involved in tumor suppression, cell cycle, DNA repair, invasion and metastasis are inactivated, tumor cells may be given a growth advantage. A number of compounds reverse HDAC activity. The reexpression of these genes can alter sensitivity to existing cancer therapies or suppress cell growth [39]. While reinactivating the expression of some genes may be useful in preventing or treating cancer, the unexpected induction of other genes may have unintended clinical consequences.

Trichostatin A (TSA) has been observed to be a potent, specific, and reversible HDAC inhibitor [40]. Suberoylanilide hydroxamic acid (SAHA), a structurally similar compound, shows strong anti-proliferative effects but low toxicity *in vivo* [41]. The antitumor activity of SAHA has been demonstrated in several animal models, and has shown promise in phase I clinical trials [42]. In phase II clinical trials, SAHA patients with prior therapy-resistant cutaneous T cell lymphomas have shown positive responses [37]. SAHA has also been tested as a chemopreventive agent. When continuously fed to rats in the diet, SAHA has been shown to reduce the development of N-methylnitrosurea (NMU) induced mammary and lung tumors [43,44].

Because CYP1A1 plays a key role in xenobiotic transformation and carcinogenesis as well as hormone metabolism, gene regulation of this protein has been studied extensively. Inducers of CYP1A1 may interact with the AhR, which dimerizes with the aryl hydrocarbon nuclear translocator (ARNT), and is transported to the nucleus [45]. This dimer associates with xenobiotic responsive elements (XREs), at least seven of which are upstream of the human CYP1A1 gene [46]. B[*a*]P causes a robust and prolonged increase in CYP1A1 protein levels and activity through this pathway. Less well understood examples of CYP1A1 induction may involve the retinoic acid receptor or protein tyrosine kinase activation [47]. Negative regulatory elements (NRE) also modulate CYP1A1 transcriptional activity [48].

Activity of the murine AHR promoter has been shown to be increased by the HDAC inhibitors *n*-butyrate and TSA [49], indicating that expression of AHR is under epigenetic influence [50], although increased AHR expression does not by itself induce or increase CYP1A1 [51]. In our study, SAHA and TSA increased CYP1A1 activity and expression in the absence of a known ligand. These results, and those of others [52], suggest that CYP1A1 as well as AHR are epigenetically regulated in MCF-7 cells.

The role of genetics in cancer has long been recognized. Recent developments have highlighted the role of epigenetic misregulation in tumor onset and progression [53].

As these mechanisms are further elucidated, new strategies for the development of chemotherapeutic treatments will be considered. HDAC inhibitors represent a diverse class of antineoplastic agents. They are of great interest because of their ability to suppress the growth of tumor cells *in vitro*, and reduce the growth of tumors in animal models. SAHA alone has been found to perturb cell cycle proteins [54,55] , downregulate survival signaling pathways [56], disrupt the cellular redox state [57], and exhibit anti-inflammatory properties [58]. Our work is the first to show that CYP1A1 and CYP1B1 expression are increased by HDAC inhibitors in the absence of AhR ligand.

In the present study, SAHA and TSA were found to induce EROD activity in intact MCF-7 cells. The ability of SAHA to induce EROD activity exceeded that of B[*a*]P. Microsomal EROD assays and RT-PCR analysis also revealed elevated CYP1A1 activity. Interestingly, both SAHA and TSA reduced the induction of CYP1A1 and CYP1B1 by B[*a*]P as measured by RT-PCR. Paradoxically, no difference in DNA binding was noted when MCF-7 cells were co-treated with B[*a*]P and the HDAC inhibitors SAHA and TSA. HDAC inhibitors appear to be promising chemotherapeutic agents, but further investigations of their roles both in cancer biology and carcinogenesis are warranted.

Proteomics analysis of MCF-7 cells treated with B[*a*]P, DB[*a,l*]P, coal tar extract, and diesel extract.

An immense body of literature has accumulated over many decades describing the effects of PAH in carcinogenesis. This work ranges from structural details of PAH-DNA binding, elucidation of PAH metabolism and characterization of the enzymes involved, to epidemiological studies relating cancer incidences to exposure and population differences. Carcinogenesis in general is not completely understood, and reviews are published periodically discussing various models for this complex process [1,59,60]. Other research groups, using DNA arrays, have treated rodents and cells

with B[*a*]P and methylchrysene (MC) [61,62]. The results reported in both these papers focus on well known P450s and GSTs.

In order to investigate possible unknown effects of PAH on gene expression simultaneously, we have undertaken a proteomic analysis of MCF-7 cells treated with B[*a*]P, DB[*a,l*]P, and standardized extracts from coal tar and diesel exhaust. Our results have shown alterations in protein expression of heat shock proteins, cytoskeletal proteins, DNA associated proteins, and glycolytic and mitochondrial proteins. The proteins that were universally increased in expression were tubulin alpha and myosin light chain alkali, cyclophilin B, heterogeneous ribonuclear protein B1 (hnRNP B1), and alpha enolase. Proteins that also exhibited altered expression compared to control included histone H2A.1, heat shock protein 70-2 (HSP70-2), galectin-3, nucleoside diphosphate kinase (NDPK), ATP synthase, and electron transfer flavoprotein (ETF). The results of our proteomic analysis indicate a need for further work in this area.

A number of cytoskeletal proteins showed altered expression in our work. Given the role of cytoskeletal proteins in cellular organization and differentiation, an expanded study of this effect is would be valuable. Several proteins involved in DNA synthesis, including histone H2A.1 and hnRNP B1 were of note. HnRNP B1 is required for mRNA maturation, and facilitates telomerase interaction with telomeres. MCF-7 cells have been shown to arrest in S phase after PAH treatment [63], and these proteins may be associated with increased DNA synthesis. HnRNP B1 has been found to be upregulated in tumors, and telomerase is dysregulated in cancer, and is a potential target of cancer therapy [64]. It has not been investigated whether or not PAH interact with telomeres. 2-DE examination of nuclear extracts could amplify proteins localized in the nucleus.

Proteins involved in metabolism also exhibited altered expression. To further examine these results, mitochondrial extracts should be isolated to enrich the populations of these proteins, and subjected to 2-DE. B[*a*]P has been found to bind extensively to

mitochondrial DNA, and may interfere with respiration by localizing to mitochondrial membranes [1]. The regulatory mechanisms of mitochondrial gene expression are poorly understood, and it is unknown how PAH might interfere with them. Mitochondria are involved in apoptosis and Ca^{2+} homeostasis, and have a role in aging and carcinogenesis. Increased mitochondrial activity increases the production of ROS, which may contribute to carcinogenesis.

Interestingly, several proteins involved in signal transduction exhibited increased expression, particularly nucleoside diphosphate kinase. NDPK has been found to inactivate oncogenic forms of Ras [65], and act as a suppressor of metastasis. B[*a*]P targets several codons in Ras genes, activating oncogenic activity [66]. Our results suggest that signal transduction proteins in addition to Ras may be affected by PAH. However, it is unlikely that these changes in expression, so soon after treatment, are a result of mutations.

Although our antisense approach to inhibiting CYP1A1 and CYP1B1 was not successful, the objectives remain worthwhile. SiRNA kits for CYP1A1 and CYP1B1 are available, and should be examined for efficacy. This approach could verify and further elucidate the roles of cytochrome P450s in PAH metabolism. Use of these inhibitors with focused 1-DE, 2-DE, or microarray experiments could prove to be a very powerful technique to understanding effects of PAH other than DNA binding. This technique could be extended to other enzymes involved in PAH metabolism, such as epoxide hydrolase, glutathione S-transferase, and the AhR as well as other receptors. Research of the effects of environmental mixtures of PAH would particularly benefit from this approach.

The increase in CYP1A1 activity and increase in CYP1A1 and CYP1B1 expression in response to SAHA and TSA prompts new questions. Are other enzymes involved in PAH metabolism under epigenetic control? SAHA and TSA had no significant impact on B[*a*]P DNA adducts. What effect does epigenetic regulation in general have on

carcinogenesis? Do PAH or any components of environmental mixtures act as HDAC inhibitors? PAH are known to bind to histones, as well as DNA. Does this interfere with DNA methylation, histone modification, and DNA organization? The reexpression of silenced genes through histone deacetylase inhibition is a potential chemotherapeutic approach. The involvement of HDACs in carcinogenesis is also likely to be a fruitful line of research.

Ongoing research in the Baird lab includes comparing our proteomics results to similar experiments using DNA microarrays. Initial results from the microarray work include increased expression of CYP1A1 and CYP1A1 and other PAH metabolizing enzymes. 2-DE proteomics is able to assess the expression of a limited number of proteins compared to microarray work, and is not be the method of choice for assaying P450 expression. An advantage of proteomics is that samples may be fractionated by cellular compartment or by physical property. As mentioned above, mitochondrial and nuclear fractions would be useful for expanding on our results. Further analysis of microarray results will also pinpoint organelles for future focus. Proteomic and DNA array analysis have the capacity to generate large amounts of data. Sifting out the patterns and relationships in the data to understand their significance is a daunting task. A large body of literature exists describing multiple aspects of chemical carcinogenesis that will make this task much easier.

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