

**TOXICITY ANALYSIS OF POLYCYCLIC AROMATIC  
HYDROCARBON MIXTURES**

A Dissertation

by

CHRISTINE SUE NASPINSKI

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Toxicology

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## ABSTRACT

Toxicity Analysis of Polycyclic Aromatic Hydrocarbon Mixtures. (May 2009)

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Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment and are generated by many sources. Though the potential of PAH-rich mixtures to cause health effects has been known for almost a century, there are still unanswered questions about the levels of PAHs in the environment, the potential for human exposure to PAHs, the health effects associated with exposure, and how genetic susceptibility influences the extent of health effects in individuals.

The first objective of this research was to quantify concentrations of PAHs in samples of settled house dust collected from homes in Azerbaijan, China, and Texas. The trends of PAH surface loadings and percentage of carcinogenic PAHs were China > Azerbaijan > Texas, indicating that the risk of health effects from exposure to PAHs in house dust is highest in the Chinese population and lowest in the Texas population. PAHs in China and Azerbaijan were derived mainly from combustion sources; Texas PAHs were derived from unburned fossil fuels such as petroleum.

The second objective of this research was to investigate the effect of pregnane X receptor (PXR) on the genotoxicity of benzo[a]pyrene (BaP). BaP treatment resulted in significantly lower DNA adduct levels in PXR-transfected HepG2 cells than in parental HepG2 cells. Total GST enzymatic activity and mRNA levels of several metabolizing enzymes were significantly higher in cells overexpressing PXR. These results suggest that PXR protects cells against DNA damage by PAHs such as BaP, possibly through a coordinated regulation of genes involved in xenobiotic metabolism.

The third objective of this research was to investigate biomarkers of exposure in house mice (*Mus musculus*) exposed to PAH mixtures *in situ*. Mice and soil were collected near homes in Sumgayit and Khizi, Azerbaijan. Mean liver adduct levels were significantly higher in Khizi than in Sumgayit. Mean lung and kidney adduct levels were

similar in the two regions. The DNA lesions detected may be a combination of environmentally-induced DNA adducts and naturally-occurring I-compounds. PAHs were present at background levels in soils from both Khizi and Sumgayit. It appears that health risks posed to rodents by soil-borne PAHs are low in these two areas.

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## NOMENCLATURE

AhR	Aryl hydrocarbon receptor
ATSDR	Agency for Toxic Substances and Disease Registry
BaA	Benz[a]anthracene
BaP	Benzo[a]pyrene
BbF	Benzo[b]fluoranthene
BkF	Benzo[k]fluoranthene
Chr	Chrysene
DBA	Dibenz[a,h]anthracene
ETS	Environmental tobacco smoke
GC/MS	Gas chromatography / mass spectrometry
GST	Glutathione <i>S</i> -transferase
HMW	High molecular weight
hPXR	Human PXR
IDP	Indeno[1,2,3-cd]pyrene
IRIS	Integrated risk information system
LMW	Low molecular weight
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo- <i>p</i> -dioxin
PXR	Pregnane X receptor
RIF	Rifampicin
ROS	Reactive oxygen species
SULT	Sulfotransferase
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TEF	Toxic equivalency factor
TLC	Thin-layer chromatography
UGT	UDP glucuronosyl transferase
USEPA	United States Environmental Protection Agency

# CHAPTER I

## INTRODUCTION

### 1.1 Overview

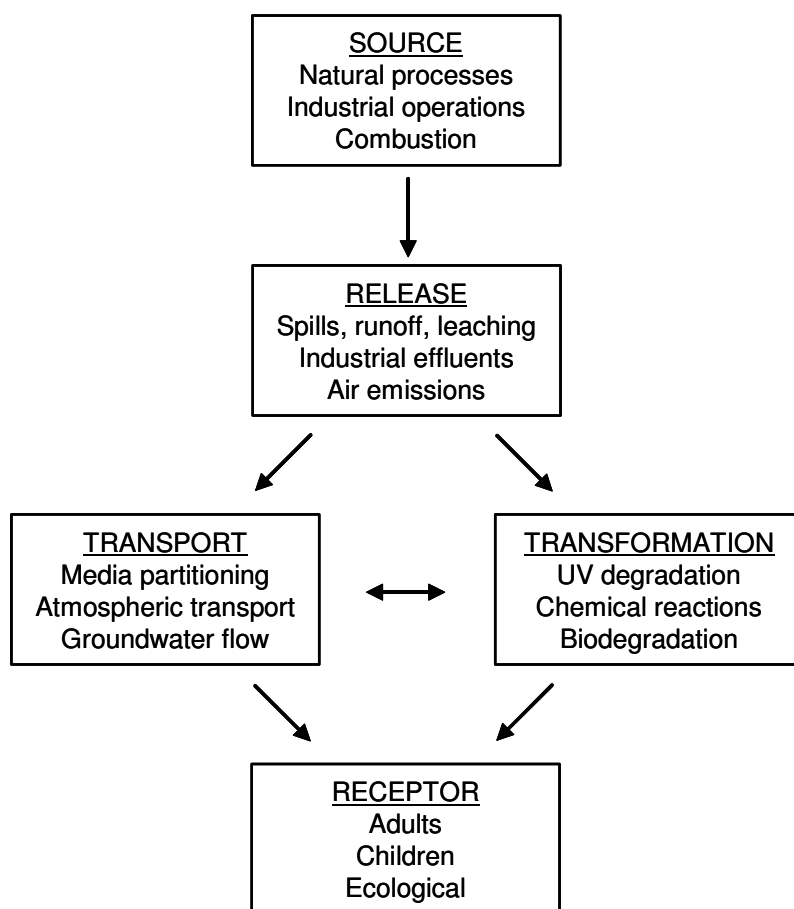
Humans are continuously exposed to complex mixtures of chemicals in their environments. Some chemical mixtures can have adverse effects on human health, ranging from acute, reversible effects to permanent disability and death. Complex chemical mixtures in the environment may contain tens, hundreds, or even thousands of individual compounds. Evaluating the risk to human health posed by environmental mixtures is challenging for many reasons, including: 1) mixture composition is often not known, 2) composition and concentration may have large temporal and spatial variability, 3) human exposure to mixtures is difficult to assess, 4) toxicity data usually are for individual compounds rather than mixtures, 5) toxicity data are often from cell culture or laboratory animals rather than humans, 6) toxicity is often evaluated for short-term high doses, rather than the long-term low doses typical of environmental exposure, and 7) individual characteristics of humans can greatly influence susceptibility to health effects. This dissertation describes research designed to address some of these challenges for mixtures of polycyclic aromatic hydrocarbons (PAHs), which are prevalent environmental contaminants.

### 1.2 Environmental Mixtures and Human Health

#### 1.2.1 Exposure to Environmental Mixtures

Complex mixtures of chemical contaminants exist everywhere in the environment, generated by a large number of diverse sources. Contaminant mixtures are present in all environmental media, including outdoor and indoor air, surface and ground waters, dust, soils, sediments, and biota. However, the presence of chemical mixtures in the environment does not necessarily lead to a human exposure. Several processes occur between the origin of the mixture and the exposure of a human or ecological receptor. A mixture is generated by a source, released into the environment, undergoes transport and/or transformation, and ultimately reaches the receptor

(Fig. 1-1). Complex chemical mixtures are generated by many sources, including natural events such as forest fires and volcanic eruptions, hundreds of industrial operations, mining activities, and combustion of fuels such as petroleum products, wood, and coal. These mixtures can then be released to the environment as air emissions, industrial effluents, or leaks and spills from improper storage and handling.



**Fig. 1-1.** Environmental exposure model.

Once a mixture is released into the environment, it is acted upon by transformation and transport processes that can change the composition of the mixture and move it far away from the point of release. Compounds may partition into other

environmental media; this partitioning will often change the mixture composition because different physicochemical properties of mixture components will cause them to partition differently. For example, components of crude petroleum have a wide range of volatility and aqueous solubility. When crude oil is spilled in a marine environment, some compounds will readily evaporate into the air, others will easily dissolve in water, and the remainder will mainly adsorb to solid particles in the water and eventually settle to the bottom sediments or be taken up by marine organisms. Mixture components may also undergo chemical reactions with other compounds and may be degraded by UV light exposure or by biological organisms. Transport processes can be as simple as movement downstream of an industrial effluent released into a river, or complex, involving multiple environmental media. Long-range atmospheric transport processes are responsible for deposition of some mixtures thousands of miles from where they were released. Examples of long-range atmospheric transport include the contamination of arctic snows with polychlorinated biphenyls (PCBs), and dust carried to the U.S. across the Atlantic Ocean from Africa and across the Pacific Ocean from Asia.

Receptors are exposed to environmental mixtures when they come into contact with contaminated environmental media. Common media of exposure for humans are indoor air, outdoor air, drinking water, house dust, and foods. Humans may be exposed at work and at home. Occupational exposures to mixtures may be significant. For example, an occupational study of soil remediation workers in a former creosote wood impregnation site demonstrated significant work-related exposure to mixtures of PAHs (Elovaara et al., 2006).

Exposures at home include mixtures generated by building materials, furnishings, clothing, cooking, combustion of fuels, tobacco and candles, and by use of personal care products, cleaning products, home maintenance products, and lawn and gardening equipment. Use of tap water for bathing, cooking, washing, and sanitation can release volatile contaminants into indoor air. Environmental tobacco smoke (ETS), the combustion product mixture that enters the surrounding air when tobacco is smoked, is a widespread contaminant of indoor air. Serum cotinine, a biomarker of ETS exposure, was detectable in 84% of a sample of approximately 4400 U.S. children aged 6 to 16 (Yolton et al., 2005).

Foods can be a major source of human exposure to environmental mixtures. Plants, livestock and wildlife which have been exposed to chemical mixtures in the environment can absorb and accumulate contaminants. Contaminated plants and animals that are used as food become a source of exposure for humans. For example, many large lakes are contaminated by pollutants such as PCBs that tend to accumulate in the fatty tissue of aquatic organisms. Fish caught from these lakes can be a significant source of food and exposure for people living near the lake.

Exposure occurs when an individual comes into superficial contact with an environmental mixture. Exposure may take place by any of three routes: inhalation, ingestion, and dermal contact. Before the mixture can cause a health effect, the mixture must be absorbed; i.e., it must cross a cell membrane and enter the body. Absorption from dermal contact can occur anywhere on the surface of the body. Absorption by inhalation can take place anywhere in the respiratory tract, from the nasal passages to the alveoli in the lungs. Likewise, by the ingestion route, absorption can occur throughout the digestive tract, from the mouth to the colon.

Absorption is a toxicokinetic process. Toxicokinetic processes determine the fate of a chemical once it comes into contact with an organism (Table 1-1). Distribution is the transport of a chemical through the body, usually by a circulatory system (blood or lymph), to tissues distant from the site of absorption. This can be an important factor in toxicity because the susceptible tissue may be different from the absorption tissue. Elimination is the process by which a contaminant leaves an organism by again crossing a cell membrane. Elimination can take place through the skin and exterior surfaces, the lungs, the kidneys, and the digestive tract.

**Table 1-1**  
Toxicokinetic processes

Process	Description
Absorption	Entry into body by crossing a cell membrane
Distribution	Transport through body
Metabolism	Chemical modification by cellular enzymes
Elimination	Exit from body by crossing a cell membrane

Metabolism, also called biotransformation, is the chemical modification of a compound by cellular enzymes. Metabolism may take place in the absorption tissue or after distribution to other tissues. Most body tissues have some metabolic capability. Major sites of xenobiotic metabolism include the liver and the small intestine. Generally, metabolism makes xenobiotics more water soluble by the addition of functional groups. This increases excretion of the xenobiotic from the cell, thus detoxifying the xenobiotic. However, some xenobiotics are also transformed by cellular metabolism into more toxic forms ('bioactivation'). The rate and extent of metabolism are often dependent on the similarity of the compound to an endogenous substrate; however, many common xenobiotic-metabolizing enzymes have broad substrate specificity.

### **1.2.2 Health Effects of Environmental Mixtures**

Linking environmental exposures to observed health effects is often challenging. The chronic, low-level doses typical of environmental exposures in the general population may cause health effects that, although significant and clinically relevant in the long-term, are subtle and difficult to measure. When health effects are detectable, establishing the level of exposure and isolating the effects of the mixture from those of concurrent exposures are additional challenges. Another level of complexity in the analysis of mixture toxicity is added because health effects may vary with exposure route.

Because of these challenges, toxicity assessment of mixtures may require a weight-of-evidence approach, in which accumulated evidence from a variety of studies is evaluated. These may include: 1) occupational epidemiological studies showing associations between worker exposure levels and disease incidence, 2) human biomarker studies linking environmental exposure and sub-clinical effects, 3) health effects in lab animals from direct administration of the mixture, and 4) *in vitro* studies demonstrating mechanisms of toxicity. While none of these studies alone may be sufficient to establish the toxicity of a mixture, when evaluated together, the combined evidence may be strong enough for conclusions to be made by regulatory agencies.

#### **1.2.2.1 Effects of Selected Common Environmental Mixtures**

The health effects of several environmental mixtures are summarized below. These mixtures are significant to human health because they are common



environmental contaminants and many people are chronically exposed to them. Toxicological Profiles of three of the mixtures have been published by the Agency for Toxic Substances and Disease Registry (ATSDR). ATSDR Toxicological Profiles are reviews of the key literature that describes a hazardous substance's toxicological properties and adverse health effects. Much of the evidence of human health effects associated with the selected environmental mixtures comes from studies of occupational or accidental exposures to relatively large amounts of the mixtures. Lab animal and *in vitro* studies provide additional evidence of toxicity.

Automotive Gasoline. Automotive gasoline is a complex mixture of refined petroleum hydrocarbons with small amounts of additives and blending agents. The composition varies seasonally and with the crude oil source and manufacturing processes. Humans are frequently exposed to gasoline by breathing gasoline vapors while filling vehicle fuel tanks. Exposure may also occur during filling and operation of gasoline-powered equipment such as lawn mowers. Significant occupational exposure may occur among workers in service stations, petroleum refineries, bulk loading terminals, marine loading docks, and among drivers of gasoline tank trucks (ATSDR, 1995a). Acute exposures to large amounts of gasoline by inhalation or ingestion have caused death in humans. Gasoline is a skin, respiratory, and gastrointestinal irritant. Neurological effects of acute exposures to gasoline vapors increase in severity with increasing duration and dose, and range from dizziness, headaches and euphoria to numbness, anesthesia and coma. Many effects of chronic exposure to gasoline have also been associated with exposure to gasoline components lead and benzene. Long-term inhalation of gasoline caused liver and kidney tumors in lab animals. In human occupational studies, there is some evidence of increased cancer risk and mortality, but it is compromised by lack of accurate exposure data for gasoline and concurrent exposures to other carcinogens, and lack of adjustment for confounding factors such as smoking (ATSDR, 1995a).

Polychlorinated Dibenzo-*p*-dioxins (PCDDs). PCDDs, also called 'dioxins', are a family of environmental contaminants that occur in mixtures. PCDDs are byproducts of production of some pesticides, chlorine bleaching processes used at pulp and paper mills, and incomplete combustion of organic matter. PCDDs occur nearly everywhere in the environment and most humans have frequent, low-level exposure to environmental

PCDD mixtures. PCDDs accumulate in living tissues and therefore in the food chain. More than 90% of PCDD exposure in the general population is through food, mainly from meat, fish, and dairy products (ATSDR, 1998). Occupational exposure to PCDDs may occur in people who work in pulp and paper mills, at hazardous waste sites, municipal and hazardous waste incinerators, and those involved in production, use, and disposal of chlorinated pesticides and herbicides. The most toxic of the PCDDs, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is the reference compound for PCDD toxic equivalency factors which are used in risk assessment (Safe, 1990). Most evidence of PCDD toxicity in humans has come from studies of occupational exposures, exposures due to accidental spills of mixtures containing PCDDs, and exposures due to large-scale aerial spraying of PCDD-contaminated herbicides by the United States in Vietnam. In these studies, exposure levels and specific routes of exposure are difficult to determine, and confounding effects of concurrent exposure to other toxic mixtures are present. PCDDs have clearly been shown to cause chloracne, a severe skin disease characterized by acne-like lesions which usually occur on the face and upper body (ATSDR, 1998). The International Agency for Research on Cancer (IARC), the U.S. Environmental Protection Agency (USEPA), and the U.S. Department of Health and Human Services recognize the likelihood that TCDD may cause cancer in humans (ATSDR, 1998). There is a large body of evidence of PCDD toxicity in laboratory animals, but toxicity varies significantly between species, and intraspecies differences in sensitivity may be related to sex, age, and strain (ATSDR, 1998; Safe, 1990). Health effects observed in lab animals exposed to high doses of PCDDs include chloracne, weight loss, mortality, reproductive toxicity, developmental toxicity, cancers of liver and thyroid, hepatotoxicity, immunotoxicity and endocrine effects (ATSDR, 1998).

Polychlorinated Biphenyls (PCBs). PCBs were manufactured, prior to being banned in the U.S. in 1977, for use in electrical transformers, capacitors, heat transfer fluids, lubricants, and many consumer products. PCB mixtures consist of molecules with varying degrees of chlorination. The same characteristics that made PCB mixtures desirable for use (chemical and thermal stability, low aqueous solubility) have made them extremely resistant to breakdown in the environment. Occupational exposures to PCBs, which may be released during repair, maintenance or disposal of items containing PCBs, usually occur by inhalation or dermal contact. Like PCDDs, PCBs are

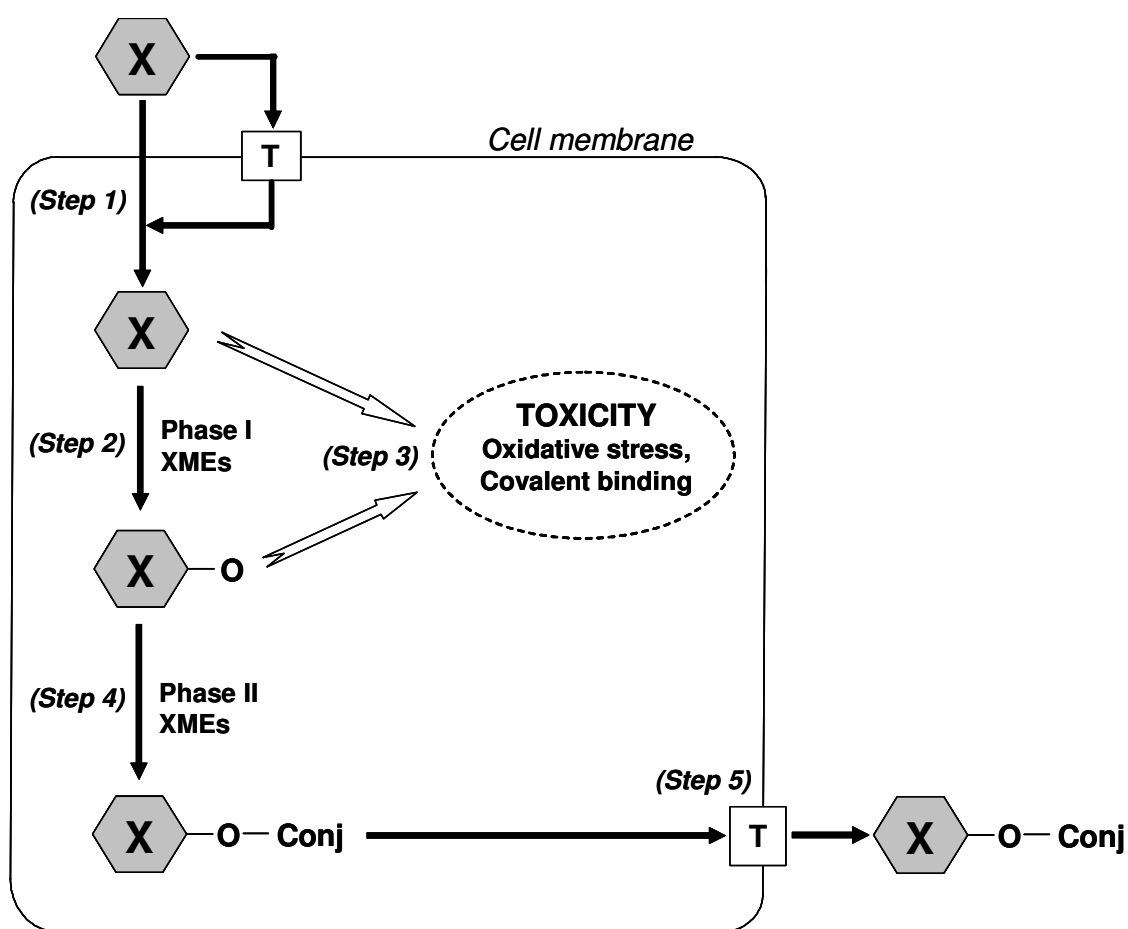
present at low levels almost everywhere in the environment and accumulate in the food chain, contributing to the exposure of the general population (ATSDR, 2000). Studies of workers exposed to PCBs have reported irritation of the nose and lungs, depression, fatigue, and gastrointestinal discomfort. Occupational studies have also associated PCB exposure with cancers of the skin, liver and biliary tract. Chronic oral exposure of rats to PCBs induced liver tumors. USEPA and IARC classify PCBs as probable human carcinogens. Developmental and immune system effects have been documented in children whose mothers were exposed to PCBs. Maternal exposure in lab animals was associated with harmful behavioral effects, prenatal death, and changes in the immune system, thyroid and reproductive system of offspring (ATSDR, 2000).

Environmental Tobacco Smoke. The health effects of active smoking have long been known, and there is also a significant body of evidence linking ETS exposure to adverse health effects in humans. ETS is a complex mixture of organic compounds and metals that results from the combination of exhaled mainstream smoke and the sidestream smoke from the burning tip of a cigarette. ETS exposure is generally an indoor exposure, occurring in workplaces and residences in which people are actively smoking. Infants whose mothers were exposed to ETS have increased risk of preterm birth and reduced birth weight (Wigle et al., 2007). Childhood ETS exposure can increase incidence and severity of asthma, recurrence of middle ear infections, and prevalence and severity of respiratory infections (Ciencewicki and Jaspers, 2007; Wigle et al., 2007). Fetal or childhood ETS exposures have been associated with adverse effects on behavior and cognitive functioning (Reardon, 2007). Evidence links ETS exposure to increased risk of chronic obstructive pulmonary disease and stroke, and workplace ETS exposure has been causally related to lung cancer risk (Jaakkola and Jaakkola, 2006). Significant reductions in worker ETS exposure due to smoke-free workplace legislation have been accompanied by increases in lung function levels and reductions in respiratory symptoms and acute myocardial infarctions (Jaakkola and Jaakkola, 2006).

#### **1.2.2.2 Toxicity Mechanisms**

Two predominant molecular mechanisms for xenobiotic toxicity are oxidative stress and covalent binding to cellular macromolecules (Nebert, 1999). Xenobiotics cause oxidative stress by undergoing redox cycling and generating damaging free

radicals. When a xenobiotic covalently binds to proteins or nucleic acids, it can disrupt the critical function of these macromolecules (Gregus and Klaassen, 2001). Oxidative stress and covalent binding can cause cell-level effects such as apoptosis, perturbation of differentiation, and alteration of metabolism, cell growth, homeostasis, and neuroendocrine signals (Nebert, 2000). When these cell-level effects continue and occur in many cells, they eventually lead to tissue-level and organ-level effects and diseases such as those discussed in the previous section.



**Fig. 1-2.** Cellular model of xenobiotic fate and toxicity. X = xenobiotic, X-O = phase I metabolite, X-O-Conj = phase II metabolite, XMEs = xenobiotic-metabolizing enzymes, T = membrane-bound transporter.

A general model of the cellular fate and toxicity of xenobiotics includes five steps (Fig. 1-2). In Step 1, the xenobiotic (X) is taken up by the cell. It crosses the cell membrane by diffusion or is assisted by a membrane-bound transport protein (T). Once inside the cell, the xenobiotic encounters phase I xenobiotic-metabolizing enzymes (XMEs) in either the cytoplasm or the endoplasmic reticulum. Step 2 is transformation by these enzymes to a phase I metabolite, which is often an oxidized form (X-O). Some xenobiotics are toxic in their unmodified (parent) form; for these compounds, phase I metabolism is a detoxification step. Other xenobiotics are activated by phase I metabolism and the phase I metabolite causes the toxic effects. Step 3 is the generation of toxic effects by the active form. Step 4 is the addition of a conjugate such as glutathione by phase II XMEs; this forms a phase II metabolite (X-O-Conj). Phase II metabolism detoxifies active phase I metabolites and prepares the compound for excretion. In Step 5 the phase II metabolite is readily excreted by a membrane-bound transporter.

### ***1.2.2.3 Mixture Interactions***

Most available toxicity data is for individual compounds. The toxicity of environmental mixtures is often assessed by determining the chemical composition of the mixture and assuming that the toxicity of individual components is additive. This approach is useful in the absence of information on whole mixture toxicity, but assumes that the full chemical composition of the mixture is known and that toxicity data is available for all the components. These criteria are difficult to meet for complex mixtures. Even if the mixture composition and component toxicities are fully characterized, interactions among the mixture components may make the overall mixture toxicity less than or more than additive.

In some cases, direct chemical interaction occurs among mixture components. For example, the genotoxicity of both 2,4,6-trinitrotoluene and benzo[a]pyrene (BaP) was suppressed when they were co-administered in a microbial bioassay (Washburn et al., 2001). Chemical analysis revealed that the compounds had formed reaction products which presumably decreased their bioavailability.

Mixture components may interact through toxicokinetics. Effects on metabolism are of particular interest because of the important role metabolism plays in both elimination and toxicity. Mixture components may increase metabolism by inducing

XMEs or decrease metabolism by inhibiting or saturating XMEs. An example is the synergistic hepatotoxicity of acetaminophen or carbon tetrachloride when combined with ethanol. The ethanol increases toxicity of acetaminophen and carbon tetrachloride by upregulating CYP2E1 and depleting glutathione (Treinen-Moslen, 2001).

Interactions of PAHs in mixtures have been evaluated both *in vivo* and *in vitro*. Most of these mixture studies involve simple mixtures of two or three compounds. Though the interactions in complex mixtures may be more complicated, these studies are a valuable starting point and, in some cases, provide information on mechanisms of interaction.

Cherng et al. (2006) found that the genotoxicity of the PAH benzo[a]pyrene (BaP) in HepG2 cells decreased with co-treatment with 1-nitropyrene (1-NP) in a dose-dependent manner. Further study revealed that co-treatment with 1-NP decreased CYP1A1 mRNA levels and increased degradation of CYP1A1 protein, an important enzyme in the bioactivation of BaP.

Genotoxicity of BaP and another genotoxic PAH, dibenzo[a,l]pyrene (DBP), alone and in mixtures was studied in human diploid lung fibroblasts (Binkova and Sram, 2004). Binary mixtures of BaP or DBP with other PAHs were less genotoxic (lower DNA binding) than BaP or DBP alone. Also, BaP administered as part of a complex mixture, extractable organic matter from respirable air particles, was significantly less genotoxic than the same dose of BaP alone. The authors suggest that the attenuation of BaP genotoxicity may result from competitive inhibition of bioactivating XMEs because the metabolic capacity of the cell system utilized is relatively low.

In an *in vivo* toxicity study, BaP and 3'-methoxy-4'-nitroflavone (MNF) were administered to C57B1/6J mice and AhR-null mice by intraperitoneal injection (Dertinger et al., 2000). Bone marrow toxicity was measured by frequency of peripheral blood reticulocytes and genotoxicity was measured by frequency of micronuclei in reticulocytes. BaP genotoxicity and bone marrow toxicity were significantly decreased by co-administration of MNF in both mouse strains. However, lethality and abnormal histology of spleen and intestine were enhanced by co-administration of MNF in both mouse strains. Further investigation of MNF *in vitro* (Hepa1c1c7 cells) demonstrated that MNF inhibited both CYP1A1/2 enzyme activity and AhR-dependent transcription of a reporter gene with a dioxin responsive element. The authors suggest that the

interactions between BaP and MNF occur through both AhR- dependent and AhR-independent mechanisms (Dertinger et al., 2000).

In an *in vivo* tumor-initiation assay, BaP and various coal-derived complex mixtures were applied to the skin of female CD-1 mice. Co-administration of BaP with each of four complex mixtures resulted in fewer skin tumors and reduced DNA binding of BaP by 50-85% (Springer et al., 1989). Hughes and Phillips (1990) reported that various binary mixtures of PAHs exhibited additive, synergistic or antagonistic effects on DNA adduct levels when applied to mouse skin.

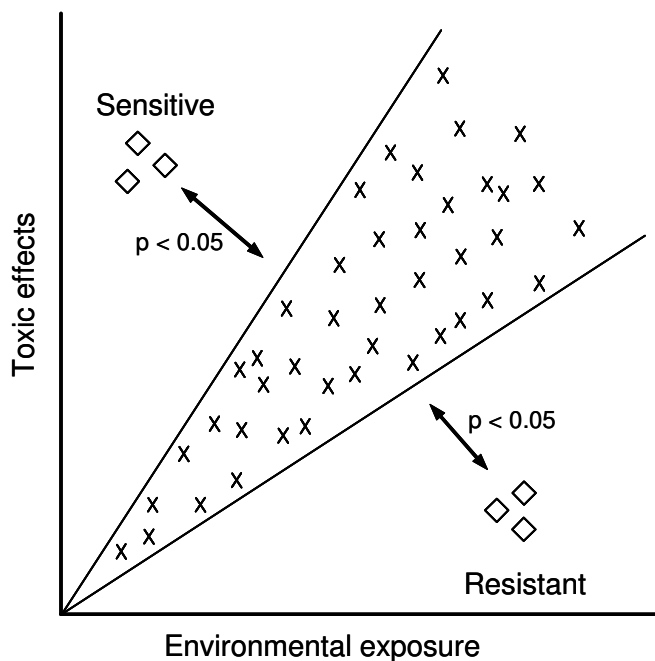
### **1.2.3 Genetic Susceptibility**

The risk of health effects from exposure to an environmental mixture depends not only on exposure levels and route, but also on individual factors such as age, sex, developmental status, general health, nutrition, concurrent exposures, and genetics. The study of genetic susceptibility (inherited variation in response) to exposure to environmental agents is part of the field of ecogenetics (Faustman and Omenn, 2001). The goal of ecogenetic studies is to establish a correlation between a quantifiable variation in response to exposure (phenotype) and genetic polymorphisms (genotype).

One of the fundamental concepts in toxicology is that of the dose-response relationship, also called the exposure-effect relationship. For most environmental toxicants, there is a gradual increase in the level of toxic effects as the environmental exposure increases. If enough data is available, a quantitative relationship between exposure and toxic effects can be developed. Fig. 1-3, adapted from Nebert (1999), is a generalized representation of a simple linear exposure-effect relationship. Data points which are statistical outliers represent separate phenotypes from the general population. Those for which a relatively large exposure results in little or no toxicity are considered to be “resistant” phenotypes, and those for which there is an exaggerated response to a relatively low exposure are considered to be “sensitive” phenotypes (Nebert, 1999). Ecogenetic studies seek to identify genetic polymorphisms that are associated with these phenotypes and to determine the underlying mechanism for the genotype/phenotype relationship.

Differences in the regulation, expression, and activity of XMEs can change tissue concentrations, bioactivation/detoxification, excretion, and, ultimately, toxic effects of a xenobiotic. Genetic polymorphisms of many XMEs have been identified and

large interindividual variation in activity of some XMEs has been reported (Autrup, 2000). Some of these changes in XME metabolic capability are significant enough to lead to measurable differences in susceptibility to environmental agents. Many ecogenetic studies have focused on polymorphisms in XME genes.



**Fig. 1-3.** Exposure-effect relationship. Adapted from Nebert (1999).

The cytochrome P450 (CYP) enzymes are a superfamily of heme-thiolate monooxygenases which are important in many cellular functions, including synthesis of steroids, cholesterol, and bile acids (Nebert and Dalton, 2006). Because of their broad substrate specificity, the CYP enzymes are also important phase I XMEs for many xenobiotics. An estimated 70-80% of phase I XMEs belong to the CYP family (Evans and Relling, 1999). Although phase I metabolism is an important step in the detoxification and excretion of xenobiotics, CYP-mediated phase I metabolism may also activate xenobiotics by transforming them into toxic reactive oxygenated intermediates (X-O in Fig. 1-2). In an extensive review of studies evaluating potential links between CYP gene polymorphisms and cancer risk, Agundez (2004) found consistent evidence



for associations between CYP polymorphisms and risk of lung, liver, and head/neck cancers. Specifically, CYP1A1 polymorphisms were associated with lung cancer risk, and CYP2D6 polymorphisms were associated with lung and liver cancer risk. Weak associations have been identified of CYP1A1 (Murata et al., 2001) and CYP1B1 (Tanaka et al., 2002) with prostate cancer risk, and CYP2A6 and CYP2C19 with colorectal cancer risk (Sachse et al., 2002).

The glutathione *S*-transferase (GST) family of enzymes is a group of phase II XMEs. GSTs detoxify and enhance excretion of xenobiotics by catalyzing the conjugation of glutathione with electrophilic phase I xenobiotic metabolites (Step 3 in Fig. 1-2). Null genotypes (gene deletion) of enzymes GSTM1 and GSTT1, which result in no enzymatic activity, have been identified in humans. In a study of Czech newborns, the GSTM1 and GSTT1 null genotypes were significantly associated with decreased birth weight among newborns whose mothers smoked (Sram et al., 2006). Each null genotype was associated with a significantly increased risk of acute lymphoblastic leukemia in a meta-analysis of 30 published case-control studies (Ye and Song, 2005). In another meta-analysis of epidemiological studies, the GSTT1 null genotype was significantly associated with risk of adult meningioma (Lai et al., 2005).

The UDP-glucuronosyl transferases (UGTs) are another family of conjugating enzymes involved in the phase II metabolism of xenobiotics. UGTs catalyze glucuronidation of phase I xenobiotic metabolites, which enhances xenobiotic detoxification and excretion. Alleles of UGT1A7 have been identified which are associated with lower glucuronidation activity than the wild-type allele. Individuals carrying any of the reduced-activity alleles were found to have a higher risk of orolaryngeal cancer than individuals with the wild-type genotype (Zheng et al., 2001). The low-activity allele UGT1A7\*3 has been significantly associated with hepatocellular carcinoma (Vogel et al., 2001) and colorectal cancer (Strassburg et al., 2002).

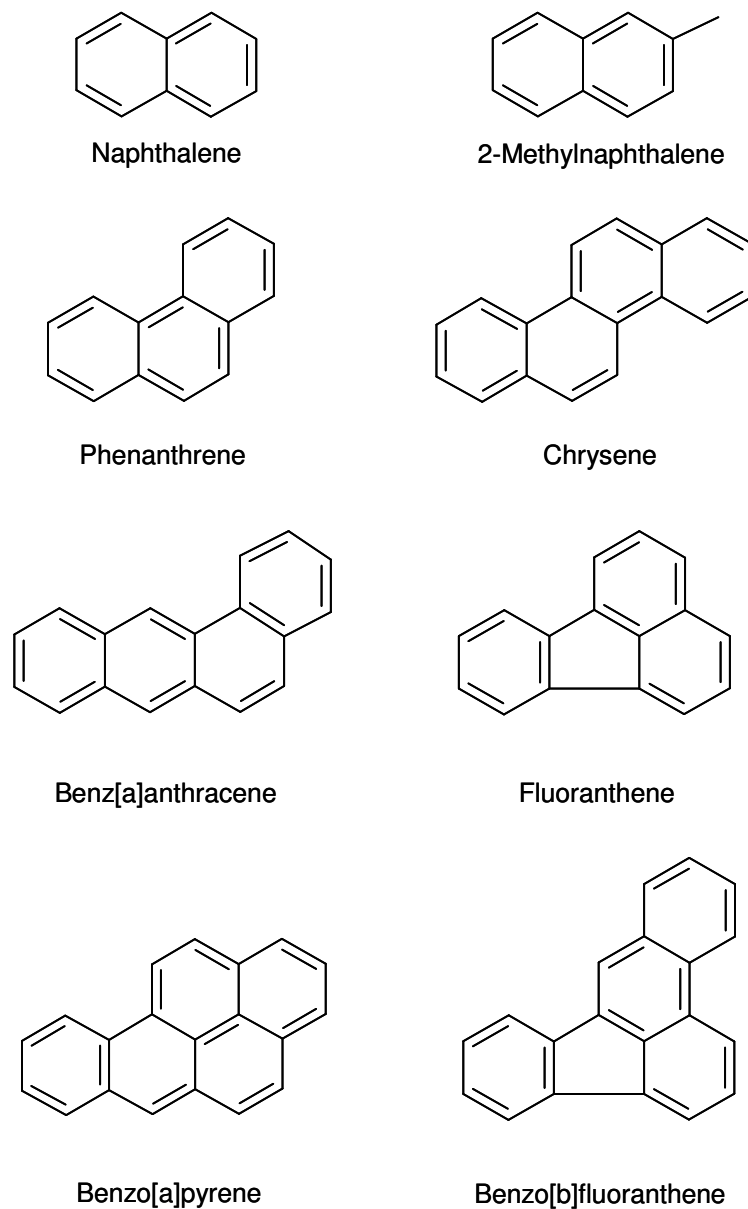
Polymorphisms in XME genes are not the only source of genetic variation in xenobiotic-metabolizing capability. In the last two decades, as knowledge of cell signaling pathways has expanded, the importance of intracellular receptors in the regulation of XME expression and activity has been increasingly recognized. Nuclear receptors such as aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) form ligand-activated transcription factor complexes which can interact with the

regulatory regions of XME genes and either induce or inhibit transcription, leading to a change in XME gene expression and cellular enzyme levels and activity. Some nuclear receptors, such as PXR and constitutive androstane receptor (CAR) also regulate expression of membrane-bound transport proteins, which can increase or decrease cellular excretion of xenobiotic metabolites. In these ways, genetic polymorphisms which affect the functioning of receptors can cause variation in susceptibility to xenobiotics. AhR regulates many XMEs, including CYP1A1, CYP1A2, CYP1B1, GSTA1, and UGT1A6/7 (Nebert and Dalton, 2006). Although evidence of the effect of AhR polymorphisms in humans is not plentiful, a few recent epidemiological studies suggest an association of various AhR polymorphisms with lung cancer risk, breast cancer risk, and survival of soft tissue sarcoma (Berwick et al., 2004; Kim et al., 2007; Long et al., 2006).

### 1.3 Polycyclic Aromatic Hydrocarbons

#### 1.3.1 Chemical Structure and Properties

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds consisting of two or more fused aromatic rings (Fig. 1-4). PAHs that consist exclusively of six-membered (benzenoid) aromatic rings are classified as alternant (Dabestani and Ivanov, 1999). Examples of alternant PAHs include phenanthrene, chrysene, benz[a]anthracene and benzo[a]pyrene (Fig. 1-4). PAHs such as fluoranthene and benzo[b]fluoranthene contain 5-membered aromatic rings and are classified as non-alternant PAHs. Another method of PAH structural classification is based on the shape formed by lines that are drawn between the centers of the rings. In *peri*-condensed PAHs, the connecting lines form cycle patterns (Ramesh et al., 2004). Examples of *peri*-condensed PAHs are fluoranthene, benz[a]anthracene, benzo[a]pyrene, and benzo[b]fluoranthene (Fig. 1-4). In *cata*-condensed PAHs such as phenanthrene and chrysene, the connecting lines form linear or angular patterns, not cycles. Because of the shape of five-membered rings, non-alternant PAHs are always *peri*-condensed (Ramesh et al., 2004).



**Fig. 1-4.** Chemical structures of representative PAHs.

Unsubstituted (parent) PAHs, consisting of only fused aromatic rings, are the basic PAH structure. Substituted PAHs have one or more chemical groups substituted for hydrogen atoms on exterior carbon atoms. An example of a parent PAH and one of

its substituted PAH derivatives are naphthalene and 2-methylnaphthalene, respectively (Fig. 1-4). The substituted groups may be alkyl chains or may contain heteroatoms such as nitrogen, sulfur, or oxygen. Substituted PAHs which contain only alkyl groups, without heteroatoms, will be referred to here as 'alkyl-substituted' or 'alkylated' PAHs. PAHs can be grouped together in "families" which consist of the parent PAH and a series of alkyl-substituted derivatives of the parent (Boehm, 2006). Common analytical techniques such as gas chromatography/mass spectrometry detect alkylated PAHs as peaks with varying numbers of alkyl carbons. For example, C<sub>2</sub>-naphthalenes include both ethyl-substituted and dimethyl-substituted naphthalenes. The naphthalene family includes the parent naphthalene, C<sub>1</sub>-naphthalenes, C<sub>2</sub>-naphthalenes, C<sub>3</sub>-naphthalenes, and C<sub>4</sub>-naphthalenes.

PAHs are chemically stable at atmospheric conditions mainly because of their aromaticity. Aromatic bonds are very stable because the orbitals have hybridized and the valence electrons are shared among the carbon atoms. At atmospheric temperature and pressure, often a concentrated energy source (e.g., UV light) or a catalytic process (e.g., biological enzymes) is required to break aromatic bonds. The aromatic structure is generally planar. Alkylation can distort the planar structure of the rings and may result in higher reactivity (Dabestani and Ivanov, 1999).

In such a large chemical class, there is a wide range of physical properties. PAHs can be roughly divided into two molecular weight groups by the number of aromatic rings: PAHs with 2 or 3 rings are low molecular weight (LMW); PAHs with four or more rings are high molecular weight (HMW). In Fig. 1-4, naphthalene, 2-methylnaphthalene and phenanthrene are LMW PAHs; the rest of the PAHs in the figure are classified as HMW. Both aqueous solubility and vapor pressure tend to decrease with increasing molecular size. LMW PAHs are sparingly soluble in water and tend to exist in the vapor phase. In general, HMW PAHs have low aqueous solubility, low vapor pressure, and high melting and boiling points (Albers, 2003).

HMW PAHs are persistent in the environment because of their physical properties as well as the chemical stability of their bonds. For example, benzene is an aromatic ring but is much more easily degraded than most PAHs because of the difference in physical properties. The low aqueous solubility of PAHs limits their bioavailability to microorganisms in the environment which could enzymatically degrade

them. It also causes them to partition out of aqueous phases to solid phases such as soils and sediments, which can limit their exposure to UV light. Uptake of PAHs by microorganisms can be limited by the size of the molecule.

### **1.3.2 Sources**

PAHs are formed by three main processes: diagenesis, petrogenesis, and pyrolysis. Diagenesis and petrogenesis are typically naturally-occurring, whereas pyrolysis can be the result of natural or anthropogenic events. Diagenesis occurs in organic matter that has been deposited in soils or sediments. In these oxygen-depleted environments, aromatization reactions occur which convert organic molecules into PAHs. These formation pathways may involve both microorganisms and non-biological chemical processes (Boehm, 2006). Diagenesis occurs at low temperature, over a span of days to years, and produces a relatively simple mixture of PAHs compared to other PAH-formation processes. PAHs formed by diagenesis may migrate from their point of origin into ground or surface water. In addition, diagenic PAHs may be distributed when soils and sediments are disturbed by excavation and dredging.

Petrogenesis is further diagenesis of organic matter in deeply buried layers of sediments at higher temperatures and pressures over the course of millions of years (Boehm, 2006). Fossil fuels petroleum and coal are formed by petrogenesis and may contain hundreds to thousands of PAH compounds of varying chemical structures. Petrogenic PAH mixtures typically contain mainly 2-ring to 6-ring PAHs. In these mixtures, alkylated PAHs are more abundant than unsubstituted PAHs (Boehm, 2006). Release of petrogenic PAHs from fossil fuels into the environment may occur naturally (e.g. petroleum seeps), or from human extraction activities such as mining and drilling. Crude oil spills in water and on land are a source of environmental PAH contamination. Petroleum refining generates products in which PAHs may be concentrated, such as diesel fuel, solvents, and lubricating oils. Use and spills of these products also release PAHs into the environment.

Complex mixtures of pyrogenic PAHs are formed by pyrolysis, a rapid process which occurs at high temperatures in oxygen-depleted environments. Pyrogenic PAH mixtures generally contain a higher ratio of unsubstituted PAHs to alkylated PAHs than do petrogenic PAH mixtures. Higher pyrolysis temperatures tend to create PAHs with more aromatic rings than do lower temperatures (Boehm, 2006). Coke production and

coal gasification are high-temperature anoxic processes that produce pyrogenic PAH mixtures. Coal tar, a by-product of these processes, is rich in PAHs. Many production sites are contaminated by coal tar that has been spilled onto soil and/or leached into groundwater. Coal tar is used for wood preservation; releases of coal tar PAHs can occur from the wood preservation process itself and when the coal tar leaches from preserved wood. Pyrogenic PAHs are also formed during high-temperature cooking of meats and oils. These PAHs enter the air as part of cooking fumes and also remain on the food.

The most common pyrolysis process is the incomplete combustion of organic matter, including fossil fuels and refined products from fossil fuels. Combustion of wood, tobacco products, and other biomass also produces PAHs. Important sources of pyrogenic PAHs released to the atmosphere include forest and prairie fires, agricultural and refuse burning, heating and power generation, industrial processes, and mobile sources (Latimer and Zheng, 2003). Motor vehicles, residential boilers, waste incinerators and industrial operations can be important sources of pyrogenic PAHs in urban environments (ATSDR, 1995b). In a study of the impact of motor vehicle traffic on residential indoor air PAH concentrations, outdoor PAH contributions were highest at the high traffic location; peaks in indoor PAH concentrations occurred on weekdays during rush-hour traffic periods (Dubowsky et al., 1999). A study of residences near an industrial source found a correlation between indoor and outdoor concentrations of 4-6 ring PAHs (Sanderson and Farant, 2004).

Pyrogenic PAHs may also be generated by indoor sources. Typical indoor sources include combustion of fossil fuels and biomass for heating and cooking; burning of tobacco products, candles and incense; and cooking fumes. Many residences rely on combustion of fuel for heating and cooking. Combustion sources include central furnaces, stoves, portable space heaters, and fireplaces for heating, as well as ovens and stovetops for cooking. Fuels used are natural gas, liquefied propane and butane, kerosene, heating oil, coal, wood, and other biomass (e.g., agricultural waste).

Indoor activities may also contribute to outdoor PAH contamination. A variety of studies in Scandinavia, Canada, the United Kingdom, and the United States highlight the role of indoor wood combustion in determining ambient particulate matter and PAH contamination levels, including evidence that residential wood combustion contributes

up to 90% of wintertime ambient  $PM_{2.5}$  in Seattle, Washington, USA (Boman et al., 2003; Prevedouros et al., 2004). Although properly maintained and functioning wood burning stoves directly contribute little to the indoor air PAH concentration of the residence in which they are used, they emit PAHs into the ambient air and thus indirectly affect the indoor air quality of homes in the surrounding area (Lewtas et al., 1992).

### **1.3.3 Environmental Distribution**

PAHs are distributed widely in the environment and occur as complex chemical mixtures. PAHs have been detected in ambient air (urban, suburban and rural), indoor air, groundwater, drinking water, surface water, atmospheric precipitation (rain, snow and fog), soil, sediments (marine and freshwater), and biota (terrestrial and aquatic plants and animals) (Menzie et al., 1992; National Library of Medicine, 2004). PAHs have been detected in at least 600 National Priorities List hazardous waste sites identified by USEPA, and fifty-four PAHs have been identified at one or more of these sites (ATSDR, 1995b). Sixteen PAHs are included in USEPA's priority pollutant list, a list mandated by provisions of the Clean Water Act of chemicals which must be monitored in wastewater.

PAHs are emitted from sources directly into various environmental media, such as combustion emissions into the ambient air, and petroleum spills into water or soil. Once the emission has occurred, environmental transport processes can distribute the PAHs far away from the source. Wind currents can carry combustion emissions many miles. A petroleum spill on land can run off into surface waters that eventually run to the ocean, or seep through the soil into flowing underground aquifers.

PAHs are also distributed by partitioning between environmental media. LMW PAHs released to the air will generally exist in the vapor phase and may be degraded by reaction with photochemically-produced radicals in a relatively short time (ATSDR, 1995b). HMW PAHs will partition almost completely into the particulate phase, adsorbed to fine particles which are suspended in the air. These particle-bound PAHs may be transferred to soil or surface waters dry or wet deposition. Long-range transport of airborne particle-bound PAHs from Great Britain to Norway and Sweden has been documented (ATSDR, 1995b).

Volatilization may be an important partitioning process for LMW PAHs released into water, but HMW PAHs do not volatilize appreciably (Dabestani and Ivanov, 1999). Due to their poor aqueous solubility, HMW PAHs in water are more likely to be adsorbed to suspended particles in the water column than to be dissolved (Latimer and Zheng, 2003). These suspended particles eventually settle to the bottom sediment, carrying the PAHs with them. In the marine environment, sediments are the primary repository of PAHs and the PAH distribution of sediment samples is dominated by 4- to 6-ring PAHs (Latimer and Zheng, 2003). PAHs in water and sediments can be sorbed to or accumulated by aquatic organisms (ATSDR, 1995b).

LMW PAHs released to or deposited on soil may volatilize appreciably or be broken down by soil microorganisms. HMW PAHs will tend to adsorb to soil particles. The physical properties of HMW PAHs limit their bioavailability and make them resistant to biodegradation (Dabestani and Ivanov, 1999). PAHs may migrate through soil into groundwater (ATSDR, 1995b).

In addition to occurring in environmental media, PAHs have also been detected in many living organisms, both plants and animals. Because of their stability and low aqueous solubility, HMW PAHs tend to accumulate in some biota. Bioconcentration depends on the metabolic capabilities of the organism (ATSDR, 1995b). Terrestrial plants take up PAHs from soil through the roots or via atmospheric deposition on the foliage. Likewise, aquatic plants may absorb PAHs from the water column or from the sediments in which they grow. Aquatic animals may take in PAHs from contact with sediments or suspended particles in the water. PAHs are also part of the food chain; however, biomagnification of PAH concentrations through the food chain appears to be limited (ATSDR, 1995b). Terrestrial animals may be exposed to PAHs by ingestion of contaminated soil or through the food chain (ATSDR, 1995b).

### **1.3.4 Health Effects**

#### **1.3.4.1 Genotoxicity**

Genotoxic chemicals affect the genetic material and/or genetic processes of cells, acting by directly damaging DNA or by altering cellular functions such as DNA repair, mitosis, and control of cell proliferation. Genotoxic endpoints such as mutations and DNA strand breaks can lead to adverse health effects, including cancer, genetic disorders (e.g., cystic fibrosis), and birth defects (Preston and Hoffmann, 2001; Rogers



and Kavlock, 2001). Chromosomal alteration of tumor-suppressor genes and proto-oncogenes has been associated with many cancers and is recognized as a possible initiating step in the three-stage model of carcinogenesis. Although carcinogenesis is a complex process, an association between mutagenicity and carcinogenicity has been demonstrated for many chemicals, including some PAHs (Preston and Hoffmann, 2001).

The *in vitro* genotoxicity of individual PAHs, especially BaP, has been studied extensively. ATSDR (1995b) has summarized studies of PAH *in vitro* genotoxicity. Several PAHs caused gene mutations in strains of *Salmonella typhimurium* and DNA damage in *Escherichia coli*. BaP caused a wide range of genotoxic effects in cultured mammalian cells, including gene mutations (mouse, hamster and rat), chromosome aberrations (hamster, mouse, rat), sister chromatic exchange (hamster, rat), transformation (hamster, mouse, rat), and DNA binding (chick embryo, hamster, mouse, rat). BaP was also genotoxic in cultured human cells, causing gene mutations, chromosome aberrations, sister chromatid exchange, unscheduled DNA synthesis, and DNA binding in various cell types, including lymphocytes, lymphoblasts, skin fibroblasts, keratinocytes, bladder, endometrial, colon and bronchus cells (ATSDR, 1995b). BaP induced DNA adducts at key mutational hotspots in the tumor-suppressor gene P53 in human HeLa and bronchial epithelial cells (Denissenko et al., 1996).

Several complex PAH mixtures are also genotoxic *in vitro*. PAH-rich fractions of coal tar, wood preserving waste, and manufactured gas plant residue produced positive results in microbial genotoxicity bioassays (Cizmas et al., 2004a; Cizmas et al., 2004b; Mayura et al., 1999). Extracts from diesel particle emissions induced DNA adducts in the human mammary carcinoma cell line MCF-7 (Kuljukka-Rabb et al., 2001). Extracts from gasoline and diesel particle emissions induced DNA adducts in the human bronchial epithelial cell line BEAS-2B (Pohjola et al., 2003). Extractable organic matter from ambient air caused DNA breakage in the human-derived cell lines HepG2 and Caco-2 (Lazarova and Slamenova, 2004).

PAHs exhibit genotoxicity in laboratory mammals *in vivo*. When applied to mouse skin, benzo[b]fluoranthene, benzo[k]fluoranthene and indeno[1,2,3-cd]pyrene induced DNA adducts (ATSDR, 1995b). Treatment of Chinese hamsters with various PAHs by intraperitoneal injection resulted in chromosome aberrations (from

benz[a]anthracene and BaP) and sister chromatid exchange (from BaP, chrysene and dibenz[a,h]anthracene) in bone marrow cells (ATSDR, 1995b). When administered to laboratory mammals, BaP has induced mutations, DNA single strand breaks, chromosome aberrations, sister chromatid exchange, and micronuclei (ATSDR, 1995b). BaP administered by oral gavage to female ICR mice induced DNA adducts in lung, liver, kidney, spleen, and white blood cells (Reddy and Randerath, 1990). Multiple intraperitoneal injections of BaP induced DNA adducts in lung, liver, spleen and peripheral blood mononuclear cells of male Sprague-Dawley rats (Qu and Stacey, 1996). Many studies also demonstrate that *in vivo* treatment of experimental animals with PAH-rich mixtures such as coal tar, wood preserving waste, manufactured gas plant residues and cigarette smoke condensate induces PAH-DNA adducts in multiple tissues (Cizmas et al., 2004b; Randerath et al., 1996; Randerath et al., 1994; Reddy and Randerath, 1990; Weyand and Wu, 1995).

Human exposure to PAH mixtures has been linked to increased levels of PAH-DNA adducts (Kriek et al., 1998). PAH-DNA adduct levels in white blood cells of coke-oven workers correlated to estimated occupational exposure level (Ovrebo et al., 1992). A meta-analysis of occupational DNA adduct studies demonstrated a significant association between DNA adduct levels and air pollution exposure in industrial and urban workers and also found a correlation between measured BaP air concentrations and DNA adduct levels (Peluso et al., 2001). However, in other studies summarized by ATSDR (1995b), the dose-response relationship between PAH and/or BaP exposure levels and genotoxic effects was not significant.

#### **1.3.4.2 Carcinogenicity in Animals**

Some of the earliest evidence of PAH carcinogenicity was obtained in the early twentieth century by K. Yamagiwa and H. Tsutsui who generated malignant skin tumors in rabbits and mice, respectively, by repeatedly applying coal tar to the skin of the animals (Phillips, 1983). However, it was not then known that PAHs in the coal tar were causative agents. In the 1920s and 1930s, E.L. Kennaway and his colleagues studied coal tar intensively, hypothesized that PAHs were the primary carcinogenic agents in coal tar, and proved the carcinogenicity of PAHs by demonstrating that several pure, chemically synthesized PAH compounds produced skin tumors in mice (Phillips, 1983).

Kennaway's group eventually isolated benzo[a]pyrene (BaP) from coal tar and found both the isolated BaP and synthetic BaP to be highly carcinogenic (Cook et al., 1933).

Largely based on evidence of carcinogenicity in experimental animals, seven PAHs have been classified as probable human (B2) carcinogens. These seven carcinogenic PAHs are listed in Table 1-2 with the abbreviations that will be used in this text. The compounds are listed in order of decreasing toxic equivalency factor (TEF) proposed by Nisbet and LaGoy (1992). TEFs are used in risk assessment to relate the toxicity of compounds that have similar chemical structures and modes of action to a reference compound. The carcinogenicity of selected PAHs was assessed relative to reference compound BaP, which was assigned a TEF of 1.0.

**Table 1-2**  
Toxic equivalency factors (TEFs) for carcinogenic PAHs

Compound	Abbreviation	TEF <sup>a</sup>
Dibenz[a,h]anthracene	DBA	5
Benzo[a]pyrene	BaP	1
Benz[a]anthracene	BaA	0.1
Benzo[b]fluoranthene	BbF	0.1
Benzo[k]fluoranthene	BkF	0.1
Indeno[1,2,3-cd]pyrene	IDP	0.1
Chrysene	Chr	0.01

a. TEFs from Nisbet and LaGoy (1992)

BaP has been widely studied and its toxicity is the most well-characterized of the PAHs. BaP has been associated with an increased incidence of tumors in multiple rodent studies; tumor locations vary with administration route and include upper respiratory tract, digestive tract, skin, and injection sites (USEPA, 1994b). In two similar studies, three consecutive doses of BaP administered by intraperitoneal injection to newborn CD-1 mice resulted in statistically significant increases of incidence of liver tumors in males, and lung tumors in both males and females (Lavoie et al., 1987; Wislocki et al., 1986). In a 2-year feeding study of female B6C3F1 mice, 3 dose levels of BaP were administered (Culp et al., 1998). Percentage survival was significantly

reduced in the medium- and high-dose groups. The incidence of papillomas and carcinomas of the forestomach, esophagus and tongue was significantly higher in BaP-treated groups and a significant dose-related effect was observed in each of these tissues (Culp et al., 1998).

Dibenz[a,h]anthracene (DBA) has the highest TEF (5.0) of the PAH B2 carcinogens. DBA administered in drinking water to DBA/2 mice induced pulmonary adenomas, pulmonary carcinomas, and mammary carcinomas (USEPA, 1994f). Gavage administration (2 doses per week, 15 weeks) of DBA to pseudo-pregnant female Balb/c mice resulted in mammary carcinomas in 13 out of 24 mice versus 2 out of 30 in the untreated control group (USEPA, 1994f). In a study designed to test skin tumor-initiating activity of various carcinogens, female Sencar mice received a single dermal application of DBA followed by 15 weeks of twice-weekly application of a promoting agent. At the end of treatment, papillomas were observed in 50% of treated mice (versus 6% control) at an average of 1.4 papillomas per mouse (Slaga et al., 1980). Subcutaneous injection of DBA (20 doses, 3 per week) induced injection-site sarcomas in 100% of female S-D rats versus 0% incidence in both the untreated and vehicle-treated control groups (Flesher et al., 2002).

Benz[a]anthracene (BaA) caused increased incidence of tumors at administration sites in mice exposed to BaA by gavage, subcutaneous injection and topical application (USEPA, 1994a). Repeated gavage with a solution containing 3% BaA caused an increased incidence of hepatomas and pulmonary adenomas in male B6AF1/J mice (Klein, 1963). In another study, newborn CD-1 mice were administered three doses of BaA by IP injection between birth and 15 days of age; survivors were sacrificed after 1 year. Statistically significant increases in tumor incidence occurred in both males (liver) and females (lung) (Wislocki et al., 1986).

Benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), and indeno[1,2,3-cd]pyrene (IDP) all contain a five-membered ring and have each been assigned a 0.1 TEF value. BbF, BkF and IDP have all generated positive results for tumor initiation in mouse skin painting assays (USEPA, 1994c; USEPA, 1994d; USEPA, 1994g). In a lifetime lung-implantation study of 3-month-old female Osborne-Mendel rats, tumors in the lung and thorax (epidermoid carcinomas and pleomorphic sarcomas) were induced in a dose-dependent manner by BbF, BkF and IDP. The percentage of animals

showing a response (tumor formation) was 37% for BbF, 44% for BkF, and 60% for IDP, versus 0% for both untreated and vehicle-treated animals (Deutsch-Wenzel et al., 1983). In another study, BbF, BkF, and IDP were administered in three consecutive doses by intraperitoneal injection to newborn CD-1 mice (Lavoie et al., 1987). The mice were sacrificed after 52 weeks and the number of mice with liver or lung tumors was determined. BbF induced liver tumors in 8 out of 15 male mice ( $p < 0.005$ ) and lung tumors in 2 of the male mice. BkF induced liver tumors in 3 out of 16 male mice, lung tumors in 1 out of 16 male mice and 3 out of 18 female mice, but these results were not statistically significant. IDP was not tumorigenic in this study (Lavoie et al., 1987).

Chrysene (Chr) has the lowest TEF (0.01) of the PAH B2 carcinogens. In skin painting assays for initiating activity, Chr produced positive results in several mouse strains when applied in combination with various promoting agents (USEPA, 1994e). For example, dermal application of Chr to female Sencar mice resulted in papillomas in 73% of treated mice (versus 10% control) at an average of 1.6 papillomas per mouse (Slaga et al., 1980). In another study, newborn CD-1 mice were administered either a low (700 nmol) or high (2800 nmol) dose of Chr three times by IP injection between birth and 15 days of age; survivors were sacrificed after 1 year (Wislocki et al., 1986). In males of the low-dose group, statistically significant increases in incidence of liver tumors and malignant lymphoma were observed. In males of the high-dose group, statistically significant increases in incidence of liver tumors and lung tumors occurred. In females, no differences were observed between either dose group and controls (Wislocki et al., 1986).

#### ***1.3.4.3 Carcinogenicity in Humans***

PAHs do not occur in the environment as individual compounds, but rather as mixtures of many PAH compounds. In addition, humans are usually exposed to PAHs which are part of complex chemical mixtures, such as tobacco smoke, containing other carcinogens. Because of this, when a complex chemical mixture is found to cause cancer in humans, establishing PAHs as the carcinogenic agents is difficult. Most evidence of PAH mixture carcinogenicity comes from occupational studies in which workers involved in coal gasification, coke production, roofing, or oil refining were exposed to PAH-rich mixtures (ATSDR, 1995b).

The 1775 report of Percival Pott on the role of soot exposure in the incidence of scrotal cancer among chimney sweeps is widely recognized as the first documentation of carcinogenicity of a mixture containing PAHs (Gallo, 2001). In a review summarizing fifty years of research on BaP, D.H. Phillips (1983) noted that, in the nineteenth century, workers in the coal tar, paraffin refining and shale oil industries were known to experience high incidences of skin cancer, although the causative agents were not identified.

A large, long-term epidemiological study of steelworkers in Allegheny County, Pennsylvania examined cancer morbidity and mortality rates of workers in the coke production areas relative to workers in all other areas of the steel plants. Increased rates of respiratory cancer were observed among coke oven workers (Lloyd, 1971). Excess mortality from lung and kidney cancer was documented among coke oven workers and digestive system cancers were significantly elevated in non-oven workers in the coke production areas (Redmond et al., 1976). In addition, evidence was found of a consistent dose-response relationship between lung cancer mortality and estimated cumulative exposure to coal tar pitch volatiles (Redmond, 1983).

Mastrangelo et al. (1996) reviewed 10 epidemiological studies of occupational PAH exposure published between 1979 and 1995. Occupations included were iron foundry worker, aluminum factory worker, miner, and steelworker. Other population-based studies included various occupations, and a population in an area with high density of chemical plants. Studies were chosen which described PAH exposure in quantitative or qualitative levels and which examined cancer as an endpoint. The authors concluded that risks of lung and bladder cancer from PAH exposure were dose-dependent (Mastrangelo et al., 1996).

Boffetta et al. (1997) reviewed cancer risk from occupational and environmental exposure to PAHs, with evidence from more than a dozen industries and occupations. The authors found that heavy occupational exposure to PAH mixtures increases the risk of lung, skin, or bladder cancer. Lung cancer risk was increased among most of the occupations. Skin cancer increased in occupations in which dermal exposure was substantial. High exposure to coal tar and pitch, such as among workers in aluminum production, coal gasification and tar distillation, was associated with increased bladder

cancer risk. There was also limited evidence of a possible link between kidney cancer and occupational PAH exposure (Boffetta et al., 1997).

A recent review (Bosetti et al., 2007) examined epidemiological cohort studies of workers exposed to PAHs which were published up until December, 2005, mainly those published after the studies covered in Boffetta et al. (1997). The review focused on risk of cancers of the respiratory and urinary tracts and included studies of workers in industries of aluminum production, coal gasification, coke production, iron and steel foundry, coal tar and related products, carbon black and carbon electrodes production. Increased risks from lung and bladder cancer were found in some of these occupations. The authors mention that data are limited from several of the industries, and estimation of exposure and effects of confounding factors and exposures other than PAHs continue to complicate the interpretation of the data. Dose-risk relationships were found in coke production workers (lung cancer) and iron and steel foundry workers (respiratory tract cancers), but effects of exposure to other potentially carcinogenic substances could not be isolated. Overall, the authors concluded that epidemiological evidence confirms an excess lung cancer risk in several PAH-related industries and an excess risk of urinary system cancers in aluminum production, coal gasification, and iron and steel foundry workers (Bosetti et al., 2007).

The relationship between PAH exposure and incidence of bladder cancer was examined in a large historical cohort (7298 men) of asphalt pavers in Denmark, Norway, Finland and Israel (Burstyn et al., 2007). A detailed procedure was used to estimate cumulative and average PAH exposure of each member of the cohort. Inhalation exposure levels were modeled for different paving operations and technologies based on previously available personal exposure measurements from asphalt industry workers. By coupling the detailed job history and the modeled exposure levels, the authors reconstructed an exposure history for each cohort member. Members were then divided in exposure quartiles. Cumulative exposure was not associated with risk of bladder cancer; the two higher quartiles of average exposure had approximately 40% excess risk of bladder cancer but there was no dose-response trend. When a 15-year lag was factored into the average exposure, there was a twofold increase in bladder cancer risk in the two higher average exposure quartiles. The work histories for subjects only included the time that each was employed in asphalt paving, and thus

could not control for PAH exposure in other occupations and lifestyle exposures (such as smoking). Also, dermal PAH exposure, which might be significant, was not estimated. The authors concluded that 1) work in asphalt paving may be associated with increased risk of bladder cancer, 2) exposure to relatively low airborne PAH levels may confer risk of bladder cancer, and 3) inability to control for sources of confounding and bias, lack of exposure-response trend, and borderline statistical significance of the data hampered interpretation of study results (Burstyn et al., 2007).

A nested case-control study examined association of prostate cancer incidence with occupational chemical exposure in workers at a nuclear energy and rocket engine-testing facility (Krishnadasan et al., 2007). A job exposure matrix was used to estimate occupational exposures to PAHs, benzene, mineral oil, hydrazine, and trichloroethylene. High levels of trichloroethylene exposure were associated with prostate cancer incidence. Increased risk of prostate cancer was observed at high levels of PAH exposure, but when the data were adjusted for other chemical exposures (PAH exposure was highly correlated with benzene and trichloroethylene exposures), only a weak association of PAH exposure with prostate cancer incidence was found. The authors note that PAH exposure levels among the workers in the study were likely lower than those in studies in which a strong association between PAH exposure and prostate cancer was found (Krishnadasan et al., 2007).

Another epidemiological study compared the use of two PAH exposure indices in aluminum smelter workers exposed to coal tar-derived substances (Friesen et al., 2007). The indices were cumulative exposure to BaP and cumulative exposure to benzene-soluble material (BSM). A job exposure matrix was developed from routine measurements of BaP and BSM at the facility, and the matrix was applied to each worker's job history to obtain cumulative exposure indices for each worker. The health outcomes evaluated were bladder cancer incidence, lung cancer incidence, and mortality due to acute myocardial infarction. Both exposure indices were strongly associated with bladder and lung cancer incidence and modestly associated with mortality due to acute myocardial infarction. The exposure-response model precision for cancer incidence was somewhat better with the BaP exposure index than the BSM exposure index (Friesen et al., 2007).



#### **1.3.4.4 Immunotoxicity**

Compromised immune system functioning increases susceptibility to bacterial and viral infections and may result in repeated, more severe, or longer infections. The immune system also plays a role in identification and rejection of neoplasms, and thus in the development of cancer. PAHs are known to be potent immunosuppressants which have effects on humoral immunity, cell-mediated immunity, and host resistance (Burns-Naas et al., 2001).

Most evidence of the immunotoxicity of PAHs is from lab animal studies and a few human cell culture studies. When applied to mouse skin, BaP suppressed humoral and cell-mediated immunity, and onset of skin tumors was accompanied by changes in Langerhans cell number, distribution, and morphology (ATSDR, 1995b). *In utero* exposure of mice to a single dose of BaP by intraperitoneal injection reduced specific T cell subsets in the thymus and spleen, and resulted in severe and sustained immunosuppression and an increase in tumor incidence later in life (Rodriguez et al., 1999; Urso and Gengozian, 1980; Urso and Gengozian, 1984; Urso and Johnson, 1987). In rats, oral administration of fluoranthene caused significant decreases in white blood cell counts in both acute and subchronic studies (Knuckles et al., 2004). Subchronic oral administration of BaP to rats resulted in immunotoxicity as measured by several parameters, including thymus and lymph node weights, and counts of spleen B cells, spleen natural killer cells, red blood cells, white blood cells, lymphocytes, and granulate eosinophils (De Jong et al., 1999).

Isolated mouse splenic macrophages were capable of metabolizing BaP *in vitro* (Ladics et al., 1992b), and repeated subcutaneous injections of BaP altered the macrophage cell population, suggesting that macrophages are a target of BaP (Ladics et al., 1992a). PAHs inhibited activation of human and rodent B and T lymphocytes and suppressed mitogenesis of human peripheral blood T cells *in vitro* (Davila et al., 1996; Romero et al., 1997). In human peripheral blood mononuclear cell lymphocytes, BaP did not deplete glutathione, but reactive BaP metabolites did (Romero et al., 1997).

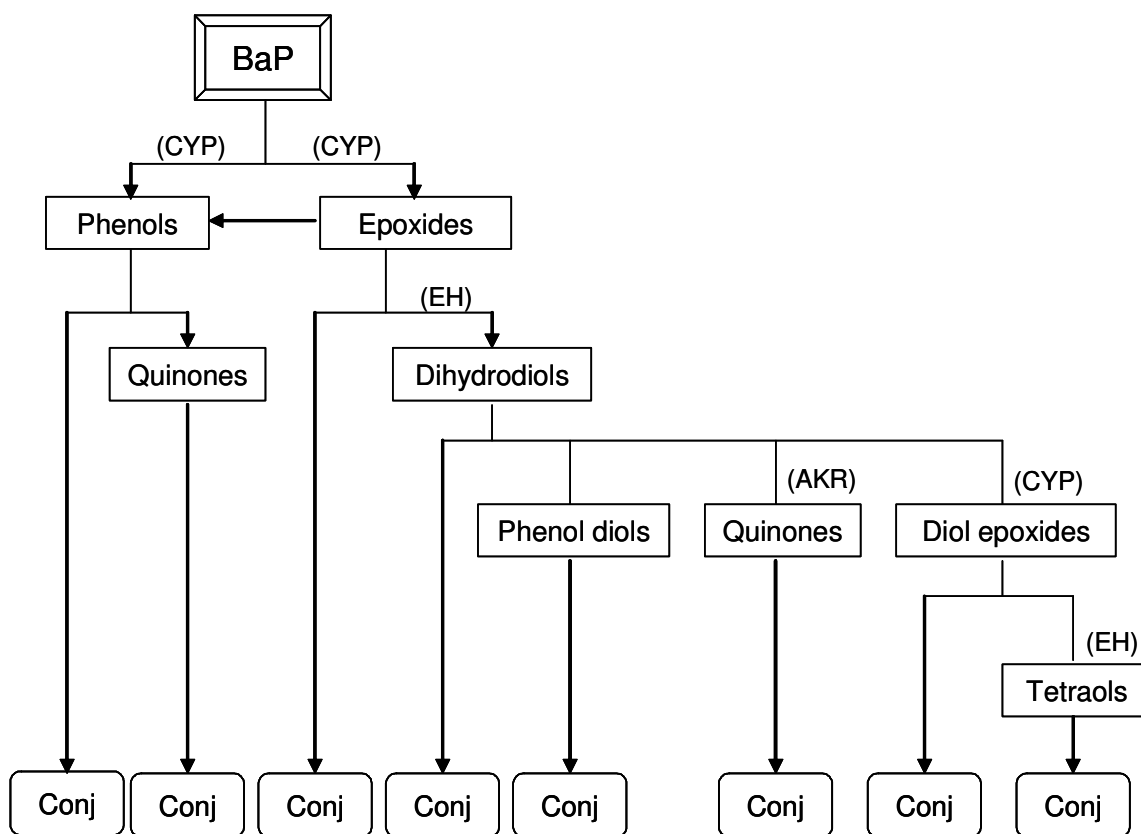
The influence of CYP enzymes on BaP immunotoxicity was investigated in short-term feeding studies of C57BL/6J mice and several knockout strains (Uno et al., 2004; Uno et al., 2006). In the wild-type animals, 18 days of a diet of food soaked with corn oil containing BaP resulted in a decrease in thymus weight. Relative to untreated

controls and treated wild-type mice, BaP-treated *Cyp1a1* knockout mice had extreme bone marrow hypocellularity and a marked decrease in blood lymphocytes, spleen weight and thymus weight (Uno et al., 2004). Thymus and spleen weights were also significantly decreased in BaP-treated *Cyp1a1/Cyp1b1* knockout mice. The authors concluded that inducible CYP1A1 is more important in detoxification than activation of ingested BaP and that “CYP1B1 in spleen and in bone marrow seems to be responsible for metabolic activation of benzo[a]pyrene, which results in immune damage in the genetic absence of CYP1A1” (Uno et al., 2006).

#### **1.3.4.5 Metabolic Activation**

PAHs are generally not toxic in their unsubstituted form; they must be transformed into toxic metabolites by cellular XMEs. However, because PAHs enter cells easily and rapidly partition into cytoplasmic membranes (Barhoumi et al., 2000), metabolism is also required to make the molecules more water soluble so that they can be excreted from the cell. Therefore, cellular XMEs are critical to both the bioactivation and detoxification of PAHs.

Unsubstituted PAHs are similar in structure and undergo similar pathways of metabolism. The metabolism of BaP is the most fully characterized of all the PAHs and is described here in Fig. 1-5, which was adapted from Ramesh et al. (2004) and Shimada (2006). The first step in BaP metabolism is transformation by CYP into either phenols or epoxides. Phenols may be further metabolized to quinones, and epoxides may be further metabolized to phenols or by epoxide hydrolase (EH) to dihydrodiols. From dihydrodiols, phenol diols are formed, as well as diol epoxides (catalyzed by CYP) and quinones which are formed by the action of aldo-keto reductase (AKR). Diol epoxides can undergo further transformation by EH to tetraols. At each step along these pathways, oxygenated metabolites may be detoxified by conjugation catalyzed by phase II XMEs such as GST, UGT, and sulfotransferases (SULT). The conjugates are then excreted from the cell and eliminated from the body by renal or biliary routes (Ramesh et al., 2004; Shimada, 2006).



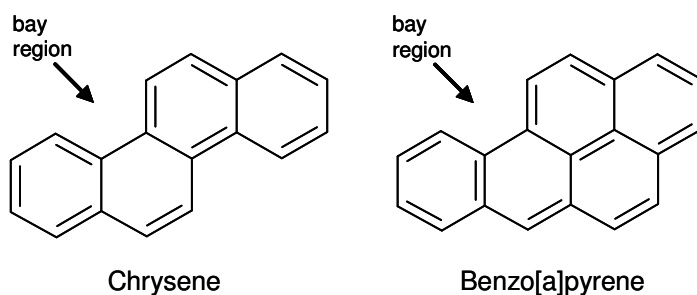
**Fig. 1-5.** BaP metabolic pathways. CYP = cytochrome P450; EH = epoxide hydrolase; AKR = aldo-keto reductase; Conj = conjugated metabolite. Adapted from Ramesh et al. (2004) and Shimada (2006).

When PAHs were isolated and identified as carcinogens, it was thought that the parent compounds themselves were biologically active; metabolism was considered to be solely a detoxification process (Phillips, 1983). The possibility that PAH metabolites were biologically active was proposed by Boyland in 1950 (Boyland, 1950). Evidence accumulated during the 1960s that covalent binding of PAHs to DNA was a critical step in carcinogenesis (Phillips, 1983). In the late 1960s, Gelboin (1969) and Grover and Sims (1968) demonstrated in *in vitro* systems that PAHs would bind to DNA only in the presence of induced rat liver microsomal fractions. Further study of PAH metabolism and DNA binding suggested that BaP-7,8-dihydrodiol had 10 times the DNA binding affinity of the parent compound (Borgen et al., 1973) and that further metabolism of the

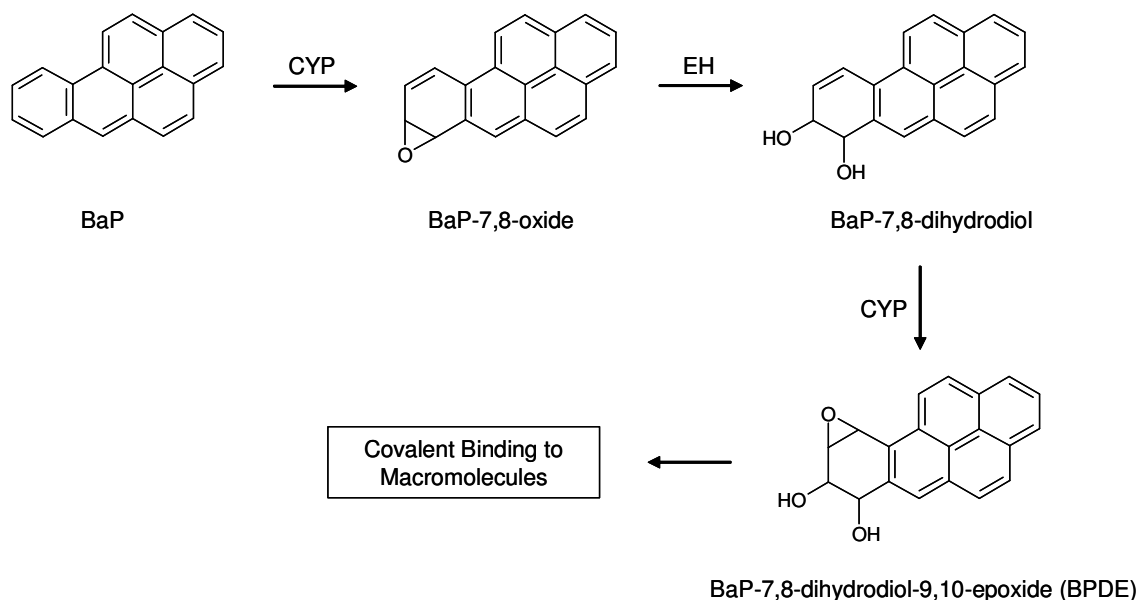
dihydrodiol produced BaP-7,8-diol-9,10-epoxide (BPDE), the ultimate carcinogen (Sims et al., 1974).

Based on these discoveries, the theory of the bay region dihydrodiol epoxide metabolic pathway was developed and was considered to be the dominant mechanism for bioactivation of carcinogenic PAHs. The bay region is a deep pocket that exists where a single benzene ring is attached to an angular ring fusion (Fig. 1-6). The formation of a bay region diol epoxide proceeds through three steps (Fig. 1-7). Step 1 is CYP-catalyzed oxidation of a double bond on the single-fused ring to an unstable arene oxide (epoxide). In Step 2, epoxide hydrolase (EH) hydrolyzes the arene oxide to a dihydrodiol. Step 3 is another CYP-catalyzed oxidation, this time of the double bond adjacent to the bay region, which forms a bay region diol-epoxide. Several PAHs are metabolized through this pathway, including BaP, chrysene, 5-methylchrysene, phenanthrene, benzo[*c*]phenanthrene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, and dibenzo[*a,l*]pyrene (Xue and Warshawsky, 2005).

When the bay region epoxide opens, a benzyl cation forms which is stabilized by the aromatic bonds around the bay region. This benzyl cation is a highly reactive electrophile which binds covalently to cellular macromolecules, including DNA. The most common binding sites of PAH-diol-epoxide DNA adducts are the exocyclic amino groups of deoxyguanosine (dG) and deoxyadenosine (dA) (Xue and Warshawsky, 2005). The major DNA adduct of BPDE, the bay region diol-epoxide of BaP, is formed by bonding of the C10 of BPDE to the N2 of dG (Cheng et al., 1989).

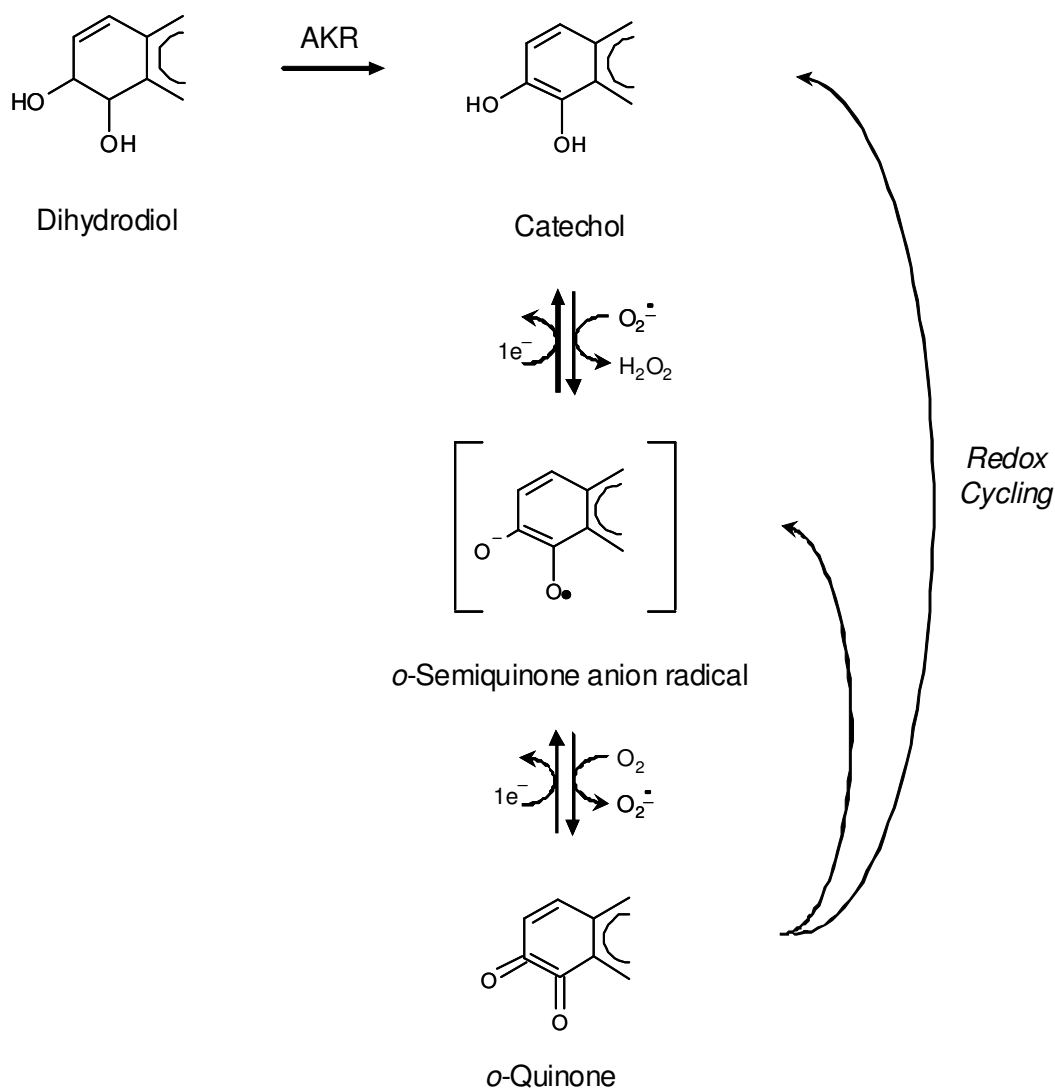


**Fig. 1-6.** Representative PAHs with bay regions.



**Fig. 1-7.** Bioactivation of BaP by bay region dihydrodiol epoxide metabolic pathway.

Another important pathway of PAH activation is through generation of reactive and redox active PAH-*ortho*-quinones. Once the PAH-dihydrodiol is formed by the combined action of CYP and EH, another set of enzymes, the dihydrodiol dehydrogenases, compete with CYP to transform the dihydrodiol. Enzymes of the AKR superfamily are responsible for human dihydrodiol dehydrogenase activity. AKR enzymes oxidize PAH dihydrodiols to unstable catechols as shown in Fig. 1-8 (adapted from Penning et al. (1999)). The catechol undergoes autoxidation in the presence of oxygen. In the first oxidation, peroxide and an *o*-semiquinone anion radical are formed. In the second oxidation, superoxide anion and an *o*-quinone are formed. The *o*-quinone can then undergo nonenzymatic reduction to either the catechol or the *o*-semiquinone anion radical, setting up redox cycling which can amplify generation of reactive oxygen species (ROS), such as peroxide and superoxide anion, all resulting from oxidation of a single molecule of PAH-dihydrodiol by AKR (Penning et al., 1999).



**Fig. 1-8.** Bioactivation of PAH by *o*-quinone pathway. Adapted from Penning et al. (1999).

PAH-*o*-quinones can react with DNA to form both stable and unstable (depurinating) adducts *in vitro*. Acellular assays have demonstrated that quinones of BaP (Balu et al., 2004; McCoull et al., 1999; Shou et al., 1993), naphthalene (McCoull et al., 1999), and phenanthrene (McCoull et al., 1999) covalently bond to DNA nucleosides. ROS can induce oxidative damage of DNA bases, forming oxidized deoxynucleosides that can cause base mismatching and result in gene mutations (Xue and Warshawsky, 2005). In both these ways, the oxidation of PAH-dihydrodiols by AKR

can lead to DNA damage which contributes to the genotoxicity of PAHs. In addition, ROS are known mitogens which enhance tumor promotion.

#### **1.4 Analysis of Environmental Mixtures**

The goal of human health risk characterization is to determine the change in risk of adverse health effects for a population of humans caused by their exposure to environmental contaminants. Toxicity assessment is an essential component of human health risk characterization. The outcome of toxicity assessment is a quantitative expression of the exposure-effect relationship. The most biologically relevant human toxicity assessment for an environmental mixture would be based on the evaluation of actual health effects in an existing exposed human population. Where this is not feasible or desirable (which is almost always the case), a variety of analysis methods may be used to assess the toxicity of an environmental mixture. These methods range from chemical analysis to toxicity testing models to complex epidemiological studies. Mixture analysis methods vary in consumption of resources (duration, size, cost), ease of data interpretation (specificity of effects, mechanistic information, statistical power), and relevance of the results to actual human biological responses and exposure conditions. Ultimately, interpretation of the combined results from multiple analysis methods will provide the most complete characterization of the human health risk of an environmental mixture.

##### **1.4.1 Chemical Analysis**

The chemical analysis approach to toxicity assessment of environmental mixtures involves determining the individual chemical components of the mixture and estimating the toxicity of the mixture based on the toxicity of individual components. In general, for chemical analysis of mixtures of hydrocarbons, a sample of the environmental medium contaminated with the mixture is collected, components of the mixture are extracted from the environmental medium, and the components of the extract are identified and quantified by analytical instrumentation. This approach relies on the availability of toxicity assessments for each mixture component and assumptions of how the toxicity of each component affects the rest of the mixture. For example, the toxicity of PAHs is usually considered to be additive, so the toxicity of each PAH is

weighted by its concentration in the mixture, and the sum of these weighted toxicities is determined to be the overall toxicity of the PAH mixture.

The resource consumption of chemical analysis is relatively low compared to other mixture toxicity analysis methods. It includes planning and execution of field sampling, processing of samples (transportation, tracking, preparation for analysis), analysis and quantitation. Analytical equipment is costly but is usually shared by many projects. Results are obtained quickly compared to animal or epidemiological studies. The low resource consumption often allows analysis of many samples, which increases statistical power. Data interpretation is relatively easy if toxicity data and interaction information are available for each component, but this is rarely the case. Because chemical analysis does not directly measure toxicity, the relevance of results of chemical analysis to actual human exposures and biological effects is low unless extensive information is available about how humans are exposed, the component toxicities, and the mixture interactions. In addition, environmental matrices can be highly heterogeneous with significant temporal and spatial variability; sometimes many samples must be collected in order to accurately represent the contamination level and potential for exposure.

#### ***1.4.1.1 PAH Analysis by Gas Chromatography/Mass Spectrometry***

USEPA Method 8270, "Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry" is an approved method for analyzing PAH concentrations in extracts from many types of solid waste matrices, soils, air sampling media and water samples (USEPA, 1996). Extracts may be prepared by various methods, including Soxhlet extraction, ultrasonic extraction, and pressurized fluid extraction (method used in this research). The prepared sample extracts are mixed with a carrier gas, heated to high temperature, and injected into a capillary column gas chromatograph (GC) for separation. A computer-controlled program gradually increases the capillary column temperature to better separate the analytes of complex mixtures with a range of boiling points. Analytes eluted from the GC then enter a mass spectrometer (MS) for detection. The MS fragments the analytes into gas-phase ions, separates these ions by mass-to-charge ratio, and measures the quantity of ions of each mass-to-charge ratio. Each PAH analyte has a unique fragmentation "fingerprint"



that can be detected by the MS. Analyte concentrations are quantitated by comparing the fragmentation spectra detected to a database of known spectra.

#### **1.4.2 Mammalian Cell Culture**

Mammalian cells can be isolated and grown in culture for use in toxicity testing. Cultured cells offer many advantages as toxicity testing models for environmental mixtures. Consumption of resources is low relative to other toxicity analysis methods. Cultured cells grow rapidly, allowing generation of large amounts of cells and many experimental groups. Cells can be frozen and stored for future use, allowing flexibility in planning of experiments. Because cells are small and experiments short, the costs of cell-based experiments are relatively low. The combination of cell culture models with powerful imaging techniques enables the collection of real-time data. The conditions under which cells are grown can be tightly controlled, minimizing variations in results and increasing statistical power. The genetic homogeneity of cell lines further reduces experimental variability, decreases the number of replications needed, and eases interpretation of results. An environmental mixture can be applied directly into the culture medium, reaching the cells almost immediately, so that cellular responses occur quickly and the dose is well controlled. Cell culture is preferred to live animal testing for ethical reasons. Because of these advantages, many assays have been developed for evaluating toxicity in cultured cells.

One other important advantage of cultured cells is that techniques have been developed that make genetic modification of cells relatively simple. Modification of mammalian cells by introduction of various gene clones can enhance the utility of the cell line in toxicity research. For example, transgenic HepG2 cells containing the entire human pregnane X receptor (hPXR) gene have been used to assess the ability of pesticides to induce CYP3A4 and CYP2B6 (Lemaire et al., 2004). Cultured mammalian cells can also be used to study the mechanisms of toxicity and to characterize dose-response relationships.

The disadvantages of mammalian cell culture for toxicity analysis are related to the relevance of the results to humans. Results of cell culture studies cannot be extrapolated directly to human toxicity assessment because of many factors, including the lack of tissue interactions and the highly simplified toxicokinetics of cells in culture (Combes, 2005). Chemicals are usually administered at a certain concentration in the

cell growth medium rather than in a measured amount, so dose determination is difficult. Gene expression in cultured cells may be different from the intact animal; this has resulted in major differences in the effects of gene expression on toxicity between cultured cells and whole animals (Nebert, 2006). It is difficult to relate the doses administered in cell culture to actual human exposures. Cellular responses to challenge with a xenobiotic may be easily measured, but extrapolating these responses to clinically-relevant, whole-organism health effects is a daunting task.

#### **1.4.2.1 HepG2 Human Cell Line**

Most cultured cells die after a finite number of divisions, but some cells, commonly those derived from tumors, have the ability to divide indefinitely and are developed into immortalized cell lines. Many mammalian cell lines have been utilized as toxicity testing models. These cell lines include those derived from mice (embryonic fibroblast 3T3, hepatoma Hepa1c1c7, lymphoma L5178Y), rats (liver epithelial Clone 9), hamsters (ovarian epithelial CHO, fibroblast BHK21), dogs (kidney epithelial MDCK) and humans (cervical epithelial HeLa, hepatocarcinoma HepG2, breast adenocarcinoma MCF-7).

The laboratory-based research described in this dissertation utilized the widely-used HepG2 cell line as a model for *in vitro* analysis of BaP genotoxicity. HepG2 is a human-derived hepatoma cell line that was isolated from a primary hepatocellular carcinoma in a young individual by Aden et al. (1979). HepG2 cells resemble liver parenchymal cells and, unlike most mammalian cell lines, have retained functionality of many phase I and phase II drug-metabolizing enzymes that are important in bioactivation and detoxification of xenobiotics (Knasmuller et al., 1998). Although activities of many enzymes in HepG2 cells are much lower than in primary human hepatocytes, substrate specificities are similar (Knasmuller et al., 1998; Rodriguez-Antona et al., 2002). In a review of the use of HepG2 cells in genotoxicity studies, Knasmuller et al. (2004) concluded that HepG2 cells are a suitable tool for genotoxicity testing and that the cell line is highly sensitive towards PAHs. HepG2 cells have been used to evaluate the genotoxic effects of PAHs with many DNA damage assays, including SCGE, micronucleus, unscheduled DNA synthesis, sister-chromatid exchange, and DNA adduct assays (Cherng et al., 2006; Knasmuller et al., 1998; Staal et al., 2006).

#### **1.4.2.2 DNA <sup>32</sup>P-Postlabeling Assay**

DNA adducts are important indicators of DNA damage and biomarkers of exposure to environmental carcinogens (Phillips, 2005). DNA adducts have been used as biomarkers of PAH exposure in environmental toxicology studies of both humans (Kriek et al., 1998) and wildlife (Shugart, 2000). DNA adducts form when genotoxins covalently bind with DNA. Many environmental contaminants are known to cause DNA adducts. Adducts can cause DNA damage which may lead to mutations if the DNA is replicated before being repaired. The formation and persistence of carcinogen-DNA adducts have been shown to be critical events for the initiation of chemical carcinogenesis in target cells.

Several methods are available for detecting DNA adducts. These include immunoassays and immunohistochemistry, fluorescence and phosphorescence spectroscopy, GC/MS, <sup>32</sup>P-postlabeling, and accelerator mass spectrometry (AMS) (Poirier et al., 2000). AMS is the most sensitive method, with a detection limit in the range of 1 in 10<sup>12</sup> nucleotides, but requires administration of radiolabeled compound (Poirier et al., 2000).

The <sup>32</sup>P-postlabeling assay is a highly sensitive and widely used method of detecting adducts that was developed in the early 1980s (Gupta et al., 1982). An enhanced procedure published a few years later (Reddy and Randerath, 1986) increased the sensitivity of the assay. The method is capable of detecting adducts at frequencies as low as 1 in 10<sup>10</sup> nucleotides using 10 µg DNA (Phillips and Arlt, 2007). <sup>32</sup>P-postlabeling can detect a wide range of adducts and does not require knowledge of adduct structure, allowing detection of unidentified carcinogens (Randerath and Randerath, 1994). This makes the method ideal for use in studies of exposures to environmental mixtures. In addition to sensitivity and broad applicability, a significant advantage of the <sup>32</sup>P-postlabeling method is that the DNA is radiolabeled after the sample is collected; it is a non-invasive technique that allows retrospective studies and eliminates radiation exposure of study participants. An important limitation of the <sup>32</sup>P-postlabeling method is that although it generates quantitative results, the efficiency of adduct labeling is uncertain. Caution must be exercised when adduct levels are compared between experiments. However, use of a synthetic standard for a specific adduct allows the efficiency of labeling to be determined (Phillips and Arlt, 2007).

In the  $^{32}\text{P}$ -postlabeling assay, digested DNA nucleotides are incubated with [ $\gamma$ - $^{32}\text{P}$ ]ATP and the radioactive orthophosphate is transferred selectively to adducted nucleotides. After the adducts are separated by three-dimensional thin-layer chromatography, the radioactivity is quantified. The relative amount of adducts is indicated by the ratio of radiolabeled nucleotides to total nucleotides (Phillips and Art, 2007; Reddy and Randerath, 1986). The research presented in this dissertation has used the DNA  $^{32}\text{P}$ -postlabeling assay to detect DNA lesions in cultured human cells treated with BaP and in wild mice exposed to environmental mixtures *in situ*.

#### **1.4.3 Laboratory Animals**

The use of laboratory animals as toxicity testing models for environmental mixtures offers some of the same advantages as cultured cells. Like cell culture experiments, in laboratory animal experiments the conditions under which the organism lives can be tightly controlled. The administered dose is known and the elapsed time between exposure and the measurement of effects is known. Route of exposure is also controlled. Confounding factors such as diet and age are the same across all experimental groups. In-bred rodent strains developed for laboratory use are genetically similar; this homogeneity reduces variability in results and increases statistical power.

The genetics of laboratory animals can also be manipulated, although this is a much more complicated and resource-intensive process than in cultured cells. Controlled genetic variation in laboratory animals has helped scientists to further understanding of molecular biology and to elucidate mechanisms of disease and toxicity. Mouse strains have been developed with naturally-occurring genetic variations which generate distinct phenotypes. For example, important advances in the understanding of PAH metabolism were made by comparing mice with a low-affinity form of the aryl hydrocarbon receptor (AhR) to those with a high-affinity form (Parkinson, 2001). Key genes may be removed from the genome; this creates a "knockout" strain that is useful for studying environmental agents (Nebert and Duffy, 1997). Important gene knockout strains for the study of PAHs include AhR, CYP1A1 and CYP1B1 knockout mice. Transgenic mouse strains carrying human copies of genes have also been developed. These "humanized" mouse strains can be especially

useful when there are distinct differences between the murine and human genes, such as with PXR (Xie et al., 2000).

An advantage of laboratory animal experiments over cell culture experiments is that they are more relevant to humans. The chemical is administered to the intact animal. Cells are supported by normal tissue architecture and have important cell-cell interactions. Whole animal toxicokinetics are in effect, including partitioning of mixture components and the formation of metabolites in one tissue that exert toxic effects in another tissue. Health effects can be assessed at the molecular, cellular, organ, tissue, and whole organism levels.

A disadvantage of laboratory animals relative to cell culture is that animal experiments usually are more resource-intensive. Animals must be cared for humanely and in accordance with regulations; animals also take up much more space and cost more to purchase. Because of toxicokinetics, effects often take longer to appear and a larger dose is often needed to generate a measurable effect. Most animal bioassays require sacrifice of the animal, whereas many cell culture assays can be performed while the cells are still alive.

Limitations exist to the application of results from laboratory animal toxicity testing to human health risk assessment. Animal studies often employ higher doses than are expected in humans and do not have adequate dose-response information for extrapolation to lower doses. Effects may be triggered at high doses that will not occur at lower doses. An example is urinary bladder tumor formation in rats that is caused by crystal precipitation of compounds in the bladder because the high dose exceeded the solubility of the compounds in urine (Faustman and Omenn, 2001). Exposures in animal experiments are often at discrete intervals, whereas many human exposures to environmental mixtures are frequent or nearly constant. The duration of exposure in animal experiments is usually shorter than the duration of typical human environmental exposures.

Significant differences in toxicokinetic processes can exist between experimental animals and humans (Kedderis and Lipscomb, 2001); these differences can lead to variation in the relationship between the administered dose and the internal dose at the target organ. Some administration methods used in animal studies, such as the intraperitoneal injection, do not occur in humans. The effects of direct oral gavage

administration on the rodent forestomach are not relevant for human exposure (Faustman and Omenn, 2001); in addition to the stress that the animal undergoes, direct gavage does not account for the dilution and interactions of the study compounds with food. Route-to-route extrapolation must take into account the differences in contact time and area, absorption rates and metabolism between different tissues. The uptake of contaminants can vary between species; for example, humans may receive smaller doses of inhaled contaminants than rats exposed to the same air concentrations because the surface area of the human olfactory epithelium is much smaller than that of the rat (Frederick et al., 1998). Metabolic capabilities, and therefore bioactivation and/or detoxification, vary between species. In some cases, different metabolizing enzymes are present in animals and humans; inducibility of enzymes may also be different between animals and humans (Parkinson, 2001). Differences in structure and function of key receptors also contribute (Barrett, 1995). Elimination of compounds occurs at varying rates and even by different routes in different species. These differences in toxicokinetic processes present a challenge for application of animal toxicity data to human health risk assessment. However, animal-to-human extrapolation can be improved by accounting for comparative physiological and toxicokinetic parameters.

#### **1.4.4 Animals *in situ***

Animals are exposed to contaminant mixtures in their environments. Humans and animals share local environments, food chains, air and water and thus share the potential for exposure to environmental contaminant mixtures. Animals have been used as sentinels for human health risk posed by environmental contaminants for more than a century; examples of these uses were compiled by van der Schalie et al. (1999). In several of these uses, animals provided effective early warning of potential risks to humans because the animals were more susceptible to the toxins. The authors identified potential applications for sentinel species, including monitoring environmental media and identifying new exposures of potential concern (van der Schalie et al., 1999). Study of sentinel animals can provide information about contaminant exposures and effects that cannot be collected from humans. For example, tissues from throughout the body of an exposed sentinel animal can be analyzed for toxicant levels and effects; this is obviously infeasible in humans (O'Brien et al., 1993). Sentinel animals also are

not subject to some confounding factors that occur in humans, such as smoking, occupational and recreational exposures. Exposures in sentinel animals may pose direct risk to humans, as in the case of fish contaminated with mercury. In this case, monitoring of exposures and effects in these animals clearly provides a benefit to human health. Rabinowitz et al. (2008) propose a concept of “shared risk” of humans and animals from hazards in the environment. The authors identify scientific knowledge gaps, such as differential disease expression across species and interspecies differences in molecular and cellular pathways for gene-environment interactions, which hamper the use of animal data as resources for human health. Two concepts suggested are “viewing a wider number of species as potential models for human environmentally induced disease, and incorporating animal sentinel data into clinical and public health decision making” (Rabinowitz et al., 2008).

Wild animals living in a contaminated area can be useful models for exposure to environmental mixtures. Field collection of animals exposed *in situ* offers an advantage over laboratory animal studies in that it is a more relevant exposure to environmental mixtures. Animals collected from a contaminated site will be exposed to the same mixtures as a human on the site. The exposures are low compared to laboratory studies and continue over time and throughout the lifespan, as is usually the case with human exposures. Similar to human *in situ* exposures, wild animal *in situ* exposures usually occur through multiple routes and from multiple environmental media.

Studies of wildlife exposed *in situ* have several limitations and disadvantages. The levels of exposure are rarely known and therefore must be estimated based on contaminant concentrations in environmental media and knowledge of animal behaviors. It is often difficult to estimate how much time the organism spends in the contaminated environment. Some species are migratory or have large home ranges; habitat and diet may vary seasonally. Because the doses are lower than in laboratory studies, effects may be more difficult to measure. The genetic variability may be far greater than in laboratory animals, and age and gender of collected wildlife may vary and be difficult to determine. Because of these variations, large numbers of field specimens may be required to gain statistical power. Capturing sufficient numbers of animals may be difficult, time-consuming and costly. These many uncertainties are

similar to those found in human epidemiological studies. Careful selection of the wildlife species to be studied can address some of these issues.

Golden and Rattner (2003) have proposed four indices for ranking the utility of a terrestrial vertebrate species for biomonitoring of specific contaminants in a particular study area. Exposure Potential estimates the likelihood that a species will be exposed to the contaminant through dermal, oral, or inhalation routes. This varies with behaviors, such as foraging technique and habitat preference, which bring the organism into contact with contaminated environmental media. Geographic Occurrence describes the range and seasonal occurrence of the species in the contaminated area. Ease of Collection is assessed by factors such as the abundance, accessibility and ease of capture of the species. Quantity of Existing Exposure and Effects Data refers to the amount of exposure and effects data available for a species relative to a particular contaminant or contaminant class. Two additional indices describe the vulnerability of the species to a particular contaminant. Sensitivity is the likelihood that an individual would sustain damage from exposure. Resilience evaluates the ability of the population to recover from exposure to the contaminant and includes the effects of abundance and reproductive potential. The authors used the proposed indices to rank a list of 25 terrestrial vertebrate species for use in biomonitoring of important environment contaminant classes in the Atlantic Coast region (Golden and Rattner, 2003).

Fox (2001) has reviewed potential wildlife sentinels for the Great Lakes-St. Lawrence Basin. Animals studied include bald eagles, herring gulls, night herons, tree swallows, snapping turtles, mink, and beluga whales. Health effects documented in these animals include reproductive impairment, developmental toxicity, genotoxicity, cancer, endocrine disorders, metabolic diseases and altered immune function. In general, severity and frequency of health effects observed in wildlife increased in the most contaminated sites (Fox, 2001).

Mink (*Mustela vison*) have been discussed as being appropriate sentinels for exposures to environmental contaminants (Basu et al., 2007). Mink are abundant, widely distributed, and regularly trapped, and are at a high trophic level. The authors noted that research groups partner with local trappers to gather biological samples from mink carcasses, providing a valuable way to obtain a steady supply of tissues (Basu et al., 2007). Factors and mechanisms influencing the exposure of terrestrial vertebrates



to environmental contaminants have been reviewed by Smith et al. (2007); for mammals, these include habitat, diet and foraging habits.

Pets are an interesting class of animals for use as sentinels because they share the human environment and have potential for similar exposures. In one study (Backer et al., 2001), biomarkers of genotoxicity were measured in pet dogs from communities near Superfund sites contaminated with organochlorine pesticides and volatile organic chemicals. Frequency of peripheral blood lymphocyte micronuclei was higher in dogs living near Superfund sites than in control animals from another community.

Multigner et al. (2008) assessed parallel exposures of wild rats and humans. The study compared reproductive effects of pesticides in male workers in banana plantations and male wild rats living on the plantations. Sperm characteristics and reproductive hormone levels were not significantly different between workers exposed to pesticides and unexposed workers. However, rats captured on the banana plantation had lower testosterone levels and gonadosomatic indices than rats captured at a control site with low potential for pesticide exposure. The authors concluded that wild rats may be more sensitive than humans to pesticide levels (Multigner et al., 2008).

#### ***1.4.4.1 Biomarkers of Exposure***

Many human health and ecological risk assessments of contaminated sites estimate exposure based on concentrations of contaminants in environmental matrices such as soil. Contaminant levels in environmental matrices are often heterogeneous, so a battery of environmental samples is collected in order to characterize an average concentration. The contaminant concentrations are then used to calculate a daily exposure value (mass contaminant per body weight per day) using assumptions for mass of environmental matrix contacted, contact frequency and duration. The amount of contaminant that actually crosses the body barriers and enters the body is estimated by the application of absorption factors. This approach presents many challenges. Environmental matrix samples may not be representative of the whole site because of temporal and spatial variations in contaminant concentrations. Matrix characteristics and contaminant physico-chemical properties may alter bioavailability. Individual differences in toxicokinetics can cause variations in the dose of contaminant that reaches the target organ(s) where health effects occur.

An alternative or complementary approach is to measure biomarkers of exposure in exposed populations. A biomarker of exposure is a biological indicator, at the molecular or cellular level, of exposure to an environmental contaminant. Biomarkers are useful tools for evaluating exposures of *in situ* populations. Biomarkers of exposure encompass all exposure routes and indicate the internal dose, including the extent of contaminant absorption, distribution, metabolism and accumulation (Vainio, 2001). Biomarkers of exposure are effective for monitoring exposure of *in situ* populations to environmental mixtures because the biomarkers account for differences in bioavailability and toxicokinetics of mixture components, in addition to any biological or toxicological interactions between mixture components (Fossi, 1998).

Biomarkers of exposure are also valuable as early warning signs that identify potentially harmful exposures before the effects are manifested at higher levels of organization (e.g., tissues, individuals, populations, or ecosystems). For example, organophosphate pesticides cause damage to the central nervous system in birds which leads to changes in behavior and mortality. Eventually, this can reduce the bird population by increased mortality or reproductive impairment. The nervous system malfunctioning is caused by inhibition of a critical enzyme, acetylcholinesterase. Acetylcholinesterase activity is therefore a biomarker of exposure at the molecular level that can be used to identify a potentially harmful exposure of bird populations to organophosphate pesticides (Fossi, 1998).

Exposure to genotoxic or carcinogenic contaminants such as PAHs can be detected by the use of DNA damage biomarkers. Types of structural DNA damage include strand breaks, altered bases, abasic sites, dimerization of pyrimidine bases, mutations, and adducts. DNA adducts have been used as biomarkers of PAH exposure in environmental toxicology studies of both humans (Kriek et al., 1998) and wildlife (Shugart, 2000). DNA adduct levels have been evaluated by the <sup>32</sup>P-postlabeling technique in a number of wildlife species, including muskrat, beluga whale, earthworm, mussel, catfish, trout, carp, and eel (Shugart, 2000). A recent biomonitoring study reported increased DNA adduct levels in juvenile Coho salmon exposed to PAH-contaminated freshwater lake sediments and confirmed the bioavailability of the contaminants (Barbee et al., 2008).

#### **1.4.4.2 House Mouse**

The house mouse (*Mus musculus*) is a useful model species for environmental monitoring for several reasons. House mice generally live commensally with humans, living in and obtaining food from human habitations, other buildings, or cultivated fields. Commensal house mouse populations are relatively stable and breeding continues throughout the year. Commensal house mice have individual home ranges of less than 10 square meters and rarely travel more than 50 feet from their established homes (Ballenger, 1999; Nowak, 1991). Therefore, the exposures of house mice are localized and easier to characterize than a species with a larger home range. House mice eating human food are exposed to the same food-borne contaminants as the humans. Mice living in human dwellings are also exposed to the same contaminants in domestic water and indoor air. House mice living outdoors in agricultural areas are exposed to agricultural chemicals and fuels used by humans.

Laboratory-bred *Mus musculus* strains have been used extensively in medical, genetic, and toxicological research for decades. A large body of knowledge has been amassed about their physiology, development, molecular biology and genome, their responses to challenge with environmental contaminants, and the variation of toxic endpoints between genetically distinct strains. Comparison of commensal mice exposed *in situ* and laboratory mice exposed in controlled experiments can be a valuable way to link the laboratory and the field. Similarly, comparison of biomarkers in humans and commensal mice living in the same area can be used to improve laboratory animal-to-human extrapolation of toxicity results.

### **1.5 Objectives**

PAHs are widely distributed in the environment and are generated by many sources. The potential of PAH-rich mixtures to cause health effects has been known for almost a century. The toxicity of both individual PAHs and PAH mixtures has been documented *in vitro*, in laboratory animals and in wild animals exposed *in situ*. Occupational epidemiological studies have provided evidence of the health effects of PAH-rich mixtures in humans.

Although PAH research has been ongoing for decades, there are still unanswered questions about the levels of PAHs in the environment, the potential for

human exposure to PAHs, the health effects associated with exposure, and how genetic susceptibility influences the internal dose and extent of health effects in individuals. The ultimate goal is to minimize health effects in exposed populations. Accomplishing this goal requires an integrated approach, one which includes field studies and laboratory experiments, *in vitro* and *in vivo* systems, ranging from molecular-level to population-level. The research documented in this dissertation includes chemical analysis of PAH concentrations in environmental media, laboratory-based studies of the role of genetic factors in PAH genotoxicity, and evaluation of biomarkers of exposure in ecological receptors.

The first objective of this research was to quantify concentrations of PAHs in samples of settled house dust, which can be a source of human exposure to PAHs. Samples of floor dust were collected from communities in three locations: Sumgayit, Azerbaijan; Shanxi Province, China; and southern Texas, USA. Organic material extracted from the dust samples was analyzed by gas chromatography/mass spectrometry to quantify thirty-six PAH analytes. Total PAH surface loading and sample variability were evaluated and compared between the locations. The PAH alkylation profile was also examined to determine the most likely sources of PAH contamination in the house dust samples.

The second objective of this research was to investigate genetic factors influencing the formation of PAH-DNA adducts in sensitive cell culture models. Pregnane X receptor (PXR) is a nuclear receptor which regulates important metabolizing enzymes. Metabolism of BaP can result in either detoxification or bioactivation to its genotoxic forms. HepG2 is a human hepatocarcinoma cell line that is sensitive to PAHs and has low constitutive expression of PXR. To evaluate the effect of PXR on BaP genotoxicity, DNA adduct formation was measured by <sup>32</sup>P-postlabeling in BaP-treated parental HepG2 cells and human PXR-transfected HepG2 cells. To further analyze PXR-regulated metabolic pathways, a panel of metabolizing genes was surveyed with real-time quantitative RT-PCR and the activity of the glutathione-S-transferase family of enzymes was measured.

The third objective of this research was to investigate DNA adduct levels in wild mice exposed to PAH mixtures *in situ*. Wild mammals may be exposed to PAHs in soil both directly (dermal contact or ingestion) and indirectly through the food chain.

Evaluation of biomarkers of DNA damage, such as DNA adducts, may function as a measure of exposure to PAHs. House mice (*Mus musculus*) were captured near homes in Sumgayit and Khizi, Azerbaijan which are part of an ongoing human health study. Lung, liver, and kidney tissues were collected from the mice; DNA adduct levels in these tissues were measured by <sup>32</sup>P-postlabeling. Soil samples collected near the mouse capture sites were analyzed by gas chromatography/mass spectrometry for PAH content.

## CHAPTER II

### A COMPARISON OF CONCENTRATIONS OF PAHS DETECTED IN DUST FROM VARIOUS REGIONS OF THE WORLD \*

#### 2.1 Introduction

Complex environmental mixtures may have a negative impact on human health. Information is needed to better quantify the composition and concentration of the components of complex mixtures in both the outdoor and indoor environment. Humans may be exposed to complex mixtures that adhere to dust particles both outside and inside their homes. Settled house dust can be a source of exposure through non-dietary ingestion and dermal contact. Children may have a higher potential for exposure to floor dust than adults because they spend much of their time on the floor and are likely to put in their mouths objects that may have picked up dust from the floor.

House dust is a complex mixture of particles of both indoor and outdoor origin, including soil, biological matter, fibers and organic particles. Environmental contaminants which have been detected in house dust include asbestos, several heavy metals, pesticides, phthalates, and polychlorinated biphenyls (Lewis et al., 1995; Liroy et al., 2002). The contaminants extracted from settled house dust collected in Ottawa, Canada and Washington, USA were shown to be mutagenic in a cell culture bioassay (Maertens et al., 2004). One of the major organic components of dust extracts is the polycyclic aromatic hydrocarbon (PAH) class of compounds. PAHs are present in coal and petroleum products, and are formed during combustion of fossil fuels and biomass. In general, PAHs have low volatility and low aqueous solubility, and are mainly adsorbed to particles, such as house dust, rather than existing in the gaseous phase. PAHs in house dust may originate from both outdoor and indoor sources. Typical indoor sources include combustion of fossil fuels and biomass for heating and cooking; burning of tobacco products, candles and incense; and cooking fumes. Seven PAHs are classified as probable human carcinogens (B2) by USEPA. Maertens et al. (2004)

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\* Reprinted with permission from "A comparison of concentrations of polycyclic aromatic compounds detected in dust samples from various regions of the world" by Naspinski C, Lingenfelter R, Cizmas L, Naufal Z, He LY, Islamzadeh A, Li Z, Li Z, McDonald T, and Donnelly KC. *Environ Int* 2008;34:988-993. doi:10.1016/j.envint.2008.03.008 Copyright [2008] by Elsevier Ltd.

estimated the excess lifetime cancer risk from non-dietary ingestion of PAH in settled house dust to exceed the acceptable range for pre-school children in homes with dust PAH loading at the 95th percentile. Chuang et al. (1999) reported that ingestion of house dust and soil contributed 24% of the estimated daily dose of B2 PAH in a study of children in low-income households.

The objective of this study was to evaluate the surface loading, variability and distribution of PAHs in residential floor dust. Study homes were located in three areas which were assumed to have broadly divergent concentrations of chemicals in dust. These sites were selected because they are locations for on-going human health studies. The first area, in south Texas, was described previously by Shalat et al. (2003). The second site is near the city of Sumgayit in the Republic of Azerbaijan, a former Soviet Union republic. The health risk of this population, described previously by Bickham et al. (2003), is of concern because they are living on contaminated land near an abandoned chemical manufacturing site. The third site is in the Shanxi Province of northern China, an area where coal is a major fuel and the prevalence of neural-tube defects is high (Li et al., 2006).

## **2.2 Materials and Methods**

### **2.2.1 Sampling Sites**

To obtain information regarding the variability of PAHs in several parts of the world, floor dust samples were collected from homes in three diverse geographic locations. These included houses in Texas, USA, Sumgayit, Azerbaijan and Shanxi Province, China. In Texas, 21 houses were sampled during a single collection period. In Azerbaijan, dust samples were collected from 19 different houses during two collection periods, one in spring (April and May) and one in autumn (September and October). Two of these houses were sampled during both collection periods; thus, a total of 21 dust samples were collected in Azerbaijan. Due to the distances between sampling locations, only 14 houses were sampled in China during two collection periods (spring and autumn).

None of the homes in China or Azerbaijan had air conditioning. Most homes in Texas were not air-conditioned. In those with air conditioning (approximately 30%), most ran the system only during the night. Thus, all of the homes in China and

Azerbaijan, and most of the homes in Texas were generally open to the environment, and dispersion of outdoor dust, during much of the year.

House dust samples in Texas were collected from a rural community bordered by a large (several hundred hectare) agricultural field. The climate is arid; there was no recorded rainfall during the month that the samples were collected. Average annual rainfall for this area is approximately 80 mm. Motor vehicle traffic is light and most homes have electricity and/or natural gas for heating and cooking. Sources of PAHs in house dust are likely to be drift of airborne combustion products from distant densely-populated areas, and occupant activities such as smoking tobacco, cooking, and using petroleum products.

Dust samples in Azerbaijan were collected from a densely populated residential area in close proximity to an industrial complex in the city of Sumgayit. Sumgayit is on the Absheron Peninsula in the Caspian Sea, north of the capital of Azerbaijan. The city was built in the early 1950's to serve as the major petrochemical production area for the Soviet Union. The community where samples were collected is adjacent to a synthetic rubber plant. Nearby industries include an electric power generating station, aluminum plant, chlor-alkali plant and pipe production facility. The climate is windy, with a mild winter and hot, dry summer. Annual rainfall averages 200-300 mm. Electricity is the main power source for heating and cooking. In addition to occupant activities, probable sources of PAHs in Azerbaijan house dust are track-in of PAH-contaminated soil and infiltration of airborne combustion products from the adjacent industrial facilities. Many of the houses have been built from scrap that was removed from the adjacent rubber plant. Thus, construction materials may be another source of PAHs in house dust.

The third location for the collection of house dust was in Shanxi Province in China. Dust samples were collected from houses that were located in small (population less than 25,000) rural villages. Shanxi Province is a major coal producing area of China. The climate is monsoonal continental, with long, cold, dry winters and mild wet summers. Annual rainfall averages 400-600 mm. Coal is used for heating and cooking, and power generation facilities in the region burn coal. The major sources of PAHs in Shanxi house dust are therefore expected to be coal dust and coal combustion products. As indicated with the other populations, occupant activities such as smoking may also contribute to the PAH load in house dust.



### **2.2.2. Dust Sample Collection**

One sample of settled dust was collected in each home on a filter cloth using a swipe method as described previously (Shalat et al., 2003). This is an inexpensive method for collecting house dust that requires minimal equipment. However, the method does not allow measurement of contaminant loading based on either mass of contaminant per mass of dust, or mass of contaminant in specific particle size of dust. Thus, in this study, contaminant loads are based on concentrations of contaminant per area sampled. The samples were collected from a non-carpeted area of the floor as close as possible to the front door. An area of approximately 1 m<sup>2</sup> was marked off (or the largest possible area if less than 1 m<sup>2</sup> of open floor surface was available). A pre-cleaned glass fiber filter cloth saturated with isopropanol was wiped over the marked area to remove floor dust. The cloth was then wrapped in aluminum foil, placed inside a plastic bag and stored on ice prior to transport to the laboratory. Upon arrival at the laboratory, samples were logged into the sample tracking record and stored at -4 °C prior to extraction and analysis.

### **2.2.3 Sample Extraction and Analysis**

To recover the organic material from the dust samples, each filter cloth was processed by pressurized fluid extraction in a Dionex ASE 200 Accelerated Solvent Extractor using a modified U.S. EPA Method 3545A (USEPA, 1998). Extraction conditions were 2 static cycles for 5 minutes each at 1500 psi and 100 °C with 100% HPLC-grade dichloromethane (EM Science, Gibbstown, NJ) solvent. Extracts were dried for 20 minutes at 54 °C in a Zymark TurboVap II evaporator, then redissolved in 1 mL of dichloromethane. An internal standard solution (Cambridge Isotope Laboratories, Andover, MA) of deuterated fluorene, pyrene and benzo[a]pyrene was added prior to analysis for quality control.

The PAH analysis was conducted on a HP 5890 II Gas Chromatograph (GC) coupled to a HP 5972A Mass Spectrometer (MS) integrated with HP MS Chemstation, using a modified U.S. EPA SW-846 Method 8270C (USEPA, 1996). The PAHs were separated using a HP-5MS, 0.25 µm film thickness, 60 m x 0.25 mm id, fused silica capillary column. The split/splitless injector port was maintained at 300 °C and the transfer line at 280 °C. The capillary column was initially at held 60 °C for 2 minutes then ramped at 5 °C/minute to 310 °C and maintained at 310 °C for 30 minutes. The mass

spectrometer was operated in the selected ion monitoring (SIM) mode and tuned with perfluorotributylamine (PFTBA) according to the manufacturer criteria. The quantification of the PAHs is accomplished by comparison to reference compounds from a standard calibration solution. Thirty-six PAH analytes were quantified, including alkylated PAHs (C1, C2, C3, and C4 derivatives of parent PAHs). The practical quantitation limit for each analyte was 10 ng per mg extract.

## 2.3 Results

### 2.3.1 PAH Surface Loadings

Dust samples were analyzed by GC/MS for 36 PAH analytes (Table 2-1). These 36 analytes include the 16 PAHs classified as priority pollutants by USEPA and two additional unsubstituted compounds, benzo[e]pyrene and perylene. The other 18 analytes are alkylated families, which represent PAH compounds with alkyl groups attached to the aromatic rings, specified by the number of carbons in the alkyl groups (C1, C2, etc.). The sixteen USEPA priority pollutant PAHs, including a subset of seven probable human carcinogens (B2 carcinogens), and the alkyl-PAH analytes are listed in Table 2-1. The number of fused 5-carbon and 6-carbon aromatic rings is listed for each analyte. The 19 analytes with four or more rings are categorized as high molecular weight (HMW) PAHs.

Surface loadings of PAHs were measured in mass of PAH per area of floor ( $\mu\text{g}/\text{m}^2$ ). The floor loadings are summarized in Table 2-2 for each location. Median floor loadings of total PAHs, HMW PAHs and B2 carcinogens were markedly different between the locations. Median loading of total PAHs in floor dust collected from homes in China was  $232 \mu\text{g}/\text{m}^2$  (Table 2-2). In Azerbaijan, median total PAH concentration in dust was  $6.1 \mu\text{g}/\text{m}^2$ , while in Texas the median concentration was  $1.34 \mu\text{g}/\text{m}^2$ . Minimum and maximum PAH concentrations in dust samples followed the same trend, with China more than an order of magnitude higher than Azerbaijan and Texas. Note also that in China the minimum PAH concentration in house dust was greater than the maximum concentration in dust from Texas homes. In many of the Texas samples, HMW PAHs were near detection limits and B2 carcinogens were undetectable. In the dust samples from China, the minimum concentration of carcinogenic PAHs was greater than the maximum concentration of carcinogenic PAHs from either Texas or Azerbaijan.

**Table 2-1**  
Individual PAH analytes quantified in house dust extracts

PAH Species <sup>a</sup>	CAS RN	Molecular Weight	No. of Rings <sup>b</sup>	
Naphthalene <sup>a</sup>	91-20-3	128	2	LMW
C1-Naphthalenes	---	142	2	
C2-Naphthalenes	---	156	2	
C3-Naphthalenes	---	170	2	
C4-Naphthalenes	---	184	2	
Acenaphthylene <sup>a</sup>	208-96-8	152	3	
Acenaphthene <sup>a</sup>	83-32-9	154	3	
Fluorene <sup>a</sup>	86-73-7	166	3	
C1-Fluorenes	---	180	3	
C2-Fluorenes	---	194	3	
C3-Fluorenes	---	208	3	
Anthracene <sup>a</sup>	120-12-7	178	3	
Phenanthrene <sup>a</sup>	85-01-8	178	3	
C1-Phenanthrene/Anthracenes	---	192	3	
C2-Phenanthrene/Anthracenes	---	206	3	
C3-Phenanthrene/Anthracenes	---	220	3	
C4-Phenanthrene/Anthracenes	---	234	3	
Fluoranthene <sup>a</sup>	206-44-0	202	4	HMW
Pyrene <sup>a</sup>	129-00-0	202	4	
C1-Fluoranthenes/Pyrenes	---	216	4	
C2-Fluoranthenes/Pyrenes	---	230	4	
C3-Fluoranthenes/Pyrenes	---	244	4	
<u>Benz[a]anthracene</u> <sup>a</sup>	56-55-3	228	4	
<u>Chrysene</u> <sup>a</sup>	218-01-9	228	4	
C1-Chrysenes	---	242	4	
C2-Chrysenes	---	256	4	
C3-Chrysenes	---	270	4	
C4-Chrysenes	---	284	4	
<u>Benzo[b]fluoranthene</u> <sup>a</sup>	205-99-2	252	5	
<u>Benzo[k]fluoranthene</u> <sup>a</sup>	205-82-2	252	5	
Benzo[e]pyrene	192-97-2	252	5	
<u>Benzo[a]pyrene</u> <sup>a</sup>	50-32-8	252	5	
Perylene	198-55-0	252	5	
Benzo[g,h,i]perylene	191-24-2	276	6	
<u>Indeno[1,2,3-c,d]pyrene</u> <sup>a</sup>	193-39-5	276	6	
<u>Dibenzo[a,h]anthracene</u> <sup>a</sup>	53-70-1	278	5	

a. Analytes classified as priority pollutants by US EPA. Analytes underlined are classified by US EPA as probable human (B2) carcinogens.

b. Low Molecular Weight (LMW): 2-3 rings; High Molecular Weight (HMW): 4+.

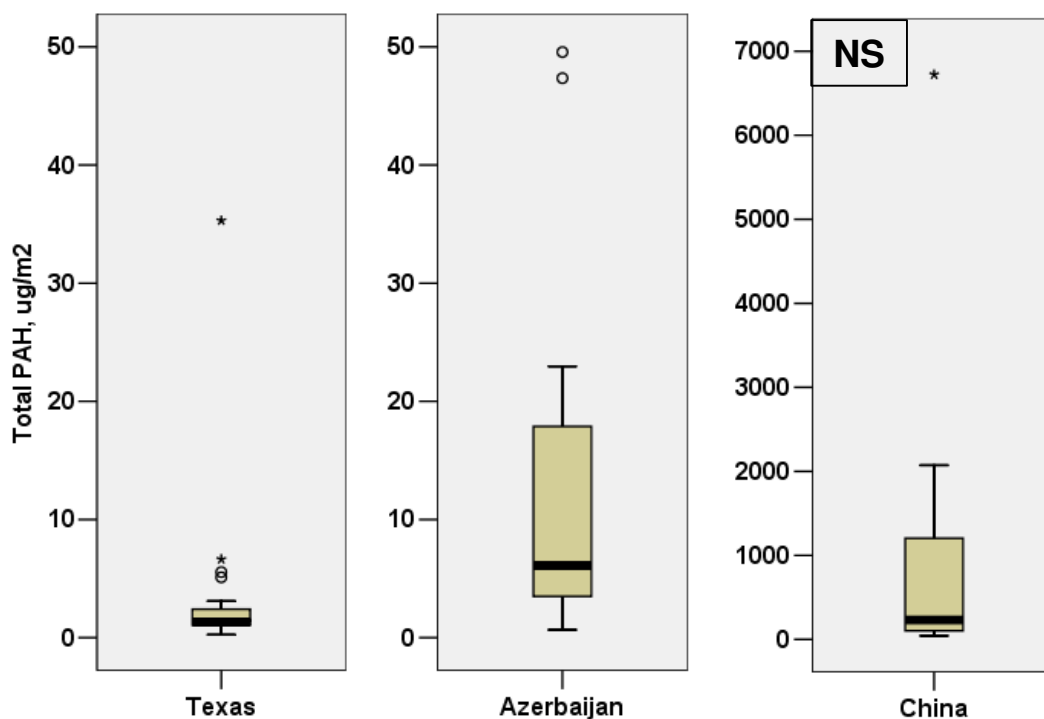
**Table 2-2**

Concentration of total and high molecular weight PAHs detected in dust samples collected from houses in Azerbaijan, China or Texas

	<u>Azerbaijan</u>	<u>China</u>	<u>Texas</u>
	n=21	n=14	n=21
	µg/m <sup>2</sup>	µg/m <sup>2</sup>	µg/m <sup>2</sup>
<b>Total PAHs</b>			
Median	6.1	232	1.34
Min	0.7	42	0.24
Max	49.6	6720	35.3
CV (%)	112	183	208
<b>High Molecular Weight PAHs</b>			
Median	2.9	162	0.11
Min	0.5	30	0.03
Max	20.0	4440	2.90
CV (%)	103	178	223
<b>EPA B2 Carcinogen PAHs</b>			
Median	1.0	53	BD <sup>a</sup>
Min	0.2	12	BD <sup>a</sup>
Max	5.4	1620	0.27
CV (%)	94	177	254

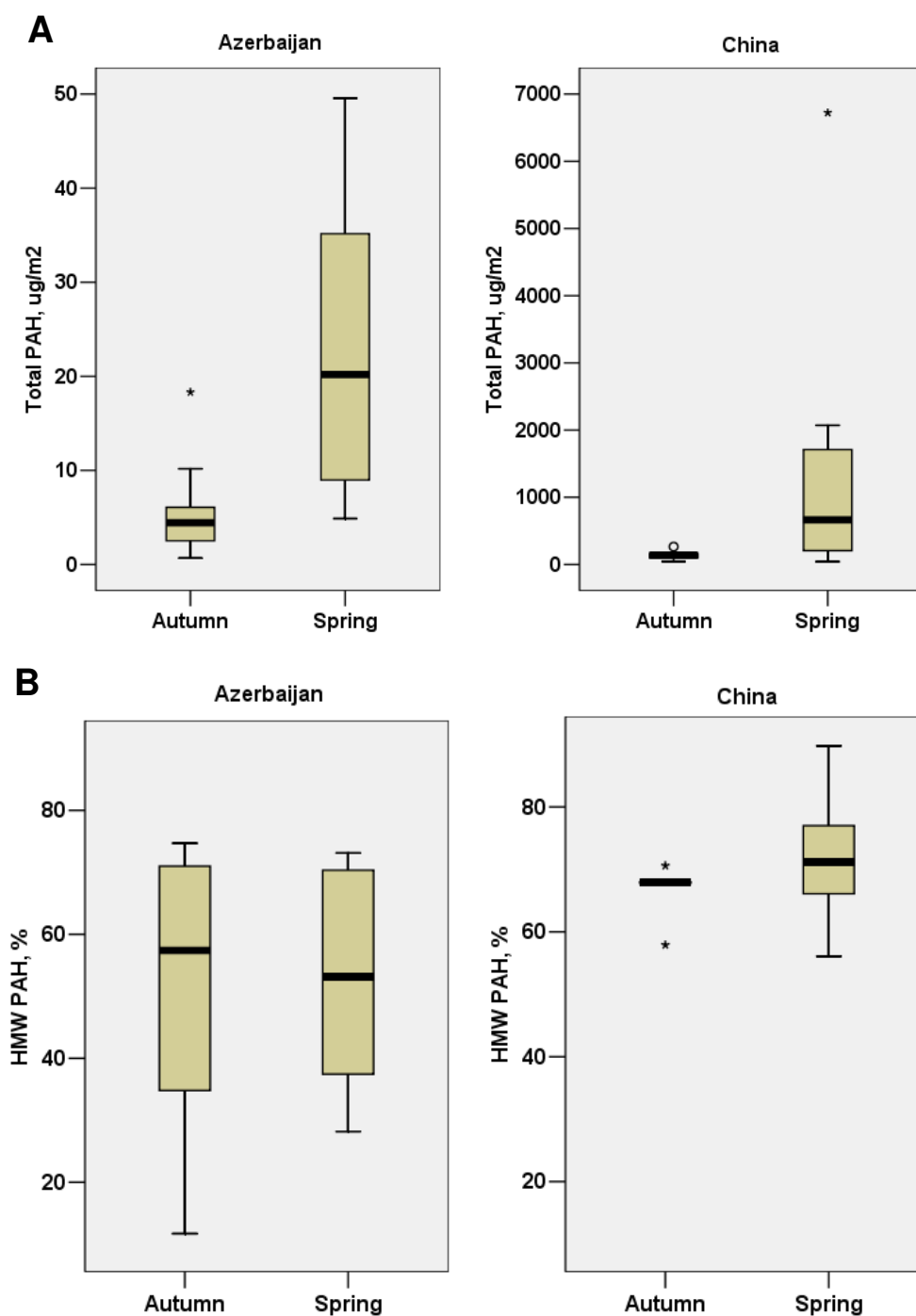
a. BD = below detection limit

Variability of floor loadings between samples within locations was high, with coefficients of variation (CV) of the mean ranging from 94% to 254% (Table 2-2). The total PAH floor loading data are also presented in boxplots in Fig. 2-1 (note different scale for China). The data distributions among the three locations are similarly right-skewed, with the majority of samples clustered near the lower end, and a few high outliers. This skewed distribution is typical for environmental contamination. There was at least one house within each of the communities where PAH concentrations in dust were appreciably elevated in comparison to the other homes in the area. The most likely explanation for these outliers is that the floor in these homes had not been cleaned as recently as in the other homes. Information was not gathered during sampling to confirm this assumption.



**Fig. 2-1.** Variability of floor surface total PAH loadings in each location. Total PAH loading represents mass of 36 PAH analytes per square meter of floor surface wiped. ○ outlier; \* extreme outlier. NS: not-to-scale (China Y-axis scale 0-7000  $\mu\text{g}/\text{m}^2$  vs. Texas/Azerbaijan 0-50  $\mu\text{g}/\text{m}^2$ ).

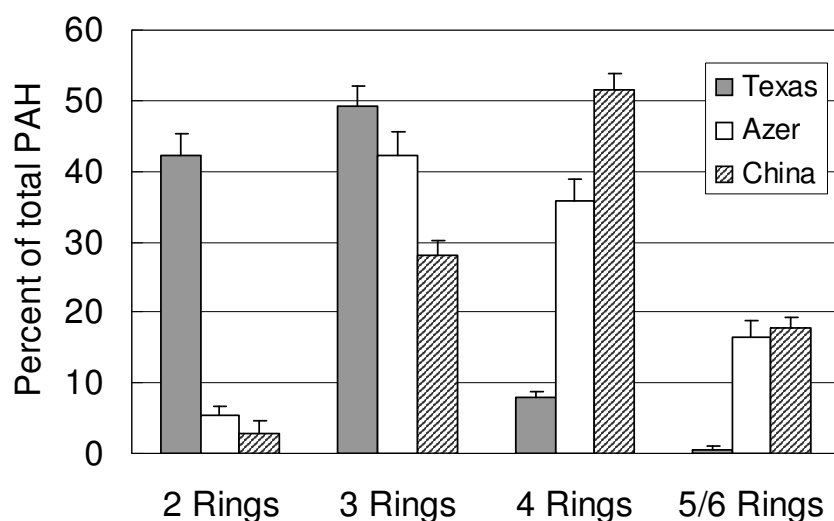
In China and Azerbaijan, samples were collected during the spring (April and May) and autumn (September and October) seasons. Some seasonal differences in floor loadings are apparent when the data are separated by season (Fig. 2-2A). In both China and Azerbaijan, concentrations of total PAHs in dust were appreciably higher in the spring than in the autumn. However, in both countries, the percentages of the HMW PAHs were comparable between seasons (Fig. 2-2B). Samples from Texas are not included in the figure because they were all collected in the same month.



**Fig. 2-2.** Seasonal variation in floor wipe samples from Azerbaijan and China. (A) Total PAH loading. (B) HMW PAH as % of total PAH. Azerbaijan: Autumn n=13, Spring n=8; two homes were sampled in both seasons. China: Autumn n=5, Spring n=9; none of the homes were repeated. o outlier; \* extreme outlier.

### 2.3.2 PAH Profile

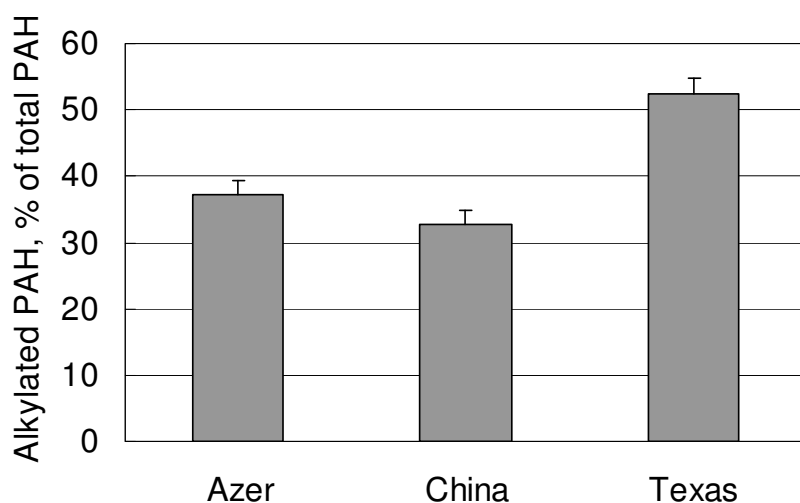
The percentage of the various PAHs in dust provides information regarding possible sources of the chemicals, as well as the potential for the components of the mixture to cause adverse health problems. The results presented in Fig. 2-3 compare the percentage of the various compounds in the dust extracts. More than 90% of the extracts of dust samples collected in homes in Texas were the smaller (2- and 3-ring) PAHs. These compounds are generally less toxic and more susceptible to degradation. In the extracts of dust from Azerbaijan and China, more than 50% of the PAHs were the larger (4-6 ring) compounds. These compounds are both more persistent in the environment and include the compounds classified by the US EPA as B2 (probable) human carcinogens.



**Fig. 2-3.** PAH profile by number of aromatic rings in floor wipes from each location. The 36 analytes were categorized by the number of fused aromatic rings in the represented compounds. Data presented are means  $\pm$  standard error.

The most abundant unsubstituted individual compounds were phenanthrene and fluoranthene in Azerbaijan, fluoranthene and chrysene in China, and naphthalene and phenanthrene in Texas (data not shown). Including alkylated analytes, the most abundant PAH families were phenanthrenes/anthracenes in Azerbaijan,

fluoranthenes/pyrenes in China, and naphthalenes in Texas. The percentage of total PAHs represented by the substituted PAHs varied appreciably among the three locations (Fig. 2-4). In Texas, where low molecular weight PAHs predominated, alkyl substituted PAHs represented more than 50% of the concentration of the total PAHs. In dust samples from China, where the high molecular weight PAHs were the predominant compounds, less than 35% of the total PAHs were alkyl-PAHs. These results likely reflect the different source of PAHs in the three locations.



**Fig. 2-4.** Percentage of alkylated analytes. Data shown are means  $\pm$  standard error.

### 2.3.3 Source Classification

The complex mixtures of PAHs associated with environmental pollution can be classified into two main categories depending on the process by which they are formed (Boehm, 2006). "Petrogenic" PAHs originated from fossil fuels such as petroleum and coal. These PAHs are formed over long periods of time at moderate temperatures. Petrogenic PAH mixtures are generally rich in the lower molecular weight PAHs (2 or 3 rings). "Pyrogenic" PAHs are present in the products of incomplete combustion of biomass and fossil fuels. Pyrogenic PAHs are formed at high temperatures and are



generally enriched in HMW PAHs (4 to 6 rings) (Boehm, 2006). The PAHs detected in dust samples from Texas appear to originate primarily from petrogenic sources. Concentrations of the low molecular weight PAHs detected in dust samples from Texas were generally more than 90% of the total PAH fraction, while the high molecular weight PAHs accounted for less than 10% of the total PAH fraction. PAHs in the extracts of dust samples from Azerbaijan and China appeared to have originated primarily from pyrogenic sources. The concentration of the 4-ring and larger PAHs for dust samples from both China and Azerbaijan was more than 50% the total PAH concentration (Fig. 2-3). It should also be noted (Fig. 2-2B), that the percentage of HMW PAHs was fairly constant between seasons in China and Azerbaijan. This suggests that there was not a shift in the source of PAHs in dust between spring and autumn.

Petrogenic and pyrogenic PAHs also differ in the degree of alkylation. One characteristic of petrogenic PAHs is that the alkylated PAHs are more abundant than the parent PAH in each family (see Boehm (2006) for more information). The high temperature processes that produce pyrogenic PAHs tend to destroy alkylated PAHs because they are more reactive; therefore, pyrogenic PAHs generally have much higher levels of parent compounds than alkylated compounds. The alkylation profile of each sample was examined for the following groups: phenanthrenes + anthracenes (PA); floranthenes + pyrenes (FP); and benzantracenes + chrysenes (BC). Based on the alkylation profile, the samples were qualitatively classified as either petrogenic or pyrogenic. The results are summarized in Table 2-3. The profiles for the FP and BC groups were all pyrogenic (the BC group was not evaluated in the Texas samples because results were near or below detection limits). In China, the PA group was heavily weighted towards pyrogenic, whereas in Texas and Azerbaijan, petrogenic and pyrogenic profiles were approximately equal in the PA groups.

**Table 2-3**

Classification of major PAH sources in dust based on alkyl PAH profile

		PA <sup>a</sup>	FP <sup>b</sup>	BC <sup>c</sup>
Texas <sup>d</sup>	Petrogenic %	52 (11/21)	0 (0/21)	----
	Pyrogenic %	43 (9/21)	100 (21/21)	----
Azer <sup>d</sup>	Petrogenic %	43 (9/21)	0 (0/21)	0 (0/21)
	Pyrogenic %	52 (11/21)	100 (21/21)	100 (21/21)
China	Petrogenic %	14 (2/14)	0 (0/14)	0 (0/14)
	Pyrogenic %	86 (12/14)	100 (14/14)	100 (14/14)

a. PA = Phenanthrene + Anthracene + C1-, C2-, C3- and C4-Phen/Anth

b. FP = Fluoranthene + Pyrene + C1-, C2-, C3-Fluor/Pyr

c. BC = Benz[a]anthracene + Chrysene + C1-, C2-, C3- and C4-BaA/Chrys

d. One sample each from Texas and Azerbaijan were not included in the PA group analysis because their PA group profiles did not clearly match a petrogenic or pyrogenic profile.

## 2.4 Discussion

The floor dust collection procedure used in this study is quick, inexpensive, easily portable, and requires no specialized equipment. It is a convenient method for remote field studies in which the use of vacuum samplers, shipment of large samples, and repeat visits for timed-accumulation sampling are not feasible. The method is semi-quantitative, yielding a measurement of contaminant surface loading (mass of contaminant per unit floor area), rather than contaminant concentration in dust. Although surface loading cannot be used directly in an exposure calculation, it is valuable for the hazard identification step of risk assessment. The results of the analysis of floor dust samples can be used to identify the major organic and inorganic chemicals in homes; and, to provide qualitative information regarding potential sources of contaminants.

The variability in PAH surface loading among the homes was high in all of the sampling locations (Table 2-2 and Fig. 2-1). PAH surface loading consists of two components: the mass of dust per unit area, and the mass of PAH per mass of dust. The mass of dust per unit area depends on the characteristics of the surface (e.g., roughness), the frequency and efficacy of cleaning, and the deposition time (time since last cleaning). In general, floors in the Texas homes were linoleum or tile, while in

Azerbaijan wood floors were most common, and in China all floors were brick. Thus, dust accumulation in the rough surface encountered in China is likely to be highest. Weather and occupant activities also can impact accumulation of dust. The mass of PAHs per mass of dust is to a large extent dependent on the characteristics of the sources and relative contributions of each source. Although the results of the current study were highly variable, they clearly establish differences between the regions from which samples were collected. PAH concentrations were lowest in the rural communities in Texas. PAH concentrations were elevated in the homes in Azerbaijan that were located in close proximity to industrial facilities. However, PAH concentrations in Texas and Azerbaijan were more than one order of magnitude lower than levels detected in the dust samples collected in China.

Seasonal changes can cause variability in PAH surface loading. Dust accumulation can be affected by ventilation and by the amount of dust in outdoor air. Also, the concentration of PAHs in dust can change depending on ventilation, home heating, etc. PAH levels in the  $PM_{2.5}$  fraction of Beijing urban outdoor air was lowest in summer and highest in winter (He et al., 2006). A study in the lower Rio Grande Valley of Texas revealed that PAH concentrations in house dust were lower in summer than in spring (Mukerjee et al., 1997). In the current study, spring surface loadings were higher than autumn in both Azerbaijan and China (Fig. 2-2A).

The distribution of PAH analytes in a sample is of significance because of the relative toxicity of the different PAHs. In general, the heavier PAHs are more toxic. All of the carcinogenic PAHs (cPAHs) contain 4 or more rings and are therefore in the HMW fraction. The percentage of HMW PAHs was highest in China and lowest in Texas. The HMW percentage did not seem to be seasonally distributed (Fig. 2-2B). The difference in PAH profiles among the three locations can also be seen in Fig. 2-3. Qualitatively, the relative carcinogenicity of the PAH mixtures in the house dust follows the trend China > Azerbaijan > Texas.

The median total PAH surface loading in China was 40-fold higher than in Azerbaijan and more than 200-fold higher than in Texas. Surface loadings of cPAHs followed the same trend, with median cPAH loading 50-fold higher in China than in Azerbaijan, and cPAHs below detection limits in most of the Texas samples. These trends in total PAH surface loading, cPAH surface loading, and relative carcinogenicity

of the PAH mixtures suggest that the risk of health effects from exposure to PAHs in house dust is highest in the Chinese population, lowest in the Texas population, and intermediate in the Azeri population.

Two main categories of PAH sources are combustion processes, which produce “pyrogenic” PAHs, and fossil fuels, which contain “petrogenic” PAHs. In the current study, the degree of PAH alkylation was used to classify the PAHs in each sample as pyrogenic or petrogenic. Phenanthrenes/anthracenes (PA) in China were mostly pyrogenic, while PA in Azerbaijan and Texas were approximately equally petrogenic and pyrogenic. Sources of petrogenic PAHs in the study locations may include use and spills of petroleum products (e.g., motor fuels and oils, solvents, degreasers) in and near the home. This is more likely in Texas, where many of the residents own motor vehicles, than in the other two locations, where most residents do not own motor vehicles. Another likely source in China is dust from unburned coal. A recent study of homes in Palermo, Italy, found that these lighter PAHs were generally petrogenic in kitchen samples and pyrogenic in samples from living rooms and bedrooms (Mannino and Orecchio, 2008).

In the current study, the fluoranthenes/pyrenes and benzanthracenes/chrysenes groups had a pyrogenic alkylation profile in all the samples. This is consistent with the Palermo study (Mannino and Orecchio, 2008), in which analysis indicated a combustion origin for the heavier PAHs in most indoor dust samples. There are many potential sources of pyrogenic PAHs, including a) unvented or poorly vented combustion for heating and cooking; b) smoke from tobacco, candles and incense; c) cooking fumes; and d) infiltration of outdoor air contaminated with motor vehicle exhaust and industrial emissions. It is difficult to distinguish these various sources by PAHs alone because the PAH profile formed during combustion often is more influenced by the characteristics of the combustion process (temperature, oxygen availability) than the combustion material (Boehm, 2006). Identification of the various pyrogenic PAH sources likely requires that PAH analysis be combined with inorganic (e.g., metals) and other organic (e.g., nicotine) source-specific markers. At this time, we are not aware of any published detailed characterization of pyrogenic PAH sources in house dust.

In conclusion, the results of this study confirm that the wipe sample collection method provides a rapid and inexpensive means for obtaining qualitative information

regarding the distribution of PAHs in house dust. The analysis of molecular weight distribution, coupled with information regarding alkylated compounds, allowed identification of the primary PAH source categories. Information regarding the distribution of high and low molecular weight compounds, as well as carcinogenic PAHs, was used to develop a qualitative ranking of health risk due to exposure to dust. Variability among the samples confirmed the need to conduct multiple sampling rounds to more accurately capture the PAH contamination spectrum. These results, coupled with ongoing investigation of appropriate PAH exposure biomarkers in humans, will guide future efforts to identify ways to reduce PAH exposures and ultimately to reduce risk.

## CHAPTER III

### THE ROLE OF PREGNANE X RECEPTOR IN BAP-INDUCED DNA DAMAGE IN HEPG2 CELLS \*

#### 3.1 Introduction

Pregnane X receptor (PXR) is a nuclear receptor with broad specificity that is activated by structurally diverse lipophilic compounds. It is expressed mainly in the liver, intestine and colon. PXR plays an important role in pharmacokinetics of a broad spectrum of endogenous and xenobiotic compounds and appears to have evolved in part to protect organisms from toxic xenobiotics (Kliewer, 2003; Xie and Evans, 2001). PXR regulates metabolism and disposition of these compounds through coordinate regulation of the gene expression of phase I and II metabolizing enzymes and transport proteins (Maglich et al., 2002; Rosenfeld et al., 2003; Staudinger et al., 2003). PXR protects the liver against toxic insult by both endogenous and xenobiotic compounds (Sonoda et al., 2005; Staudinger et al., 2001; Xie et al., 2001).

Benzo[a]pyrene (BaP) is a common environmental contaminant found in air, water, soil, sediment and cooked foods. BaP is a well-established animal carcinogen and a probable human carcinogen; human exposure to mixtures containing BaP has been linked with an increased risk of cancer (Boffetta et al., 1997; Mastrangelo et al., 1996; Mumtaz et al., 1996; Sasco et al., 2004). A critical step in the induction of cancer by BaP is bioactivation by phase I metabolizing enzymes, most notably by members of the cytochrome P450 (CYP) superfamily (Ramesh et al., 2004). These electrophilic metabolites are capable of reacting with nucleophilic sites of DNA to form DNA adducts. The formation and persistence of carcinogen-DNA adducts have been shown to be critical events for the initiation of neoplasia in target cells. However, phase I metabolism, when coupled with phase II conjugation and subsequent elimination by membrane-bound transporters, is also an important mechanism for detoxifying BaP. The toxicity of BaP in a particular organism, tissue, or cell type is determined by the balance among these competing metabolic pathways.

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\* Reprinted with permission from "Pregnane X receptor protects HepG2 cells from BaP-induced DNA damage" by Naspinski C, Gu X, Zhou GD, Mertens-Talcott SU, Donnelly KC, and Tian Y. *Toxicol Sci* 2008;104:67-73. Copyright [2008] by Society of Toxicology.

In this study, the major goal was to test the hypothesis that PXR could modulate the genotoxicity of BaP. A cell culture model was developed based on the well-characterized human hepatoma cell line HepG2. HepG2 cells possess many functional xenobiotic-metabolizing enzymes and are sensitive to PAHs (Knasmuller et al., 2004). However, constitutive expression of PXR is low or undetectable in HepG2 cells. PXR expression was restored by stable transfection of human PXR (hPXR) expression plasmid. The effect of PXR on BaP-induced DNA damage was investigated by comparison of DNA adduct formation in the parental cells and the hPXR-enhanced cells. The results demonstrate that PXR decreases BaP genotoxicity in this cell model.

The second goal of the study was to test the hypothesis that PXR attenuates BaP genotoxicity by regulation of metabolizing enzymes involved in detoxification pathways. The gene expression of 20 BaP-metabolizing enzymes and 5 PXR-regulated transport proteins was measured by quantitative RT-PCR. In addition, the total GST enzymatic activity was measured. The results of the gene expression and enzyme activity experiments suggest that PXR reduces BaP genotoxicity by upregulating metabolizing enzymes that contribute to detoxification.

## **3.2 Materials and Methods**

### **3.2.1 Materials**

The human HepG2 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). LipofectAMINE and oligonucleotides used as PCR primers were obtained from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) was from HyClone. Fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA). Plasmid DNA purification kits, rifampicin, nuclease P1, spleen phosphodiesterase, micrococcal endonuclease, and potato apyrase were from Sigma (St. Louis, MO). Polynucleotide kinase was from USB Corp. (Cleveland, OH). [ $\gamma$ -<sup>32</sup>P]-ATP was from MP Biomedicals (Irvine, CA). Benzo[a]pyrene (BaP) was obtained from Sigma and 5000x solutions were prepared in ACS-grade dimethyl sulfoxide (DMSO) from EMD Science (Gibbstown, NJ).

### **3.2.2 Stable Transfection**

The expression vector for hPXR, p3XFlag-hPXR (Fig. 3-1A), was constructed as described previously (Gu et al., 2006). The PCR primers were designed based on the

published hPXR sequence (Lehmann et al., 1998). HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic in 5% CO<sub>2</sub> at 37°C. For transfection, cells were seeded onto 6-well plates, and when cell density reached 50% confluency, transfected with PXR expression plasmid (0.5 µg/well) by lipofection for five hours using LipofectAMINE according to manufacturer's instructions. After the transfected cells were cultured for two days, G418 (1 mg/ml) was added to the medium for an extended selection period. The G418-resistant colonies were pooled and further selected with medium containing 400 µg/ml G418. Surviving single colonies were cloned, expanded, and tested for PXR expression and ligand responsiveness.

### **3.2.3 Cell Culture and Treatment**

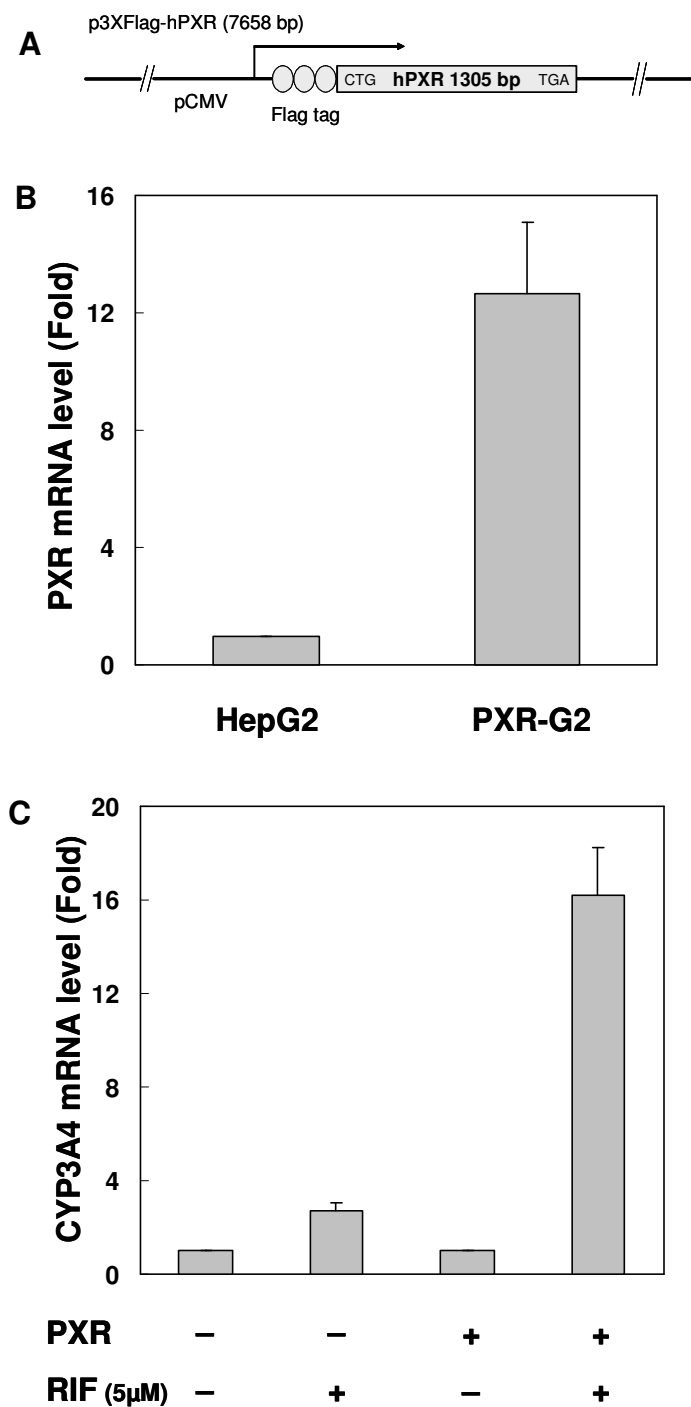
Parental and hPXR-enhanced cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B). Cells were pretreated with 5 µM rifampicin (RIF) for 24 hours, then treated with BaP and collected after 24 h. RIF and BaP controls consisted of an equivalent volume of DMSO.

### **3.2.4 DNA Adduct Assay**

Cells were cultured in 150x25 mm culture dishes. BaP was administered to the cells at three doses (0.5, 2, and 5 µM). DNA was isolated from the cells using QIAGEN 100/G Genomic-tips (QIAGEN, Valencia, CA) according to the manufacturer's protocol.

DNA adducts were quantified by the nuclease P1-enhanced <sup>32</sup>P-postlabeling assay (Phillips and Arlt, 2007) using 6 µg DNA. Multidirectional anion-exchange thin-layer chromatography (conditions described previously in (Cizmas et al., 2004b)) was used to separate <sup>32</sup>P-labeled DNA adducts on polyethyleneimine-cellulose sheets. Profile and radioactivity of DNA adducts from individual samples were determined by Instant Imager (PerkinElmer). Student's t-test was used to evaluate the effects of cell type and inducer treatment on adduct levels at each dose.





**Fig. 3-1.** Establishment of stable cell line expressing hPXR. (A) Schematic of hPXR expression plasmid 3XFlag-hPXR. (B) hPXR expression in HepG2 and stable transfected (PXR-G2) cells by RT-PCR. (C) CYP3A4 induction by RT-PCR in HepG2 (PXR<sup>-</sup>) and PXR-G2 (PXR<sup>+</sup>) cells treated with 5  $\mu$ M RIF (RIF<sup>+</sup>) or DMSO (RIF<sup>-</sup>).

### **3.2.5 Real-time Quantitative RT-PCR**

Cells were seeded in 12-well plates at 25% confluency and treated as described above. BaP was administered at 5  $\mu$ M (24 h). Total RNA was isolated from the cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RNA from three replicate wells was pooled. cDNA was synthesized by reverse transcription using Invitrogen's M-MLV Reverse Transcriptase according to the manufacturer's instructions. PCR primers were designed based on published sequences and purchased from Invitrogen. Real-time quantitative PCR was performed on a 7900HT Real Time PCR system from Applied Biosystems (Foster City, CA) using SYBR Green Master Mix (Applied Biosystems). Results were normalized to  $\beta$ -actin.

### **3.2.6 GST Assay**

Cells were cultured as described above and treated with RIF (5  $\mu$ M, 24 h) or DMSO. The total activity of glutathione S-transferase (GST) was determined using the Glutathione S-Transferase Assay Kit (Cayman Chemicals, Ann Arbor, MI) following the manufacturer's protocol. The conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione was measured spectrophotometrically (320 nm) and linear regression was performed to calculate a rate (slope). Student's t-test was used to evaluate the effects of cell type and inducer treatment on the slopes.

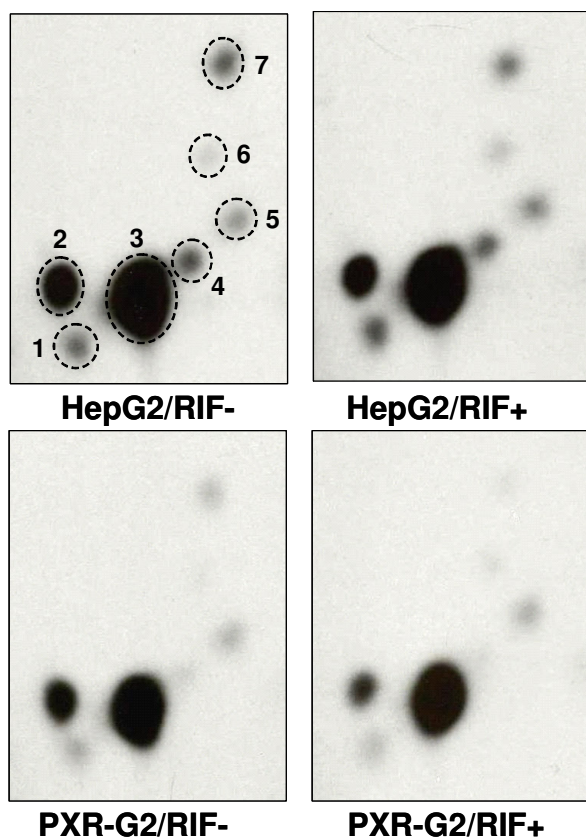
## **3.3 Results**

### **3.3.1 Establishment of Stable Transfected Cell Line**

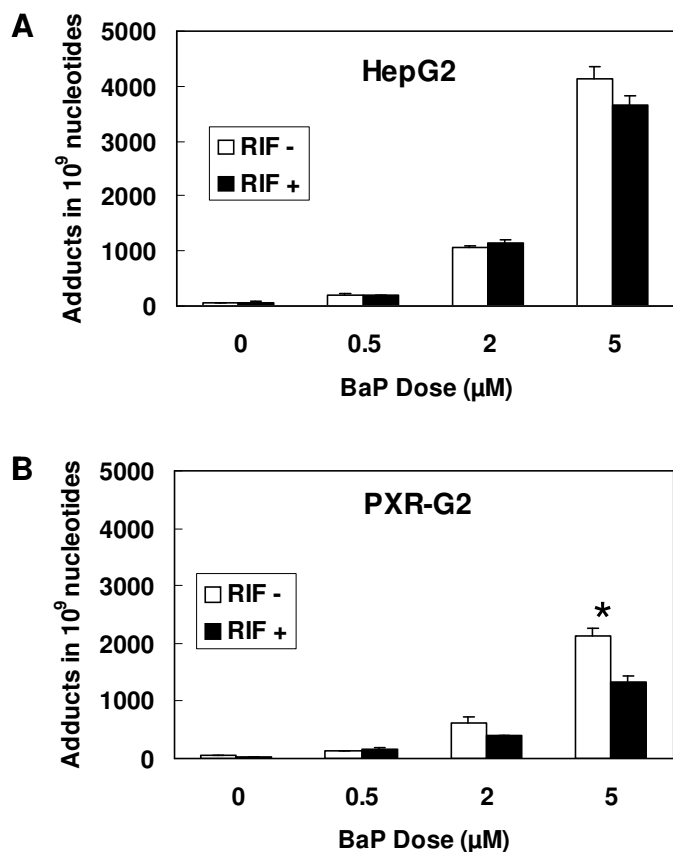
A modified HepG2 cell line, PXR-G2, was produced for this study using an hPXR expression plasmid, p3XFlag-hPXR (Fig. 3-1A). Stable transfected colonies were selected with G418 and expanded. The expression of transfected hPXR was confirmed by quantitative RT-PCR (Fig. 3-1B). PXR mRNA level was 13-fold higher in the stable transfected PXR-G2 cells relative to the parental HepG2 cells. The presence of transfected hPXR in the PXR-G2 cells increased the responsiveness of CYP3A4 to RIF (Fig. 3-1C). Induction of CYP3A4 in response to RIF treatment (5  $\mu$ M) was measured in both cell lines by real-time quantitative RT-PCR. Response to RIF over DMSO control increased from 2-fold to 16-fold with the presence of transfected hPXR.

### 3.3.2 PXR Impact on BaP-induced DNA Adduct Formation

The effect of hPXR expression on BaP-induced genotoxic effects in HepG2 and PXR-G2 cells was investigated. DNA adduct formation was measured by  $^{32}\text{P}$ -postlabeling in both cell lines, with or without RIF (5  $\mu\text{M}$ ) pretreatment, at three BaP doses (0.5, 2, and 5  $\mu\text{M}$ ). HepG2 and PXR-G2 cells exhibited qualitatively similar profiles of BAP-induced DNA adducts. The DNA adduct patterns with or without RIF in each cell line were also comparable (Fig. 3-2). The major spot, Spot 3, was characterized in previous  $^{32}\text{P}$ -postlabeling analyses (Lu et al., 1986) as BaP-diol-epoxide-N2-deoxyguanosine (BPDE-dG).



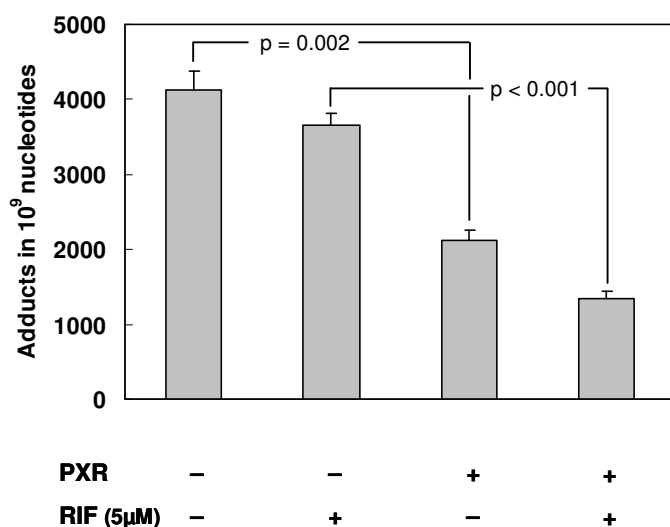
**Fig. 3-2.** Autoradiographic profiles of DNA adducts in parental HepG2 and hPXR-enhanced (PXR-G2) cells treated with BaP (5  $\mu\text{M}$ , 24h). Cells were pretreated with inducer RIF (RIF+) or DMSO control (RIF-) for 24 hours. Screen-enhanced autoradiography was performed with Kodak XAR-5 film at  $-80^{\circ}\text{C}$  for 1.5 h.



**Fig. 3-3.** Comparison of levels of DNA adducts in parental HepG2 cells (A) and hPXR-enhanced (PXR-G2) cells (B) treated with BaP. Cells were pretreated with inducer RIF (RIF+) or DMSO control (RIF-) for 24 h, then treated with BaP (0.5, 2, 5  $\mu\text{M}$ ) for 24 h. Cells were collected and DNA adducts were detected by  $^{32}\text{P}$ -postlabeling assay. Adduct levels are means of triplicate treatments (except HepG2/RIF-/2  $\mu\text{M}$ , which was duplicate) with S.E.M. error bars. Data are representative of two independent experiments. \* indicates statistically significant difference ( $p < 0.01$ ) between RIF- and RIF+.

Treatment with BaP, both with and without RIF pretreatment, resulted in formation of DNA adducts in a dose-dependent manner in both HepG2 and PXR-G2 cells (Fig. 3-3).  $4121 \pm 248$  adducts per  $10^9$  nucleotides were detected in HepG2 cells, whereas  $2128 \pm 134$  adducts per  $10^9$  nucleotides were detected in PXR-G2 cells at the highest BaP dose (Fig. 3-4). The presence of transfected hPXR had a statistically significant effect on adduct levels at 5  $\mu\text{M}$  BaP both without inducer treatment

( $p = 0.002$ ) and with inducer treatment ( $p < 0.001$ ). Treatment with inducer significantly ( $p = 0.008$ ) decreased adduct levels in PXR-G2 cells (37%), but decreased adducts levels in HepG2 cells by only 11%. BPDE-dG (Spot 3) levels were significantly lower in PXR-G2 cells both without inducer pretreatment ( $p = 0.003$ ) and with inducer ( $p < 0.001$ ) (data not shown). These results indicate that PXR protects the cells from genotoxic effects of BaP.



**Fig. 3-4.** Effect of hPXR expression on BaP-DNA adduct formation. Parental HepG2 (PXR-) and hPXR-enhanced HepG2 (PXR+) cells were treated with 5  $\mu$ M BaP (24 h), without (RIF-) or with (RIF+) rifampicin pretreatment. Adduct levels are means of triplicate treatments with S.E.M. error bars. Data are representative of two independent experiments.

**Table 3-1**  
Effects of transfected PXR and BaP treatment on gene expression

	Change in mRNA Level <sup>a</sup>		
	PXR- HepG2 vs. HepG2	HepG2	PXR- HepG2
	DMSO	BaP 5 $\mu$ M vs. DMSO	BaP 5 $\mu$ M vs. DMSO
<i>Phase I Metabolizing Enzymes</i>			
CYP1A1	2.5	350	98
CYP1A2	8.8	120	130
CYP1B1	ND <sup>b</sup>	ND <sup>b</sup>	ND <sup>b</sup>
CYP2B6	3.6	2.7	1.1
CYP3A4	1.2	2.0	2.3
AKR1C2	16	3.5	0.7
AKR1C3	3.0	1.5	1.1
EPHX1 (mEH)	4.0	1.6	1.1
EPHX2 (sEH)	0.6	0.6	0.9
<i>Phase II Conjugating Enzymes</i>			
GSTA1	12	0.8	0.4
GSTA2	16	1.0	0.6
GSTA4	1.7	0.8	0.7
GSTM1	140	1.6	1.2
GSTP1	0.6	51	260
GSTT1	ND <sup>b</sup>	ND <sup>b</sup>	ND <sup>b</sup>
UGT1A6	200	3.2	0.8
UGT2B10	0.8	0.8	0.8
UGT2B15	ND <sup>b</sup>	ND <sup>b</sup>	ND <sup>b</sup>
SULT1A1	2.3	1.2	1.5
SULT1A3/4/5	3.3	2.6	1.3
<i>Transporters</i>			
MDR1 (ABCB1)	0.8	1.3	1.0
MRP1 (ABCC1)	1.2	1.2	0.7
MRP2 (ABCC2)	4.0	1.2	0.4
MRP3 (ABCC3)	3.7	1.6	0.8
BCRP (ABCG2)	7.2	2.1	3.6

<sup>a</sup> Data are representative of three independent experiments.

<sup>b</sup> Gene expression not detectable.

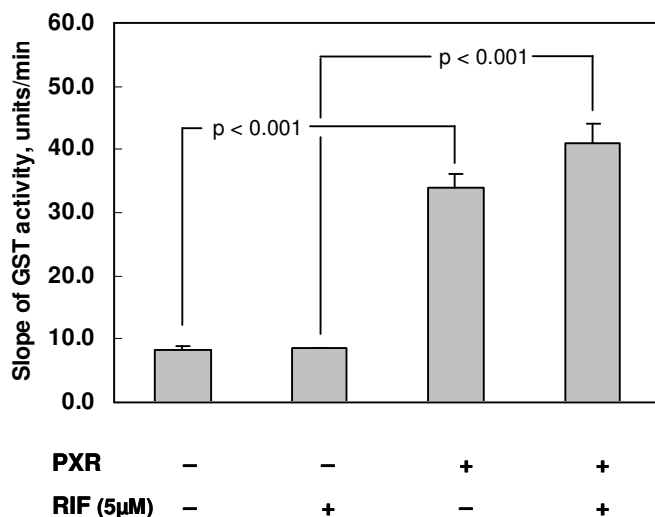
### 3.3.3 PXR Regulation of Xenobiotic Metabolism and Elimination Genes

The effects of BaP and PXR on expression of a panel of 25 genes were investigated by quantitative RT-PCR. The products of these selected genes, which include metabolizing enzymes and drug transporters, are involved in xenobiotic pharmacokinetics. Relative to parental HepG2 cells, stable PXR-HepG2 cells exhibited higher constitutive mRNA expression of many phase I and II xenobiotic enzymes including CYPs, GSTs and UGTs in the absence of BaP (Table 3-1). Most notably, mRNA levels of CYP1A2 increased 8.8-fold, GSTM1 increased 140-fold and UGT1A6 increased 200-fold. In addition, mRNA levels of breast cancer resistance protein (BCRP), a transporter that excretes BaP-sulfate metabolites in colon cells (Ebert et al., 2005), increased in PXR-HepG2 cells. Upregulation of these genes in cells overexpressing PXR may reduce BaP toxicity by enhancing detoxification pathways.

Following exposure to BaP, there was an increase in mRNA levels of some phase I and II enzymes in HepG2 cells, notably CYP1A1, CYP1A2, and GSTP1 (Table 3-1). A similar increase in mRNA levels of phase I and II enzymes was observed in the PXR-HepG2 cells following BaP exposure, although there was a notably higher increase in GSTP1 and a lower increase in CYP1A1 (Table 3-1).

### 3.3.4 Effect of PXR on GST Enzymatic Activity

The total activity of GST (cytosolic and microsomal) was assessed to determine the GST enzymatic levels. The rate of CDNB conjugation, which is directly proportional to the GST activity, was measured in both cell lines, with or without RIF treatment (5  $\mu$ M, 24h). The GST activity level was  $8.23 \pm 0.61$  units per minute in HepG2 cells and  $34.0 \pm 2.3$  units per minute in PXR-G2 cells (Fig. 3-5), a 4-fold increase. The presence of transfected hPXR had a statistically significant effect ( $p < 0.001$ ) on GST activity both without rifampicin pretreatment and with rifampicin pretreatment. Treatment with rifampicin had no effect on GST activity in HepG2 cells, but increased GST activity in PXR-G2 cells by 21% ( $p = 0.13$ ). These results, considered with the increased mRNA levels of GSTM1, GSTA1 and GSTA2, suggest that PXR increases the enzyme levels of GSTs in liver cells through transcriptional regulation of GST gene expression.



**Fig. 3-5.** Effect of hPXR expression on total GST enzymatic activity. Parental HepG2 (PXR<sup>-</sup>) and hPXR-enhanced HepG2 (PXR<sup>+</sup>) cells were treated with DMSO (RIF<sup>-</sup>) or 5  $\mu$ M rifampicin (RIF<sup>+</sup>) for 24 h. Total GST enzymatic activity was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. Slopes of GST activity are means of triplicate treatments with S.E.M. error bars.

### 3.4 Discussion

PXR is a broad specificity, low affinity nuclear receptor that transcriptionally regulates many genes associated with xenobiotic metabolism and clearance, and therefore performs an important function for detoxification of xenobiotics (Kliwer, 2003). PXR is considered to be a xenosensor because of its ability to respond to many xenobiotics, including pharmaceuticals, dietary nutrients, and environmental contaminants. Consistent with this protective role, PXR is mainly expressed in the liver, intestine and colon, which are sites of first-pass elimination. Because PXR regulates a wide range of the genes expressed in the hepato-intestinal system, it may affect the pharmacokinetics/toxicokinetics of environmental contaminants. The major goal of this study was to investigate the role of PXR in the genotoxicity of BaP, a well-known carcinogen and environmental contaminant. Although PXR can be activated by many therapeutic drugs, naturally-occurring chemicals and environmental contaminants, its role as a sensor of BaP and effector on BaP detoxification has not been well understood.



In the liver, PXR upregulates multiple phase I and phase II enzymes which metabolize BaP. Metabolism is important in both the detoxification of BaP and its transformation to genotoxic metabolites. For example, recent *in vivo* studies utilizing knockout mouse strains (Sagredo et al., 2006; Uno et al., 2004; Uno et al., 2006; Uno et al., 2001) indicate that CYP isoforms involved in phase I metabolism of BaP contribute to both the protective and potentiating pathways. In the current model of BaP bioactivation, BaP is transformed to highly electrophilic dihydrodiol-epoxides (BPDEs), which then can form DNA adducts, through a series of steps mediated by phase I enzymes. The formation of an epoxide is catalyzed by CYPs, followed by conversion to a dihydrodiol by epoxide hydrolases (EH), and further conversion by CYPs to BPDE (Ramesh et al., 2004). Reactive *ortho*-quinones, which are also capable of forming DNA adducts, can be produced by oxidation of BaP-dihydrodiols by aldo-keto reductases (AKR) (Penning et al., 1999). Phase I enzymes also contribute to detoxification by alternate reactions that form non-toxic or less toxic compounds, such as the conversion of BPDEs to tetraols by EH (Ramesh et al., 2004).

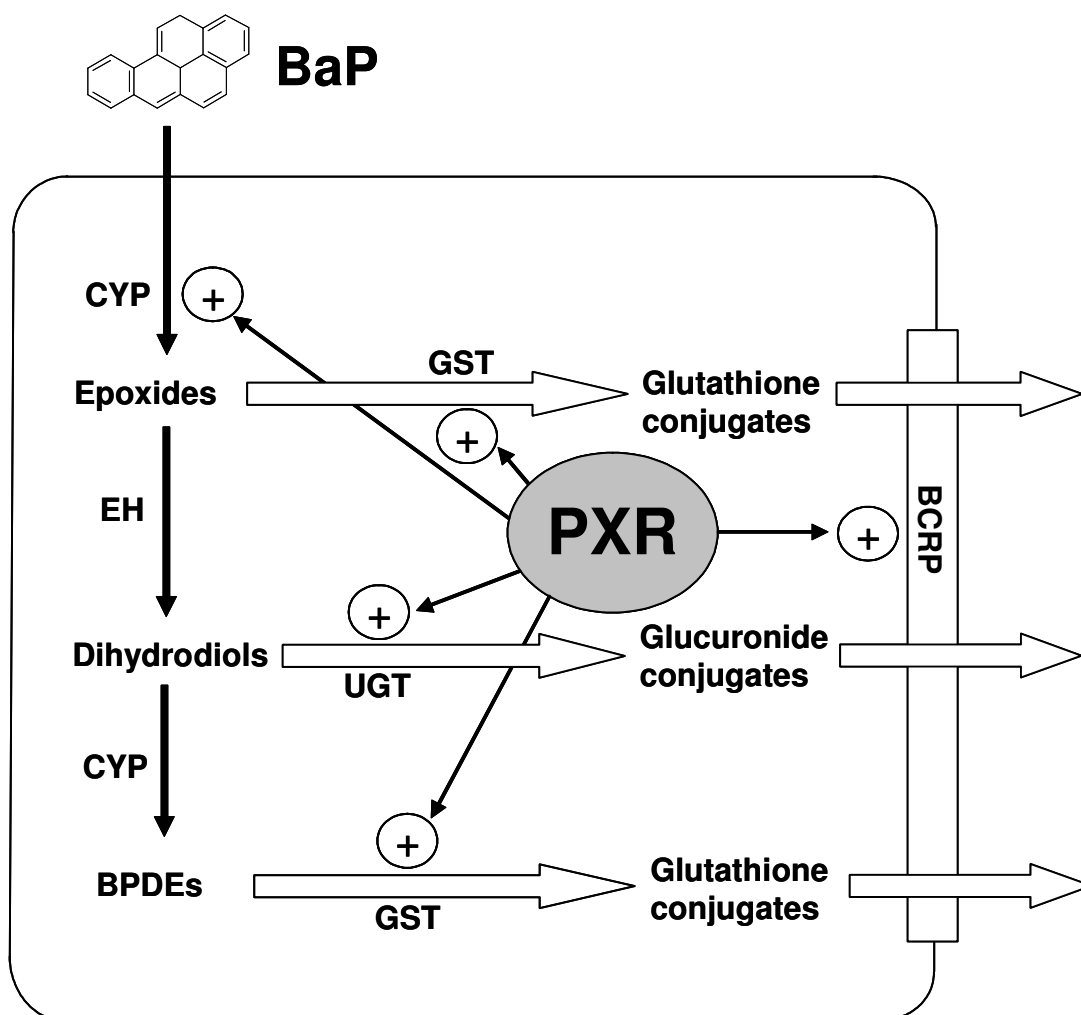
Phase I metabolism adds functional groups which allow conjugation by phase II enzymes, a step that effectively detoxifies BaP by preventing further transformation to carcinogenic metabolites, and facilitates excretion from the cell. In a discussion of the paradoxical effects of the CYP1 enzymes on BaP toxicity (Nebert et al., 2004), the authors suggest that detoxification may be enhanced in cells, such as hepatocytes and gastrointestinal epithelial cells, in which the CYP1s are tightly coupled to phase II enzymes.

To analyze the role of PXR in BaP detoxification, we created a cell culture model using HepG2 cells, which lack PXR, and PXR-transfected HepG2 cells. Levels of DNA adducts were significantly decreased by the expression of PXR and, in cells overexpressing PXR, by pretreatment with RIF. Levels of BPDE-dG, the major adduct, were significantly lower in cells overexpressing PXR. Although the other spots have not been identified, they seem to be BPDE-related adducts because they display a similar pattern to adducts detected in cells treated with pure BPDE (Li et al., 2001). These results demonstrate that PXR protects the cells from the genotoxic effects of BaP, and suggest that PXR may reduce the formation of BPDE.

In our cell culture model, CYP1A2, GSTM1, UGT1A6, GSTA1, and GSTA2 were upregulated by PXR, independent of BaP (Table 3-1). Consistent with the GST gene expression results, total GST activity was significantly increased by PXR, independent of BaP (Fig. 3-5). GSTs catalyze the formation of glutathione conjugates of primary epoxides and BPDEs; UGTs catalyze the formation of glucuronide conjugates of dihydrodiols (Ramesh et al., 2004). Following exposure to BaP, there was an increase in mRNAs of phase I and II enzymes in HepG2 cells (Table 3-1). A similar increase in mRNAs of phase I and II enzymes was observed in PXR-HepG2 cells following BaP exposure, although there were notably higher increases in mRNAs of CYP1A2 and GSTP1 (Table 3-1). As expected, the Ah receptor agonist BaP caused large increases in CYP1A1 mRNA in both HepG2 and PXR-HepG2 cells; interestingly, the increase was attenuated in PXR-transfected cells. The mechanism for this attenuation is unclear and it is conceivable that a squelching mechanism may play a role where PXR competes with the Ah receptor for the common transcriptional co-regulators.

mRNA levels of membrane-bound drug transporter BCRP, which has been shown to be regulated by PXR in mice (Anapolsky et al., 2006), increased 12-fold in the hPXR-enhanced cells. BCRP actively excretes sulfate metabolites of BaP in Caco-2 cells (Ebert et al., 2005) and has been shown to transport glutathione, glucuronide and sulfate conjugates of other compounds (Adachi et al., 2005; Suzuki et al., 2003). BCRP may contribute to detoxification by removing BaP conjugates from the cell and thus decreasing bioavailability.

Our results strongly suggest a mechanism in which PXR coordinately regulates phase I and II enzymes and transporters that are relevant for BaP metabolism and disposition, leading to BaP detoxification (Fig. 3-6). Upregulated CYP increases initial BaP metabolism to epoxides. A higher level of GST favors rapid conjugation of the epoxides rather than further metabolism to dihydrodiols by EH, which was not affected by PXR. Higher levels of UGT increase conjugation of dihydrodiols. Dihydrodiols are converted to BPDEs by upregulated CYP, but increased GST also favors rapid conjugation of BPDEs, reducing the amount available to bond to DNA. Finally, upregulated BCRP enhances elimination of conjugated species from the cell.



**Fig. 3-6.** PXR-regulated detoxification of BaP: a possible metabolic mechanism. PXR upregulates CYP, GST, and UGT enzymes and the membrane-bound transporter BCRP. BaP enters the cell and is transformed through a cascade of phase 1 reactions. These metabolites are conjugated and transported out of the cell. These upregulated detoxification pathways decrease bioavailability, and therefore genotoxicity.

In conclusion, this research demonstrates that PXR decreases the formation of BaP-induced adducts in an hPXR-enhanced liver cell line, indicating that PXR plays an important role in protecting these cells from genotoxicity induced by exposure to PAHs. The gene expression of several BaP-metabolizing phase I and phase II enzymes and one PXR-regulated drug transporter was increased in the hPXR-enhanced cells. Enzymatic activity of glutathione S-transferase, an important family of phase II

enzymes, was significantly higher in HepG2 cells overexpressing PXR. Taken together, these data suggest that PXR may effectively decrease bioavailability of carcinogenic BaP metabolites by coordinately regulating a pathway of biphasic metabolism and excretion that reduces intracellular concentrations of BaP and pro-carcinogenic phase I metabolites. These findings further our understanding of factors influencing the genotoxicity of BaP and can guide the design of *in vivo* studies that may help improve risk assessment of PAHs.

## CHAPTER IV

### MEASUREMENT OF DNA ADDUCT LEVELS IN MICE EXPOSED TO PAH MIXTURES *IN SITU*

#### 4.1 Introduction

##### 4.1.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a common class of environmental contaminants which are present in coal and petroleum products and are formed during combustion of fossil fuels and biomass. PAHs are introduced into the environment by both natural (e.g., forest fires) and anthropogenic (e.g., fossil fuel extraction and use) sources. Most sources produce a complex mixture of hundreds of PAH compounds with many other hydrocarbons, making toxicity assessment difficult. Decades of research have accumulated substantial evidence that occupational exposure to PAH-rich mixtures such as coal tar and soot cause cancer in humans. In addition, several individual PAH compounds are known animal carcinogens. A commonly accepted mechanism of PAH carcinogenicity is induction of mutations by PAH-DNA adducts which are formed when electrophilic PAH metabolites covalently bond to DNA.

##### 4.1.2 Exposure Assessment and Biomarkers

Many human health risk assessments of contaminated sites assume that the source of human exposure to the onsite contaminants is through intake of contaminated soil. Soil contaminant concentrations are measured by chemical analysis of soil samples, and a daily human exposure value (mass contaminant per body weight per day) is calculated using assumptions for mass of soil contacted, contact frequency and duration. The amount of contaminant that actually crosses the body barriers and enters the body is estimated by the application of absorption factors. This approach presents many challenges. Soil samples may not be representative of the whole site because of temporal and spatial variations in contaminant concentrations. Soil characteristics and contaminant physico-chemical properties may alter bioavailability. Individual differences in toxicokinetics can cause variations in the dose of contaminant that reaches the target organ where health effects occur. An alternative or complementary approach is to measure biomarkers of exposure in exposed populations. A biomarker of exposure is a biological indicator, at the molecular or cellular level, of exposure to an

environmental contaminant. Biomarkers of exposure encompass all exposure routes and indicate the internal dose, including the extent of contaminant absorption, distribution, and accumulation (Vainio, 2001). Biomarkers are useful tools for evaluating exposures of *in situ* populations.

Exposure to genotoxic contaminants such as PAHs can be detected by the use of DNA damage biomarkers. Types of DNA damage include strand breaks, altered bases, mutations, and adducts. DNA adducts have been used as biomarkers of PAH exposure in environmental toxicology studies of both humans (Kriek et al., 1998) and wildlife (Shugart, 2000).

#### **4.1.3 Study Location: Azerbaijan**

The sampling sites for this study are located in two regions of the Republic of Azerbaijan, a former Soviet Union republic. Our research group is conducting a human health study in these regions. The health risk of the human population in the Sumgayit area, described previously by Bickham et al. (2003), is of concern because they are living on contaminated land near chemical manufacturing sites. A series of ecotoxicology studies have documented genotoxic effects in amphibians and reptiles captured from contaminated wetlands near Sumgayit (Matson et al., 2006; Matson et al., 2005a; Matson et al., 2005b; Swartz et al., 2003). Genotoxic effects in wild mammals have not yet been measured.

#### **4.1.4 Study Objectives**

This is a preliminary study to determine if soil contamination and biomarkers of exposure in wild mammals are detectable in the study area. Wild house mice (*Mus musculus*) were used as a model mammalian species for environmental monitoring. The objectives of this study were to: 1) quantify DNA adduct levels and variability in wild house mice, 2) quantify PAH levels in soil near the mouse capture areas, and 3) investigate the relationship between media contaminant level and biomarkers of exposure.

## **4.2 Materials and Methods**

### **4.2.1 Study Sites**

House mice and soil samples were collected from four study sites in Azerbaijan during May, 2006. Three sites (S-1, S-2 and S-3) are located in a densely populated

residential area in close proximity to an industrial complex in the city of Sumgayit. Sumgayit is on the Absheron Peninsula on the western shore of the Caspian Sea, and north of Baku, the capital of Azerbaijan. The city was built in the early 1950's to serve as a major petrochemical production area for the former Soviet Union. The community where samples were collected is adjacent to a synthetic rubber plant. Nearby industries include an electric power generating station, aluminum plant, chlor-alkali plant and pipe production facility. Each study site is outside a home in the community.

The fourth study site (K-1) is in a rural village approximately 70 km north of Sumgayit. Samples were collected from a barn near one of the houses.

#### **4.2.2 Mouse Collection**

Mice were collected following procedures described in Animal Use Protocol # 2004-161. Sherman live traps were set at the study sites and left overnight. Captured mice were collected the following day, sacrificed by cervical dislocation, and frozen whole. Mice were shipped on ice to the laboratory. Upon arrival at the laboratory, samples were logged into the sample tracking record and stored at -80 °C. The mice were later thawed and dissected to obtain skin, lung, liver, and kidney tissues. The tissues were frozen and stored at -80 °C prior to DNA isolation. Four mice were collected in Sumgayit and five mice were collected in Khizi (Table 4-1). Four mice were male and three mice were female (two of which were pregnant); sex of the other two mice could not be determined.

**Table 4-1**  
Description of wild house mice collected in Azerbaijan

Mouse	Area	Site	Description
MS1	Sumgayit	S-1	M
MS2	Sumgayit	S-2	Sex not determined
MS3	Sumgayit	S-3	Sex not determined
MS4	Sumgayit	S-3	F, pregnant
MK1	Khizi	K-1	F, pregnant
MK2	Khizi	K-1	M
MK3	Khizi	K-1	F
MK4	Khizi	K-1	M
MK5	Khizi	K-1	M

#### 4.2.3 DNA Isolation and Adduct Analysis

DNA was isolated from the mouse tissues following the protocol by Gupta (1984). Minced tissue (0.2 g to 0.5 g) was homogenized in a solution of 1% SDS/1mM EDTA, then incubated for 40 min at 38°C with 100 to 150 µL proteinase K (15 mg/mL) to digest proteins. DNA/RNA was extracted from the digested sample into an aqueous phase using a three-step phenol/chloroform extraction: 1) phenol saturated with Tris-HCL; 2) 1:1 mixture of saturated phenol and SEVAG (24:1 chloroform/isoamyl alcohol); and 3) SEVAG. The DNA/RNA was precipitated and washed with ethanol, then dissolved in diluted SSC (1.5 mM NaCl, 150 µM Na citrate) and incubated with 15 µL RNase A (10 mg/mL) and 16.5 µL RNase T1 (5000 units/mL) for 40 min at 38°C to remove RNA. DNA was again extracted from the digested sample into an aqueous phase by SEVAG, precipitated and washed with ethanol, then partially dried and redissolved in SSC to a concentration of approximately 2 µg/µL. DNA concentration and purity were verified by UV/Vis spectrophotometer. Isolated DNA was stored at -80°C prior to DNA adduct labeling.

DNA adducts were quantified by the nuclease P1-enhanced <sup>32</sup>P-postlabeling assay (Phillips and Arlt, 2007; Reddy and Randerath, 1986), a four-part assay. The first step was digestion, in which 10 µg DNA was incubated with a mixture of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD), enzymes which cleave the DNA



strands into deoxyribonucleoside 3'-monophosphates, both of normal nucleotides (Np) and adducted nucleotides (Xp). Step 2, enrichment, enhanced the sensitivity of the assay by using nuclease P1 to cleave the phosphate group preferentially from the normal nucleotides (Np cleaved to N + p), leaving a solution of normal nucleotides (N), free phosphate groups (p), and adducted nucleoside 3'-monophosphates (Xp). In the labeling reaction, Step 3, polynucleotide kinase (PNK) was used to radiolabel adducted nucleotides by transferring a radioactive phosphate group (\*p) from [ $\gamma$ - $^{32}$ P]ATP to the 5' end of the adducted nucleotides (\*pXp). Apyrase was used at the end of this step to remove excess [ $\gamma$ - $^{32}$ P]ATP. The fourth step, resolution of adducts, was accomplished by multidirectional anion-exchange thin-layer chromatography (TLC) on polyethyleneimine-cellulose sheets. TLC was done in three parts, which are summarized here; detailed chromatography conditions were described in (Reddy and Randerath, 1986). The labeled sample was applied to the first sheet and D1 solvent (2.3 M NaH<sub>2</sub>PO<sub>4</sub>, pH 5.7) was used to remove impurities including most of the free phosphate (\*p), minimizing background radioactivity. The remaining labeled adducted nucleotides were cut out and transferred to 2D maps. The 2D maps were developed vertically in D3 solvent (6.75 M urea, 3.82 M lithium formate, pH 3.35), washed, dried, and then developed horizontally in D4 solvent (0.72 M NaH<sub>2</sub>PO<sub>4</sub>, 0.45 M Tris-HCl, 7.65 M Urea, pH 8.20) to separate the adducted nucleotides. Radioactivities of the adduct spots from individual mice were determined by Instant Imager (PerkinElmer). The detection limit of this assay is approximately 1 adducted nucleotide in one billion normal nucleotides.

#### **4.2.4 Soil Collection**

At each study site, three soil samples were collected near the areas from which mice were collected. Approximately 250 g of the upper layer of soil was obtained for each sample. The samples were placed in plastic bags and shipped on ice to the laboratory. Upon arrival at the laboratory, samples were logged into the sample tracking record and stored at -4°C prior to extraction and analysis.

#### **4.2.5 Soil Extraction and Analysis**

Soil samples were air-dried to reduce moisture, ground, and sieved. Approximately 10 g of soil from each sample was extracted with HPLC-grade dichloromethane (EM Science, Gibbstown, NJ) in a Dionex ASE 200 Accelerated

Solvent Extractor. Extracts were dried in a Zymark TurboVap II evaporator, then redissolved in 1 mL of dichloromethane. An internal standard solution (Cambridge Isotope Laboratories, Andover, MA) of deuterated fluorene, pyrene and benzo[a]pyrene was added prior to analysis for quality control. The PAH content of the prepared extracts was quantified using a modified SW-846 Method 8270C (USEPA, 1996) on an HP-5890 series II gas chromatograph (GC) with an HP5-MS capillary column (60m, 0.25 mm ID) coupled to an HP-5972 mass spectrometer (MS) operated in selected ion monitoring (SIM) mode. Thirty-six PAH analytes were quantified, including alkylated PAHs (C1, C2, C3, and C4 derivatives of parent PAHs). The practical quantitation limit for each analyte was 10 µg per kg soil.

## **4.3 Results**

### **4.3.1 Mouse DNA Adduct Levels**

A total of nine mice were dissected and their tissues isolated for DNA adduct labeling (Table 4-1). Four mice were from sites in Sumgayit: one from S-1, one from S-2, and two from S-3. Five mice were from K-1 in Khizi. Tissues isolated were skin, lung, liver, and kidney. Each tissue was processed to extract DNA, and the DNA adducts were radio-labeled. DNA adducts were detected in all liver, lung, and kidney samples; adducts were not detected in any skin samples.

Lung, kidney, liver and total (sum of the three tissues) DNA adduct levels are presented in Table 4-2 for individual mice in both regions. In the overall data set, tissue DNA adduct levels ranged from 5.8 to 34.1 adducts per billion nucleotides. Total DNA adducts ranged from 32.1 to 85.0 adducts per billion nucleotides. Variability of adduct levels in each tissue, quantified by relative standard error (data not shown), was less than 20%. The maximum total adducts and maximum adducts in each tissue were detected in individuals from Khizi. The minimum total, lung, and liver adduct levels were found in individuals from Sumgayit, whereas the minimum kidney adduct level was in a mouse from Khizi.

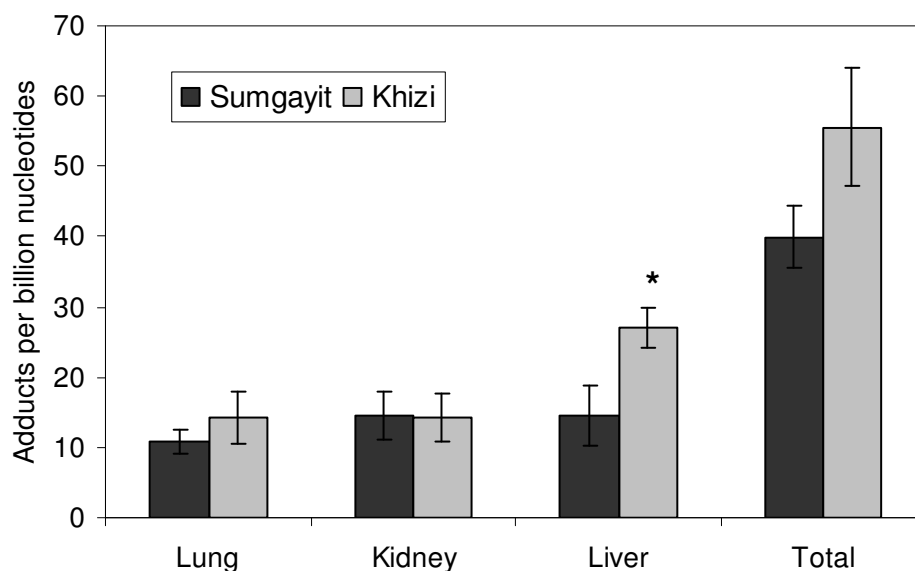
Mouse DNA adduct levels grouped by region are presented both in Table 4-2 and in Fig. 4-1 as means  $\pm$  standard error (SE). In the Khizi region, mean adduct levels in liver were highest, with similar lower levels in lung and kidney. In the Sumgayit group, liver and kidney mean adduct levels were similar, and lung levels were lowest.

Mean liver adduct level in Khizi was significantly higher than in Sumgayit ( $p = 0.041$ ). Lung and total adduct levels were also higher in Khizi, but the difference was not statistically significant. Kidney adduct levels were similar in the two regions.

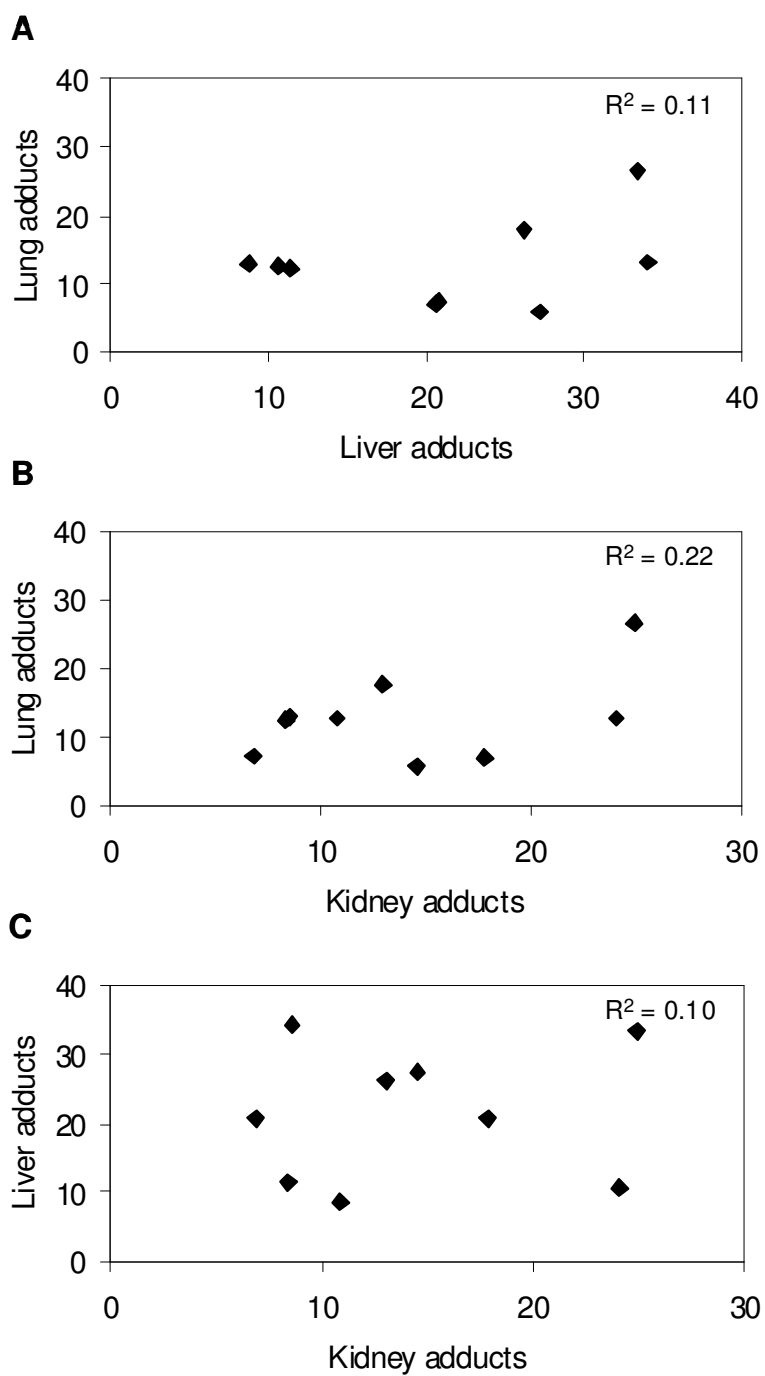
**Table 4-2**

DNA adduct levels detected in tissues of wild house mice (adducts / billion nucleotides)

Region:	Sumgayit					Khizi					
Mouse:	MS1	MS2	MS3	MS4	Mean $\pm$ SE	MK1	MK2	MK3	MK4	MK5	Mean $\pm$ SE
Lung	5.8	12.9	12.6	12.3	10.9 $\pm$ 1.7	13.0	26.6	17.8	7.0	7.2	14.3 $\pm$ 3.7
Kidney	14.6	10.8	24.1	8.4	14.5 $\pm$ 3.4	8.5	24.9	13.0	17.8	6.9	14.2 $\pm$ 3.3
Liver	27.2	8.8	10.6	11.4	14.5 $\pm$ 4.3	34.1	33.5	26.2	20.6	20.7	27.0 $\pm$ 2.9
Total	47.6	32.5	47.3	32.1	39.9 $\pm$ 4.4	55.7	85.0	57.0	45.4	34.7	55.6 $\pm$ 8.4



**Fig. 4-1.** Adduct levels detected in mice from locations in Sumgayit and Khizi. Data are presented as means  $\pm$  SE. \* Liver adduct levels were significantly higher in Khizi mice ( $p = 0.041$ ). Sumgayit  $n=4$ ; Khizi  $n=5$ .



**Fig. 4-2.** Comparison of tissue adduct levels (adducts in  $10^9$  nucleotides) in individual mice. X-Y plots of DNA adducts in A) lung versus liver; B) lung versus kidney; and C) liver versus kidney.

If the mice had one main source of PAH exposure, trends in DNA adduct levels between tissues would most likely be similar. For example, if soil were the overriding source of PAH exposure, mice with higher exposure levels would be expected to have higher total adduct levels and higher levels in each tissue than mice with lower exposure levels. For evaluation of trends between tissues, adduct data matched by mouse were graphed in X-Y plots of pairs of tissues (Fig. 4-2). No obvious patterns were observed in these graphs. For all three pairs of tissues, R-squared values obtained from linear regression were less than 0.25. The data were also evaluated statistically by paired-sample t-test. No correlation between tissues was found. Adduct levels in one tissue were not predictive of adduct levels in another tissue from the same mouse.

#### **4.3.2 Soil PAH Levels**

Three soil samples from each of the four sites were analyzed by GC/MS for 36 PAH analytes. These analytes were described in detail in a previous study (Naspinski et al., 2008). Eighteen of the analytes are individual, unsubstituted PAHs. The other 18 analytes are alkylated families, which represent PAH compounds with alkyl groups attached to the aromatic rings, specified by the number of carbons in the alkyl groups (C1, C2, etc.).

PAH concentrations in all samples were at the low end of the detection limit of the analytical method. In nine of the twelve samples, concentrations of each of the 36 analytes were below the PQL of 10 µg/kg. Detection frequencies of the 2-ring and 3-ring PAHs were lower than those of the larger PAHs. Naphthalene was detected in only 2 of the 12 samples; alkylated naphthalenes were detected in 7 of the samples. Acenaphthene was detected in 3 samples and acenaphthylene was detected in 9 samples. Fluorene was detected in 5 samples; alkylated fluorenes were detected 7 samples. All other analytes were detected in at least 10 of the samples with the exception of the alkylated chrysenes (C2-chrysenes in 7 samples; C3- and C4-chrysenes not detected in any sample). The seven carcinogenic PAHs were detected in all 12 samples. In both Sumgayit and Khizi, the most abundant unsubstituted tPAH analytes were carcinogens: benzo[b]fluoranthene and chrysene. The most abundant PAH families (i.e., unsubstituted parent compounds plus their alkylated analytes) in both regions were fluoranthenes/pyrenes, followed by anthracenes/phenanthrenes.

PAH levels found in the soil samples are summarized in Table 4-3. The sum of all 36 analytes is reported as total PAH (tPAH). A subset of seven PAHs considered by USEPA to be probable human (B2) carcinogens is reported as carcinogenic PAHs (cPAH). In Table 4-3, the data are presented by individual site and also grouped by region. Individual sample tPAH levels ranged from 15 µg/kg to 100 µg/kg and cPAH levels ranged from 7 µg/kg to 41 µg/kg. Mean levels of tPAH were highest at S-1 and S-2, lower at S-3, and lowest at K-1. A similar trend occurred in cPAHs. Variability of tPAH levels at individual sites ranged from 7 to 18 µg/kg (standard error). Variability of cPAH ranged from 2 to 8 µg/kg. Mean cPAH levels as a percentage of tPAH ranged from 30% at S-3 to 42% at S-1. When grouped by region, mean tPAH and cPAH levels were significantly ( $p < 0.05$ ) higher in Sumgayit than in Khizi. The fraction of cPAH was similar in the two regions: 36% in Sumgayit and 40% in Khizi.

**Table 4-3**

PAH levels in extracts of soil samples collected from sites in Azerbaijan

	S-1 n=3	S-2 n=3	S-3 n=3	K-1 n=3	Sumgayit Overall n=9	Khizi Overall n=3
<b>Total PAHs, µg/kg</b>						
Mean	71	69	46	23	62	23*
SE	18	8	18	7	9	7
Min	37	57	20	15	20	15
Max	100	84	80	37	100	37
<b>Carcinogenic PAHs, µg/kg</b>						
Mean	30	25	11	9	22	9*
SE	8	2	2	2	4	2
Min	14	21	8	7	8	7
Max	41	28	14	13	41	13
<b>Carcinogenic PAHs, % of Total PAH</b>						
Mean	42	37	30	40	36	40

\* denotes that Sumgayit mean is significantly higher ( $p < 0.05$ ) than Khizi mean.

#### 4.4 Discussion

The house mouse (*Mus musculus*) generally lives commensally with humans, living in and obtaining food from human habitations, other buildings, or cultivated fields. House mice dwelling in buildings such as houses, barns and granaries will often nest in a hidden spot near a source of food. House mice living outdoors will nest in cracks in rocks or walls or build underground burrows. Some individuals live outside during the summer but overwinter in buildings (Ballenger, 1999). Commensal house mouse populations are relatively stable; breeding continues throughout the year and in suitable conditions a female produces 5 to 10 litters per year. Sexual maturity is reached at 5 to 7 weeks of age (Nowak, 1991). Commensal house mice have individual home ranges of less than 10 square meters and rarely travel more than 50 feet from their established homes. House mice in human habitations eat any accessible human food, but will also consume household materials including paste, glue and soap. Outdoors, house mice eat many kinds of plant matter, including seeds, fleshy roots, leaves and stems. They also consume insects such as beetle larvae, caterpillars and cockroaches, and carrion, when available (Ballenger, 1999; Nowak, 1991).

Commensal house mice are a useful model species for environmental monitoring for several reasons. House mice eating human food are exposed to the same food-borne contaminants as the humans. Mice living in human dwellings are also exposed to the same contaminants in domestic water and indoor air. House mice living outdoors in agricultural areas are exposed to agricultural chemicals and fuels used by humans. In addition, laboratory-bred *Mus musculus* strains have been used extensively in medical, genetic, and toxicological research for decades. A large body of knowledge has been amassed about their physiology. Comparison of commensal mice exposed *in situ* and laboratory mice exposed in controlled experiments could be a valuable way to link the laboratory and the field.

In this study, DNA adducts were detected by <sup>32</sup>P-postlabeling assay in liver, lung and kidney of all collected wild house mice. Liver adduct levels were significantly higher, and lung adduct levels were somewhat higher, in Khizi than in Sumgayit; kidney adduct levels were similar in the two regions. The chromatographic properties of the detected spots were typical for bulky, nonpolar adducts such as those induced by PAHs (Randerath et al., 1997). Further analysis to identify the chemical structure of specific

spots was not conducted, so positive identification of PAH-DNA adducts is not possible. Other environmental contaminants may form bulky nonpolar DNA adducts.

In addition, the detected spots could be bulky covalent DNA modifications known as type I I-compounds, some of which have similar chromatographic properties to PAH-DNA adducts. Type I I-compounds are derived from endogenous DNA-reactive intermediates of nutrient and oxygen metabolism and represent useful biomarkers in carcinogenesis and cancer prevention studies. Type I I-compound levels vary depending on age, species, strain, tissue and gender and are also affected by diet as well as exposure to chemicals and hormones. Numerous experiments have shown that many carcinogens and tumor promoters significantly reduce I-compound levels gradually during carcinogenesis and suggest that certain type I I-compounds may protect against carcinogenesis and age-related degenerative processes (Randerath et al., 1999). Dietary restriction and food deprivation have been shown to increase levels of type I I-compounds in various rodent organs, especially in liver (Randerath et al., 1999; Randerath et al., 1993; Zhou et al., 1999).

House mouse exposure to contaminants in soil may be direct or indirect. Direct dermal contact with soil occurs almost constantly in mice living outdoors. Direct ingestion of soil occurs during grooming. House mice are also indirectly exposed to contaminants in soil through their food sources: plants and insects that have taken up soil-borne contaminants during their life cycles.

PAHs were detected in all soil samples collected from the Sumgayit and Khizi regions. The mean concentrations of both tPAH and cPAH were statistically higher in Sumgayit than in Khizi. However, the number of sampling sites was small (1 in Khizi, 3 in Sumgayit), detection frequencies of some of the analytes were less than 50%, and the PAH concentrations were below the practical quantitation limit of the analytical method. Care must be taken to not over interpret differences between small numbers. We can conclude that there is little or no difference in PAH levels in the two regions.

A large body of soil PAH concentration data exists in the literature. Comparison of concentrations between studies can be problematic because of the many different methods used for soil collection, extraction and analyte quantification. However, order-of-magnitude comparisons can be useful. A common method for reporting "total PAH" concentrations is the sum of concentrations of sixteen unsubstituted PAHs classified as



priority pollutants by USEPA (16PAH). Table 4-4 presents the 16PAH concentrations reported in several recent studies of soils in various European countries. These soils include those in rural, urban, and industrial areas. The Azerbaijan samples from the current study ranged from 10 to 68  $\mu\text{g}/\text{kg}$ . The most comparable concentrations were reported by Crnkovic et al. (2007) for rural soils near Belgrade, Serbia. Rural soils in Switzerland (Bucheli et al., 2004) were somewhat higher. Mean PAH levels measured in urban and industrial soils (Crnkovic et al., 2007; Maliszewska-Kordybach, 1996; Morillo et al., 2007; Saltiene et al., 2002) were an order-of-magnitude or more higher than those found in the current study. Maliszewska-Kordybach (1996) has proposed classifying soils by 16PAH levels. Soils which contain less than 200  $\mu\text{g}/\text{kg}$  are classified as “not contaminated”. The Azerbaijan samples clearly fall into this category. All but two of the Azerbaijan samples also meet the Dutch government target of 20-50  $\mu\text{g}/\text{kg}$  for unpolluted soil (Crnkovic et al., 2007).

If the observed DNA adducts in the mice are PAH-DNA adducts, and soil is the main source of PAH exposure, we would expect adduct levels to be similar in the two regions because soil PAH levels are similar. Kidney and lung adduct levels were similar. Liver adducts, however, were significantly higher in mice from the Khizi site. It is possible that the liver adduct levels are influenced by another environmental contaminant or by PAHs in another medium such as food or air. PAHs in food have been determined to pose an appreciable health risk to the Azeri human population (Nwaneshiudu et al., 2007). Food as a source of PAHs would affect liver adduct levels. However, we do not have information about differences in food sources or food PAH levels between Khizi and Sumgayit.

Conversely, if the detected DNA modifications are type I I-compounds, the levels in Sumgayit could be decreased by exposure of the mice to non-PAH carcinogenic or tumor-promoting contaminants present in the area. Levels of I-compounds in Khizi could also be increased if mice in Khizi were food-deprived compared to those in Sumgayit. Type I I-compound levels may also be different because of different age and gender distributions between the two regions.

**Table 4-4**  
PAH concentrations in soils of several European countries

Country	Type	16PAH, µg/kg <sup>a</sup>			Reference
		Mean	Min	Max	
Azerbaijan	mixed	32	10	68	current study
Serbia	rural	18	1	62	Crnkovic <i>et al.</i> , 2007
Serbia	urban	375			Crnkovic <i>et al.</i> , 2007
Switzerland	rural		60	310	Bucheli <i>et al.</i> , 2004
Poland	industrial	260	28	2450	Maliszewska-Kordybach, 1996
Slovenia	urban	990	220	4490	Morillo <i>et al.</i> , 2007
Italy	urban	1990	150	3410	Morillo <i>et al.</i> , 2007
Estonia	urban	1020	250	4140	Saltiène <i>et al.</i> , 2002
Lithuania	urban	650	400	1060	Saltiène <i>et al.</i> , 2002

a. Sum of concentrations of USEPA 16 priority pollutant PAHs, except for Morillo *et al.* (2007), which excludes acenaphthylene.

In summary, we found that bulky DNA lesions were present in house mice from Sumgayit and Khizi; DNA lesion levels in liver were higher in Khizi than in Sumgayit. These DNA lesions may be a combination of environmentally-induced DNA adducts and naturally-occurring I-compounds. The effects of environmental carcinogens on DNA lesion levels are complex because increased exposure tends to increase carcinogen-DNA adduct levels but tends to decrease type I I-compound levels. Therefore, no conclusions could be made about the relative risk posed by exposure of mice to environmental carcinogens in the two regions. PAHs were present at background levels in soils from both Khizi and Sumgayit. Based on the soil data, it appears that health risk posed to rodents by soil-borne PAHs is low in these two areas.

## CHAPTER V

### SUMMARY

PAHs are widely distributed in the environment and are generated by many sources. The potential of PAH-rich mixtures to cause health effects has been known for almost a century. The toxicity of both individual PAHs and PAH mixtures has been documented *in vitro*, in laboratory animals and in wild animals exposed *in situ*. Occupational studies have provided evidence of the health effects of PAH-rich mixtures in humans.

Although PAH research has been ongoing for decades, there are still unanswered questions about the levels of PAHs in the environment, the potential for human exposure to PAHs, the health effects associated with exposure, and how genetic susceptibility influences the internal dose and extent of health effects in individuals. The ultimate goal is to minimize health effects in exposed populations. Accomplishing this goal requires an integrated approach, one which includes field studies and laboratory experiments, *in vitro* and *in vivo* systems, ranging from molecular-level to population-level. The research documented in this dissertation included chemical analysis of PAH concentrations in environmental media, laboratory-based studies of the role of genetic factors in PAH genotoxicity, and evaluation of biomarkers of exposure in ecological receptors.

The first project of this research quantified concentrations of PAHs in samples of settled house dust. Settled house dust can be a source of human exposure to toxic PAHs through non-dietary ingestion and dermal contact. Information regarding the concentrations of various contaminants in house dust would be useful in estimating the risk associated with exposure to these compounds. This study reported on the surface loading, variability and distribution of PAHs in settled house dust collected from homes in three locations: Sumgayit, Azerbaijan; Shanxi Province, China; and southern Texas, United States. Organic material extracted from the dust samples was analyzed by gas chromatography/mass spectrometry to quantify thirty-six PAH analytes. Total PAH surface loading and sample variability were evaluated and compared between the locations. The highest PAH floor surface loadings were observed in China, followed by Azerbaijan and Texas. Median concentrations of high molecular weight (four ring and

larger) PAHs ranged from a low of 0.11  $\mu\text{g}/\text{m}^2$  in Texas, to 2.9  $\mu\text{g}/\text{m}^2$  in Azerbaijan and 162  $\mu\text{g}/\text{m}^2$  in China. These trends in total surface loading and relative carcinogenicity indicate that the risk of health effects from exposure to PAHs in house dust is highest in the Chinese population and lowest in the Texas population. As anticipated, variability among dust samples from different houses within the same region was high, with coefficients of variation greater than 100%. Alkylated PAHs comprised 30% to 50% of the total mass of PAHs. Based on a comparison of the composition of specific components, PAHs in China and Azerbaijan were determined to be derived mainly from combustion sources rather than from unburned fossil fuels such as petroleum.

The results of the house dust study, coupled with ongoing investigation of appropriate PAH exposure biomarkers in humans, will guide future efforts to identify ways to reduce exposures in the study areas. The substantially higher surface loadings in China suggest that this area would benefit most from further research. Future work would benefit from use of a quantitative dust sampling method so that the results could be used in a human health risk assessment. In addition, quantification of PAHs in samples of air and food would provide more information to evaluate inhalation and ingestion exposure pathways.

The second project of this research investigated genetic factors influencing the formation of PAH-DNA adducts in sensitive cell culture models. Pregnane X receptor (PXR) is a nuclear receptor that coordinately regulates transcriptional expression of both phase I and phase II metabolizing enzymes. PXR plays an important role in the pharmacokinetics of a broad spectrum of endogenous and xenobiotic compounds and appears to have evolved in part to protect organisms from toxic xenobiotics. Metabolism of benzo[a]pyrene (BaP), a well-established carcinogen and ubiquitous environmental contaminant, can result in either detoxification or bioactivation to its genotoxic forms. Therefore, PXR could modulate the genotoxicity of BaP by changing the balance of the metabolic pathways in favor of BaP detoxification. To examine the role of PXR in BaP genotoxicity, BaP-DNA adduct formation was measured by  $^{32}\text{P}$ -postlabeling in BaP-treated parental HepG2 cells and human PXR-transfected HepG2 cells. The presence of transfected PXR significantly reduced the level of adducts relative to parental cells by 50% to 65% ( $p < 0.001$ ), demonstrating that PXR protects liver cells from genotoxicity induced by exposure to BaP. To analyze potential PXR-

regulated detoxification pathways in liver cells, a panel of genes involved in phase I and phase II metabolism and excretion was surveyed with real-time quantitative RT-PCR. The mRNA levels of CYP1A2, GSTA1, GSTA2, GSTM1, UGT1A6, and BCRP (ABCG2) were significantly higher in cells overexpressing PXR, independent of exposure to BaP. In addition, the total GST enzymatic activity, which favors the metabolic detoxification of BaP, was significantly increased by the presence of PXR ( $p < 0.001$ ), independent of BaP exposure. Taken together, these results suggest that PXR plays an important role in protection against DNA damage by PAHs such as BaP, and that these protective effects may be through a coordinated regulation of genes involved in xenobiotic metabolism.

The role of PXR in protecting cells from environmental contaminants is an intriguing area for future research. Metabolism studies which identify and quantify BaP metabolites generated by hPXR-enhanced cells, imaging studies which track the uptake and distribution of BaP in the cells, and investigation of cross-talk with other receptors and signalling pathways would further our understanding of the mechanism by which PXR reduces BaP-induced DNA damage. In addition, studies in the recently-developed PXR-humanized mouse strain would shed light on the impact of PXR on *in vivo* toxicokinetics and genotoxicity of BaP.

The third project of this research investigated DNA adduct levels in wild mice exposed to PAH mixtures *in situ*, quantified PAH levels in soil, and evaluated the link between mouse DNA adduct levels and potential PAH exposure. Wild mammals may be exposed to PAHs in soil both directly (dermal contact or ingestion) and indirectly through the food chain. Evaluation of biomarkers of DNA damage may function as a measure of exposure to PAHs. House mice (*Mus musculus*) were captured near homes in Sumgayit and Khizi, Azerbaijan which are part of an ongoing human health study. Lung, liver, kidney, and skin were collected from the mice; DNA adducts in these tissues were measured by  $^{32}\text{P}$ -postlabeling. Soil samples collected near the mouse capture sites were analyzed by gas chromatography/mass spectrometry for PAH content. DNA adducts were detected in all lung, liver, and kidney samples; adducts were not detected in any skin samples. In the overall data set, tissue DNA adduct levels ranged from 5.8 to 34.1 adducts per billion nucleotides. Mean liver adduct levels were significantly higher in Khizi than in Sumgayit. Mean lung adduct levels and kidney

adduct levels were similar in the two regions. Adduct levels in one tissue were not predictive of adduct levels in another tissue from the same mouse. The DNA lesions detected may be a combination of environmentally-induced DNA adducts and naturally-occurring I-compounds. The effects of environmental carcinogens on DNA lesion levels are complex because increased exposure tends to increase carcinogen-DNA adduct levels but tends to decrease type I I-compound levels. Therefore, no conclusions could be made about the relative risk posed by environmental carcinogens to mice in the two regions. PAHs were present at background levels in soils from both Khizi and Sumgayit. Based on the soil data, it appears that health risk posed to rodents by soil-borne PAHs is low in these two areas.

Further research is needed to better evaluate the impact of environmental genotoxins on mice in these two areas of Azerbaijan. Samples of air and food could be collected and analyzed for PAH concentrations to quantify potential exposures from these sources. The research would benefit from larger mouse sample groups to increase statistical power and provide more representation of various ages and both genders. A measure of PAH body burden in the mice would provide more evidence of possible PAH exposure. Finally, inclusion of laboratory mice as a control group in the PAH body burden and DNA adduct experiments would allow comparison to a relatively unexposed population.

There is still much to be learned about the potential for exposure to PAHs, the health effects from exposure, and the most effective ways to characterize and mitigate health risk from environmental exposures. Additional research in these areas, especially field sample collection from Azerbaijan and China, is resource-intensive and may be difficult in the current funding environment. However, innovation and collaboration have been hallmarks of the field of environmental toxicology. Application of innovative sampling and analysis techniques and strengthening of collaborative relationships with scientists in China and Azerbaijan will facilitate the future work in these areas.

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