Phenanthrene Tetraols as Probes for the Metabolic Activation of Carcinogenic Polycyclic Aromatic Hydrocarbons in Humans

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Dedication

This dissertation is dedicated to my father, Caigao Zhong, and my mother, Aiyun Gong.

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are believed to be among the causative agents for lung cancer in smokers. Benzo[*a*]pyrene (BaP) is a prototypic carcinogenic PAH, which requires metabolic activation to elicit its detrimental effects. The major end product of its diol epoxide metabolic activation pathway is *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, anti*-BaPT). Individual differences in exposure to, and metabolic activation of, carcinogenic PAH may influence cancer risk. Measurement of PAH metabolites in human urine could provide a direct way to assess individual differences in susceptibility to PAH-related cancer. We developed a sensitive and reliable method for quantitation of *trans, anti*-BaPT in human urine using gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS).

A unique phenotyping strategy using $[D_{10}]$PheT as probes have been developed, allowing direct assessment of metabolic activation of carcinogenic PAH in humans. We investigated $[D_{10}]$phenanthrene metabolism in smokers after administration by inhalation in cigarette smoke or orally. Sixteen smokers received 10 μg $[D_{10}]$phenanthrene in a cigarette or orally. Plasma and urine samples were analyzed for $[D_{10}]$*r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ($[D_{10}]$PheT), the major end product of the diol epoxide pathway, by gas chromatography-negative ion chemical ionization-tandem mass spectrometry. The ratios of $[D_{10}]$PheT (oral dosing: inhalation) in 15 smokers were 1.03 ± 0.32 and 1.02 ± 0.35, based on plasma $AUC_{0-\infty}$ and total 48 hr urinary excretion, respectively. Overall there was no significant difference in the extent of $[D_{10}]$PheT

formation after the two different routes of exposure in smokers. Its application in clinical studies has approved, for the first time, that there is large inter-individual variation in PAH metabolic activation in smokers. One striking finding is that the formation of PAH diol epoxides, the ultimate carcinogen, occurs astonishingly rapidly in smokers, particularly after inhalation of cigarette smoke. The pharmacokinetics parameters of PAH metabolites in humans have also been presented. The results have established the basics for using [D_{10}]PheT as a biomarker for PAH metabolic activation in future large-scale risk assessment studies in smokers.

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Chapter 1 Introduction

1. Tobacco Smoking and Risk of Lung Cancer

Lung cancer is the leading cause of cancer-related mortalities in both men and women (*1-3*). It accounts for 17% of all new cases and 23% of total cancer deaths in the world (*2*). Lung cancer kills more than 3000 people in the world each day, and the numbers are still increasing (*4*). Lung cancer has one of the lowest survival outcomes of any cancer, mainly because of late-stage detection and a paucity of late-stage treatments. The five-year relative survival rate of lung cancer patients post-diagnosis in the U.S. is approximately 17% (*5*).

Tobacco smoking is the single most important risk factor for lung cancer. Cigarettes are the main type of tobacco product worldwide. Other tobacco products include cigars, pipes, and smokeless tobacco. Lung cancer incidence was rare at the beginning of the 20th century, and increased dramatically after World War II (*6*). As early as 1950, Wynder and Graham in the U.S. published the first large-scale case-control study which suggested that excessive and prolonged use of tobacco might be an important factor in the induction of bronchigenic carcinoma(*7,8*). Numerous large international prospective epidemiologic studies and case-control studies repeatedly provided the evidence on the association between tobacco smoking and lung cancer (*9*). It was shown that increases in lung cancer death rates parallel increases in cigarette consumption with an approximately 20-year lag time, which is conceivable to be the latency period for the development of smoking-caused lung cancer. About 90% of

current male lung cancer deaths and 75%-80% of female lung cancer deaths in the U.S. are caused by tobacco smoking (*10-12*).

The current population of smokers is still very large. There are approximately 1.3 billion smokers in the world and hundreds of millions of smokeless tobacco users (*13*). China alone has more than 300 million male smokers, comparable to the whole population of the U.S. (*14*). Globally, about 57% of men and 10% of women smoke tobacco products. About 1,000 cigarettes are consumed for every person on Earth every year (*14*).

The devastating link between tobacco smoking and human cancers results from a powerful alliance of two factors: nicotine and carcinogens (*15*). Nicotine is addictive, and cause people to continue to use tobacco products. While nicotine itself is not believe to cause cancer, each cigarette contains more than 70 established carcinogens and numerous other toxicants (*4,15,16*).

The strongest determinants of lung cancer risk in smokers are believed to be duration of smoking and the number of cigarettes smoked per day (*4*). Dose response relationships of cigarettes with the lung cancer risk in smokers have been shown. Smoking cessation avoids further increase in risk of lung cancer at any age, but not during the first 5 years after cessation, and the relative risk never returns to that of a nonsmoker. Even after more than 15 years since quitting smoking, the relative risk of developing lung cancer among ex-smokers to lifelong non-smokers has ranged from 1.1 to 5.0 as shown in three cohort studies (*6,9*). Smoking increases the risk of all histological types of lung cancer including squamous cell carcinoma, small cell

carcinoma, adenocarcinoma (including bronchiolaveolar carcinoma) and large cell carcinoma (*6*). In addition to lung cancer, at least 18 other types of cancer are caused by tobacco products. These cancers occur in larynx, oral cavity, esophagus, pancreas, kidney, liver, bladder, stomach, and colon (*16*). Involuntary exposure to tobacco smoke or secondhand smoke is an important cause of lung cancer as well (*4,17*).

Preventing people from starting to smoke is believed to be the most effective way to reduce tobacco smoking related cancer mortality. Most smokers become addicted to nicotine usually at a young age, when peer pressure and advertising cause them to experiment with cigarettes. Remarkable progress has been achieved in tobacco control in the second half of the 20th century. In the U.S., the prevalence of smoking decreased from 42.4% in 1965 to 25.5% in 1990, which drove a corresponding decline of lung cancer mortality rate among men (*18*). However, the rate of decrease in the prevalence of smoking in the U.S. has reached a plateau in recent years. There are still 45.3 million adult smokers, about 21% of the adult population (*19,20*). This stall has been attributed to a plateau in smoking-cessation success (*21*). Although 70% of smokers attempt to quit each year, less than 5% succeed (*22*). The average success rates at 6 months after quitting were reported as less than 25% in 2008 (*23*). The remaining population of smokers may be hardcore and either unwilling or unable to quit. These addicted smokers as well as ex-smokers who have succeeded in quitting are at high risk for lung and other type of carcinomas.

The overall goal of our research is to elucidate mechanisms by which tobacco causes cancer, and to apply this knowledge to the future prevention and treatment of tobacco-induced cancer.

2. Tobacco Carcinogens, Carcinogenesis Mechanisms and Carcinogen Biomarkers

Tobacco carcinogens form the link between nicotine addiction and lung cancer in smokers. While nicotine addiction is the reason that people continue to smoke, it is these carcinogens that are responsible for cancer caused by tobacco products. Passed in 2009, the Family Smoking Prevention and Tobacco Control Act empowers the U.S. Food and Drug Administration to regulate tobacco products. Harmful and potential harmful substances in tobacco products will be monitored in the U.S. going forward. Biomarkers, which have been developed to quantitate levels of carcinogen and toxicant exposure and/or their metabolism, are likely to play an important role in evaluating potential tobacco-related harm in the coming era of tobacco product regulation.

A. Carcinogens in tobacco products

The definition of a carcinogen is any agent, chemical, physical or viral that causes cancer or increases the incidence of cancer (*6*). Unburned tobacco contains fewer carcinogens than cigarette smoke because most carcinogens are formed during combustion. Seventy two (72) carcinogens in cigarette smoke have been evaluated by the International Agency for Research on Cancer (IARC) as having sufficient evidence for carcinogenicity in either laboratory animals or humans (*4*). All the compounds are carcinogenic to animals. Sixteen (16) of them are rated as carcinogenic to humans

(Group 1) including certain polycyclic aromatic hydrocarbons (PAH), *N*-nitrosamines, aromatic amines and aldehydes {International Agency for Research on Cancer, 2004 8002910 /id}. These strong carcinogens exist in smaller amounts (1-200 ng per cigarette) while some of the weaker carcinogens such as acetaldehyde (Group 2B: possibly carcinogenic to human, nearly 1 mg per cigarette) exist in much larger amounts (*6*).

Among these carcinogens, PAH and *N*-nitrosamines are widely considered as major etiological agents for lung cancer in smokers (*12*,*15*). PAH are formed in the incomplete combustion of organic matter in tobacco. Levels of PAH in unburned tobacco are typically very low. PAH are usually locally acting carcinogens (*24*). A prototypic PAH, benzo[a]pyrene (BaP), is a powerful carcinogen, and can readily induce tumors in various tissues such as the lung and skin at relatively low doses upon local administration or inhalation (*25*,*26*). When administrated systemically, it causes lung tumors in mice, but not in rats (*25*,*26*). BaP is considered carcinogenic to humans (Group 1) by IARC (*25*). PAH carcinogens could also induce laryngeal, oral and cervical cancer in smokers (*27*,*28*).

Tobacco-specific *N*-nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN), are formed from tobacco alkaloids during the curing and processing of tobacco. They are found only in tobacco products. NNK and NNN are the most prevalent strong carcinogens in unburned or smokeless tobacco products such as oral snuff and chewing tobacco (*29*,*30*). The levels of NNK and NNN in smokeless tobacco products are hundreds to thousands of times higher than those of carcinogenic nitrosamines in any other consumer product for oral ingestion (*30*).

Although the cancer risk from smokeless tobacco products is believed to be less than that from cigarettes, smokeless tobacco products are known to cause oral cavity cancer and pancreatic cancer (*31*,*32*). *N*-Nitrosamines are potent systematic carcinogens that induce tumors independently of their route of administration (*33*). NNK causes lung tumors in all species tested, mainly adenoma and adenocarcinoma (*33*). NNK also induces tumors of the pancreas, nasal cavity, and liver. NNN causes lung tumors in mice and esophageal and nasal tumors in rats (*33*). NNK and NNN are considered as carcinogenic to humans (Group 1) by IARC (*34*).

Other compounds in cigarette smoke which may be involved in lung cancer etiology include benzene, isoprene, 1,3-butadiene, ethylene oxide, ethyl carbamate, and aldehydes, but their carcinogenic effects are not as strong as those of PAH and NNK (*12*). Cigarette smoke also contains co-carcinogens and tumor promoters that could increase carcinogenic activity when administrated simultaneously with or after carcinogens, respectively (*35*). Examples of co-carcinogens in cigarette smoke are catechol and methycatechols, while tumor promoters in tobacco products are mainly uncharacterized (*15*).

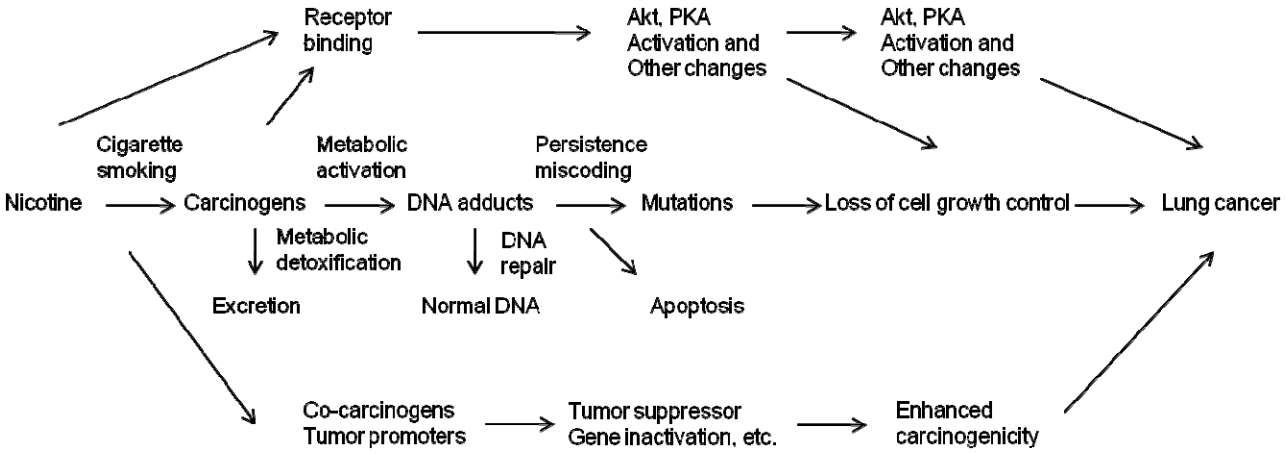
B. Mechanisms of tobacco smoke-induced carcinogenesis

It has been estimated that an average of one mutation is formed for every 15 cigarettes smoked (*36*). Cancer is driven by mutations. Recent new-generation sequencing technologies promise the capacity to identify important common somatic

mutations in human lung cancers (*36*,*37*). Ding *et al.* have identified more than 1,000 somatic mutations in 188 human lung adenocarcinomas (*37*). Sequencing of a small-cell lung cancer genome found more than 22,000 somatic substitutions with a complex signature of tobacco exposure (*36*). A comparison of two genomes between primary lung tumor and adjacent normal tissue from a lung cancer patient has revealed more than 50,000 single nucleotide somatic variants including one in the *KRAS* proto-oncogene (*38*). These somatic mutations only occur in somatic cells and are not inheritable. They can be generated from DNA-interaction with chemical carcinogens in tobacco smoke.

Dr. Stephen Hecht has previously proposed a conceptual model for tobacco smoke-induced lung carcinogenesis (Figure 1.1) (*6*,*15*). In this model, conversion of parent carcinogens to reactive metabolites can result in formation of DNA adducts, which are responsible for many of the genetic changes underlying lung cancer. This model is completely consistent with the somatic mutation theory of cancer.

Figure **1.1** Model linking nicotine addiction and lung cancer via tobacco smoke carcinogens



Each puff of cigarette smoke delivers more than 72 established carcinogens. Most carcinogens require metabolic activation to become electrophilic intermediates, which can covalently bind to DNA and form DNA adducts (*39*,*40*). DNA adducts can cause somatic mutations during DNA replication when DNA polymerase enzymes insert a wrong nucleotide into a sequence. G to T and G to A mutations are most frequently observed (*12*). If these mutations occur in crucial genes, such as oncogenes and tumor suppressor genes, the consequence could be interruption of cell-cycle checkpoints, chromosomal instability and development of cancer (*41*). This model is supported by a recent DNA sequencing study, which reveals that 26 genes including the tumor suppressor gene *TP53* and the oncogene *KRAS* were mutated at significantly high frequencies in 188 human lung carcinomas (*37*).

Considerable evidence has shown that DNA adducts play a central role in tobacco induced carcinogenesis. PAH-DNA adducts have been detected in human lung samples, and the spectrum of *TP53* mutations from lung tumors is similar to that produced in vitro or in cell culture by PAH carcinogens (*27*,*42*,*43*). Lung DNA adducts derived from NNK and NNN are present at higher levels from lung cancer patients than those from cancer free controls (*44*).

Metabolic activation of parent tobacco carcinogens is generally initiated by cytochrome P450 enzymes (P450), a diverse family of enzymes catalyzing Phase I oxidation of xenobiotics including drugs and carcinogenic chemicals. Subfamilies P4501A1, 1B1, and 1A2 are important enzymes in the metabolic activation of PAHs; while CYP2A13 and CYP2B6 are responsible for human NNK metabolic activation (*40*).

CYP1A1 and CYP1B1 can be greatly induced in humans. A broad range of carcinogenic PAH such as BaP are able to significantly increase the expression levels of *CYP1A1* and *CYP1B1* genes in liver, lung, and most extrahepatic tissues because of their strong affinity for the arylhydrocarbon receptor (AhR), a member of the Per-Arnt-Sim, basic-helix-loop-helix family of transcription factors (*45*). The inducibility of these P450s is believed to be a critical factor for cancer susceptibility in smokers. Competing with metabolic activation is metabolic detoxification of tobacco related carcinogens, which results in excretion of most carcinogen metabolites in hydrophilic forms. Metabolic detoxification of tobacco carcinogens is catalyzed by a variety of enzymes including P450s, glutathione-S-transferases and UDP-glucuronosyl transferases (*46*,*47*). The balance between metabolic activation and detoxification varies among individuals. It has been hypothesized that smokers who can metabolically activate carcinogens more effectively and/or detoxify carcinogens less effectively are at higher risk of developing lung cancer.

Cellular repair systems can remove DNA adducts, and return the sequence of DNA to the correct one. Mechanisms of DNA repair include direct base repair by alkyltransferases, excision of DNA damage, mismatch repair, and double strand repair. However, some DNA adducts escape these mechanisms and become permanent mutations. These altered nucleotide sequences can be passed down from one cellular generation to the next, leading to a higher probability of cancer development. Furthermore, when the genes for the DNA repair enzymes themselves become mutated, mistakes begin accumulating at a much higher rate (*48*). Polymorphisms in some DNA

repair enzymes have been identified. Their relationship to cancer risk has stimulated significant interest in the field of molecular epidemiology (*6*). Another cellular protective mechanism is apoptosis or programmed cell death, which could remove permanently damaged cells. Apoptosis also has a major impact on tumor growth (*49*).

Beyond the central pathway proceeding through genetic damage, epigenetic modification also contributes to lung carcinogenesis. Enzymatic hypermethylation of promoter regions of genes can result in gene inactivation. It has been shown that the aberrant methylation of the tumor suppressor gene *p16* and the DNA repair gene O^6-*methylguanine-DNA methyltransferase* are common in squamous cell lung carcinoma, and thus likely contribute to carcinogenesis, probably due to gene inactivation and unregulated proliferation (*50*).

Tobacco smoke also acts as a tumor promoter after malignant initiation. One possible mechanism of tumor promotion is though binding to nicotinic-acetylcholine and other cellular receptors by nicotine, NNK, and their metabolites. This process can activate the serine/threonine kinase AKT (also known as protein kinase B), resulting in decreased apoptosis, increased angiogenesis, and promoted tumor growth (*51*,*52*). A recent study has demonstrated that repetitive exposure to tobacco smoke promotes tumor development in both chemically and genetically initiated lung cancers in mice (*53*). This tumor-promoting activity of tobacco smoke depends on IKKβ and JNK1 activity, suggesting that induction of inflammation might play an important role in lung tumorigenesis(*53*).

Collectively, cigarette smoke acts as a perfect storm for lung carcinogenesis, with each puff delivering a mixture of more than 72 established carcinogens, along with numerous toxicants, tumor promoters, co-carcinogens, oxidants, free radicals and inflammatory agents to the lung. Tobacco control and smoking cessation are the most effective ways to prevent lung cancer, but they are only partially successful, especially in developing countries where tobacco use is increasing. An understanding of tobacco induced carcinogenesis can provide new insights for blocking key steps of carcinogenesis, and hopefully will contribute to reducing lung cancer incidence and mortality in the future.

C. Tobacco Carcinogen biomarkers

A biomarker is defined as a distinctive biological or biologically derived indicator of a process, event, or condition in Merriam-Webster's Collegiate Dictionary. Tobacco carcinogen biomarkers are any quantifiable substance in human body fluids or tissues, such as a metabolite, that can be specifically related to a given tobacco carcinogen (*15*). Three well established types of carcinogen biomarkers include parent compounds or metabolites in human body fluids or tissues, carcinogen-DNA adducts, and carcinogen-protein adduct (*15,54*). In most cases, the levels of these biomarkers are increased in individuals who have been exposed to tobacco products.

Parent carcinogens or their metabolites are found in human urine, blood, breath, nails, and hair. Urinary metabolites are probably the most practical biomarkers for

tobacco carcinogens, because urine is readily obtained and metabolites are generally present in high concentration for reliable quantification. In addition, urinary metabolites biomarkers can provide information about not only carcinogen exposure but also metabolic activation and detoxification of carcinogens. Commonly used urinary biomarkers include metabolites of PAH, tobacco-specific nitrosamines, benzene, and acrolein (*55*).

Among PAH metabolites, 1-hydroxypyrene (1-HOP), a metabolite of pyrene, is probably the most extensively used biomarker for PAH exposure. Pyrene is present in almost all PAH mixtures, and is considered non-carcinogenic. One-HOP can be readily quantified by HPLC with fluorescence detection. The concentration of 1-HOP is generally 2-3 times higher in smokers than in non-smokers (*56*). Besides 1-HOP, *r*-1, *t*-2,3-*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (Phe-T) and *r*-7, *t*-8,9-*c*-10-tetrahydroxy-1,2,3,4-tetrahydrobenzo[a]pyrene (BaP-T), the metabolic activation products of Phe and BaP, have been used as biomarkers to assess PAH exposure plus metabolic activation (*57*,*58*). Both urinary Phe-T and BaP-T can be reliably measured by gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS). The levels of Phe-T and BaP-T are 2-3 times higher in smokers than in non-smokers (*57*,*58*). In addition to higher PAH exposure, the induction of P450s 1A1, 1B1, and 1A2 also cause higher levels of these biomarkers in smokers (*59*).

The most important biomarkers of NNK exposure are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (sum of which is denoted as total NNAL). Total urinary NNAL is a very powerful biomarker for lung carcinogen uptake

due to the tobacco specificity of NNK (*60*). NNAL can be readily quantified in human urine by gas chromatography with nitrosamine selective detection or by combined liquid chromatography-electrospray ionization-tandem mass spectrometry (*61*). Due to its sensitivity and specificity, urinary NNAL is the only tobacco carcinogen biomarker so far consistently elevated in nonsmokers including fetus and newborns exposed to secondhand smoke (*62*).

Mercapturic acids of benzene and acrolein are also highly specific and practical biomarkers of exposure. They are formed from glutathione conjugation of bezene and acrolein metabolites, and can be readily quantified in human urine (*63,64*). Both of these mercapturic acids occur in significantly higher amounts in smokers than in nonsmokers (*63,64*).

Measurement of carcinogen-DNA adducts in humans is possibly the most direct approach to examine carcinogen metabolic activation. The quantitation of DNA adducts of PAH, tobacco-specific nitrosamines, alkylating agents, aldehydes, and products of oxidative damage such as 8-oxo-dGuo has been reported previously (*42,65-69*). Adduct levels are generally higher in lung tissues of smokers than nonsmokers but studies using blood DNA samples have given inconsistent results (*55*). A meta-analysis and a pooled analysis have tested the hypothesis that the presence of a high level of DNA adducts in tissues is associated with an increased risk of cancer in humans, using data from several case control studies and prospective studies (*70,71*). The results suggest that carcinogen-DNA adducts are associated with lung cancer arising in current smokers after a follow-up of several years (*70,71*). However, DNA adduct quantification has a major disadvantage

in that their levels are extremely low, ranging from 1 per 10^6 to 1 per 10^8 normal bases in humans, and the tissue samples containing adducts are often available in very small quantities (*72*). Methods such as immunoassays and ^{32}P postlabeling for DNA adducts are relatively nonspecific, and quantitation can be unreliable (*73*). Highly sensitive analytical methods such as HPLC-fluorescence and GC-NICI-MS are quantitative but usually detect DNA-adducts in only some human tissues and blood samples (*42*). Carcinogen-hemoglobin (Hb) adducts and serum albumin adduct measurements have been used as surrogates for DNA adduct quantification, since carcinogens that react with DNA can also react with these proteins to some extent (*74,75*). Advantages of using carcinogen-protein adducts as biomarkers include the relatively large amounts of Hb in blood and the relatively long lifetime of erythrocytes, providing an opportunity for adducts to accumulate (*76*).

D. Applications of tobacco carcinogen biomarkers

There are three major applications of tobacco carcinogen biomarkers.

First, biomarkers can be used to assess exposure to tobacco carcinogen in smokers, smokeless tobacco users, and non-smokers. One of the most useful application of total NNAL has been in the studies of lung carcinogen uptake by nonsmokers exposed to second-hand cigarette smoke based on the tobacco specificity of NNK (*62*). As an example, the results from one study of 79 children of cigarette smokers strikingly demonstrated that levels of total NNAL in the urine of children exposed to secondhand

smoke were generally higher than levels in adult non-smokers, due mainly to exposure from parent smokers in their homes(*77*). In addition, biomarkers of NNK and PAH have been used to monitor smoking behavior and smoking cessation. One important finding was that there was not a corresponding decrease in carcinogen uptake after smokers reduced their number of cigarettes per day or switched from regular to light cigarettes. This is because smokers most likely would compensate for reduced nicotine delivery by altering their manner of smoking (*78,79*).

The application of tobacco carcinogen biomarkers is essential to the ongoing tobacco product regulation. The tobacco industry has been introducing a wide variety of new tobacco products with claims of exposure and harm reduction (*80*). However, reducing the toxicant and carcinogen content in tobacco products does not necessarily reduce people's exposure to toxicants and carcinogens. Furthermore, exposure reduction does not necessarily result in harm reduction. Therefore, suitable and valid biomarkers sampled from smokers are needed urgently for assessing the accuracy of harm reduction claims in new products and/or treatments. A wide variety of biomarkers for tobacco exposure or harm have been developed. These biomarkers include specific chemical components of tobacco and their metabolites; early biochemical, histological, or physiological effects; and early health effects (*80*).

Second, tobacco carcinogen biomarkers can help us further understand mechanisms of lung carcinogenesis. It is believed that individual differences in carcinogen metabolism can affect cancer risk by affecting the amount of reactive metabolites that bind to DNA, influencing the resulting mutation frequency (*15*). In the

case of Phe, its urinary metabolite Phe-T is a practical biomarker for Phe metabolic activation, while urinary phenanthrols (HO-Phe) are a detoxification biomarker (*57*,*81*). The ratio of PheT over HO-Phe has been used to assess an individual's capacity to metabolic activate PAH, corrected for exposure (*82*). This ratio has been found significantly higher in smokers than in non-smokers, suggesting that the diol epoxide metabolic activation pathway is induced in smokers (*82*).

Third, measurement of tobacco carcinogen biomarkers is believed to provide a useful determinant for individual cancer susceptibility. A recent epidemiology study directly links a urinary tobacco carcinogen biomarker to lung cancer in humans. The results from this nested case-control study including 246 lung cancer cases and 245 matched controls have shown a statistically significant, dose-dependent association between pre-diagnostic urinary total NNAL and increased risk of lung cancer among smokers with comparable smoking histories (*83*). Biomarkers, such as urinary total NNAL, are promising cancer risk biomarkers reflecting extent of carcinogen uptake as well as that of metabolic activation in smokers.

In summary, urinary biomarkers of tobacco are readily measured and have been applied in numerous fields nowadays. They are expected to play an important role in lung cancer risk prediction, lung cancer prevention, and tobacco product regulation.

3. Inter-individual variation and assessing cancer susceptibility

We ask the question why only 11-15 of every 100 cigarette smokers die of lung cancer, some die of chronic obstructive lung diseases, while others are unaffected by similar extensities of smoking. How can we know where smokers as individuals stand, in regard to lung cancer risk? The identification of smokers with higher cancer susceptibility is a challenging and worthwhile goal, as they could be targeted for lung cancer prevention activities and early detection.

It is believed there is large inter-individual variation in response to tobacco carcinogenic agents among smokers. The hypothesis is that individuals who metabolically activate tobacco smoke carcinogens more extensively will be at higher risk for cancer (*84*). The long term goal is to identify the group of smokers at high-risk for lung cancer, and further understand carcinogenesis mechanisms. Genotyping and phenotyping have been the two major strategies to understand inter-individual variation in lung cancer susceptibility in the last decade of molecular cancer epidemiology.

A. Background on molecular epidemiology

Epidemiology is the science of studying the distribution of diseases in populations (descriptive epidemiology), and the determinants or risk factors of disease (analytical epidemiology). Cohort and case-control methodologies are two main study designs in epidemiology research. A cohort study works prospectively tracking data from exposure to clinical responses. A cohort always starts with a large group of healthy subjects and

follows them over time for disease incidence. It asks the question how many exposed subjects developed disease in comparison with the unexposed. A case-control study works retrospectively. It starts with the subjects with a specific disease who are called "cases" and healthy subjects as control. A case-control study asks the question how many cases with the disease have been exposed compared with healthy controls (*85*).

The traditional epidemiology study design is based on questionnaires. It has tried to answer many important questions such as what are the causes of lung cancer, whether the causes are environmental or genetics or a mixture of two; whether the exposure to particles from cigarettes or traffic exhausts causes lung cancer. Traditional epidemiology studies has shown that the drastic increase in cigarette consumption in the 20th century is highly correlated with the lung cancer death rate with about a 20-year lag time (*6*). Numerous large international prospective epidemiology studies and case-control studies involving millions of subjects repeatedly confirmed the association between smoking and lung cancer, originally demonstrated in case-control studies by Wynder and Graham, and Doll and Hill. However, not all issues related to exposure and effects can be estimated by a questionnaire. For example, estimation of PAH exposure though cigarette smoke is very difficult to obtain from a questionnaire.

From the early 1980s, molecular epidemiology has been introduced into cancer research and made significant contributions since then. Molecular epidemiology has evolved from traditional epidemiology, sharing the same goal and the same study design. What makes it distinctive is its ability to look inside the "black box" of the continuum between exposure and disease by applying biologic measurements made at the molecular

level (*85*). The hypothesis of molecular epidemiology is that the continuum of molecular alterations leads to disease. Molecular epidemiology therefore is an extension of the increasing use of biologically based measures in epidemiology research (*86*). The major goals of molecular epidemiology include better estimates of exposure including "internal doses" which actually enter the body, further understand of individual genetic susceptibility, and ultimately to indentify risk factors for disease. Besides traditional tools such as questionnaires, job-exposure matrices, data from environmental monitoring, and routine health data, molecular epidemiology emphasizes the application of biologic or analytic chemical measurements made at the molecular level to test epidemiologic hypotheses.

The black box between initial exposure to final disease status always includes multiple continuous events under the hypothesis of molecular epidemiology. The sequential events thus include exposure, internal dose, biologically effective dose, early biological effect, altered structure function, and consequent clinical disease. In the case of carcinogenic PAH from tobacco, exposure is cigarette smoke. Internal dose is the actual amount of PAH compounds that are formed during burning of tobacco and are metabolized in the human body. We could measure the internal dose as plasma or urinary metabolites of PAH such as Phe-T or phenols. The biologically effective dose is the amount of PAH metabolites that bind to DNA and have potential mutagenic effects. PAH DNA-adducts are the most direct biomarker of the biologically effective dose. Early biological effects of tobacco carcinogenic PAH include somatic mutations after incorrect DNA replication, chromosomal aberrations, sister chromatid exchanges and

others. If the permanent mutations occur in certain important genes such as *p53* and *RAS,* and escape from DNA repair and apoptosis, this pathway will result in altered cell structure or function such as malignant transformation, and finally, lead to lung cancer development.

The nested case-control study design is often used in molecular epidemiology. It is a case-control study done in the population of an ongoing or established cohort study. The methods include the measurement of an exposure retrospectively in the cohort. The endpoint is to test whether the distribution of the risk factor is different among cases and controls. Compared with case-control study, nested case-control study can reduce recall bias and temporal ambiguity; while compared with cohort study, it can reduce cost and save time.

In summary, molecular epidemiology applies molecular techniques to measure exposure, early biologic responses, or host susceptibility. It incorporates biomarkers to reveal mechanisms and events occurring along the continuum between exposure and disease (*80*).

B. Genotyping and phenotyping

Genotyping and phenotyping are two major strategies of molecular epidemiology in cancer research. The goal is to improve cancer prevention by identifying human cancer risk and to further understand carcinogenesis mechanisms. These two strategies share the general epidemiology study designs: case-control and population cohort studies.

Genotyping looks into an individual's DNA to test the hypothesis that genetic polymorphisms are significantly associated with cancer risk in individuals. Several groups of genes play important roles in regulating biological pathways of tobacco induced carcinogenesis. These include metabolism genes, DNA repair genes, genomic stability genes, and other cellular pathway genes. Some polymorphisms in genes such as CYP1A1 and GSTM1, contribute to the metabolic activation and detoxification of carcinogens. These genes have low penetrance which means that a genotype is not always associated with an observable phenotype, and the phenotype demonstration always requires environmental exposure. Ideally, a practicing physician could use highly accurate DNA tests to measure multiple genotypes to identify individuals at higher risk and initiate individualized preventive therapies. However, this task has turned out to be far more challenging than anticipated. The major obstruction has been the difficulty in determining specific genotype-phenotype associations. There are few examples in the published literature in which a single DNA variant or a combination of several polymorphisms are always associated with a particular trait within human populations (*87*). Numerous factors can override the penetrance of any single nucleotide polymorphism (SNP) and its association with a given trait. These factors include but are not limited to gene-gene interactions, gene conversion, gene silencing, transcriptional mutagenesis, genomic imprinting, epigenetics, alternative splicing, tissue-specific codon usage and gene expression, and ethnic differences (*87*). In the case of gene-gene interactions, one allele of gene "A" may affect the expression of gene "B" with a particular SNP, whereas a different allele of gene "A" may affect gene "B" differently,

thus overriding that SNP in gene "B". Gene silencing can occur due to DNA hypermethylation and RNA interference. Transcriptional mutagenesis results in mutated mRNA and therefore in altered protein, but the corresponding DNA doesn't carry any mutations. Furthermore, codon usage and gene expression were shown to be tissue specific. SNPs in some codons could alter gene expression in some tissues but not others. The polymorphism frequencies vary by ethnicity too. Therefore, the true complexity of genomics must be taken into account in the future if DNA array techniques can accurately identify protein-coding genes. In summary, DNA testing alone may be incapable of prediction and preventing environmentally-caused diseases, such as tobacco smoke induced lung cancer, on an individual basis with current knowledge and technologies.

Phenotype refers to any biochemical, physiological, morphological or behavioral characteristic or trait of an organism, including environmental toxicity or disease as an outcome. In environmental toxicology, phenotype denotes "any defined disease or abnormal toxic response to a given exposure of an environmental agent" (*87*). Individual response to environmental chemicals including carcinogens is highly variable with a broad continuous gradient, similar to measurements of height and weight within populations. Thus these phenotypes or traits are quantitative rather than qualitative. In the last decade, many analytical or biological approaches have been developed to better understand these phenotypes in populations. Biomarkers play a critical role in phenotyping strategy. These biomarkers could be used in the early stages of tobacco use to assess exposure to tobacco toxincants and carcinogens. They are also expected to

predict increased cancer risk resulting from both genetic and environmental factors. Our hypothesis is that levels of phenotypic biomarkers are associated with increased lung cancer risk. Three major types of tobacco carcinogen phenotype biomarkers are urinary metabolites, DNA adducts, and protein adducts.

Genotypic and phenotypic approaches have their own specific advantages and disadvantages (Table 1). With advanced PCR and gene array techniques, genotyping for SNPs is faster, less complex, and less expensive. Phenotyping is more time consuming and more expensive, because it requires complicated procedures of method development and biomarker validation and characterization. The major disadvantage of genotyping, as mentioned previously, is that the conclusions from previous genotyping studies were varied and inconsistent as a result of genomic complexity. Phenotyping, on the other hand, directly assesses the effects generated from both genetics and environmental influences. The results from phenotyping studies may be more accurate and reliable.

Table **1.1**

Comparison between genotypic and phenotypic approaches

	Advantages	**Disadvantages**
Genotyping	Faster, less complex and expensive than phenotyping, not variable	Inconsistency
Phenotyping	Objective measurement, captures all genetic and environmental factors	Time and labor consuming and expensive, may be variable over time

4. PAH and PAH metabolism

Polycyclic aromatic hydrocarbons (PAHs) are among the strongest carcinogens in cigarette smoke, and are believed to be one of the major causes of lung cancer in smokers. PAH carcinogenesis has been extensively studied. Numerous PAH metabolite biomarkers have been developed.

A. Background on PAH

As indicated in the name of "polycyclic aromatic hydrocarbons", compounds in this family are composed of different numbers of fused benzene rings with conjugated double bonds, and containing only C and H atoms.

PAH are ubiquitous environmental carcinogens. They are formed from incomplete combustion of virtually any organic matter. PAH can be commonly found in polluted air and water, tobacco smoke, broiled and smoked foods, automobile exhaust, and in certain occupational environments such as coke production from coal and other processes involving soot and tar (*88-91*). PAH are readily absorbed into the body through the lung, gastrointestinal tract, and skin. Many PAH are potent locally acting carcinogens. Fractions of cigarette smoke condensate enriched in these compounds are tumorigenic in animals (*92*).

PAH is one of the oldest topics in cancer research. The history of PAH research could be tracked back about one hundred years. By 1907 it was officially recognized that occupational exposure to pitch, coal tar and related materials is associated with skin cancer based on thousands of cases in certain factories of Great Britain (*93*). In 1915, the

Japanese pathologist Katsusaburo Yamagiwa and his assistant Koichi Ichikawa demonstrated that repeated application of coal tar to the ears of rabbits produced malignant epithelial tumors (*92*). This was the first step into the modern era of experimental cancer research. At that time, identification of a single carcinogenic compound was extremely difficult due to the PAH mixture's complexity. By 1930, Sir Ernest Kennaway and his co-workers at the Royal Cancer Hospital in London first succeeded in the synthesis of single higher molecular weight PAHs, such as dibenz[*a,h*]anthracene. They also showed that their repeated application could induce malignant skin tumors in mice, which was the first example of tumor induction by a pure chemical (*94*). Following this, many PAHs were synthesized and characterized for carcinogenicity. The prototype of PAH, BaP, was isolated from coal tar pitch, and its carcinogenicity in mouse skin was demonstrated in 1933 (*93*). In 1935, it was proposed by Eric Boyland and Alfred A. Levi that carcinogenic PAHs might either be transformed into more active intermediate or detoxified in vivo (*95*). About 20-25 years later, it was shown that PAH can induce metabolizing enzymes in rat liver in vivo (*96*). The epoxide was shown to be one of the most important metabolites during the biotransformation of PAH (*97*). In 1964, a correlation between DNA-binding level and carcinogenicity of six PAH was demonstrated (*98*). In 1974, Sims *et al.* demonstrated the first evidence that the vicinal "bay-region" BaP diol epoxide is the ultimate DNA-binding metabolite of BaP (*99*). The enzymatic conversion of BaP is stereoselective. In 1976, it was shown that biotransformation of BaP predominately leads to the 7*R*, 8*S*-diol-9*R*, 10*S*-epoxide, *trans,anti*-BaP diol epoxide (BPDE) (*100*). Subsequent work then confirmed a central

role of *trans, anti*-BPDE in the initiation of BaP tumorigenesis by showing that binding of BaP to DNA in vivo occurs predominantly through this enantiomer (*101*).

The rapid development of molecular techniques and transgenic mouse technology since the 1980s provided further evidence for the mechanism of PAH carcinogenesis. In 1983, it was shown that mouse skin carcinomas induced in vivo by carcinogenic PAH contain an activated *RAS* oncogene (*102*). In 1996, Denissenko *et al*. demonstrated a direct etiological link between BaP exposure and human lung cancer based on mutations at hotspots in the tumor suppressor gene *p53* (*103*). In the early 2000s, a number of studies using knockout mice showed that disruption of genes that encode enzymes that are involved in PAH metabolic activation pathways, such as P450 1B1, epoxide hydrolase (mEH), and AhR, can protect mice from PAH induced tumorigenesis (*104-106*). It is well established now that PAH-mediated carcinogenesis requires a series of steps of metabolic activation as well as the presence of a functional AhR.

B. Metabolic activation and detoxification of PAH

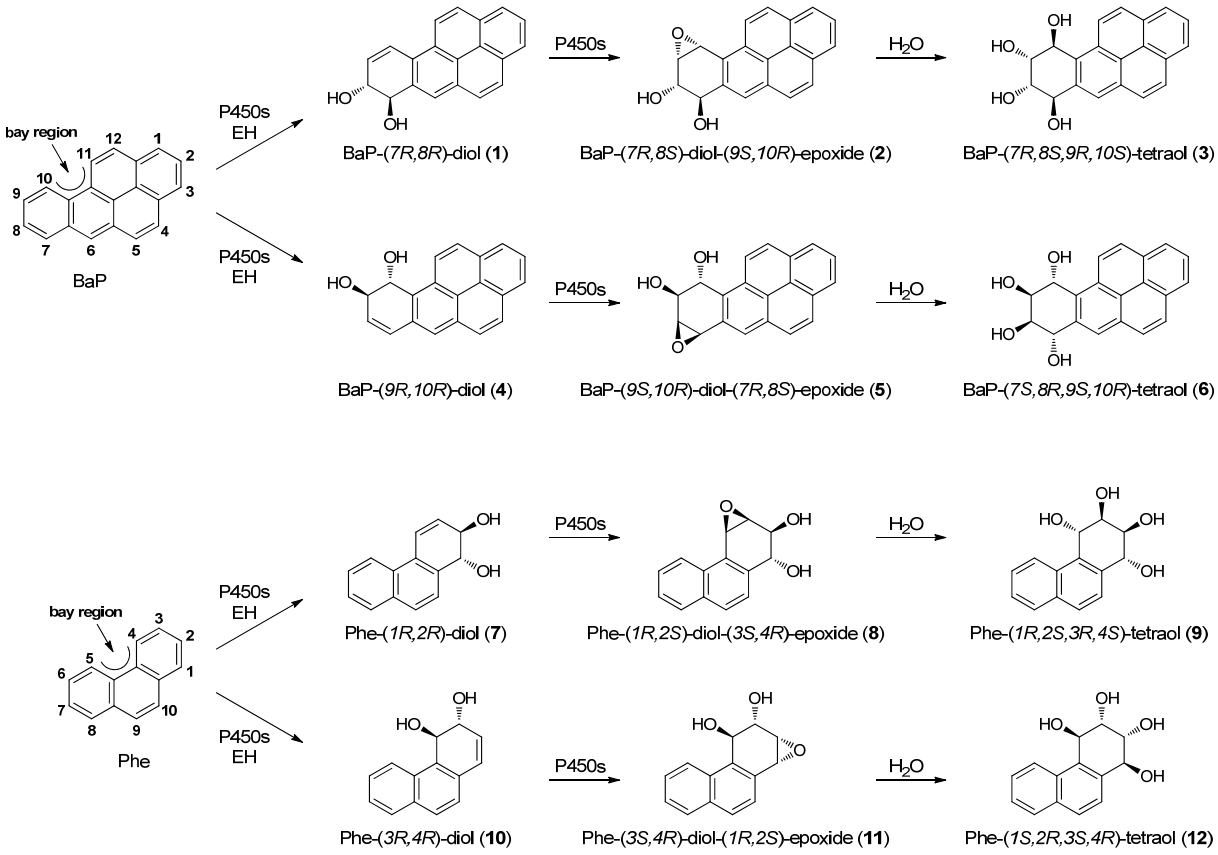
PAH themselves are pro-carcinogens and quite unreactive. After they are taken into the human body, PAH can be activated or detoxificated by competing pathways. To exert its carcinogenic effect, a pro-carcinogenic PAH requires metabolic activation to convert it to reactive electrophiles. DNA-adducts generated by covalently binding of electrophiles with DNA are central in the PAH carcinogenesis process because they convey miscoding potential to human cells. If miscoding events occur in critical regions

of important genes such as the tumor suppressor gene *p53* or the oncogene *KRAS*, the process of tumorigenesis will be initiated and promoted. DNA-adducts can be repaired by DNA repair systems. DNA repair deficiency syndromes are shown to lead to extraordinarily high cancer risks (*107*). In summary, without metabolic activation and formation of DNA adducts, original PAH compounds are not mutagenic and carcinogenic. Competing with metabolic activation, enzymatic metabolic detoxification protects DNA from potential mutations and development of cancer.

The major established route of metabolic activation of BaP and other carcinogenic PAH to DNA adducts is the bay region diol epoxide pathway (Figure **1.2**). For the example of BaP, this pathway starts with the formation of BaP-7,8-epoxide, catalyzed by cytochrome CYP1A1, 1B1, 1A2 and possible others. This is followed by epoxide hydrolase-catalyzed hydration to produce BaP-7,8-diol (*108-110*). This metabolite is stable and nonreactive, but has an isolated double bond in its terminal ring. This bond is readily epoxidized at the 9,10-position, and generates BPDE (*111,112*). Four stereoisomers are possible, but the *trans, anti*-BPDE from *trans*-ring opening of the epoxides ring that is *anti*- to the 7-hydroxyle group is the predominated product (*113*). BPDE is considered to be a major ultimate carcinogen of BaP because it readily reacts with DNA producing the same adducts as those observed in biological systems exposed to BaP (*114-116*). The major DNA adduct results from *trans*- addition of the exocyclic amino group of 2'-deoxyguanosine (dG) to the 10-position of the epoxide ring of BPDE. If not repaired, the resulting *trans, anti*-BPDE-N^2-dG adduct can induce mutations, such as G to T transversions, during DNA replication. Most BPDE then undergo hydrolysis

producing predominantly BaP-(7*R*,8*S*,9*R*,10*S*)-tetraol, which is one enantiomer of *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans*, *anti*-BaPT) (*57,58,117*). BaPT is non-reactive and is the end product of this bay region diol epoxide pathway. This pathway is generalizable to many other PAHs. Phe, the simplest PAH with the bay region, adopts the same pathway utilizing similar metabolic enzymes (*57*).

Figure **1.2** Metabolism of BaP and Phe to trans,anti-tetraols via the bay region diol epoxides (2 and 8) and reverse diol epoxides (5 and 11).



Two other pathways of PAH metabolic activation have been proposed. One involves oxidation of BaP-7,8-diol to a catechol and subsequent formation of redox-active o-quinones by dihydrodiol dehydrogenase members of the aldo-keto reductase (AKR) superfamily, resulting in oxidative damage to DNA (*118*). The other one involves the generation of electrophiles via one-electron oxidation by P450 enzymes, which can result in a variety of DNA adducts (*119*). These pathways have been studied in vitro and in some animal studies, but evidence in human is lacking. Up until now only the diol-epoxides of PAH have been examined for their ability to generate *p53* mutations in humans.

Competing with the metabolic activation of PAH, there are a variety of detoxification pathways (*114,115*). BaP can be detoxified to phenol isomers by P450s. BaP epoxides and BPDE can be detoxified by glutathione conjugation catalyzed by glutathione-*S*-transferase. Epoxides can also form diols by hydration. These diols can be detoxified by glucuronidation and sulfation (*114*). Several isomers of BaP quinones are also considered to be detoxification products.

C. Genotyping individual variation in PAH metabolism

There is large interindividual variation in the human metabolism of PAH (*120-124*). Beginning with the work in the 1960s and continuing to the present, numerous studies based on genotyping have been conducted to investigate the interindividual variation in PAH metabolism and its relationship to cancer.

Most tobacco carcinogens are very hydrophobic, thus they are good substrates for the CYP1 enzymes (CYP1A1, CYP1A2 and CYP1B1) (*59*). CYP1A2 could also catalyzes the metabolism of hydrophilic amines, while CYP1A1 and CYP1B1 metabolize the exceptionally hydrophobic PAHs and polyhalogenated aromatic hydrocarbons. Tobacco PAH are potent inducers of CYP1 enzymes through their strong binding to the transcription factor AhR, which activates the expression of *CYP1* genes. Polymorphisms of the *CYP1A1* gene and their relationship to lung cancer risk have been investigated extensively. The expression of CYP1A1 in mammalian tissues is absent or at very low levels under normal conditions, but can be greatly induced by environmental substances such as cigarette smoke (*59*). The human *CYP1A1* gene has at least 12 variant alleles. Two major polymorphisms in human *CYP1A1* gene include an *MspI* polymorphism due to a T-to-C substitution within the 3' noncoding region of the gene (*CYP1A1*2A*) and an A-to-G substitution in exon 7 resulting in the substitution of valine for isoleucine (*CYP1A1*2C*). These polymorphisms are more common in Asian populations than in Caucasians (*125*). Previous studies found modest or no differences in CYP1A1 enzyme activities associated with any of its polymorphisms (*59*). A meta-analysis in 2000 found little support for the association of these polymorphisms and lung cancer risk (*125*). However, a pooled analysis published in 2003 including 22 case-control studies has shown a significant association between the homozygous *CYP1A1*2A* variant and increased lung cancer risk in Caucasian smokers, but not in Asians (*126*). Another pooled analysis has demonstrated a significant effect of the heterozygous *CYP1A1*2C* variant on lung cancer risk in non-smokers, but not of *CYP1A1*2A* (*127*). CYP1B1 is the

newest member in the CYP1 family. It is also highly regulated by AhR. The human *CYP1B1* gene is expressed at significant levels in almost all organs including brain, placenta, fetal, lung and kidney, whereas its expression in liver is rather low. It has about 24 known allelic variants, but their association with cancer risk is unclear (*106*). The human *CYP1A2* gene is highly unpredictable with more than 60-fold interindividual differences in hepatic constitutive expression (*59*). At least 33 allelic variants of the *CYP1A2* gene have been reported. A recent study analyzed *CYP1A2* SNPs from 280 non-smokers, and concluded that genotyping for predicting the CYP1A2 metabolic phenotype in an individual patient is not informative (*128*).

Genetic variation in human GST enzymes has also been intensively studied. Human GST enzymes detoxify the reactive electrophilic metabolites such as epoxides of PAH by conjugating them with glutathione (*114*). Five classes of GST enzymes in humans are known to be important in the metabolism of environmental carcinogens: GSTA (α), GSTM (μ), GSTP (π), GSTS (σ), and GSTT (θ) (*129*). The most common genetic variants of *GST* include *GSTM1, GSTT1,* and *GSTP1*. These have been extensively studied in relation to lung cancer risk (*130*). Among them, *GSTM1* is particularly relevant in the deactivation of carcinogenic intermediates of PAH. The most common polymorphism of *GSTM1* is a genetic deletion, which leads to a lack of function and decreased ability to detoxify electrophilic PAH metabolites (*131*). The resulting non-functional genotype *GSTM1* null is expressed in about 50% of Caucasians, and has been linked genetically to human malignancies of the lung, colon, and bladder. Its association with lung cancer has been reviewed in meta-analyses and a pooled analysis. The results

have shown little evidence that the *GSTM1* null genotype is associated with risk of lung cancer, either among ever-smokers or never-smokers (*132-134*).

The relationship between polymorphisms of the AhR gene and lung cancer risk in smokers has been also investigated. About 10% of the Caucasian population has a high-inducibility phenotype for AhR-regulated metabolic enzymes, which co-segregates with the polymorphism of *CYP1A1MspI* (*135*). However, because AhR inducibility affects both the metabolic activation and detoxification of PAH, the studies on its association with lung cancer risk have shown conflicting results (*59,122,136*).

Collectively, these previous results indicate that the effects of polymorphisms of genes such as *CYP1A1* and *GSTM1* on lung cancer risk are modest and not straightforward, presumably because PAH metabolism involves multiple genes, some of which participate in both metabolic activation and detoxification.

5. PAH Metabolite Phenotyping to Assess Individual Variation in Lung Cancer Susceptibility

Phenotyping, which directly measures the levels of carcinogens and/or their metabolites, is proposed to provide more comprehensive and reliable information on an individual's susceptibility to cancer with respect to carcinogen exposure and metabolism than genotyping methods (*137*). A series of biomarkers have been developed for PAH metabolite phenotyping to assess inter-individual variation in lung cancer risk.

A. Methods for analysis of BaP metabolites in humans

BaP is the prototype of the PAH family. This five-ring PAH is present in virtually all PAH mixtures and is one of the most carcinogenic of those commonly detected. It has been demonstrated in laboratory animal studies that BaP can readily induce tumors in various tissues such as lung and skin at relatively low doses (*26*). BaP is rated as carcinogenic to humans by the International Agency for Research on Cancer (*25*). BPDE is considered to be a major ultimate carcinogen generated from its bay-region diol epoxides metabolic activation pathway (*114,116*). BPDE mainly reacts with water, producing predominantly *trans, anti*-BaPT. Thus *trans, anti*-BaPT is the major end product of BPDE metabolic activation pathway and its levels would represent the extent of BaP metabolic activation in humans.

Only a few studies have previously described methods for the analysis of BaPT in human urine. Weston *et al*. and Bowman *et al*. analyzed BaPT in human urine using immuno-affinity chromatography for sample purification and synchronous fluorescence spectroscopy for quantitation (*138,139*). They reported BaPT levels of 240-3120 fmol/mL in the urine of four individuals who were highly exposed to PAHs in their diet and 150 fmol/mL BaPT in psoriasis patients receiving coal tar therapy. Simpson *et al*. developed a method to quantify human urinary *trans, anti*-BaPT by GC-NICI-MS-SIM (*117*). The urine was treated with β-glucuronidase and sulfatase, and the analyte BaPT was enriched by reverse-phase and phenylboronic acid solid-phase extraction. The resulting mixture was treated with sodium hydride and methylmethane sulfonate to convert BaPT to the corresponding tetramethyl ethers (BaP-TME). The fraction was then

purified by normal-phase HPLC prior to GC-NICI-MS-SIM. This study reported 16 fmol/mL *trans, anti*-BaPT from psoriasis patients, 4.1 fmol/mL from exposed steel workers, and 0.5 fmol/mL from 9 of 21 smokers, with an on-column limit of detection of 1 fmol (*117*). The urine of steel workers was also analyzed for *cis, anti*-BaP-tetraol and *cys, syn*-BaP-tetraol, but neither was found.

Although the above methods for the quantitation of BaP metabolites in human urine were highly sensitive, they would not be practical for large-size clinical and epidemiology studies. The major challenge is that the levels of BaP metabolites are so low (1-100 fmol/mL urine, even in some highly exposed individuals) that multiple purification steps were needed for GC-MS analysis. The metabolites from other smaller PAH such as Phe exist at much higher levels in human urine. The urinary levels of *trans, anti*-PheT are approximately 10,000 times higher than those of *trans, anti*-BaPT. This is partly because the level of Phe in mainstream cigarette smoke is about 30 times higher than that of BaP (*90,140*). More important, Phe metabolites are excreted mainly in urine, while BaP metabolites are mainly excreted in feces (*141-143*).

Therefore, we focused our attention on Phe in PAH metabolism phenotyping to investigate individual variation in lung cancer susceptibility, simply because Phe's metabolic activation is similar to that of carcinogenic BaP, and Phe metabolites exist at much higher levels in human urine which would facilitate large scale analysis.

B. Methods for analysis of Phe metabolites in humans

Phe is the simplest PAH with a bay region, but it is not carcinogenic (*144*). Neither Phe nor its metabolites exhibit significant tumorigenicity in newborn mice, which are highly sensitive to BPDE and other carcinogenic PAH diol epoxides (*145,146*). The lack of carcinogenicity of Phe is probably because it has only three rings, and its bay-region diol epoxides have extremely weak affinity to DNA.

Several studies have clearly demonstrated the similarities in metabolism between Phe and other bay region carcinogenic PAH such as BaP, chrysene, and benz[α]anthracene (*147-149*). These results show that Phe metabolic activation through the diol epoxide pathway closely mimics that of BaP. Structurally similar intermediates are formed by some of the same cytochrome P450 enzymes, leading to the formation of bay-region diol epoxides with *R,S,S,R*- absolute configuration from dihydrodiols with *R,R*- configuration. Therefore, we propose that Phe metabolites in human urine could be effective surrogates to assess the metabolic activation of BaP and other carcinogenic PAH via diol epoxide formation.

In 2003, Hecht *et al*. reported a simple and sensitive method for analysis of urinary *trans, anti*-PheT, the end product of Phe diol epoxide pathway (*57*). The urine sample was treated with β-glucuronidase and sulfatase to release any conjugated PheT. The analyte was enriched by solid phase extraction, and further purified by high-performance liquid chromatography collection. Then the appropriate residue was silylated and analyzed by GC-NICI-MS-selected ion monitoring at *m/z* 273. The resulting chromatographic traces were extraordinary clean. The quantitation limit of this

method was about 0.2 fmol of *trans, anti*-PheT per mL urine, allowing readily quantitation of this analyte in all human urine samples. Mean levels of *trans, anti*-PheT were 4.58 pmol/mg creatinine in 31 smokers and 1.51 pmol/mg creatinine in 30 non-smokers. An important feature of *trans, anti*-PheT as a biomarker is that it integrates all of the genetic, enzymatic, and environmental factors that impact the Phe metabolic activation pathway.

A similar method for analysis of PheT in human plasma has also been developed (*150*). The steps were similar except the solid phase extraction was performed on a mixed mode cation exchange cartridge. The plasma PheT analysis was shown to be sensitive (limit of quantitation 13 fmol/mL), accurate, and precise. Levels of PheT in plasma in 16 smokers averaged 95 ± 71 fmol/mL, which is about 1% to 2% of the amount found in urine.

Levels of PheT are influenced by both exposure and metabolic activation. To explore solely inter-individual variation in metabolic activation, Hecht's lab developed a method for quantitation of HO-Phe, the detoxification product of Phe, in human urine (*81*). It was proposed to create a metabolic activation: detoxification ratio to correct for exposure. *Trans, anti*-PheT would be in the numerator of this ratio, while HO-Phe would be in the denominator. To measure urinary HO-Phe, the urine sample was first treated by β-glucuronidase and arylsulfatase. A fraction enriched in HO-Phe was prepared by partitioning and solid phase extraction, and HO-Phe was silylated and analyzed by GC-PIC-MS. Accurate quantitation of four HO-Phe (1-HO-Phe, 2-HO-Phe, 3-HO-Phe, 4-HO-Phe) was readily achieved in all human urine samples. There were no significant

difference between the levels of any HO-Phe in smokers and non-smokers' levels were about 2-3 pmol/mg creatinine. Total levels of all HO-Phe were highly correlated with those of 3-HO-Phe. There was about 10-fold spread of the ratios of *trans, anti*-PheT divided by 3-HO-Phe in 46 individuals in this study, suggesting that this ratio may be a useful phenotyping parameter for PAH metabolic activation (*81*).

Another intensively-studied pathway of PAH detoxification involves the conjugation of the epoxides and diol epoxides by reaction with glutathione, catalyzed by glutathione-*S*-transferases (GSTs). Numerous molecular epidemiology studies have examined the relationship between polymorphisms in GST genes and cancer incidence in people exposed to PAH, using genotyping methods. In 2006, Upadhyaya *et al.* reported a method for quantitation of a Phe epoxide mercapturic acid (PheO-NAC), the end product of the reaction of phenanthrene-9,10-epoxide with glutathione (*151*). PheO-NAC was detected in the urine of 46 of 104 smokers with an average amount of 57.9 fmol/mL urine, which is considerably lower than the levels of PheT or HO-Phe. It was found that PheO-NAC was detected significantly more frequently in *GSTM1* positive subjects than in *GSTM1* null subjects, and the levels of PheO-NAC were significantly higher in the *GSTM1* positive subjects. No significant relationships were found between PheO-NAC levels and the occurrence of *GSTP1* polymorphisms. In 2008, Hecht *et al.* reported the identification of human urinary mercapturic acids that result from Phe diol epoxides (*152*). Interestingly, the only detectable mercapturic acid was derived from the "reverse diol epoxide", *anti*-Phe-3,4-diol-1,2-epoxide, and not from the bay region diol epoxides, *syn*- or *anti*-Phe-1,2-diol-3,4-epoxide. Furthermore, incubation of Phe diol epoxides and

reverse diol epoxides in human hepatocytes has shown that the reverse diol epoxides were far better substrates for GSH conjugation than were the bay region diol epoxides (*153*). Consistent with these results from Phe, it was demonstrated in 2010 that the reverse diol epoxides rev-BPDE undergoes GSH conjugation in human hepatocytes, but the carcinogenic bay region diol epoxide, BPDE, does not (*154*). Reverse diol epoxides such as rev-BPDE generally have little or no carcinogenic effect in contrast to bay region diol epoxides. Collectively, these results indicated that GSTs are unlikely to drive carcinogenic PAH diol epoxide detoxification in humans. The assumption underlying GST genotyping studies may be incorrect.

C. Longitudinal consistency of urinary Phe metabolite ratios and the relationship between phenotyping and genotyping data

As described above, it was proposed to use a ratio of PheT (as a biomarker of metabolic activation) to HO-Phe (as a biomarker of metabolic detoxification) to assess a given individual's ability to metabolically activate PAH. In a longitudinal study, the consistency of this ratio over time was examined, and the ratios in smokers and non-smokers were compared (*82*). Twelve (12) smokers and 10 non-smokers were included in the study. First morning urine samples were collected daily for 7 days, then weekly for 6 weeks. The results showed that levels of PheT, HO-Phe, and PheT/HO-Phe ratios were relatively constant in both smokers and non-smokers over 49 days, with mean coefficients of variation ranging from 29.3% to 45.7%. PheT/HO-Phe ratios were

significantly higher in smokers than in non-smokers, suggesting that smoking can induce the diol epoxide metabolic activation pathway of Phe in humans. Consistent with the previous study, the levels of HO-Phe were not significantly different between smokers and non-smokers, likely because the diol epoxide metabolic activation pathway of Phe was induced to a greater extent than the HO-Phe detoxification pathway in smokers. The results from this longitudinal study have indicated that a single sampling of urine is sufficient for comparing among different groups of subjects, and sampling should be done at about the same time of day.

A subsequent study investigated the relationship of polymorphisms in PAH metabolizing genes with PheT:HOPhe ratios in smokers (*155*). The goal was to examine whether genetic polymorphisms, individually or in combination, could predict metabolic activation capacities of individuals as represented by PheT/HOPhe ratios. In this study, urinary PheT/HOPhe ratios in 346 smokers were calculated. A blood sample was collected from each smoker and genotyping was carried out for 11 polymorphisms in genes involved in PAH metabolism: *CYP1A1MspI, CYP1A1I462V, CYP1B1R48G, CYP1B1A119S, CYP1B1L432V, CYP1B1N453S, EPHX1Y113H, EPHX1H139R, GSTP1I105V, GSTP1A114V, and GSTM1 null.* The distribution of PheT:HOPhe ratios was shown to be a bell shaped curve skewed to the left. The geometric mean of PheT/HOPhe ratio was 4.8. Ten percent of the smokers had a PheT/HOPhe ratio greater than 9.9, and it was proposed that this small group of smokers should be at higher risk for lung cancer. After comparing genotyping and phenotyping results, a significant association between the presence of the *CYP1A1*I462V polymorphism and high

PheT/HOPhe ratios was found. In contrast, the *CYP1B1*R48G and *CYP1B1*A119S polymorphisms were associated with significant lower ratios, particularly in Blacks. No significant effects of any of the other polymorphisms on PheT/HOPhe ratios were found. The highest 10% PheT/HOPhe ratios could not be predicted by the presence of any of the 11 polymorphisms individually or by certain combinations. Collectively, the results of this comparison study indicate that genotyping data cannot accurately predict the metabolic activation of Phe, and likely the metabolism of other PAH, in smokers. This conclusion was not a surprise, because enzyme activities are not solely determined by genotype, and other factors beyond genotype such as gene expression, induction, and inhibition can affect PAH metabolism as well.

D. Analysis of the minor enantiomer of PheT and its correction with BaPT

It has long been known that PAH metabolism through the bay region diol epoxide pathway is highly stereoselective, but their enantiomeric composition hasn't been addressed before in human in vivo. This is important because different enantiomers would result from the bay region diol epoxide or "reverse" diol epoxide pathways, which are associated with strong or weak carcinogenicity respectively.

The diol epoxide and "reverse" diol epoxide pathways of BaP and Phe are shown in Figure **1.2**. Diol epoxide **2** is formed from the oxidation occurring in the bay region. It can react easily with DNA and is highly carcinogenic in newborn mice. Thus diol

epoxide **2** is considered to a major ultimate carcinogen of BaP. The reaction of diol epoxide **2** with H_2O produces predominantly BaP-(7*R*,8*S*,9*R*,10*S*)-tetraol (**3**). A "reverse" diol epoxide pathway of BaP generate diol epoxide **5** (an enantiomer of **2)** and BaPT **6**. However, much less is known about the carcinogenicity of **5** and the conversion of diol **4** to diol epoxides **5** and BaPT **6**. The "reverse" diol epoxide pathway is believed to be associated with weak mutagenicity and carcinogenicity. Analogous pathways have been observed in the metabolism of Phe. Metabolic activation of Phe diols **7** and **10** followed by hydrolysis gives mainly the tetraols arising from *trans* ring opening of the *anti*-diol epoxides **8** and **11**. Therefore, PheT **9** is formed from the bay region diol epoxide pathway while PheT **12** is from the "reverse" diol epoxide pathway. In earlier studies, total racemic tetraols from BaP and Phe have been quantified in humans. No attempt has been made to distinguish BaPT enantiomers **3** and **6**, or PheT enantiomers **9** and **12**.

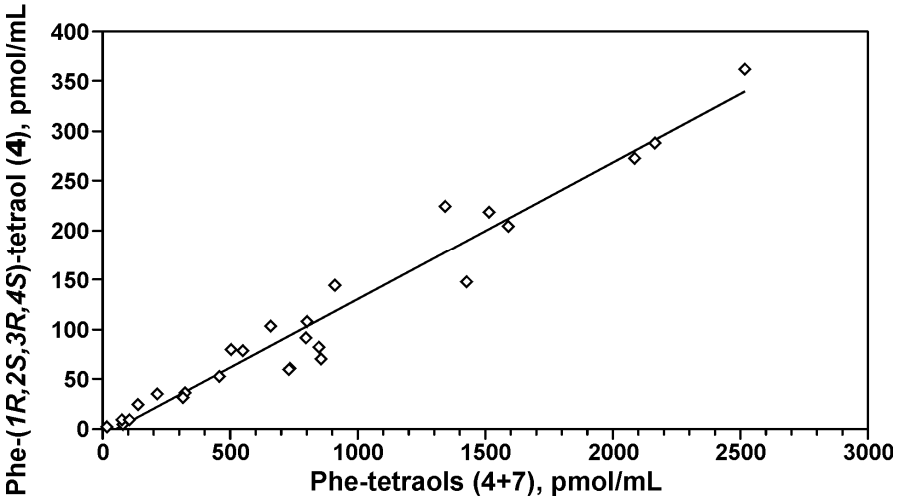
To address BaPT and PheT enantiomeric composition, Hecht *et al*. supplemented the previous analysis with chiral HPLC separations and successfully resolved the enantiomers (*113*). Urine samples from smokers were analyzed for PheT enantiomers. The results demonstrated that greater than 96% of PheT in smokers' urine was enantiomer **12**, resulting from the "reverse" diol epoxide pathway. Urine from creosote workers, who were highly exposed to PAH, was similarly analyzed for BaP enantiomers. In contrast to PheT results, 78% of BaPT in these urine samples were BaPT **3** resulting from bay region diol epoxide pathway.

The result of the BaPT enantiomeric composition study is fully consistent with the previous belief that BaP is metabolically activated in humans to mainly bay region diol epoxides. However, the results demonstrated a key difference in Phe and BaP metabolism, and raised some question about the utility of PheT as a surrogate for carcinogenic PAH metabolic activation.

The next important question was whether the measurement of overall unresolved PheT would still accurately represent the extent of the bay region diol epoxide metabolic activation of carcinogenic PAH such as BaP.

In a subsequent study, the minor enantiomer of PheT was specifically quantified with chiral HPLC separations (*156*). The correlation of levels of Phe-(1*R*,2*S*,3*R*,4*S*)-tetraol (**4**) to total PheT (**4**+**7**) was tested in urine samples from cigarette smokers and highly PAH-exposed creosote workers. The levels of **4** were also compared to those of BaPT and 1-HOP in the same samples. The results demonstrated that levels of PheT minor enantiomer **4** were highly correlated with those of total PheT (**4**+**7**) as shown in Figure **1.3**. Strong correlations of PheT minor enantiomer and total PheT with BaPT and 1-HOP were also observed. The results demonstrated therefore that practical and rapid measurement of total PheT in human urine, without enantiomeric resolution, is an excellent indicator of PAH exposure and metabolic activation by the bay region diol epoxide pathway.

Figure **1.3** Relationship of levels of Phe-(1R,2S,3R,4S)-tetraol (4) to Phe-tetraols (4 + 7) in creosote workers' urine.



E. Application of Phe metabolite phenotyping

Our ultimate goal of PAH metabolite phenotyping is to test the hypothesis that individual differences in PAH metabolism are related to lung cancer risk in smokers. How would this hypothesis be tested in the real world? There are stored urine and plasma samples in a number of very large epidemiologic studies in which cancer outcomes are known. The hypothesis could be directly tested in such epidemiology cohorts using the nested case-control study design, which analyzes and compares the levels of biomarkers in pre-diagnostic samples between lung cancer cases and controls.

Yuan *et al*. reported urinary levels of PheT in relation to lung cancer development in cigarette smokers of Shanghai cohort study (cancer research in press). The Shanghai Cohort is consisted of 18,244 men enrolled from more than 20 years ago. In addition to in-person interview for cigarette smoking and other lifestyle factors, baseline blood and urine samples were collected from all participants. In this nested case control study, more than 400 lung cancer cases and matched controls who smoked cigarettes at urine collection were included in this study. Lung cancer free controls were matched to lung cancer cases by age at enrollment, date of biospecimen collection and neighborhood of residence at recruitment. Pre-diagnostic urinary levels of total PheT, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides (total NNAL), and cotinine and its glucuronides (total cotinine) were quantified. The results demonstrated that urinary levels of total PheT, total NNAL, and total cotinine were significantly higher in cases than in controls, in a dose-dependent manner. When all three biomarkers were simultaneously considered in the risk model, each of them was significantly and

independently associated with lung cancer risk. Total cotinine had the strongest association with the risk of lung cancer, followed by total PheT and total NNAL. Findings of this study directly link PAH exposure to lung cancer development in humans. It is also suggested that these urinary biomarkers might be used as the starting point of an individual-based, predictive model for lung cancer risk in a smoker.

Phe metabolite phenotyping could also be used to monitor the effect of chemoprevention therapy at the individual level. One ongoing study utilized PheT phenotyping methods to investigate the potential chemopreventive effect of indole-3-carbinol (I3C) in smokers (unpublished data). I3C is enzymatically derived from cruciferous vegetables, particularly of the brassica genus. It has been shown that I3C can inhibit NNK and BaP-induced lung carcinogenesis in A/J mice (*157*), but it was unclear whether I3C induces the metabolic activation, detoxification, or both of PAH in smokers. In this clinical study, smokers received isotope labeled deuterated Phe in cigarettes twice on two separate visits, before one of which subjects were instructed to take I3C for 7 days. Urinary and plasma levels of deuterated PheT were measured as metabolic activation biomarkers. Within individual comparisons of PheT formation between two visits (with or without I3C therapy) were performed to test the hypothesis that I3C induces PAH detoxification in the lung, and therefore may protect smokers from lung cancer. The analysis of the preliminary results of this study is in progress.

In summary, a number of previous studies support the hypothesis that inter-individual differences in PAH metabolism are related to lung cancer risk in smokers. There are several models in the literature for the identification of susceptible smokers, but all include retrospective analysis of years of smoking (*158-161*). Our ultimate goal is to develop a prospective model of susceptibility which could identify the small group of smokers with higher risk at a young age. This model would include important metabolite biomarkers derived from tobacco-related carcinogens. It is conceivable that the PAH metabolism phenotype would be an important part of such a model, and might be helpful in encouraging susceptible smokers to quit smoking or take chemoprevention therapies in the future.

Chapter 2

Analysis of *r*-7,*t*-8,9,*c*-10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in Human Urine: A Biomarker for Directly Assessing Carcinogenic Polycyclic Aromatic Hydrocarbon Exposure plus Metabolic Activation

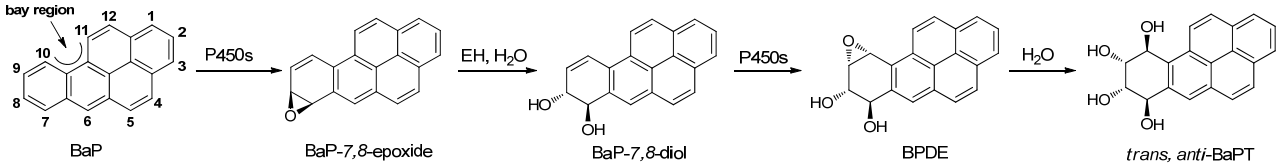
1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large class of compounds mainly formed by incomplete combustion of organic matter. They are commonly found in polluted air and water, tobacco smoke, broiled and smoked foods, and in certain occupational environments such as coke production from coal and other processes involving soots and tars (*4,88-92,162*). Humans readily absorb PAH into the body through the lung, gastrointestinal tract, and skin. Many PAHs are carcinogens, and are believed to be causative agents for various types of human cancers, including lung cancer in smokers (*4,12,15,25*). Benzo[*a*]pyrene (BaP), a prototypic PAH, is rated as carcinogenic to humans by the International Agency for Research on Cancer (*25*). This five-ring PAH is present in virtually all PAH mixtures, and is one of the most carcinogenic of those commonly detected. It has been conclusively demonstrated in laboratory animal studies that BaP is a powerful carcinogen, which readily induces tumors in various tissues such as lung and skin at relatively low doses (*12,25,26,89,163*).

PAHs including BaP are procarcinogens that require metabolic activation to elicit their carcinogenic effects (*25,114*). Considerable evidence supports the view that these carcinogens act as tumor initiators after metabolic oxidation to reactive electrophiles by the bay region diol epoxide pathway (Figure **2.1**) (*114-116*). This pathway starts with an initial cytochrome P450s-catalyzed epoxide formation at the 7,8-position followed by epoxide hydrolase -catalyzed hydration (*108-110*). Subsequent epoxidation at the 9,10-position then predominantly generates BaP-(7*R*,8*S*)-diol-(9*S*,10*R*)-epoxide (BPDE) (Figure **2.1**) (*111,112,114-116*). BPDE is considered to be a major ultimate carcinogen

of BaP because it is carcinogenic and readily reacts with DNA to produce the same adducts as observed in biological systems exposed to BaP (*111,112,114-116*). The major reaction of BPDE in biological systems is hydrolysis producing predominantly BaP-(*7R,8S,9R,10S*)-tetraol, which is one enantiomer of *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, anti*-BaPT). Competing with metabolic activation, detoxification pathways of BaP include phenol formation, glutathione conjugation, glucuronidation, and sulfation (*12,114,115*).

Figure **2.1** Metabolism of benzo[a]pyrene (BaP) to trans, anti-BaPT via the bay region diol epoxide BPDE. P450, cytochrome P450; EH, epoxide hydrolase.



Approximately 11-24% of lifelong smokers develop lung cancer over their lifetimes (*4*). It would be important to identify individuals in this group with apparently higher cancer susceptibility. Our working hypothesis is that people who metabolically activate tobacco smoke carcinogens more extensively should have higher cancer risk. Large inter-individual differences in the metabolic activation of carcinogenic PAHs have been demonstrated (*121,123,164,165*). Multiple studies have used genotyping methods to examine the relationship between lung cancer risk and polymorphisms in genes such as *CYP1A1* and *GSTM1* which code for enzymes involved in PAH metabolism (*70,120,125,133,134,166-169*). But the results remain varied and inconsistent, likely because of the complexity of PAH metabolism (*155*). We propose that a phenotyping strategy, with direct measurement of metabolites generated by the metabolic activation pathways, can provide more comprehensive and reliable information on an individual's susceptibility to cancer (*15,137*). With this goal in mind, we developed and optimized a gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) method to quantify racemic *trans, anti*-BaPT in human urine.

2. Materials and Methods

Chemicals, Enzymes and Apparatus. All BaP metabolites used in this study were racemic. *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, anti*-BaPT), *r*-7,*t*-8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*cis, anti*-BaPT), *r*-7,*t*-8,,*c*-9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*cis, syn*-BaPT), *r*-7,*t*-8,*c*-

9,*t*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*trans, syn*-BaPT), *trans*-4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene (BaP-4,5-diol), *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene (BaP-7,8-diol) and *trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene (BaP-9,10-diol) were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO). The internal standard, [$^{13}C_6$]*trans, anti*-BaPT was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). Purities of these standards were >99%, as determined by HPLC analysis. β-Glucuronidase and arylsulfatase (from *Helix pomatia*) were obtained from Roche Diagnostics Corp.(Indianapolis, IN). Strata-X polymeric reversed phase SPE cartridges (200 mg/6 mL, #8B-S10-FCH) were procured from Phenomenex (Torrance, CA). Bond Elute phenylboronic acid SPE cartridges (100 mg/1 mL, # 12102018) were from Varian, Inc (Palo Alto, CA). *bis*-Trimethylsilyltrifluoroacetamide (BSTFA) was purchased from Regis Technologies (Morton Grove, IL). Removal of solvents was carried out with an SVC-200 Speedvac (Thermo Savant, Holbrook, NY). Gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) was carried out with a TSQ Quantum instrument (Thermo Scientific).

Urine Samples. This study was approved by the University of Minnesota Institutional Review Board. Urine samples from 30 smokers (11 females) were obtained from ongoing studies at the University of Minnesota Tobacco Use Research Center. Current smoking status was confirmed by CO and cotinine levels. Thirty non-smokers

(16 females) were laboratory personnel. All urine samples were stored in a -20 ºC freezer before analysis.

Determination of urinary creatinine. The creatinine content in urine was determined at the University of Minnesota Medical Center, Fairview, Diagnostic Laboratories, using Vitros CREA slides.

Analysis of Urine for *trans, anti*-BaPT. A 1.5 mL aliquot of urine was placed in a 10 mL centrifuge tube containing 780 µL of 0.5M NaOAc buffer, pH 5, β-glucuronidase (3,500 units) and arylsulfatase (28,000 units). [$^{13}C_6$]*trans, anti*-BaPT (20 fmol) in 2 µL CH_3CN was added as internal standard. The mixture was incubated in a water bath overnight with shaking at 37 °C. A Strata-X cartridge was preconditioned with 5 mL of CH_3OH, then with 5 mL of H_2O. The urine sample was applied to the cartridge slowly, along with two 1 mL H_2O washings of the urine sample tubes. The cartridge was washed with 5 mL of 1% NH_4OH in 50% CH_3OH, then with 0.5 mL CH_3OH. *trans, anti*-BaPT was then eluted with 2 mL CH_3OH. This fraction contained the analyte and internal standard. Solvents were removed by overnight concentration on a Speedvac. The residue was dissolved in 1 mL of 30% aq CH_3OH with sonication and loaded onto a 100 mg/1 mL phenylboronic acid cartridge, which had been preconditioned with 1 mL of CH_3OH and then with 1 mL of H_2O. The cartridge was then washed with 100 µL of 30% CH_3OH in H_2O, placed under vacuum overnight to remove residual H_2O , and then washed twice with 1 mL acetone which had been dried with Na_2SO_4. *trans,*

anti-BaPT was eluted with 1 mL of 80% CH_3OH in H_2O. The solution was concentrated to dryness. The residue was dissolved in 200 µL of CH_3OH with sonication and vortexing, transferred to an insert vial, speed vacuumed to dryness, and dissolved in 10 µL of BSTFA. The samples were heated at 60 °C for 60 min with periodic mixing by vortexing, and 2.5 µL was injected into the GC-NICI-MS/MS system.

GC-NICI-MS/MS Analysis. This was modified from that described previously (*113*). The GC was fitted with a 0.25 mm (inside diameter) × 30 m, 0.15-µm film thickness, DB-17MS column (Agilent Technologies, Palo Alto, CA), and a 0.53 mm (inside diameter) × 2 m deactivated fused silica precolumn. The oven temperature program was as follows: 80 °C for 1 min, then 80 to 200 °C at 35 °C/min, then 200 to 215 °C at 3 °C/min, then 215 to 320 °C at 35 °C/min, then hold for 3 min. The injection port temperature was 250 °C, and the MS transfer line temperature was 320 °C. The flow rate was 1 mL/min He. The injector was operated in the splitless mode; the evaporation temperature was 80 °C for 2 min, then 80 to 260 °C at 5 °C/sec, then hold for 1 min. The NICI-MS/MS conditions were as follows: CI gas, methane at 2.0 mL/min; source temperature, 200 °C; emission current, 500 µA. Selected reaction monitoring (SRM) with a collision energy of 18 eV, electron energy of -150 eV, and Ar collision gas pressure of 1.0 mTorr was used to detect *trans, anti*-BaPT-tetratrimethylsilyl ether (*trans, anti*-BaPT-TMS) and [$^{13}C_6$]*trans, anti*-BaPT-TMS at *m/z* 446 → *m/z* 255 and *m/z* 452 → *m/z* 261, respectively.

Hepatocyte Incubations. Primary human hepatocytes were purchased from Cellzdirect (St. Louis, MO), and prepared as described before (*154*). Hepatocytes (approximately 0.12 mg protein per well) were incubated with 10 μM of BaP-4,5-diol, BaP-7,8-diol or BaP-9,10-diol, or H_2O as a control. Each diol was dissolved in 20 μL DMSO and added to the 2 mL incubation mixtures. One mL of media was collected at 24 hrs. Samples were stored at -80 °C until analysis.

Statistical Analysis. The hypothesis that the level of *trans, anti*-BaPT in the urine of smokers (n=30) was significantly higher than that in non-smokers (n=30) was tested using one-sided two-sample *t* test.

3. Results

The analytical method is outlined in Figure **2.2**. After the addition of [$^{13}C_6$] *trans, anti*-BaPT as the internal standard to a 1.5 mL urine sample, the mixture was incubated with β-glucuronidase and aryl sulfatase to release conjugated BaPT. The samples were purified by solid-phase extraction using polymeric reversed phase cartridges, and then phenylboronic acid cartridges which retain *cis*-hydroxy groups with high specificity (*113*). *trans, anti*-BaPT was derivatized with BSTFA to produce the corresponding tetra-TMS ethers. A full scan NICI-MS of BaPT-TMS showed a base peak at *m/z* 446 [M − $(OSi(CH_3)_3 + Si(CH_3)_3)]^-$ with little or no molecular ion (*m/z* 608). We used GC-NICI-MS/MS-SRM to monitor the transition *m/z* 446 → *m/z* 255 for *trans, anti*-BaPT-TMS,

and *m/z* 452 → *m/z* 261 for the internal standard [$^{13}C_6$]*trans, anti*-BaPT-TMS. The daughter ion peak at *m/z* 255 corresponds to the loss of [$HOSi(CH_3)_3$ + $(CH_3)_3Si$ + CO] from *m/z* 446 (Figure **2.3**).

Figure **2.2** Analytical scheme for quantitation of *trans, anti*-BaPT in human urine. IS, internal standard; SPE, solid phase extraction; BSTFA, *bis*-trimethylsilyltrifluoroacetamide; GC-NICI-MS/MS, gas chromatography-negative ion chemical ionization-tandem mass spectrometry.

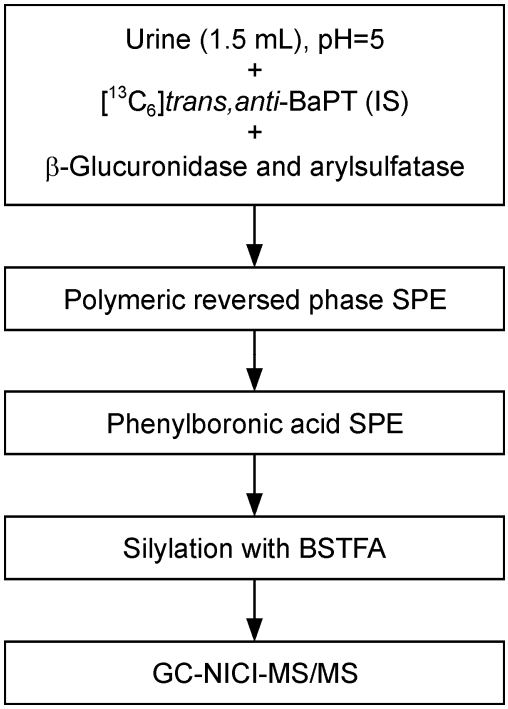
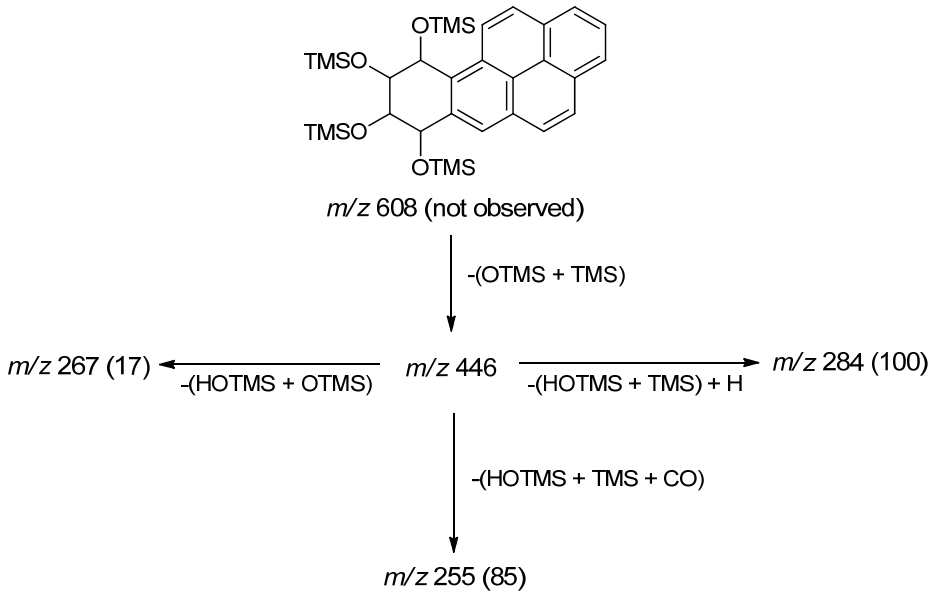


Figure **2.3** Fragmentation pattern and relative intensities (in parentheses) in the daughter ion spectrum of m/z 446 of trans, anti-BaPT-TMS. TMS = (CH3)3Si



Typical GC-NICI-MS/MS chromatograms obtained upon analysis of *trans, anti*-BaPT in the urine of a smoker and a non-smoker are shown in Figure **2.4**. The internal standard [$^{13}C_6$]*trans, anti*-BaPT-TMS eluted at the same retention time as *trans, anti*-BaPT-TMS. The identity of *trans, anti*-BaPT-TMS was verified by co-injection with a standard. To further confirm the identity of the analyte peak in the urine samples, three different transitions, from *m/z* 446 to daughter ions of *m/z* 255, *m/z* 267, and *m/z* 284 were monitored and compared to standards (Figure **2.3**). As shown in Table **2.1**, the ratios of the integrated peaks from monitoring the three transitions in a urine sample were very close to those in the standards. The GC-NICI-MS/MS method proved to be highly sensitive. The on-column detection limit was about 20 amol of *trans, anti*-BaPT-TMS. The limit of quantitation of the assay was less than 0.05 fmol *trans, anti*-BaPT per mL urine. A calibration curve demonstrating linearity when 0.05 – 10 fmol of *trans, anti*-BaPT was derivatized is illustrated in Figure **2.5**.

As multiple tetraols could be formed from BaP, we sought further confirmation of the identity of *trans, anti*-BaPT. Incubation of primary human hepatocytes with BaP-7,8-diol produced four isomers of BaPT, as summarized in Table **2.2**. Each had a retention time identical to those of the TMS derivatives of the corresponding standards - *trans, syn*-BaPT, *trans, anti*-BaPT, *cis, anti*-BaPT, and *cis, syn*-BaPT. *trans, anti*-BaPT was the predominant tetraol formed. Incubation of BaP-9,10-diol with human hepatocytes produced two tetraols which co-eluted with standards – *trans, syn*-BaPT and *trans, anti*-BaPT. There were also two minor products which did not co-elute with *cis*, *anti*-BaPT and *cis, syn*-BaPT, which was expected since the 9,10-hydroxyl groups in these

compounds are *cis*-, not *trans*- as in BaP-9,10-diol. The two minor products probably resulted from *cis*- ring opening of the *syn*- and *anti*-9,10-diol-7,8-epoxides, but this was not pursued. A small peak at the same retention time as *trans, anti*-BaPT was also observed in the incubations of BaP-4,5-diol with human hepatocytes, but the yield of this product was at least 10,000 times lower than those from the two other diols. No tetraol peaks were observed in control incubations.

The intraday precision of the assay was determined by analyzing seven aliquots of a smoker's urine. The results were 1.01 ± 0.05 fmol *trans, anti*-BaPT/mL urine (relative SD, 4.8%). The interday precision based on analyses of a smoker's urine (2 per set as positive controls in four sets of assays) was 15.2% (relative SD). Assay accuracy was assessed by the standard addition method. One mL of the positive control urine, which contained 1.01 fmol/mL *trans, anti*-BaPT, was enriched with 0.1, 0.2, 0.5, 1, and 2.5 fmol *trans, anti*-BaPT. The results of this experiment are presented in Figure **2.6**. The added and measured levels of *trans, anti*-BaPT were highly correlated (r = 0.998) and the y intercept was 0.98 fmol/mL urine, in excellent agreement with the amount of *trans, anti*-BaPT in the untreated sample. Recoveries of internal standard were generally good, averaging 44 ± 19% (n = 80).

The method was applied to the analysis of urine samples from 30 non-smokers and 30 smokers. The results are summarized in Table **2.3.** The mean level of *trans, anti*-BaPT (0.71 ± 0.64 fmol/mg creatinine, n = 30) in the urine of smokers was significantly higher ($P = 0.0018$) than that in non-smokers (0.34 ± 0.20 fmol/mg creatinine, n = 30).

No gender differences in the amounts of this analyte were found among either smokers or non-smokers.

Figure **2.4** Chromatograms from GC-NICI-MS/MS SRM analysis of trans, anti-BaPT in urine of a smoker (A and B) and a non-smoker (C and D). The indicated peaks are TMS derivatives of trans, anti-BaPT (A and C) and internal standard [13C6]trans, anti-BaPT (B and D).

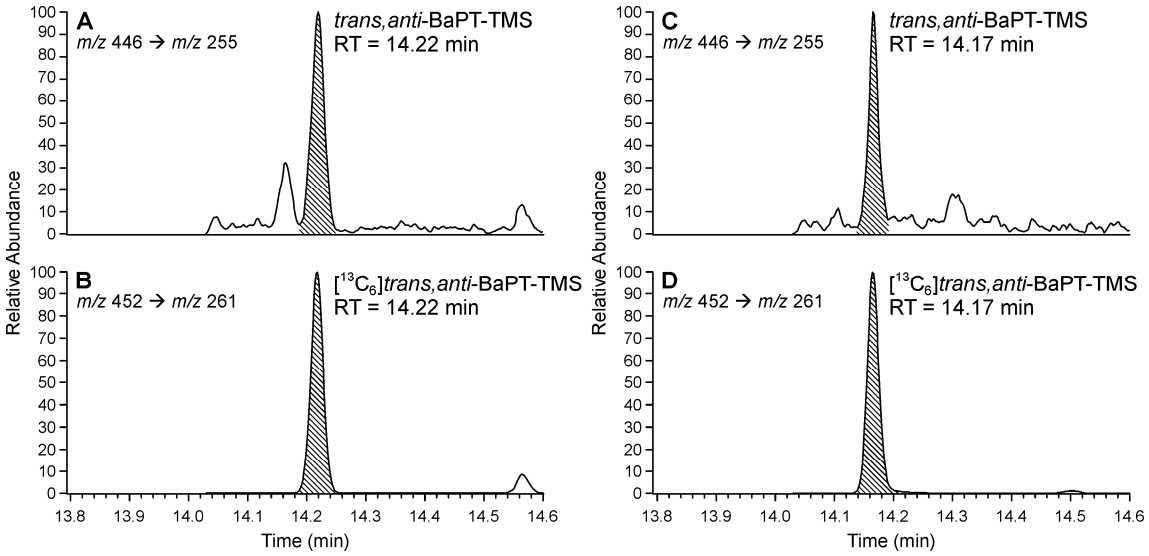


Figure **2.5** Calibration curve for trans, anti-BaPT-TMS. The amount of standard trans, anti-BaPT was increased from 0.05 fmol to 0.1, 0.5, 2.5, 5, and 10 fmol, with a constant amount of internal standard [13C6]trans, anti-BaPT (20 fmol). The calibration curve shows the concentration ratio (analyte/internal standard) versus their area ratio.

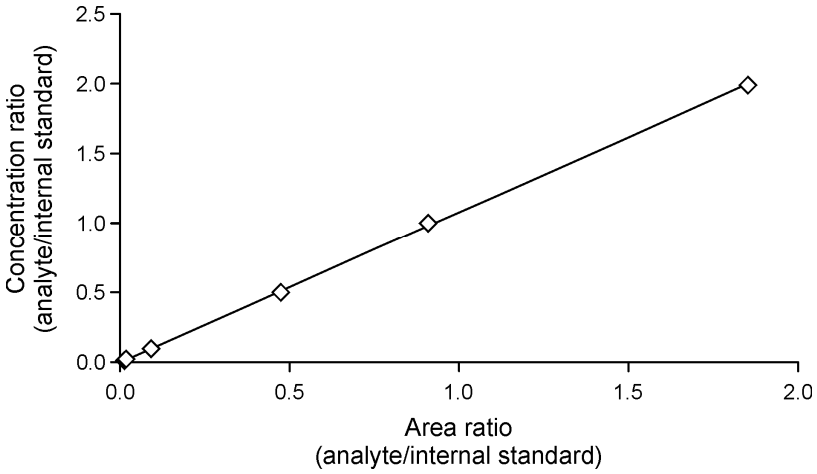


Figure **2.6** Relationship between levels of *trans, anti*-BaPT added to a urine sample and levels measured (r=0.998). Points, mean of duplicate determinations.

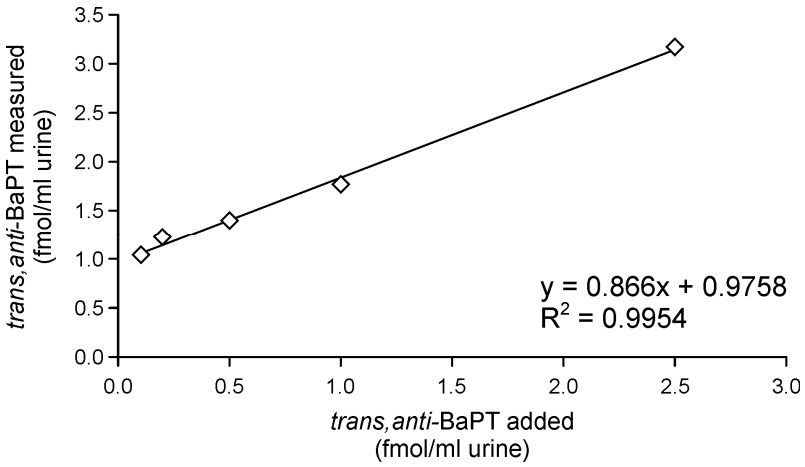


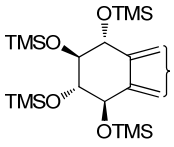
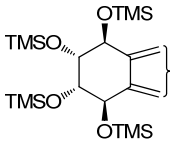
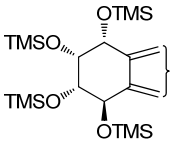
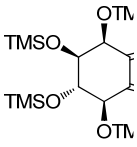
Table **2.1**

Ratios of major *trans, anti*-BaPT-TMS fragment ion intensities from analysis of a human urine sample, compared to *trans, anti*-BaPT-TMS and [$^{13}C_6$]*trans, anti*-BaPT-TMS standards.

	m/z 446 → *m/z* 255	*m/z* 446 → *m/z* 267	*m/z* 446 → *m/z* 284
trans, anti-BaPT-TMS from analysis of human urine	1.0	0.2	1.3
trans, anti-BaPT-TMS standard	1.0	0.2	1.1
	m/z 452 → *m/z* 261	*m/z* 452 → *m/z* 273	*m/z* 452 → *m/z* 290
[$^{13}C_6$]*trans, anti*-BaPT-TMS	1.0	0.2	1.2

Table **2.2**

Summary of retention time (min) and yield (pmol/mL media) of BaPT isomers from incubation of three BaP-diols (10 μM) with human hepatocytes, and analyzed as their TMS derivatives by GC-NICI-MS/MS.

	trans, syn- BaPT-TMS	*trans, anti-* BaPT-TMS	*cis, anti-* BaPT-TMS	*cis, syn-* BaPT-TMS	[$^{13}C_6$]*trans,anti-* BaPT-TMS
					
tetraol standards	13.72	13.93	14.25	14.51	13.93
tetraols from BaP-7,8-diol	13.72 (0.4)[a]	13.93 (18.1)	14.25 (1.2)	14.51 (0.8)	13.93
tetraols from BaP-9,10-diol	13.72 (5.6)	13.93 (7.0)	ND[b]	ND	13.93
tetraols from BaP-4,5-diol		13.93 (0.7×10^{-3})			13.93

[a] retention time (yield)

[b] ND, not detected. Two minor peaks from BaP-9,10-diol were not identified.

Table **2.3**

(a) Urinary *trans, anti*-BaPT concentrations in non-smokers.

Non-smokers			*trans, anti*-BaPT	
Subject no.	age	gender	fmol / ml urine	fmol / mg creatinine
1	28	F	0.37	0.38
2	23	F	0.31	0.25
3	25	F	0.21	0.21
4	22	M	0.23	0.45
5	25	M	0.27	0.99
6	24	M	0.24	0.31
7	28	F	0.21	0.63
8	22	M	0.22	0.26
9	24	M	0.22	0.13
10	25	F	0.22	0.75
11	22	M	0.28	0.20
12	24	F	0.22	0.27
13	60	M	0.28	0.18
14	27	F	0.26	0.35
15	25	F	0.26	0.55
16	57	M	0.24	0.19
17	41	M	0.34	0.51
18	29	F	0.21	0.24
19	24	F	0.26	0.44
20	50	F	0.20	0.26
21	24	F	0.24	0.20
22	52	M	0.28	0.14
23	66	M	0.25	0.39
24	39	M	0.36	0.36
25	28	F	0.26	0.31
26	23	M	0.25	0.10
27	23	M	0.25	0.21
28	28	M	0.25	0.21
29	29	F	0.24	0.31
30	75	M	0.24	0.57
		Mean ± SD	0.26 ± 0.04	0.34 ± 0.20
		Range	0.20 - 0.37	0.10 – 0.99

(b) Urinary *trans, anti*-BaPT concentrations in smokers.

Smokers (n=30)				*trans, anti*-BaPT	
Subject no.	age	gender	cigs/day	fmol / ml urine	fmol / mg creatinine
1	24	F	14	0.61	1.38
2	20	F	12	0.60	0.69
3	53	M	15	0.51	0.65
4	24	M	16	0.50	0.74
5	35	F	12	1.20	1.14
6	44	M	20	0.73	0.50
7	66	M	20	0.41	0.35
8	57	M	30	0.62	0.28
9	43	F	15	1.31	0.60
10	56	M	10	0.93	0.33
11	24	F	20	0.56	0.34
12	28	M	24	0.40	0.57
13	20	M	20	0.47	0.47
14	56	M	20	0.62	0.56
15	53	M	25	0.62	0.23
16	49	M	8	0.55	0.33
17	55	M	40	0.36	0.41
18	50	F	20	0.33	0.33
19	43	M	30	0.27	0.34
20	30	F	20	0.45	0.31
21	42	F	20	0.29	0.23
22	54	F	20	0.29	0.93
23	42	M	10	0.64	0.86
24	27	M	40	0.36	1.07
25	38	M	12	0.38	0.42
26	30	M	20	0.28	0.73
27	43	F	15	1.61	0.86
28	40	F	15	1.38	1.74
29	47	M	23	0.28	0.63
30	38	F	14	0.98	3.62
			Mean ± SD	0.64 ± 0.34	0.71 ± 0.64
			Range	0.27 - 1.61	0.23 – 3.63

4. Discussion

We present a highly selective and sensitive GC-NICI-MS/MS-SRM method for quantitation of *trans, anti*-BaPT in human urine. The method is precise, accurate, yet relatively simple with only two SPE purification steps. The GC-NICI-MS/MS-SRM traces illustrated in Figure **2.4** are quite clean. The sensitivity of the method, with a quantitation limit of 0.05 fmol of *trans, anti*-BaPT/mL urine, was essential for its success. Due to its relative simplicity, this analytical method might be applicable to large numbers of samples in epidemiology studies in the future. A potentially significant advantage of this method is that it measures exposure *plus* metabolic activation of BaP, and therefore may be related to cancer risk.

A major challenge for quantitation of BaP metabolites in human urine is that their levels are extremely low compared to those of smaller PAH such as phenanthrene. The urinary levels of *trans, anti*-BaPT are, for example, approximately 10,000 times lower than those of the corresponding metabolite of phenanthrene, *r*-1,*t*-2,3-*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (*trans, anti*-PheT), which we have previously quantified in the urine of smokers (*57,117*). The substantially lower levels of *trans, anti*-BaPT in human urine compared to those of *trans, anti*-PheT results from differences in exposure, metabolism, and excretion. The level of BaP in mainstream cigarette smoke is about 30 times lower than that of phenanthrene (5-10 ng vs. 150-300 ng/cigarette) (*90,140*). BaP metabolites are mainly excreted in feces while phenanthrene metabolites are excreted mainly in urine, based on studies in laboratory animals (*141,142,170,171*). Metabolism

of phenanthrene to *trans, anti*-PheT may exceed metabolism of BaP to *trans, anti*-BaPT, but further studies are required on this point.

Only a few studies have previously described methods for the analysis of BaP metabolites in human urine. 3-Hydroxy BaP was quantified as a biomarker of exposure in industrial workers (*172,173*). Weston et al. and Bowman et al. analyzed BaPT in human urine using immuno-affinity chromatography for sample purification and synchronous fluorescence spectroscopy for quantitation (*138,139*). They reported BaPT levels of 240-3120 fmol/mL in the urine of four individuals who were highly exposed to PAHs in their diet, and 150 fmol/mL BaP-T in psoriasis patients receiving coal tar therapy. Our laboratory determined urinary *trans, anti*-BaPT by GC-NICI-MS-SIM following three steps of SPE purification, and reported 16 fmol/mL *trans, anti*-BaPT from psoriasis patients and 0.5 fmol/mL from 9 of 21 smokers, with an on-column limit of detection of 1 fmol (*117*). The lower sensitivity in that study was due to the lower sensitivity of the MS system available at that time. As shown in this paper, with the modified SPE preparation and high performance GC-NICI-MS/MS-SRM, we were able to achieve an ultra low detection limit of 20 amol, at least 50 times greater than our previous method for *trans, anti*-BaPT analysis. Thus, in contrast to our previous study, we were able to detect *trans, anti*-BaPT in 100% of the urine samples from smokers and non-smokers. In addition, the urinary *trans, anti*-BaPT level in smokers we reported here (mean 0.64 fmol/mL urine, or 0.71 fmol/mg creatinine, n = 30) is quite consistent with our previous data for the samples in which quantitation was possible (mean 0.5 fmol/mL urine, n = 9).

Our results clearly demonstrate, apparently for the first time, that cigarette smokers have significantly higher levels of *trans, anti*-BaPT, the end-product of the carcinogenic diol epoxide metabolic activation pathway of BaP, in their urine than do non-smokers. Besides higher BaP exposure in smokers from cigarettes (5-10 ng/cigarette, and mean = 19 cigarettes per day per person, in this study) (*90,140*), this difference may also result from the induction of P450s 1A1, 1A2, and 1B1, known to occur in smokers (*59*), as these enzymes are involved in producing *trans, anti*-BaPT. Non-smokers are exposed to BaP probably from polluted air and food. Specifically, our results have shown that smokers excreted approximately twice as much *trans, anti*-BaPT as did non-smokers. This is quite consistent with previous results comparing urinary metabolites from two other PAH components - pyrene and phenanthrene -between smokers and nonsmokers. Levels of 1-hydroxypyrene, a urinary metabolite of pyrene, are about twice as high in smokers' urine as in non-smokers urine, in most studies (*137,174,175*). Our laboratory has reported that smokers have about three times higher levels of urinary *trans, anti*-PheT than do non-smokers (*57*).

In this study, we quantified racemic *trans, anti*-BaPT. One enantiomer of this tetraol would be expected to arise from hydrolysis of BPDE, based on known stereoselectivity in the metabolic formation of BPDE, as illustrated in Figure **2.1** and summarized previously (*113*). The opposite enantiomer would be expected from hydrolysis of the reverse diol epoxide, BaP-(*9S,10R*)-diol-(*7R,8S*)-epoxide. Thus, racemic *trans, anti*-BaPT measured here could result from both pathways. We have previously shown that, in the urine of creosote workers exposed to relatively high levels

of BaP, 78% of *trans, anti*-BaPT results from hydrolysis of BPDE. Whether that is also the case in smokers and non-smokers, as in this study, requires further investigation.

Measurement of BPDE-DNA adducts in humans may be a more direct approach to examine BaP metabolic activation than that described here. However, methods such as immunoassays and ^{32}P postlabelling for BaP-DNA adduct have limitations, and quantitation can be unreliable (*73*). Highly sensitive analytical methods such as HPLC-fluorescence and GC-NICI-MS are quantitative, but usually detect BPDE-DNA adducts in only some human tissues and blood samples (*42*). The urinary metabolite *trans, anti*-BaPT may be a more practical biomarker because it can be reliably quantified and detected in all samples (*54,137*).

In summary, we have developed a relatively simple and ultra-sensitive method for the analysis of *trans, anti*-BaPT in human urine. Average levels of this metabolite were 0.34 fmol/mg creatinine in non-smokers, and 0.71 fmol/mg creatinine in smokers. This biomarker can potentially provide a critical component of a carcinogen metabolite phenotyping model that may eventually be used to determine individual susceptibility to PAH-induced human cancer.

Chapter 3

Immediate Consequences of Cigarette Smoking: Rapid Formation of Polycyclic Aromatic Hydrocarbon Diol Epoxides

1. Introduction

Lung cancer kills an average of 3,000 people in the world each day (*176*). An estimated 90% of this horrific toll is due to cigarette smoking, which also causes at least 18 other types of cancer (*16*). Among the multiple carcinogens and toxicants in cigarette smoke, polycyclic aromatic hydrocarbons (PAH) are widely viewed as important causative agents for lung cancer (*15,27,103*). PAH are formed in the incomplete combustion of organic matter, including tobacco, and always occur as mixtures (*25*). Some PAH are potent carcinogens, readily inducing tumors of the lung, skin, and other organs upon treatment of laboratory animals (*177*). One member of this class of compounds, benzo[*a*]pyrene (BaP), is classified as "carcinogenic to humans" by the International Agency for Research on Cancer (*25*).

PAH require metabolism to exert their mutagenic and carcinogenic effects (*177,178*). The formation of metabolites that bind covalently to DNA producing potentially mutagenic DNA adducts that can cause miscoding and permanent mutations is an accepted mechanism by which PAH initiate the carcinogenic process (*25*). Multiple studies clearly demonstrate that certain diol epoxide metabolites of PAH bind readily to DNA and are highly tumorigenic in animal models (*25,111,114,177*). Further evidence shows that diol epoxide - DNA adducts are present in human lung tissue, including that obtained from smokers, and that diol epoxides of BaP and other PAH can cause mutations in the *p53* tumor suppressor gene similar to those found in lung tumors from smokers (*27,42,76,179,180*).

Formation of diol epoxides from phenanthrene (Phe), the simplest PAH with a bay region, is illustrated in Figure **3.1** (*113,148,149,181,182*). Phe is generally considered non-carcinogenic but it has structural features similar to those of other PAH such as BaP (Figure **3.2**). The first step is cytochrome P450-catalyzed formation of an epoxide, followed by epoxide hydrolase-catalyzed hydration to yield diols such as Phe-(*1R,2R*)-diol (**1**) and Phe-(*3R,4R*)-diol (**4**) (Figure **3.1**). The diols can then undergo a second epoxidation to produce diol epoxides **2** and **5**. Previous metabolic studies of Phe have shown that both Phe bay region diol epoxide (**2**) and Phe reverse diol epoxide (**5**) are formed in humans, with the latter predominating (*113*). Phe bay region diol epoxide (**2**) is structurally analogous to the highly carcinogenic bay region diol epoxide of BaP (BPDE, Figure **3.1**), considered to be a major ultimate carcinogen of BaP (*25*). Many PAH diol epoxides react readily with DNA, but their major fate is rapid and facile reaction with H_2O, producing tetraols. In the case of Phe, tetraol enantiomers **3** and **6**, collectively termed PheT and usually quantified together, are formed (*57,82,155*). Tetraols such as PheT are excellent biomarkers for the PAH diol epoxide metabolism pathway because they cannot be formed any other way. In previous studies, we have developed methods for quantitation of PheT in human urine and plasma (*57,82,150*). PheT, which is found in all human urine samples due to environmental and dietary exposure to Phe, is present in much greater quantities than the corresponding tetraol derived from BPDE, and is therefore a more practical biomarker of PAH exposure plus metabolic activation. Levels of PheT are higher in smokers than in non-smokers (*82*).

Many studies have investigated PAH metabolism in human tissues in vitro, and some have also quantified PAH metabolites in human urine and blood as well as PAH-DNA adducts in human tissues including lung tissue from smokers (*25,42,137,178,183*). However, there are to our knowledge no reports in the literature on human PAH pharmacokinetics after exposure by inhalation, from a cigarette or from any other source. Indeed, a recent comprehensive review concludes that "most of the available data on toxicokinetic parameters for PAHs derive from studies… in animals" (*25*). In the study reported here, we used a double stable isotope labeling strategy to assess the fate of $[D_{10}]$Phe inhaled by a cigarette smoker. Smokers smoked cigarettes to which $[D_{10}]$Phe had been added, and $[D_{10}]$PheT as well as unlabelled PheT were quantified in plasma, using $[^{13}C_6]$PheT as internal standard. The results demonstrate the remarkable rapidity of Phe diol epoxide formation in smokers.

Figure **3.1** Formation of diol epoxides and tetraols in the metabolism of Phe. Three steps are required for diol epoxide formation, catalyzed sequentially by cytochrome P450s, epoxide hydrolase (EH), and cytochrome P450s.

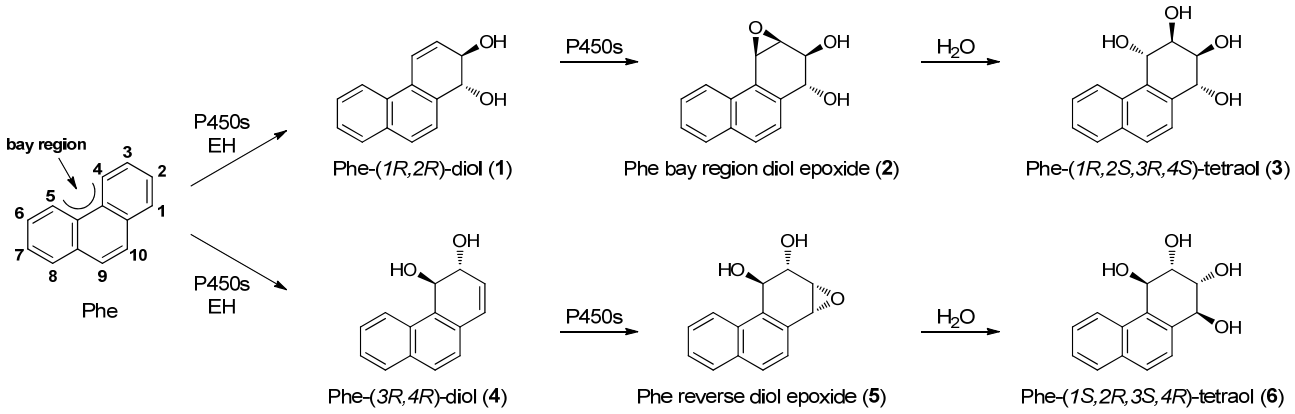
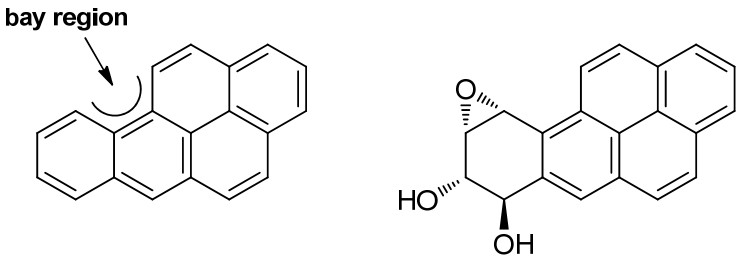


Figure **3.2** Structures of BaP and BPDE



BaP BPDE

2. Materials and Methods

Chemicals. [D_{10}]Phe (98%, containing 2% non-deuterated Phe) and [$^{13}C_6$]Phe were purchased from Cambridge Isotope Laboratories. [$^{13}C_6$]PheT was prepared by in vitro metabolism and oxidation of [$^{13}C_6$]Phe (**add – orig 427**). PheT was kindly provided by Drs. Donald M. Jerina and Haruhiko Yagi.

Purification of [D_{10}]Phe. [D_{10}]Phe was re-purified in the University of Minnesota Molecular and Cellular Therapeutics GMP facility by normal phase HPLC followed by recrystallization from ethanol, m.p. 102 °C. Its chemical purity was greater than 99% as established by normal and reverse phase HPLC, UV, MS, and ^{1}H- and ^{13}C-NMR. Its isotopic purity was 98% [D_{10}]Phe, 2% Phe, as determined by ^{1}H-NMR.

Cigarettes containing [D_{10}]Phe. Using a specially designed cigarette spiking instrument which distributes an additive uniformly along the tobacco rod, [D_{10}]Phe, dissolved in 20 μl ethanol, was added to each of 20 Marlboro cigarettes at each of the following levels (μg): 0, 25, 50, 100, and 200. The cigarettes were sent to Dr. David Ashley at the Centers for Disease Control and Prevention, Atlanta, GA, where they were smoked on a machine. Ten cigarettes at each spike level were smoked under ISO conditions (puff volume 35 ml; puff interval 60 sec; puff duration 2 sec; no vent blocking) and 10 were smoked under Health Canada "intense" conditions (puff volume 55 ml; puff interval 30 sec; puff duration 2 sec; ventilation holes blocked). Mainstream

smoke total particulate matter from 3 cigarettes was collected on one Cambridge filter pad, and the pads were sent back to the University of Minnesota, where they were analyzed by GC-MS, essentially as described (*140*), for [D_{10}]Phe, using [$^{13}C_6$]Phe as internal standard. Levels of added [D_{10}]Phe were plotted against levels of [D_{10}]Phe detected in mainstream smoke. The Health Canada intense smoking regimen was chosen for this study because it more closely approximated the subjects' natural smoking pattern.

Study Design. This study was approved by the University of Minnesota Institutional Review Board and the U.S. Food and Drug Administration. Cigarette smokers were recruited using advertisements on the radio, television or in metropolitan and campus newspapers. The specific inclusion criteria included: smoking at least 10 cigarettes daily for the past year; being in good physical health with no unstable medical condition; having stable mental health e.g. not currently experiencing unstable or untreated psychiatric diagnosis including substance abuse as determined by the DSM-IV criteria; in the case of female subjects, not pregnant or nursing.

Cigarette smokers who were interested in the study called the clinic, were informed about the study, and were screened to determine whether they met specific inclusion criteria. Subjects meeting these criteria were asked to come into the clinic for an orientation visit, to provide informed consent, and to undergo a more thorough screening. They filled out a questionnaire including information on age, gender, medical history, medication use, and smoking history including number of years of smoking, age at onset of smoking, number of cigarettes per day, and brand of cigarettes used.

Pregnancy tests were done. Subjects were informed that the study would examine ways in which their bodies metabolized toxic constituents of cigarette smoke. Subject recruitment incentives were used, with an average of $500 per subject for completing the study.

Subjects were asked to smoke the cigarette containing [D_{10}]Phe through a smoking topography device which recorded the number of puffs, puff duration, and puff volume. Subjects first underwent an adaptation trial prior to smoking the cigarette containing [D_{10}]Phe. They were given specific instructions on puff number, inter-puff interval, puff duration and puff volume to achieve the correct conditions. They then engaged in a practice session with a Marlboro cigarette. Puff volume was assessed by the topography device, and when a specific puff volume and duration were attained, the subject was given a signal to exhale.

Subjects reported to the clinic at 7:00 a.m. and were fitted with an intravenous catheter or heparin lock to allow serial blood sampling without the need for multiple venipunctures. Blood samples of 10 ml each were taken 30 min before smoking the cigarette and 15, 30, 45, 60, 90, 120, 150, 240, 360, 540 or 720, and 1440 min after the completion of smoking the cigarette. Blood samples were collected into anticoagulant-containing vacuum collection tubes and centrifuged to obtain plasma which was frozen at -20 °C until analysis.

Analysis of Plasma. The method was modified from that described previously (*150*). A mixture of 0.8 ml plasma, 3 ml saline, 12,000 units of β-glucuronidase

(recombinant from *E. coli* BL21, Sigma-Aldrich catalog # G8295), and 25 pg (100 fmol) of [$^{13}C_6$]PheT (internal standard) was incubated overnight at 37 °C, and then applied to a preconditioned Oasis MCX mixed mode cation exchange solid-phase extraction cartridge (60 mg, Waters, Mi(*58*)lford, MA). The PheT-containing fraction was eluted with 4 ml 40% CH_3OH in H_2O, concentrated to dryness, and passed through a 0.45-μm nylon filter into an HPLC vial (Whatman, Clifton, NJ) with 16.7% CH_3OH. To the vial was added 6 μg of *trans*-1,2-dihydroxynaphthalene as an HPLC UV marker (retention time: 12.5 min). The apparatus for HPLC was the same as reported before (*150*). The column was eluted with an acetonitrile/1% aq formic acid gradient at 1.0 ml/min. The gradient program was as follows (percentage 1% aq formic acid, time): 95% to 93%, 0 to 8.2 min; 93%, 8.2 to 9.5 min; 93% to 0%, 9.5 to 10 min; 0%, 10 to 13 min. The UV/Vis detector was set at 254 nm. HPLC eluant was collected for 5 min before the peak of the UV marker appeared (collection time: 7.5 – 12 min). The retention time for PheT, [D_{10}]PheT and [$^{13}C_6$]PheT was about 9 min. The collected HPLC fraction was dried, transferred to a glass insert vial with CH_3OH, dried again, and dissolved in 10 μl of *bis*-trimethylsilyltrifluoroacetamide plus 1% trimethylchlorosilane (BSTFA, Regis Technologies, Morton Grove, IL). The samples were heated to 60 °C for 1 h. Two μl were injected on GC-NICI-MS/MS using the splitless mode.

GC-NICI-MS/MS analysis was carried out with a TSQ Quantum instrument (Thermo Scientific, San Jose, CA) with a 0.25 mm (inside diameter) × 30 m, 0.15-μm film thickness, DB-17MS column (Agilent Technologies, Palo Alto, CA), and a 0.53 mm (inside diameter) × 2 m deactivated fused silica pre-column. The analytes were detected

as their trimethylsilyl derivatives. The oven temperature program was as follows: 80 °C for 1 min, then 80 to 200 °C at 35 °C/min, then 200 to 215 °C at 3 °C/min, then 215 to 320 °C at 35 °C/min, then hold for 4 min. The injector was operated in the splitless mode. The injection port temperature was 250 °C and the MS transfer line temperature was 320 °C. The flow rate was 1 ml/min He. The NICI-MS/MS conditions were as follows: CI gas, methane at 1.5 ml/min; source temperature, 200 °C; emission current, 350 μA. Selected reaction monitoring with a collision energy of 12 eV, electron energy of -150 eV, and Ar collision gas pressure of 1.0 mTorr was used to detect PheT, [$^{13}C_6$]PheT, and [D_{10}]PheT at *m/z* 372 → *m/z* 210, *m/z* 378 → *m/z* 216, and *m/z* 382 → *m/z* 220 respectively. Quadrupoles 1 and 3 were operated at a resolution of 0.7 amu.

Pharmacokinetic Analysis. Based on the concentration-time data, a noncompartmental analysis was carried out using Phoenix WinNonlin v6.1 (PharSight, Cary, NC). The area under the concentration-time curve (AUC_{0-t}) was calculated with the linear trapezoidal rule up to the last measured time point. The remainder of the AUC ($AUC_{t-\infty}$) was calculated by dividing the last measured time point by the rate constant associated with the terminal mono-exponential phase, λ. Summation of the two portions of the AUC results in $AUC_{0-\infty}$, which is a measure of the exposure of the body to [D_{10}]Phe diol epoxides, assessed by [D_{10}]PheT. The half-life associated with the terminal phase was determined by dividing 0.693 by λ. The apparent clearance (CL) was calculated by dividing the inhaled dose (10 μg [D_{10}]Phe) by the $AUC_{0-\infty}$.

3. Results

In preliminary experiments, we determined the approximate delivery of [D_{10}]Phe in mainstream smoke of cigarettes to which various amounts of [D_{10}]Phe had been added to the tobacco. These cigarettes were smoked on a machine, using either the International Organization of Standards (ISO) conditions or the "intense" conditions favored by Health Canada to evaluate smoke emissions. Plots of added [D_{10}]Phe versus mainstream smoke levels of [D_{10}]Phe were linear. Using these plots, we determined that a cigarette to which 80 μg of [D_{10}]Phe had been added delivered approximately 10 μg [D_{10}]Phe in its mainstream smoke under Canadian intense conditions. These smoking conditions were duplicated to the extent possible by each subject, by monitoring their smoking with a topography apparatus, such that the inhaled dose of [D_{10}]Phe was approximately 10 μg. Blood samples were drawn at baseline and at intervals beginning 15 min after each subject finished smoking the single cigarette, which took approximately 5 min for about 8 puffs.

For the analysis of plasma samples, [$^{13}C_6$]PheT was added as the internal standard. After enrichment steps, quantitation of PheT, [D_{10}]PheT, and [$^{13}C_6$]PheT were accomplished by gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) with selected reaction monitoring. The transition monitored for PheT was *m/z* 372 → *m/z* 210 which corresponds to loss of [$OSi(CH_3)_3$ + $Si(CH_3)_3$] from its base peak. The corresponding transitions for [D_{10}]PheT and [$^{13}C_6$]PheT were *m/z* 382 → *m/z* 220 and *m/z* 378 → *m/z* 216, respectively. Typical chromatograms from this analysis are illustrated in Figure **3.3A,B**. The analysis at

baseline, 30 min before a subject smoked the cigarette containing [D_{10}]Phe, shows peaks for PheT and the internal standard [$^{13}C_6$]PheT, but no detectable peak for [D_{10}]PheT (Figure **3.3A**). The origin of PheT, which is detected in all human urine samples, is Phe in cigarette smoke, the diet, and the general environment. The analysis of the sample collected 15 min. after the subject smoked the cigarette containing [D_{10}]Phe shows a clear peak for [D_{10}]PheT (Figure **3.3B**). Clean, readily quantifiable peaks such as these were observed in all samples. Positive and negative control samples were included with each set of analyses, and produced the expected values.

The results summarized in Table **3.1** show that levels of [D_{10}]PheT reached their maximum in each subject's plasma within 15-30 min after smoking the cigarette, and decreased thereafter. This is illustrated for one subject in Figure **3.4**, which also shows that levels of PheT remained relatively constant throughout the time period studied, as would be expected due to steady state exposure to Phe. Plots such as that in Figure **3.4** for the other 11 subjects were similar.

The pharmacokinetic results are shown in Table **3.2**. The AUC ranged from 6561 to 92155 fmol-min/ml with a terminal elimination half-life of 290-681 min. The apparent clearance ranged from 577 – 8108 ml/min.

Figure **3.3** GC-NICI-MS/MS-SRM analysis of samples containing Phe and [D_{10}]Phe metabolites isolated from plasma of smokers: (A) 30 min prior to smoking a cigarette to which [D_{10}]Phe had been added; and (B) 15 min after smoking that cigarette. The transitions illustrated were monitored to analyze for PheT and [D_{10}]PheT in plasma, and internal standard [$^{13}C_6$]PheT which was added to all plasma samples. Shaded peaks correspond to the target analytes. PheT is present in all samples due to exposure of the smoker to Phe from cigarette smoke and environmental or dietary sources. [D_{10}]PheT is present only after smoking the cigarette containing [D_{10}]Phe. The internal standard for the analysis is [$^{13}C_6$]PheT.

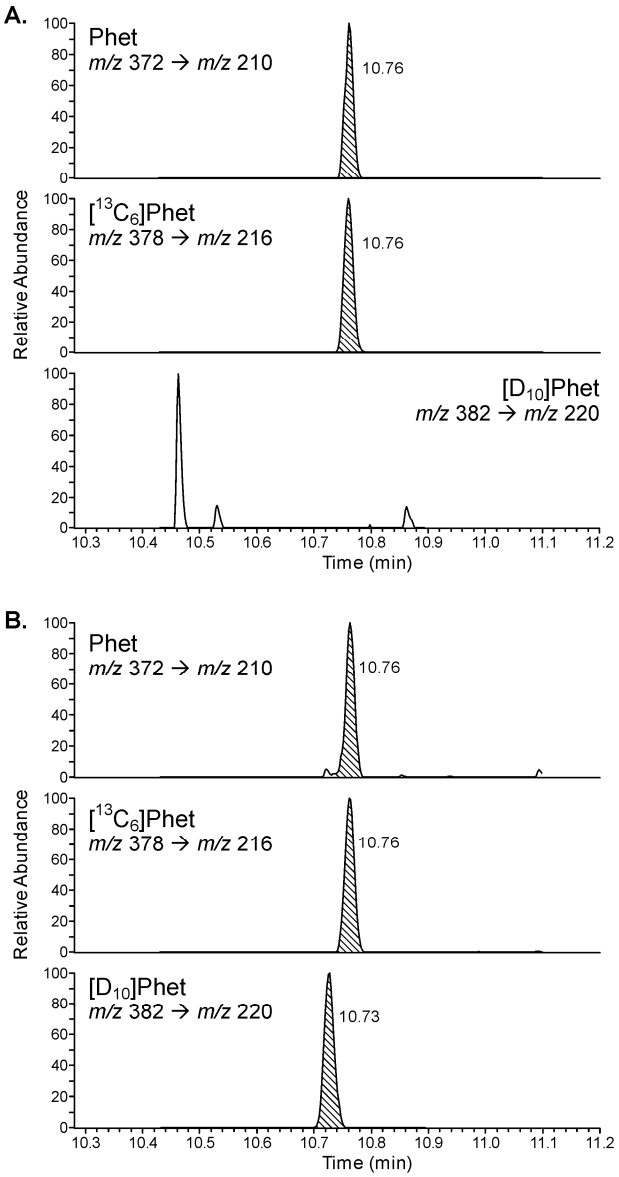


Figure **3.4** Levels of [D_{10}]PheT (closed triangles) and PheT (open circles) in the plasma of subject 1 at various intervals after smoking a cigarette containing [D_{10}]Phe.

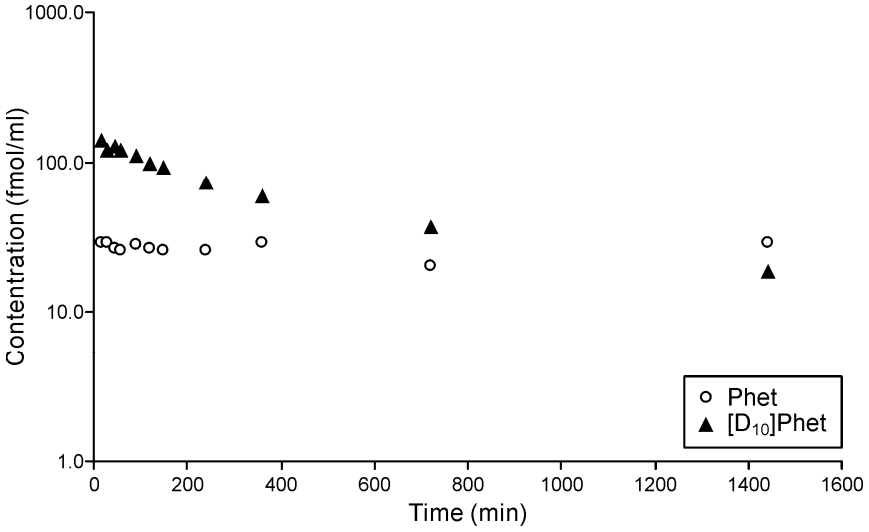


Table **3.1**

Levels of [D_{10}]PheT in plasma of 12 subjects (S1-S12) at various times after smoking a cigarette containing [D_{10}]Phe.

Time point (min)	[D_{10}]PheT (fmol/ml plasma)											
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**
-30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	142.0	11.0	70.2	48.1	76.1	203.5	50.3	62.1	134.5	44.9	67.6	44.9
30	121.0	8.8	64.4	62.4	76.6	190.4	39.5	56.5	142.3	48.6	70.8	38.4
45	127.3	9.4	67.3	42.0	64.5	127.9	37.4	56.9	122.7	36.0	60.4	39.8
60	120.4	8.3	63.3	36.8	61.1	158.0	43.8	51.0	98.3	40.9	72.8	38.9
90	110.9	7.9	55.5	36.2	51.7	136.8	39.4	40.7	88.5	49.1	52.2	37.5
120	99.0	8.5	57.7	34.0	53.2	116.7	42.6	50.8	64.1	43.5	54.3	32.8
150	91.2	7.5	57.1	33.4	48.5	105.7	48.0	35.9	63.1	43.3	42.8	31.4
240	73.8	6.2	50.6	24.4	N/A[a]	89.5	37.9	32.9	51.4	36.0	41.0	27.5
360	59.3	5.8	44.5	20.1	27.5	66.6	20.3	19.6	38.3	27.0	39.5	26.9
540 or 720	36.4	3.1	30.1	8.6	12.6	43.5	23.2	16.9	12.1	20.6[b]	29.8[b]	18.8[b]
1440	18.4	1.2	14.9	3.8	ND[c]	16.1	5.8	4.1	3.2	3.4	3.3	4.9

a. N/A, Not available
b. 540 min
c. ND, not detected, detection limit 0.05 fmol/ml.

Table **3.2**

Pharmacokinetics parameters of [D_{10}]PheT in 12 subjects after smoking a cigarette containing 10 µg (53.2 nmol) [D_{10}]Phe

	AUC (min*fmol/ml)	Terminal half-life (min)	CL/F (ml/min)
Subject 1	85900	651	619
Subject 2	6560	489	8110
Subject 3	63500	681	837
Subject 4	22800	402	2330
Subject 5	29000	290	1840
Subject 6	92200	523	577
Subject 7	35700	483	1490
Subject 8	29700	385	1790
Subject 9	40200	295	1320
Subject 10	30500	354	1750
Subject 11	39600	295	1340
Subject 12	29200	447	1830
Mean	**42100**	**441**	**1990**
S.D.	25600	132	2000

4. Discussion

This study is unique. It is the first to investigate human metabolism of a PAH specifically delivered by inhalation in cigarette smoke, without interference by other sources of exposure such as air pollution or the diet. The use of cigarettes containing [D_{10}]Phe made this possible.

Our results clearly demonstrate that the formation of Phe diol epoxides occurs rapidly in smokers. The biomarker [D_{10}]PheT, which results from hydrolysis of Phe diol epoxides, was readily detected in all plasma samples after subjects smoked a cigarette containing [D_{10}]Phe, and its levels were maximal 15-30 min after the subjects finished smoking the cigarette. These results are significant because PAH diol epoxides react readily with DNA, induce mutations, and are considered to be ultimate carcinogens of multiple PAH in cigarette smoke (*25*).

While previous studies indicate that PAH diol epoxides are formed in humans and that their levels may be higher in smokers than in non-smokers, their rates of formation in smokers were unknown (*42,82,113*). As shown in Figure **3.1**, diol epoxide formation requires three steps catalyzed by P450s and epoxide hydrolase. Multiple competing reactions such as glutathione-*S*-transferase-catalyzed detoxification of the initially formed epoxides, or glucuronidation of the diol metabolites could retard or even prevent diol epoxide formation (*178*). Our results suggest that these competing reactions have relatively little impact on rates of diol epoxide formation.

One striking finding is the large variability in the exposure of the subjects to [D_{10}]Phe diol epoxides. Subjects 2 and 4 had the lowest exposures (as measured by the

AUC $_{0-\infty}$) which resulted in very high apparent clearances, indicating that the mechanisms which remove [D_{10}]PheT from the body are relatively efficient. On the other hand, Subjects 1 and 6 had very high AUCs, indicating that these subjects have greater overall exposure to [D_{10}]Phe diol epoxides. It is unlikely that this wide range of exposure is due to differences in dosing, considering the sophisticated and well-monitored means by which the cigarettes were smoked. It is much more likely that the difference in exposures is due to the subjects' relative abilities to form and eliminate the metabolites of Phe.

Stable isotope labeling has been used previously in studies of nicotine and cotinine metabolism in tobacco users (*184*). These studies have defined pharmacokinetic aspects of nicotine metabolism and have probed the utility of metabolic ratios in predicting nicotine dependence in smokers. A deuterium labeled tobacco-specific nitrosamine has also been added to cigarettes to investigate its metabolic products in smokers (*185*). However, no previous studies have examined PAH metabolism in smokers using a deuterated probe compound.

The results reported here should serve as a stark warning to those who are considering starting to smoke cigarettes: PAH diol epoxide formation occurs immediately and is not a theoretical long term effect. PAH diol epoxides are DNA damaging compounds which can cause genetic damage and initiate the carcinogenic process.

This study has some limitations. We used [D_{10}]Phe as a representative PAH because Phe is generally considered non-carcinogenic. Addition of a carcinogenic PAH to a cigarette would be potentially hazardous and possibly unethical. There are some potential differences in the metabolism and toxicokinetics of Phe versus other PAH that

could affect the interpretation of our results. First, metabolism of Phe by the angular ring diol epoxide pathway occurs mainly via Phe reverse diol epoxide (**5**) in humans and to a much smaller extent by Phe bay region diol epoxide (**2**)(Figure **3.1**)(*113*), although our unpublished data indicate that measurements of PheT and diol epoxide **2** specifically are highly correlated. It is the bay region diol epoxide which, strictly speaking, would represent the potentially carcinogenic pathway. Second, the rates of tissue distribution and metabolism of Phe and higher molecular weight carcinogenic PAH such as BaP might be quite different, as highly lipophilic PAH such as BaP diffuse slowly into tissues (*25*). Thus, the results for Phe might not be generalizable to higher molecular weight PAH.

In summary, we demonstrate that the formation of Phe diol epoxides, as represented by the biomarker PheT, is rapid in smokers. These results provide the first direct evidence for human metabolic activation of a PAH delivered in cigarette smoke and indicate the potential for immediate genetic damage in a cigarette smoker.

Chapter 4

Metabolism of [D_{10}]Phenanthrene to Tetraols in Smokers for Potential Lung Cancer Susceptibility Assessment: Comparison of Oral and Inhalation Routes of Administration

1. Introduction

Lung cancer kills more than 3000 people in the world each day (*4*). About 90% of lung cancer mortality is due to cigarette smoking, entailing exposure to more than 70 established carcinogens (*4,15,16*). Polycyclic aromatic hydrocarbons (PAHs) are among the strongest carcinogens in cigarette smoke, and are believed to be major causes of lung cancer in smokers (*15*). Benzo[*a*]pyrene (BaP), a representative PAH, is "carcinogenic to humans" according to the International Agency for Research on Cancer (*25*).

PAH require metabolic activation for carcinogenicity (*114,177*). One important pathway proceeds through diol epoxides, which react with DNA producing adducts, causing permanent mutations and initiating carcinogenesis (*114,177*). BaP diol epoxide-DNA adducts are present in smokers' lung tissue (*76*). This pathway includes initial epoxide formation catalyzed by cytochrome P450s, hydration catalyzed by epoxide hydrolase, and further oxidation by P450s. The resulting diol epoxides react with DNA, but even more readily with H_2O, producing tetraols (*114*). These tetraols may be excellent biomarkers for PAH metabolic activation (*149*). Competing with this metabolic activation pathway, oxidation to phenols is a representative detoxification pathway (*81,114*). Detoxification by glutathione conjugation, glucuronidation, and sulfation of PAH metabolites are also observed (*114,178*).

Figure **4.1** illustrates the diol epoxide pathways of phenanthrene (Phe), the simplest PAH with a bay region, a feature closely associated with carcinogenicity. Phe is generally considered non-carcinogenic, but its metabolism to diol epoxides closely parallels that of BaP (*147-149*). Our group has developed methods for quantitation of

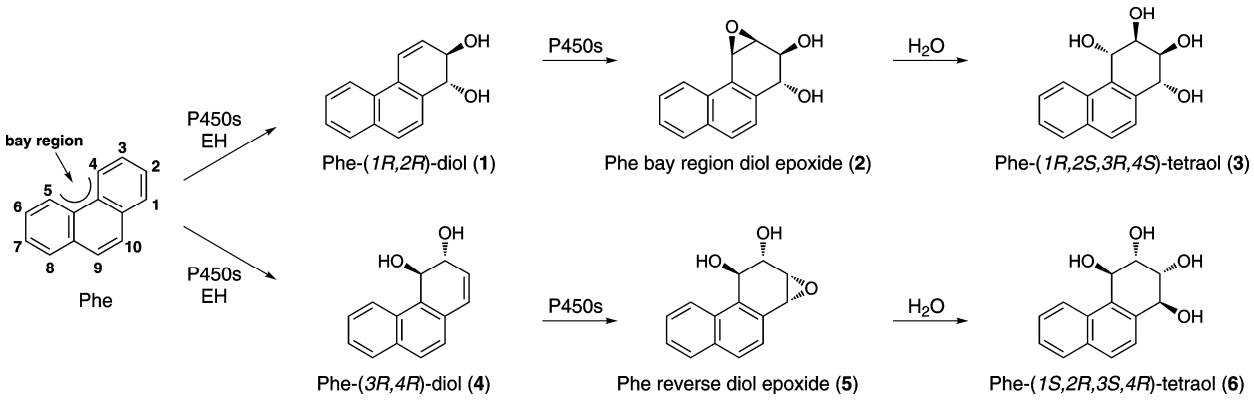
total *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT, the sum of Phe-(1*R*,2*S*,3*R*,4*S*)-tetraol (**3**) and Phe-(1*S*,2*R*,3*S*,4*R*)tetraol (**6**) in Figure **4.1**) in human urine and plasma (*57,150*). We have demonstrated correlations of urinary PheT with its bay region diol epoxide-derived enantiomer **3** and with the corresponding tetraol derived from BaP (*156*). Since PheT is found in all human urine samples in much greater quantities than tetraols derived from carcinogenic PAH (*57,58*), we have proposed that PheT could be an indicator of PAH exposure plus metabolic activation by the bay region diol epoxide pathway.

Approximately 11% - 24% of lifelong smokers develop lung cancer over their lifetimes (*4*). The identification of smokers with higher cancer susceptibility is a challenging and worthwhile goal, as they could be targeted for lung cancer prevention activities and early detection. Our hypothesis is that individuals who metabolically activate tobacco smoke carcinogens more extensively will be at higher risk for cancer (*84*). Induction of P450s 1A1, 1A2, and 1B1 is apparently related to higher lung cancer risk in smokers (*121,123*). Polymorphisms in genes involved in PAH metabolism may also influence lung cancer risk, but the results of these studies are inconsistent (*121,125,134,169*). We propose that carcinogen metabolite phenotyping has the potential to specifically identify those individuals at high risk. The approach described in this study is unique, involving administration of the stable isotope labeled compound $[D_{10}]$Phe. The use of $[D_{10}]$Phe allows us to quantify individual differences in metabolism, without complication by environmental exposure to Phe, which is ubiquitous. In the study reported here, we compared levels of $[D_{10}]$PheT in the plasma

and urine of smokers who were given [D_{10}]Phe either by inhalation in cigarette smoke or by oral administration. This study was crucial for developing a practical yet accurate route of administration for use in potentially large phenotyping studies. A primary objective was to determine and compare the pharmacokinetics of [D_{10}]PheT following the two routes of exposure. A preliminary report of plasma levels of [D_{10}]PheT in smokers given [D_{10}]Phe by inhalation has been published(*186*).

The results of the study reported here reveal, for the first time, the comparative metabolic activation profiles of a bay-region PAH administered by different routes to humans and provide direct evidence for significant inter-individual variation in PAH diol epoxide metabolism in smokers.

Figure **4.1** Formation of PheT through diol epoxide pathways in the metabolism of Phe. Three steps are required for diol epoxide formation, catalyzed sequentially by cytochrome P450s, epoxide hydrolase (EH), and cytochrome P450s.



2. Materials and Methods

Chemicals, Enzymes, and Chromatography Supplies. [D_{10}]Phe (98%) was purchased from Cambridge Isotope Laboratories (Cambridge, MA). [D_{10}]Phe for oral dosing was re-purified as described before in the University of Minnesota Molecular and Cellular Therapeutics GMP facility by normal phase HPLC followed by recrystallization from ethanol. It had >99% chemical purity and 98% isotopic purity (*186*). All PheT used in this study was racemic. PheT was kindly supplied by Drs. Donald M. Jerina and Haruhiko Yagi, National Institutes of Health (Bethesda, MD). [$^{13}C_6$]PheT was prepared by hydrolysis in 50/50 THF/H_2O of [$^{13}C_6$]Phe(1*R*,2*S*)diol-(3*S*,4*R*)epoxide (*152*). [D_{10}]PheT was prepared by *in vitro* metabolism of [D_{10}]Phe, obtained from Cambridge Isotope Laboratories, as described before (*150*). 1-Hydroxyphenanthrene (1-HOPhe, synthesized), 2-HOPhe (synthesized), 3-HOPhe (Chiron AS, Trondheim, Norway), 4-HOPhe (Chiron), and 9-HOPhe (Sigma-Aldrich, St. Louis, MO) were either synthesized by Jerina and Yagi or purchased. [$^{13}C_6$]3-HOPhe was procured from Cambridge Isotope Laboratories (Cambridge, MA). 2,7-Dihydroxynaphthalene was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All stock solutions were prepared in acetonitrile.

β-Glucuronidase and arylsulfatase (from *Helix pomatia*, catalog no. 127-698) were obtained from Roche Diagnostics Corp. (Indianapolis, IN). *bis*-Trimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane was purchased from Regis Technologies, Inc. (Morton Grove, IL). Strata-X polymeric SPE cartridges (200 mg/6 mL, #8B-S10-FCH) were obtained from Phenomenex (Torrance, CA). ChemElut diatomaceous earth partitioning cartridges (5 mL) were procured from

Varian, Inc. (Walnut Creek, CA). Blue rayon was purchased from MP Biomedicals (5X5 GM, catalog no. 808688) and 100 mg was placed in an empty 6 mL Bond Elute solid phase extraction cartridge (Varian) between two polyethylene frits. Oasis MCX mixed mode cation exchange solid-phase extraction cartridges (60 mg) were purchased from Waters, Inc (Milford, MA). Envi-Chrom P SPE columns (styrene-divinylbenzene copolymer resin, Supelclean Envi Chrom P: 0.1 g) were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Oral doses and cigarettes containing [D_{10}]Phe. [D_{10}]Phe oral dose solutions were prepared by dissolving 4 mg of purified [D_{10}]Phe in 400 mL of 100% ethanol and delivering 1 mL aliquots of the solution into amber dosing bottles in the Fairview Compounding Pharmacy (Minneapolis, MN), such that each bottle contained 10 μg of [D_{10}]Phe. All oral doses were stored at 4 ºC before administration. On the day of dosing, 4 mL of drinking water was added to the amber dosing bottle which was swirled to achieve a composition of 20% ethanol (this was necessary because storage in 20% ethanolic H_2O resulted in decreased concentrations of [D_{10}]Phe). Cigarettes containing [D_{10}]Phe were prepared to deliver approximately 10 μg [D_{10}]Phe in the mainstream smoke as described before (*186*). Before giving to a smoker, the spiked cigarette was conditioned in a humidity chamber at 60% relative humidity for 1-2 days.

Subject recruiting and screening. This study was approved by the U.S. Food and Drug Administration and the University of Minnesota Institutional Review Board.

Cigarette smokers were recruited and screened as described before (*186*). The specific inclusion criteria were: smoking at least 10 cigarettes daily for the past year; and being in good physical and mental health. Female subjects who were pregnant or nursing were excluded.

Eligible subjects were invited into the clinic for an orientation visit and to sign an informed consent. A questionnaire including information on age, gender, medical history, medication use, and smoking history including number of years of smoking, age at onset of smoking, number of cigarettes per day, and brand of cigarettes used was completed. Blood pressure, heart rate, height, weight, and carbon monoxide (CO) level were measured. Pregnancy tests were done. Selected subjects were then asked to complete a brief physical exam. Laboratory tests including blood counts, and liver and kidney function were performed to verify health status. Sixteen eligible subjects were each scheduled to attend two sessions.

Study design. This study was a randomized, open-label, single-dose, nonblinded, within-subject crossover study. Sixteen subjects received 10 μg [D_{10}]Phe orally or by smoking a cigarette containing [D_{10}]Phe. The order of administration was randomized and each dose was separated by a washout period of at least one week. For the oral dose, each subject was given 10 μg [D_{10}]Phe in 5 mL of 20% ethanol in water, as described above. The dosing bottle was rinsed twice with water, and the rinsings administered to ensure that the subjects consumed the entire dose. Administration via cigarette smoking was carried out as described before (*186*). Briefly, subjects were asked to smoke the

cigarette containing [D_{10}]Phe through a CReSS desktop smoking topography device (Plowshare Technologies) which recorded the number of puffs, puff duration, and puff volume, as employed in the Health Canada intense smoking conditions, resulting in delivery of approximately 10 μg [D_{10}]Phe in mainstream smoke.

The clinical visit procedure was essentially identical to that described before (*186*). Blood samples of 10 mL each were taken prior to dosing and 15, 30, 45, 60, 90, 120, 150, 240, 360, 540 or 720, and 1440 min after the completion of administration. Blood samples were collected into EDTA-containing vacuum collection tubes and centrifuged to obtain plasma which was frozen at -20 °C until analysis. Urine collections were obtained pre-dosing and at the following intervals post-dosing: 0-30, 30-60, 60-120, 120-360, 360-720, 720-1440, 1440-2880 min. The volume of each urine collection was measured and an aliquot of 50 mL was frozen at -20 °C until analysis. Adverse effect reports, blood pressure, and heart rate were taken several times during the inpatient stay. Subjects were asked not to smoke during the stay and remain abstinent until after the 24 hr blood draw the next morning.

Analysis of [D_{10}]PheT in urine and plasma. [D_{10}]PheT in urine and plasma were analyzed by gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS) as described previously, without the resolution of enantiomers (*150,186*).

Analysis of total [D_9]HOPhe in urine. The procedure was modified from a previous study (*81*). A deuterium is lost in the metabolism of [D_{10}]Phe to phenols;

therefore the analyte is [D_9]HOPhe**.** A 3 mL aliquot of urine was placed in a 10 mL centrifuge tube containing 970 μL of NaOAc buffer (0.5 M, pH 5), β-glucuronidase (2,000 units) and arylsulfatase (16,000 units). [$^{13}C_6$]3-HOPhe (1 ng) in 10 μL CH_3CN was added as internal standard. The mixture was incubated in a water bath overnight with shaking at 37 °C. The sample was loaded directly on a ChemElut cartridge and allowed to stand for 2 min. The HOPhe were eluted with three 8 mL portions of toluene into a 50 mL centrifuge tube, to which was added 5 mL of H_2O. The toluene was removed on a Speedvac for about 3.5 hr. Methanol (0.5 mL) was added to the aqueous solution. A blue rayon solid phase extraction cartridge (100 mg) was preconditioned with 5 mL of CH_3OH, then with 5 mL of H_2O. The sample was applied dropwise to the cartridge, along with 2 mL H_2O washings of the urine sample tubes. The cartridge was washed with 7 mL of CH_3OH/ H_2O (25:75), which was discarded. Phenanthrols were then eluted with 8 mL CH_3OH. This fraction contained the analyte and internal standard. The solution was concentrated to dryness on a Speedvac overnight in the dark. The residue was dissolved in 200 μL of CH_3OH with sonication and vortexing, transferred to a 0.1 mL insert vial, and concentrated to dryness. To this residue, 9 μL BSTFA and 0.6 ng 1-hydroxybenz[*a*]anthracene (external standard) in 3 μL CH_3CN were added. The samples were heated at 60 °C for 60 min with periodic mixing, and 1 μL was injected into the gas chromatography-electron impact-tandem mass spectrometry (GC-EI-MS/MS) system.

GC-EI-MS/MS was carried out with a TSQ Quantum instrument (Thermo Scientific, San Jose, CA). The GC was fitted with a 0.25 mm (inside diameter) × 30 m,

0.15-μm film thickness, DB-17MS column (Agilent Technologies, Palo Alto, CA), and a 0.53 mm (inside diameter) × 2 m deactivated fused silica pre-column. The oven temperature program was as follows: 80 °C for 2 min, then 80 to 225 °C at 25 °C/min, then hold for 5 min, then 225 to 310 °C at 25 °C/min, then hold for 2 min. The injector was operated in the splitless mode at 250 °C and the MS transfer line temperature was 310 °C. The flow rate was 1 mL/min He. The EI-MS/MS conditions were as follows: source temperature, 200 °C; emission current, 110 μA. Selected reaction monitoring (SRM) with a collision energy of 14 eV, electron energy of -55 eV, and Ar collision gas pressure of 1.0 mTorr was used to detect HOPhe-tetratrimethylsilyl ether (HOPhe-TMS), [$^{13}C_6$]3-HOPhe-TMS, and [D_9]HOPhe-TMS at *m/z* 266.1 → *m/z* 251.1, *m/z* 272.1 → *m/z* 257.1 and *m/z* 275.1 → *m/z* 260.1, respectively. External standard 1-hydroxybenz[*a*])anthracene was detected at *m/z* 316.1 → *m/z* 301.1.

Analysis of unchanged [D_{10}]Phe in plasma and urine. [D_{10}]Phe analysis was performed essentially as described, with modifications (*187*). A 5 mL aliquot of urine or 1 mL aliquot of plasma was placed in a 10 mL centrifuge tube containing 50:50 (vol/vol) of cyclohexane/ethyl acetate and [$^{13}C_6$]Phe (1 ng) as internal standard. After 30 min gently shaking followed by 20 min centrifugation (2000 g at 15 °C), the supernatant was collected and concentrated to dryness, and 600 μL cyclohexane was added. An Envi-Chrom P solid-phase extraction (styrene-divinylbenzene copolymer resin, Envi Chrom P: 0.1 g) column was conditioned with 1 mL of H_2O, 1 mL of methanol and twice with 1 mL of cyclohexane. The sample was applied to the column, which was then washed

with 600 μL of cyclohexane, then with 1.8 mL of cyclohexane:ethyl acetate (50:50; vol/vol) to elute [D_{10}]Phe. The solution was evaporated to dryness (at 37 °C using a stream of N_2), and 100 μL of cyclohexane was added and mixed by vortexing at ambient temperature. After centrifugation, the sample was transferred into a 0.1 mL insert vial, and concentrated to dryness on a Speedvac. Twenty μL of cyclohexane was added to the residue before GC-EI-MS/MS analysis.

GC-EI-MS/MS quantitation for [D_{10}]Phe was performed on a TSQ Quantum instrument (Thermo Scientific, San Jose, CA) with a 0.25 mm (inside diameter) x 30 m, 0.15 μm film thickness, DB-17MS column (Agilent Technologies, Palo Alto, CA). The splitless injector was maintained at 250 °C, and the MS transfer line temperature was 320 °C. The EI-MS/MS conditions were as follows: source temperature, 220 °C; emission current, 500 μA. Selected reaction monitoring (SRM) with a collision energy of 18 eV, electron energy of -70 eV, and Ar collision gas pressure of 1.2 mTorr were used to detect Phe, [$^{13}C_6$]Phe, and [D_{10}]Phe at *m/z* 178.1 → *m/z* 152.1, *m/z* 184.1 → *m/z* 158.1 and *m/z* 188.1 → *m/z* 160.1, respectively.

The recovery of internal standard was 55% in plasma and 80% in urine. The limit of quantification was 5 pg / mL urine or 25 pg / mL plasma.

Analysis of [D_{10}]*N*-acetyl-*S*-(*r*-4,*t*-2,3-trihydroxy-1,2,3,4-tetrahydro-*c*-1-phenanthryl)-L-cysteine (*anti*-[D_{10}]PheDE-1-NAC) in urine. This was carried out as previously described (*152*).

Determination of urinary creatinine. The creatinine concentration in each urine aliquot was determined at the University of Minnesota Medical Center, Fairview, Diagnostic Laboratories, using Vitros CREA slides.

Pharmacokinetic Analysis. Pharmacokinetics parameters were calculated using non-compartmental methods with Phoenix WinNonlin v6.1 (PharSight, Cary, NC). The trapezoidal rule was used to calculate the area under the concentration-time curve (AUC_{0-t}). The remainder of the AUC ($AUC_{t-\infty}$) was calculated by dividing the last measured time point by the rate constant associated with the terminal mono-exponential phase, λ. $AUC_{0-\infty}$, was estimated by combining the two above AUC portions. The half-life associated with the terminal phase was determined by dividing 0.693 by λ.

Statistical Analysis. Ninety percent confidence intervals (CI) for the geometric means of ratios of pharmacokinetic parameters (plasma $AUC_{0-\infty}$ and total urinary excretion) were calculated and evaluated using the paired t-test and Pearson correlation coefficients. Grubb's test was used to test for outliers. Two-way ANOVA was used to determine the effects of route of administration and type of sampling on the elimination half-life of $[D_{10}]$PheT.

3. Results

Sixteen subjects (6 males) were enrolled in the single-dose (10 μg [D_{10}]Phe) randomized crossover study. The mean age (± S.D.) was 37.9 ± 10.1 years, with a mean weight (± S.D.) of 85.0 ± 17.0 kg. Nine were Caucasian (1 male), 6 were African American (4 male), and 1 was of mixed race (male). All subjects received the dose of 10 μg [D_{10}]Phe orally or though cigarette smoking in two separate visits with at least a one week washout period in between. Smoking conditions were monitored by a topography apparatus so that the inhaled dose of [D_{10}]Phe was approximately 10 μg (*186*). Blood samples were drawn at baseline before dosing, and at a series of time points from 15 min until 24 hr after dosing. Urine samples were collected at baseline and at intervals during a 48 hr post-dose period. All subjects completed the entire protocol, and did not experience any significant adverse effects.

Our major endpoint was [D_{10}]PheT, a biomarker of the diol epoxide metabolism pathway of [D_{10}]Phe. Quantitation of plasma and urinary [D_{10}]PheT were accomplished by GC-NICI-MS/MS with selected reaction monitoring using [$^{13}C_6$]PheT as the internal standard as reported before (*150,186*). The transitions monitored for PheT, [D_{10}]PheT and [$^{13}C_6$]PheT were *m/z* 372 → *m/z* 210, *m/z* 382 → *m/z* 220 and *m/z* 378 → *m/z* 216, respectively. The analysis of baseline samples showed peaks for natural PheT and the internal standard [$^{13}C_6$]PheT, but no detectable peak for [D_{10}]PheT. Natural PheT arises from exposure to Phe in cigarette smoke, the diet, and the environment. Clean, readily quantifiable peaks for [D_{10}]PheT were observed as early as the first time points after both oral and smoking doses in all subjects: at 15 min post-dose for plasma samples and 0-30

min interval for urine samples. Positive and negative control samples produced the expected values.

Typical plasma concentration-time profiles following a single dose of 10 μg $[D_{10}]$Phe administrated orally or through cigarette smoking are illustrated in Figure **4.2**. In order to determine whether the route of administration affected the extent of $[D_{10}]$PheT formation, ratios ($[D_{10}]$PheT from oral dose divided by $[D_{10}]$PheT from smoking dose for each subject) of plasma $AUC_{0\text{-}\infty}$ and amount excreted in 48 hr urine were calculated. The results are summarized in Table **4.1**. Subject 7, with ratios of 10.06 and 3.05, was determined to be an outlier (*** $p < 0.001$). The ratios in the other 15 subjects were 1.03 ± 0.32 (90% CI 0.84-1.14) and 1.02±0.35 (0.83-1.13), for plasma $AUC_{0\text{-}\infty}$ and urinary excretion, respectively, showing that excretion of $[D_{10}]$PheT after administration of $[D_{10}]$Phe either orally or in a cigarette was the same. Furthermore, a two-sided paired t-test of all subjects comparing oral and smoking doses indicated that there was no statistically significant difference in either plasma $AUC_{0\text{-}\infty}$ or the total urinary excretion of $[D_{10}]$PheT between the two routes of administration ($p = 0.42$ and 0.51, respectively). Figure **4.3** shows plasma $AUC_{0\text{-}\infty}$ of $[D_{10}]$PheT and the total urinary excretion of $[D_{10}]$PheT over 48hr following the two routes of administration for 16 subjects, ranking from low to high by the level of $[D_{10}]$PheT after smoking administration.

Plasma levels of $[D_{10}]$PheT after smoking doses were maximum at early time points examined in all subjects, 15-30 min after smoking the cigarette containing $[D_{10}]$Phe, and decreased thereafter, as we have published previously for 12 of these subjects (*186*). Oral administration produced delayed maximum blood concentrations of

[D_{10}]PheT, from 30 min to 6 hr post-dose (Figure **4.2**). However, there was no significant difference in the elimination half-life of [D_{10}]PheT, determined by the slope of the terminal phase of the plasma concentration-time profile and the urinary excretion rate-time profile, between the two routes of administration ($p = 0.43$). The mean elimination half-lives (± S.D.) of [D_{10}]PheT in 16 subjects determined by the plasma concentration-time profiles were 8.93 ± 5.40 hr after the oral dose, and 7.69 ± 1.99 hr after the smoking dose. The mean elimination half-lives (± S.D.) determined by urinary excretion rate-time profiles were 7.78 ± 1.85 hr after the oral dose, and 7.95 ± 1.04 hr after the smoking dose.

The plasma concentration-time profile provides a direct measurement of systemic exposure to [D_{10}]PheT. However, a measurement of the urinary level of [D_{10}]PheT will be preferred for future phenotyping clinical studies as it is non-invasive and simpler. We thus examined whether total urinary excretion can substitute for plasma $AUC_{0\text{-}\infty}$ to predict individual metabolic activation. The results demonstrated that total 48-hr urinary excretion of [D_{10}]PheT as percent of dose administrated was highly correlated with plasma $AUC_{0\text{-}\infty}$ of [D_{10}]PheT, either after smoking or oral dose (r = 0.95 and 0.81, respectively), as illustrated in Figure **4.4**. Furthermore, 2-hr and 6-hr post-dose amounts of [D_{10}]PheT excreted in urine were significantly correlated with the 48-hr urinary excretion (r = 0.83 and 0.95, respectively, *** $p < 0.001$), as shown in Figure **4.5**.

We observed a large interindividual variation among the 16 subjects in their capacity to metabolize [D_{10}]Phe to [D_{10}]PheT. Amounts of urinary [D_{10}]PheT excreted in 48 hr ranged from 0.54% to 12.0% of the dose after oral dosing, and from 0.74% to

15.1% after smoking dosing (Figure **4.3B**). These results show that the extent of metabolism of [D_{10}]Phe by the diol epoxide pathways varies by as much as 20 fold among 16 smokers after both routes of administration. There was a large interindividual variation in natural urinary PheT in these 16 smokers as well, ranging from 0.17 – 5.03 pmol/mg creatinine. However, there was a weak correlation between the urinary excretion of [D_{10}]PheT (% of dose) and urinary level of natural PheT ($r = 0.42$, * $p < 0.05$), probably because the PheT level is also affected by Phe exposure.

The PheT:HOPhe ratio in urine has been used as a measurement of an individual's capacity to metabolize Phe, since this ratio presumably would not be influenced by variations in Phe exposure (*82*). We further analyzed the level of urinary total HOPhe and total [D_9]HOPhe in 3 subjects who had the highest levels of urinary excretion of [D_{10}]PheT and 3 with the lowest levels. PheT and HOPhe were detected in all samples, and remained relatively constant throughout the 48 hr period. Table **4.2** summarizes the levels of total 48 hr urinary excretion of [D_{10}]PheT, total [D_9]HOPhe and their ratios after oral and smoking dosing, compared with the PheT:HOPhe ratios in these 6 subjects' urine samples. Levels of [D_{10}]PheT were not correlated with the ratios of [D_{10}]PheT: [D_9]HOPhe ($r = 0.52$, $N = 12$, $p = 0.08$). However, there was a statistically significant correlation of [D_{10}]PheT with PheT:HOPhe ratios ($r = 0.71$, $N = 12$, ** $p < 0.01$). The ratios [D_{10}]PheT: [D_9]HOPhe and PheT:HOPhe were strongly correlated ($r = 0.91$, $N = 12$, *** $p < 0.001$), as illustrated in Figure **4.6**. The ratios [D_{10}]PheT: [D_9]HO-Phe among individuals showed a 50-100 fold spread, much greater than the variation in [D_{10}]PheT and PheT:HOPhe ratios.

Table **4.2** shows that the group including subjects 1, 6, and 14 who formed the largest amounts of [D_{10}]PheT excreted similar amounts of [D_9]HOPhe as did subjects 12, 7, and 2 who excreted the lowest levels of [D_{10}]PheT. To further investigate the reason for the interindividual variation in [D_{10}]PheT formation between these two groups, we analyzed plasma and urinary levels of [D_{10}]Phe and urinary levels of *anti*-[D_{10}]PheDE-1-NAC in these 6 subjects. Levels of [D_{10}]Phe in both plasma and urine samples were below our limit of quantitation (5 pg / mL), which suggests that unmetabolized [D_{10}]Phe comprised less than 0.2% of the dose in all six subjects. There was no significant difference in the 24 hr urinary excretion of *anti*-[D_{10}]PheDE-1-NAC between the two groups: 0.018 ± 0.021% of the dose for the group forming higher amounts of [D_{10}]PheT (subjects. 1, 6, and 14) and 0.020 ± 0.021% of the dose for the lower [D_{10}]PheT forming group (subjects 12, 7, and 2). Collectively, these results demonstrate that the difference in [D_{10}]PheT excretion between the high excretion group (subjects 1, 6, and 14) versus the low excretion group (subjects 12, 7, and 2) was not due to differences in extents of formation of other metabolites (e.g. [D_9]HOPhe or *anti*-[D_{10}]PheDE-1-NAC) or to differences in unmetabolized [D_{10}]Phe.

Figure **4.2** (A) Plasma concentration-time plots for [D_{10}]PheT in subject 3 after smoking a cigarette containing [D_{10}]Phe (closed triangles) or receiving an oral dose of [D_{10}]Phe (open circles); (B) Urinary excretion rate-time plots for [D_{10}]PheT in subject 3 after smoking dose (closed triangles) or the oral dose (open circles). Plasma data following the smoking dose have been previously published for 12 of the subjects (*186*); one is included in (A) for comparison to the oral dose.

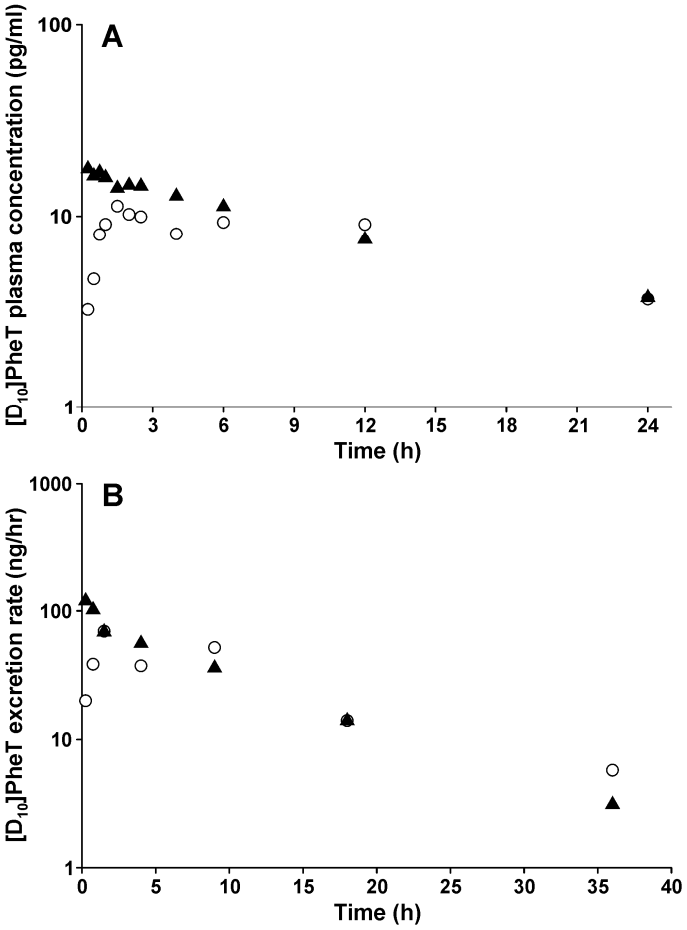


Figure **4.3** (A) Plasma AUC0-∞ of [D_{10}]PheT following oral dose (open bars) and smoking administration (closed bars) in 16 subjects, ranking from low to high by AUC0-∞ of [D_{10}]PheT after smoking dose (p = 0.42); (B) Total 48 hr urinary excretion of [D_{10}]PheT following oral dose (open bars) and smoking administration (closed bars) for 16 subjects, ranking from low to high by the level of [D_{10}]PheT after smoking administration (p = 0.51). Plasma data for 12 subjects following the smoking dose have been previously published and are included here for comparison (186).

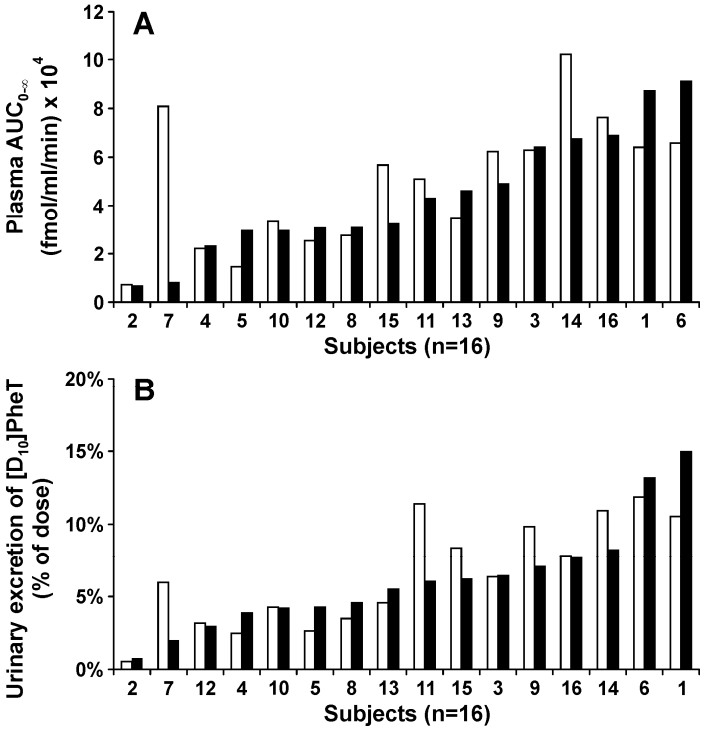


Figure **4.4** Relationship of total 48-hr urinary excretion of [D_{10}]PheT as percent of dose to plasma AUC0-∞ of [D_{10}]PheT, after smoking (A) or oral dose (B) (N =16).

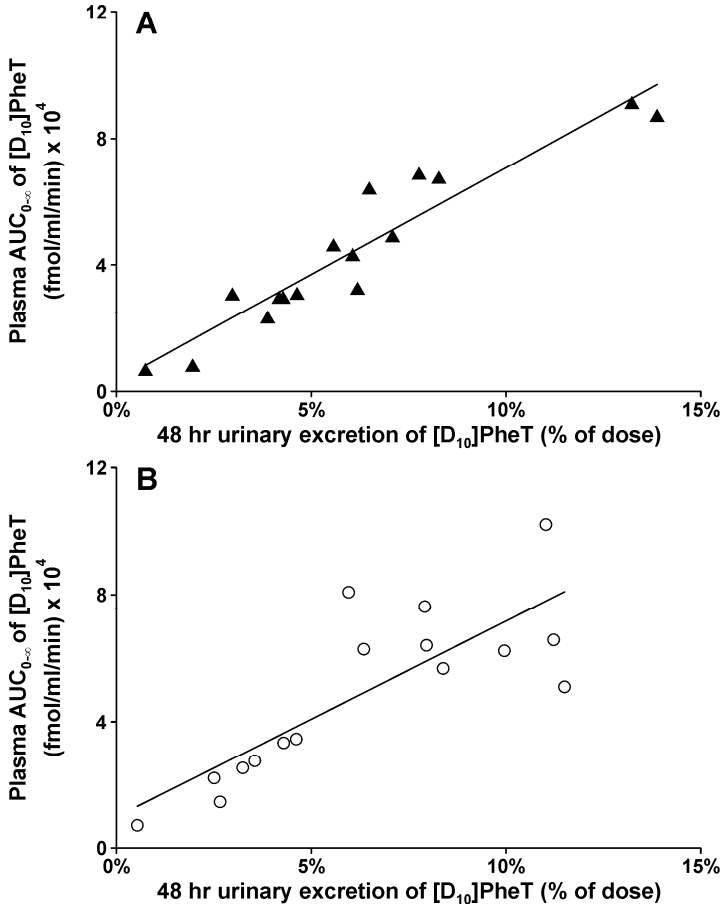


Figure **4.5** Relationship of 48-hr urinary excretions of [D_{10}]PheT as percent of dose to 2-hr (A) and 6-hr (B) post-dose urinary excretion of [D_{10}]PheT (N = 32). Closed triangles, smoking dose; open circles, oral dose.

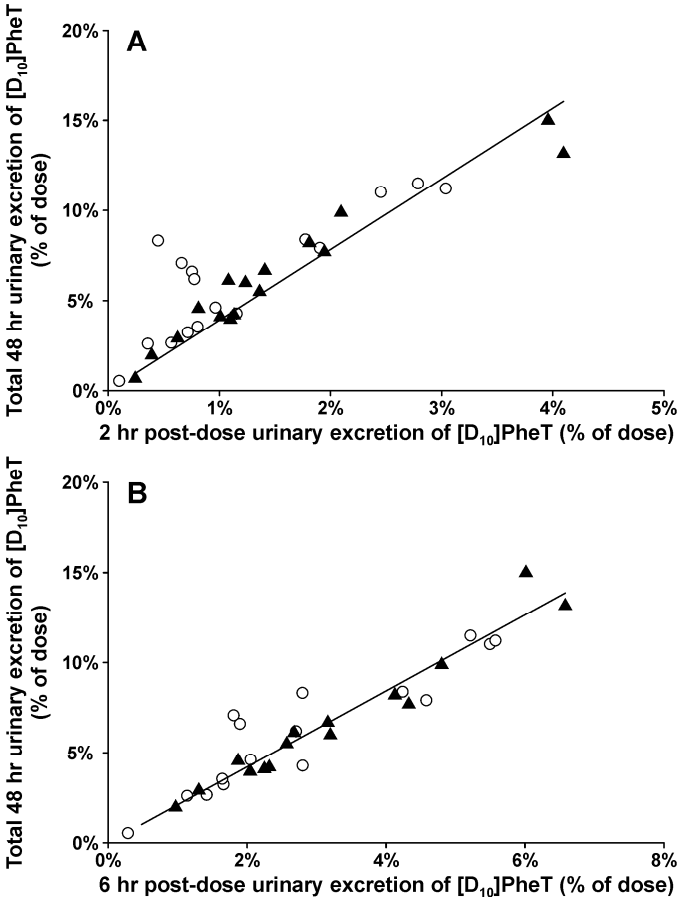


Figure **4.6** Relationship of the ratios of [D_{10}]PheT: [D_9]HOPhe to natural PheT:HOPhe (N = 12); closed triangles, smoking dose; open circles, oral dose.

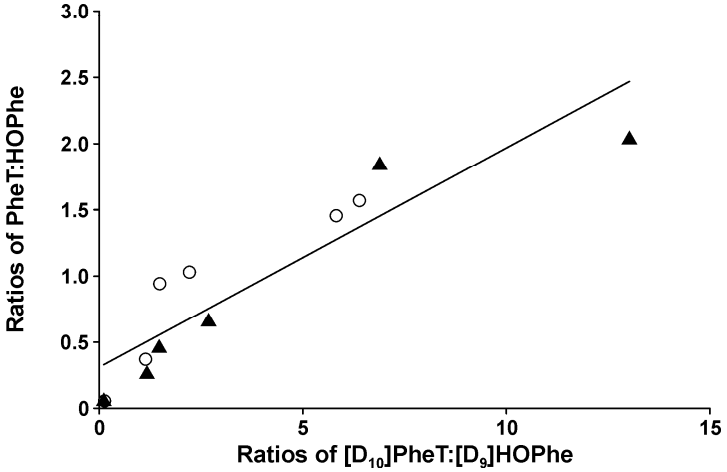


Table **4.1**

Ratios (oral dose/smoking dose) of geometric means of two pharmacokinetic parameters for [D_{10}]PheT after oral and smoking administration of 10 µg [D_{10}]Phe to 16 healthy smokers.

Subject (n=16)	Ratios of plasma $AUC_{0-\infty}$	Ratio of total urinary excretion
1	0.73	0.7
2	1.08	0.66
3	0.98	0.98
4	0.96	0.65
5	0.49	0.66
6	0.72	0.91
7	10.06	3.05
8	0.9	0.77
9	1.27	1.41
10	1.13	1.03
11	1.18	1.9
12	0.83	1.09
13	0.75	0.83
14	1.51	1.33
15	1.75	1.36
16	1.11	1.02
Mean±SD	1.59±2.28	1.15±0.61
90% CI	(0.85, 1.52)	(0.86, 1.26)
Mean±SD [a]	1.03±0.32	1.02±0.35
90% CI [a]	(0.84, 1.14)	(0.83, 1.13)

[a] without subject 7

Table **4.2**

Levels of urinary [D_{10}]PheT, total [D_9]HOPhe, and the ratios of [D_{10}]PheT: [D_9]HOPhe and natural PheT:HOPhe in subjects 1, 6, and 14 (with high [D_{10}]PheT formation) and 12, 7, and 2 (with low [D_{10}]PheT formation).

				% of dose administrated				Ratios			
Subject no.	**gender**	**age**	**CPD[a]**	**[D_{10}]PheT**		**[D_9]HOPhe[b]**		**[D_{10}]PheT:[D_9]HOPhe**		**PheT:HOPhe**	
				smoking	**oral**	**smoking**	**oral**	**smoking**	**oral**	**smoking**	**oral**
1	F	40	15	15%	11%	5.6%	1.8%	2.7	5.8	0.67	1.5
6	M	54	25	13%	12%	1.9%	5.4%	6.9	2.2	1.8	1.0
14	F	25	12	8.2%	11%	0.6%	1.7%	13	6.4	2.0	1.6
12	F	28	25	3.0%	3.2%	2.6%	2.8%	1.2	1.1	0.27	0.37
7	F	31	13	2.0%	5.9%	1.4%	4.0%	1.5	1.5	0.47	0.94
2	M	47	23	0.8%	0.5%	7.2%	4.0%	0.11	0.13	0.063	0.056

[a] CPD = cigarette per day

[b] Total HOPhe = 1-HOPhe + 2-HOPhe + 3-HOPhe + 4-HOPhe

4. Discussion

This study used a unique strategy for phenotyping of PAH metabolic activation in humans. Urinary biomarkers of exposure *plus* metabolism of PAHs such as PheT, BaP-tetraol, and 1-hydroxypyrene have been described previously (*57*,*186*,*188*). However, the levels of these biomarkers reflect individual differences in both exposure and metabolic activation of PAHs. All humans – smokers and non-smokers – are exposed to PAHs through the diet and the general environment. The use of deuterium labeled [D_{10}]Phe makes it possible to exclude the contribution of environmental or dietary exposure. Thus we were able to specifically track diol epoxide formation following a single PAH dose. This approach allows us to directly assess in vivo interindividual variation in PAH metabolism in smokers.

Only a fraction of lifelong smokers develop lung cancer, while others with comparable smoking histories remain free of lung cancer. This large inter-individual variation is believed to be influenced not only by the extent of tobacco carcinogen exposure but also by differences in carcinogen metabolic activation or detoxification, among other factors. While the extent of carcinogen exposure in a smoker is clearly important, this can be estimated by measurement of urinary nicotine metabolites and other tobacco-specific compounds. The approach described here potentially will allow us to determine inter-individual differences in PAH metabolic activation in smokers, unconfounded by dietary or environmental exposures which may not be relevant to lung cancer. We hypothesize that differences in PAH metabolic activation will be related to lung cancer risk. Our ultimate goal is to identify those smokers who are at highest risk

for lung cancer, so they can be targeted for preventive measures. This clinical study was designed to establish the basics for using [D_{10}]PheT as a biomarker for PAH metabolic activation in future large-scale risk assessment studies in smokers.

A main aim of this study was to determine and compare the pharmacokinetic profile of [D_{10}]PheT after two routes of administration of [D_{10}]Phe: oral ingestion and inhalation though cigarette smoke. This information is essential for developing a reliable phenotyping method. If the two routes of administration produce markedly different results, smoking administration will be preferred because it mimics the major PAH exposure route in smokers. If there are no significant differences between the two routes of administration, an oral dose will be used, as it is simpler and more practical. Several previous studies have examined the bioavailability and urinary excretion of BaP and pyrene metabolites following oral, intravenous, cutaneous, intraperitoneal or intrapulmonary administration to rats (*189-191*). They found that the formation of metabolites varied greatly after different administration routes. In contrast to these results in animal studies, we found comparable plasma AUC, half life, and total urinary excretion of [D_{10}]PheT after oral and cigarette smoking administration. There was no significant difference in the rate or extent of metabolism of [D_{10}]Phe to diol epoxides between the two routes of exposure. This supports the use of oral dosing in future phenotyping studies. [D_{10}]PheT was formed rapidly after the smoking dose and was already present at its highest levels in plasma at 15-30 min post-dose in all subjects, as we have previously reported (*186*). We found here in the oral dosing arm that the [D_{10}]PheT level peak time varied from 30 min to 6 hr. These results suggest that there

was a significant "first-pass" biotransformation of [D_{10}]Phe in the lung as well as in the liver. Absorption in the gastrointestinal tract is probably related to our observation that the [D_{10}]PheT peak was reached more slowly after the oral dose than after the smoking dose in some subjects, but this requires further study.

Our results indicate that 6-hr urinary excretion of [D_{10}]PheT was sufficient to reflect the extent of metabolism to [D_{10}]PheT in smokers, as it was highly correlated with 48-hr excretion. After both routes of administration, excretion of [D_{10}]PheT was almost complete over the 48 hr period, which is consistent with the 24-48 hr period reported in animal studies (*190*). By 6 hr after administration, about half of [D_{10}]PheT transformed from the parent dose had already been excreted in urine. The 6 hr time period will be practical for phenotyping studies in smokers.

Many previous studies clearly demonstrate large inter-individual differences in PAH metabolism in humans (*121,165*), leading to the logical hypothesis that individuals who extensively metabolically activate these carcinogens are at a higher risk for cancer. Beginning with the work of Conney and co-workers in the 1960s (*192*) and continuing to the present, these studies have employed a variety of in vitro approaches, most commonly using mitogen-activated human lymphocytes to investigate the inducibility of PAH metabolism via the aryl hydrocarbon receptor (AhR) which regulates the *CYP1A1*, *1A2*, and *1B1* genes, coding for cytochromes P450 1A1, 1A2, and 1B1, which are involved in PAH metabolism (*59,193*). About 10% of the Caucasian population has a high inducibility phenotype. It is well established that cigarette smoking induces CYP1A and CYP1B enzymes, most likely through the binding of PAH and other smoke constituents

to the AhR (*4,59,193-196*). The relationship between AHH inducibility and lung cancer risk in smokers has been examined with conflicting results (*4,59,122,194*), possibly because AHH inducibility affects *both* the metabolic activation and detoxification of PAH. In contrast to these studies, our approach is apparently the first to use a PAH for in vivo phenotyping of smokers.

While our approach was different, our results are consistent with these previous studies. A 20-fold interindividual variation in the formation of [D_{10}]PheT was observed after both oral and smoking dosing. This is a striking finding considering that only 16 smokers were included in this study. We investigated other pathways of [D_{10}]Phe metabolism in the subjects with the 3 highest and 3 lowest levels of urinary [D_{10}]PheT. The differences in [D_{10}]PheT excretion were not explained by differences in levels of [D_{10}]HOPhe or *anti*-[D_{10}]PheDE-1-NAC, nor could we detect [D_{10}]Phe in the urine or plasma of these subjects. Apparently, there are other pathways of Phe metabolism that differ in the subjects with high vs. low amounts of excreted [D_{10}]PheT. One possibility is Phe-diols, which have previously been identified in human urine (*197*), but we did not investigate their levels in this study. There may be uncharacterized routes of Phe metabolism as well.

One limitation of this study is that the dose of [D_{10}]Phe (10 μg) used is about 2-3 times as much as the amount of natural Phe a regular smoker (20 cigarettes/day) receives per day from smoking cigarettes, based on machine measurements (*140*). The 10 μg dose was used to facilitate analysis of [D_{10}]PheT in plasma and urine. Our plasma data suggest that Phe metabolizing enzymes were not saturated by this dose. Unchanged

[D_{10}]Phe was not detected in urine after either route of administration, which suggests a complete and rapid metabolism of Phe in our subjects**.** The metabolites quantified here in six of our subjects - [D_{10}]PheT, [D_9]HOPhe, and *anti*-[D_{10}]PheDE-1-NAC - together accounted for about 3% to 21% of the dose of [D_{10}]Phe (Table **3.2**). Previous studies have identified two other types of Phe metabolites in humans – dihydrodiols, formed in amounts similar to HOPhe, and a mercapturic acid resulting from conjugation of Phe-9,10-epoxide with glutathione, formed in amounts similar to *anti*-[D_{10}]PheDE-NAC (*151,197*). Including these metabolites, the percent of dose in these six subjects might increase by a few percent, but there is still a considerable amount of unknown metabolism. We speculate that this might result from further oxidation of Phe-9,10-diol to Phe-9,10-quinone and subsequent ring cleavage, but further studies are required to more fully characterize Phe metabolism in smokers. The study reported here focused mainly on the diol epoxide pathway believed to be critical in the induction of cancer by PAH.

A second limitation of our approach is related to [D_{10}]Phe itself. As it is a relatively low molecular weight PAH, its pharmacokinetics and metabolism may be quite different from those of carcinogenic PAH such as BaP but it would be unethical to give a carcinogenic PAH to smokers. Phe is generally considered non-carcinogenic and we have reported a correlation between levels of PheT and BaP-tetraols in human urine (*156*). Also, the approach described here using [D_{10}]Phe as a surrogate for carcinogenic PAH does not reveal any information about differences in uptake or metabolism of other carcinogens in tobacco smoke such as tobacco-specific nitrosamines or volatile organic

compounds. Alternate techniques would be required to assess the contributions of these compounds to individual susceptibility.

In summary, we demonstrate that the metabolic activation of Phe, as represented by the biomarker [D_{10}]PheT, is comparable after ingestion and inhalation in smokers. Our approach is apparently the first to directly phenotype PAH metabolism in vivo in humans. This non-invasive phenotyping method potentially can become part of an individual-based, predictive model for lung cancer risk assessment in smokers in the future.

Chapter 5

Summary and Perspective

A unique phenotyping strategy using [D_{10}]PheT as probes has been developed, allowing direct assessment of metabolic activation of carcinogenic PAH in humans. The study design is based on accurate instrumental analysis of urinary biomarkers BaP-T and Phe-T, the end products of the conventional PAH diol epoxide pathway. Its application in clinical studies has approved, for the first time, that there is large inter-individual variation in PAH metabolic activation in smokers. One striking finding is that the formation of PAH diol epoxides, the ultimate carcinogen, occurs astonishingly rapidly in smokers, particularly after inhalation of cigarette smoke. The pharmacokinetics parameters of PAH metabolites in humans have also been presented. The results have established the basics for using [D_{10}]PheT as a biomarker for PAH metabolic activation in future large-scale risk assessment studies in smokers.

This phenotyping study will be continued with a large collaborative clinical trial in smokers to test the hypothesis that higher levels of [D_{10}]PheT correlate with higher incidences of bronchoepithelial metaplasia and dysplasia, which are thought to be precancerous histological changes in the tracheobronchial tree. The combined use of autofluorescence and standard white light bronchoscopy allows direct visualization of small neoplastic lesions and track their progress. To determine the relationship between phenotypes of Phe metabolic activation and lung cancer risk, it will be tested whether smokers with dysplasia or smokers with both metaplasia and dysplasia have higher levels of [D_{10}]PheT after they receive [D_{10}]Phe as described previously.

The results reported here also brought up a few interesting questions which could lead to future investigation. First of all, what is the metabolites profile of [D_{10}]Phe in humans? Would it be different between oral and inhalation administration routes? The majority of metabolites from [D_{10}]Phe dose are still unclear. It would be possible in the future to identify these unknown metabolites from [D_{10}]Phe, the isotope labeled dose, with advanced Orbitrap instrument. Second, what is the plasma concentration of [D_{10}]PheT before 15 minutes after dosing? In the aforementioned studies, we started to collect blood samples at 15 minutes post-dose, and found that in most of cases [D_{10}]PheT has already reached the maximum levels in the circulation at this point. It would be interesting to know what the exact time point of the maximum level (Tmax) of plasma [D_{10}]PheT is. Third, would the capacity to metabolically activate PAH decrease after quitting smoke. If yes, what is the timeline for the change? It could be done by combining [D_{10}]PheT dosing with the smoking cessation programs. Fourth, can we combine NNK and PAH phenotyping methods, for example, smoking cigarettes containing both deuterated NNK and Phe, to assess an individual's lung cancer susceptibility, since they are two major types of carcinogens in tobacco products for lung cancer? How a given individual responds to NNK and PAH respectively at the same time has not been shown in previous studies. The results would contribute significantly to the proposed prospective model for lung cancer risk prediction. All the above proposals aim to further understanding the mechanisms of tobacco-induced cancer and apply this knowledge to cancer prevention.

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