

Phenanthrene Metabolism in Smokers: Use of a Two-Step Diagnostic Plot Approach to Identify Subjects with Extensive Metabolic Activation

Jing Wang, Yan Zhong, Steven G. Carmella, J. Bradley Hochalter, Diane Rauch, Andrew Oliver, Joni Jensen, Dorothy K. Hatsukami, Pramod Upadhyaya, Stephen S. Hecht, and Cheryl L. Zimmerman

Department of Pharmaceutics (J.W., C.L.Z.) and Masonic Cancer Center (Y.Z., S.G.C., J.B.H., D.R., A.O., J.J., D.K.H., P.U., S.S.H.), University of Minnesota, Minneapolis, Minnesota

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke are among the most likely causes of lung cancer. PAHs require metabolic activation to initiate the carcinogenic process. Phenanthrene (Phe), a noncarcinogenic PAH, was used as a surrogate of benzo[α]pyrene and related PAHs to study the metabolic activation of PAHs in smokers. A dose of 10 μ g of deuterated Phe ($[D_{10}]Phe$) was administered to 25 healthy smokers in a crossover design, either as an oral solution or by smoking cigarettes containing $[D_{10}]Phe$. Phe was deuterated to avoid interference from environmental Phe. Intensive blood and urine sampling was performed to quantitate the formation of deuterated *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ($[D_{10}]PheT$), a biomarker of the diol epoxide metabolic activation pathway. In both the oral and smoking arms approxi-

mately 6% of the dose was metabolically converted to diol epoxides, with a large intersubject variability in the formation of $[D_{10}]PheT$ observed. Two diagnostic plots were developed to identify subjects with large systemic exposure and significant lung contribution to metabolic activation. The combination of the two plots led to the identification of subjects with substantial local exposure. These subjects produced, in one single pass of $[D_{10}]Phe$ through the lung, a $[D_{10}]PheT$ exposure equivalent to the systemic exposure of a typical subject and may be an indicator of lung cancer susceptibility. Polymorphisms in PAH-metabolizing genes of the 25 subjects were also investigated. The integration of phenotyping and genotyping results indicated that *GSTM1*-null subjects produced approximately 2-fold more $[D_{10}]PheT$ than did *GSTM1*-positive subjects.

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Introduction

According to the International Agency for Research on Cancer (2004), tobacco smoking is associated with 90% of the lung cancer deaths in the United States. Polycyclic aromatic

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; BaP, benzo[α]pyrene; Phe, phenanthrene; PheT, *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene; $[D_{10}]Phe$, deuterated Phe; $[D_{10}]PheT$, deuterated PheT; $[D_{10}]PheDE$, deuterated *anti*-1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrophenanthrene; BPDE, benzo[α]pyrene-7,8-diol-9,10-epoxide; GST, glutathione transferase; *GSTM1*, GST mu 1; PK, pharmacokinetic; AUC, area under the curve; CL, clearance; SBE, single-base extension; EPHX1, epoxide hydrolase 1; A_{act} , total amount of $[D_{10}]PheDE$ formed during metabolic activation; $A_{(Phe,abs),t}$, amount of $[D_{10}]PheDE$ at the site of absorption; $A_{(Phe,abs),t=0}$, amount of $[D_{10}]PheDE$ at the site of absorption at time 0; $A_{(Phe,abs),t=T}$, amount of $[D_{10}]Phe$ at the site of absorption at time *T*; $A_{(PheDE),t}$, amount of $[D_{10}]Phe$ in the body; $A_{(PheDE),t=0}$, amount of $[D_{10}]PheDE$ in the body at time 0; $A_{(PheDE),t=T}$, amount of $[D_{10}]PheDE$ in the body at time *T*; $A_{(PheDE),t=\infty}$, amount of $[D_{10}]PheDE$ in the body at time infinity; $A_{(PheT),t}$, amount of $[D_{10}]PheT$ in the body; $A_{(PheT),t=0}$, amount of $[D_{10}]PheT$ in the body at time 0; $A_{(PheT),t=T}$, amount of $[D_{10}]PheT$ in the body at time *T*; $A_{(PheT),t=\infty}$, amount of $[D_{10}]PheT$ in the body at time infinity; $AUC_{(Phe,abs),t}$, area under the $C_{(Phe,abs)}$ -time curve from time 0 to *T*; $AUC_{(Phe)}$, area under the $C_{(Phe)}$ -time curve from time 0 to infinity; $AUC_{0(Phe)}$, area under the $C_{(Phe)}$ -time curve from time 0 to *T*; $AUC_{(PheDE),t}$, area under the $C_{-(PheDE)}$ -time curve from time 0 to infinity; $AUC_{0(PheDE)}$, area under the $C_{(PheDE)}$ -time curve from time 0 to *T*; $AUC_{(PheT),t}$, area under the $C_{(PheT)}$ -time curve from time 0 to infinity; $AUC_{0(PheT)}$, area under the $C_{(PheT)}$ -time curve from time 0 to *T*; $C_{(Phe,abs)}$, concentration of $[D_{10}]Phe$ at the site of absorption; $C_{(Phe)}$, plasma concentration of $[D_{10}]Phe$; $C_{(PheDE)}$, plasma concentration of $[D_{10}]PheDE$; $C_{(PheT)}$, plasma concentration of $[D_{10}]PheT$; f_a , the fraction of dose absorbed; f_{act} , the fraction of dose converted to $[D_{10}]PheDE$ during first-pass activation; f_m , the fraction of $[D_{10}]Phe$ converted to $[D_{10}]PheDE$ in the systemic circulation; $Xu_{(PheT),t}$, cumulative amount of $[D_{10}]PheT$ collected in the urine; $Xu_{(PheT),t=T}$, amount of $[D_{10}]PheT$ collected in the urine at time *T*; $Xu_{(PheT),t=48}$, amount of $[D_{10}]PheT$ collected in the urine at 48 h; $Xu_{(PheT),t=\infty}$, amount of $[D_{10}]PheT$ collected in the urine at time infinity.

hydrocarbons (PAHs) are among the strongest carcinogens in cigarette smoke and are considered to be a major etiological factor in lung cancer (Pelkonen and Nebert, 1982; Hecht, 2003). PAHs are very lipophilic and could theoretically accumulate in tissues and reach toxic concentrations (Lang and Pelkonen, 1999). Thanks to evolution, humans have developed the capacity to detoxify foreign chemicals, including carcinogens, by converting them to more water-soluble metabolites, which may enhance their elimination from the body. Enzymes involved in the detoxification of PAHs include cytochromes P450 1A1 (CYP1A1) and 1B1 (CYP1B1), glutathione transferases (GSTs), UDP-glucuronosyltransferases, and sulfotransferases (Shimada, 2006; Hecht, 2010). However, at the cost of solving the acute problem (lethal accumulation of xenobiotics), a small portion of PAHs is transformed to electrophilic intermediates. These intermediates do not cause lethal hazards in the short term, but may initiate carcinogenesis in the long term by attacking nucleophilic macromolecules of the cell, causing gene mutations that may eventually lead to the development of tumors (Hecht, 1999). The conversion of PAHs to active intermediates is called metabolic activation or bioactivation. Enzymes involved in metabolic activation include CYP1A1, CYP1A2, and CYP1B1, as well as epoxide hydrolase (Shimada, 2006).

Fewer than 20% of lifelong smokers develop lung cancer, indicating that some individuals are more susceptible than others (International Agency for Research on Cancer, 1986; Peto et al., 2000). How to identify susceptible subjects is still not clear. Numerous epidemiological studies have been conducted to investigate the relationship of cancer susceptibility to genetic polymorphisms in PAH-metabolizing enzymes (Kawajiri et al., 1990; Nakachi et al., 1991; Tefre et al., 1991; Shields et al., 1993; Okada et al., 1994; Xu et al., 1996; Ishibe et al., 1997; Persson et al., 1999; Bartsch et al., 2000; Williams, 2001; Kiyohara et al., 2002; Shi et al., 2008). However, the results of those studies were mixed and sometimes even contradictory, perhaps because of the complexity of PAH metabolism and the multiple factors involved. For example, these studies have not considered gene-environment interactions and tissue-specific metabolic enzyme expression. Subjects with a higher expression of activation enzymes and lower expression of detoxification enzymes would theoretically have larger systemic exposure to active intermediates than other subjects. In addition, if the pulmonary enzymes of

these subjects played a major role in activation, then lung exposure would be substantial, which could lead to an increased susceptibility to lung cancer. Because of the important role of metabolic activation of PAHs in lung carcinogenesis, the present study focused on identification of subjects with large systemic and local (lung) exposure and presumably high lung cancer risk.

Benzo[α]pyrene (BaP) is found in cigarette smoke and is a prototypic and widely studied compound for the investigation of carcinogenesis by PAHs (Straif et al., 2005; Uno et al., 2006; Jiang et al., 2007). A major bioactivation pathway of BaP (Fig. 1) is its conversion to *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(α)pyrene via the formation of the "bay region diol epoxide," BaP-7,8-diol-9,10-epoxide (BPDE), which reacts readily with DNA and is mutagenic and carcinogenic (International Agency for Research on Cancer, 2010). BPDE is thought to be one of the electrophilic reactive intermediates responsible for BaP mutagenesis and carcinogenesis (Conney, 1982; Cooper et al., 1983; Geacintov et al., 1997; Szeliga and Dipple, 1998; Hecht, 1999), and related bay region diol epoxides are considered to be major ultimate carcinogens of a number of other PAHs (International Agency for Research on Cancer, 2010). Because most PAHs, such as BaP, are carcinogenic and cannot be administered to humans, there have not been any detailed pharmacokinetic (PK) studies of PAHs in humans. To address this issue, a novel biomarker approach has been proposed: the use of phenanthrene (Phe) as a surrogate of BaP (Hecht et al., 2003). Phe is a noncarcinogenic PAH ubiquitous in the environment that can be safely administered to human subjects because all humans are exposed to Phe. The conversion of Phe to *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT) mimics the formation of the diol epoxide metabolic activation of BaP, including the intermediates formed and enzymes involved (Fig. 1) (International Agency for Research on Cancer, 1983; Carmella et al., 2004), although there are some differences (Hecht et al., 2010).

In the present study, deuterated Phe ($[D_{10}]$ Phe) was administered to 25 subjects, and the metabolism of $[D_{10}]$ Phe to deuterated PheT ($[D_{10}]$ PheT) was characterized to evaluate each individual's capacity to carry out the diol epoxide metabolism pathway. We used $[D_{10}]$ Phe to avoid interference from ubiquitous exposure to environmental Phe. An intermediate formed during the activation of $[D_{10}]$ Phe was deuter-

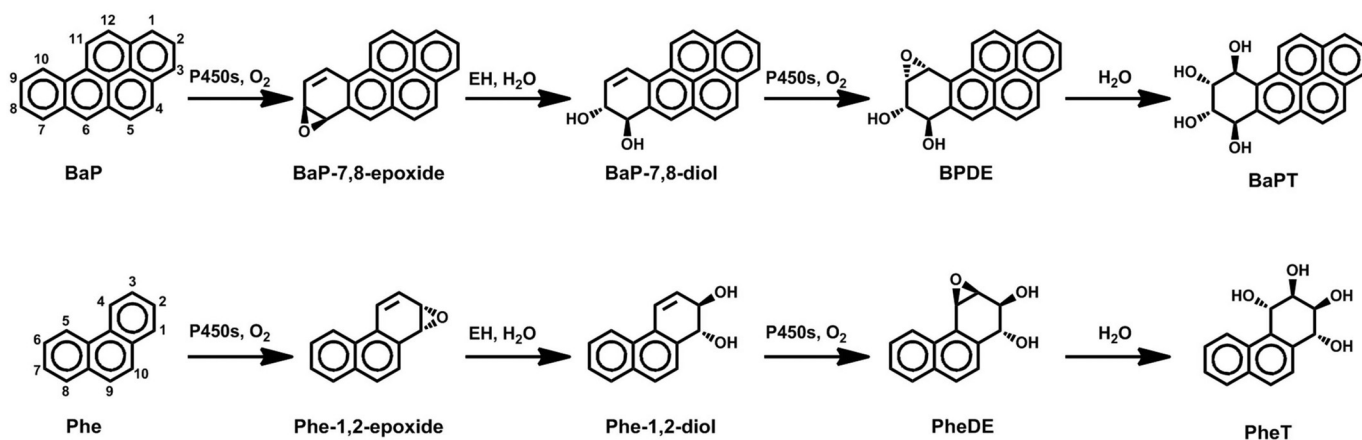


Fig. 1. Metabolic activation pathways of BaP and Phe (modified from Carmella et al. (2004)). BaPT, *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(α)pyrene.

ated *anti*-1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrophenanthrene ($[D_{10}]PheDE$), a biomarker of the diol epoxide pathway leading to BPDE from BaP (Fig. 1). The primary objective of our study was to quantify the amount of $[D_{10}]PheDE$ formed in the metabolism of $[D_{10}]Phe$. $[D_{10}]PheDE$ cannot be quantified directly because it reacts rapidly with H_2O , producing $[D_{10}]PheT$, which was measured in this study (Carmella et al., 2004).

Preliminary reports from a subset of subjects demonstrated the rapid formation of diol epoxides and potential immediate negative health consequences of smoking (Zhong et al., 2011a) and examined the metabolism of $[D_{10}]Phe$ administered to 16 smokers either in a cigarette or orally as a biomarker of the activation pathway (Zhong et al., 2011b). With the completion of the clinical trial, a comprehensive analysis of the bioactivation of Phe is now reported.

Materials and Methods

Clinical Study Design. The study was approved by the U.S. Food and Drug Administration and the University of Minnesota Institutional Review Board. Details on the recruitment of subjects and dosing protocols were reported previously (Zhong et al., 2011a,b). Subjects were recruited by using advertisements on the radio, television, or metropolitan and campus newspapers. Volunteers interested in the study called the University of Minnesota Transdisciplinary Tobacco Use Research Center and were informed about the study. The preliminary screening was performed over the phone to select subjects meeting the following specific inclusion criteria: the smoking of at least 10 cigarettes daily for the past year and good physical and mental health. Female subjects who were pregnant or nursing were excluded. Eligible subjects were further invited to the clinic site for an orientation visit to fill out a detailed questionnaire regarding their smoking and medical history. Pregnancy tests were also conducted. Subject recruitment incentives were used, and an average of \$500 was paid to each subject for the completion of the study.

A total of 25 eligible subjects, eight men, were recruited between April 2008 and September 2010. The subjects were between 23 and 54 years with a mean age (\pm S.D.) of 36.2 ± 10.4 years. Their weights ranged from 61 to 113 kg with a mean weight (\pm S.D.) of 87.4 ± 15.4 kg. Twelve subjects were white, eight were African-American, and five reported being multiracial.

A dose of $10 \mu g$ (53.2 nmol) $[D_{10}]Phe$ was administered to 25 subjects, either as an oral solution or by smoking cigarettes to which $[D_{10}]Phe$ had been added. $[D_{10}]Phe$ (98%; containing 2% nondeuterated Phe) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA), and then repurified in the University of Minnesota Molecular and Cellular Therapeutics GMP facility. The study had a randomized, open-label, single-dose, crossover design. The order of administration was randomized, and each dose was separated by a washout period of at least 1 week. For the oral dose, each subject was given $10 \mu g$ of $[D_{10}]Phe$ (5 ml of 20% ethanol-80% water solution). The dosing bottle was rinsed twice with water to ensure accurate dosing. For the inhalation dose, subjects followed a standard smoking protocol designed to deliver $10 \mu g$ of $[D_{10}]Phe$, as monitored by a smoking topography device (Plowshare Technologies, Baltimore, MD) (Zhong et al., 2011a). The administration of $[D_{10}]Phe$ by smoking was performed in a specially ventilated room at the Transdisciplinary Tobacco Use Research Center. Subjects underwent an adaptation trial before smoking the cigarettes containing $[D_{10}]Phe$. Specific instructions about the puff volume (55 ml), puff duration (2 s), and puff interval (30 s) were given to the subjects. Subjects smoked the cigarettes through the smoking topography device, which recorded the puff volume, puff duration, and puff number. A Marlboro cigarette (Philip Morris, Richmond, VA) was used in the

practice session, after which subjects were then allowed to smoke the cigarette containing $[D_{10}]Phe$. The smoking process was also observed by the clinician to ensure good compliance. The smoking session usually lasted approximately 4 to 5 min.

Blood samples of 10 ml each were taken before dosing and 15, 30, 45, 60, 90, 120, 150, 240, 360, 540, 720, or 1440 min after completion of administration. Blood samples were centrifuged to obtain plasma, which was stored at $-20^\circ C$ until analysis. Urine samples were obtained predosing and at the following postdosing intervals: 0 to 30, 30 to 60, 60 to 120, 120 to 360, 360 to 720, 720 to 1440, and 1440 to 2880 min. The volume of each urine collection was measured, and an aliquot of 50 ml was stored at $-20^\circ C$ until analysis. Gas chromatography-mass spectrometry was used to analyze the plasma and urine samples as described previously (Zhong et al., 2011a). It should be noted that because approximately 90% of the PheT in human urine exists as sulfate and glucuronide conjugates (Hecht et al., 2003), plasma and urine samples were incubated with β -glucuronidase and arylsulfatase before quantitation. Hence the reported level of $[D_{10}]PheT$ in both plasma and urine is the sum of the free $[D_{10}]PheT$ and its conjugates.

Pharmacokinetic Analysis. Noncompartmental analysis was performed with the use of Phoenix WinNonlin (version 6.1; Pharsight, Mountain View, CA) to calculate the area under the $[D_{10}]PheT$ plasma concentration-time curve ($AUC_{(PheT)}$) and the elimination half-life ($t_{1/2}$) of $[D_{10}]PheT$.

The clearance of $[D_{10}]PheT$ (CL_7 ; Fig. 2) was estimated from the slope of the urinary excretion rate versus midpoint plasma concentration curve:

$$\Delta X/\Delta t = CL_7 \times C_{p_{mid}} \quad (1)$$

where $C_{p_{mid}}$ was the plasma concentration of $[D_{10}]PheT$ at the midpoint of the urine collection interval. The urinary excretion rate ($\Delta X/\Delta t$) was determined from the amount of $[D_{10}]PheT$ excreted in urine at each interval (ΔX) divided by the length of the collection interval (Δt). Urine collections at 720 to 1440 and 1440 to 2880 min were not included in the estimate of CL_7 because very low concentrations were present in urine in those intervals, and each of those intervals was >1 half-life of $[D_{10}]PheT$ (Gibaldi and Perrier, 1982).

The total amount of $[D_{10}]PheDE$ formed during activation (A_{act}) was estimated by two methods (eqs. 2 and 3; see Appendix for detailed derivation of the equations). The first method was using the product of $AUC_{(PheT)}$ and CL_7 :

$$A_{act,plasma} = AUC_{(PheT)} \times CL_7 \quad (2)$$

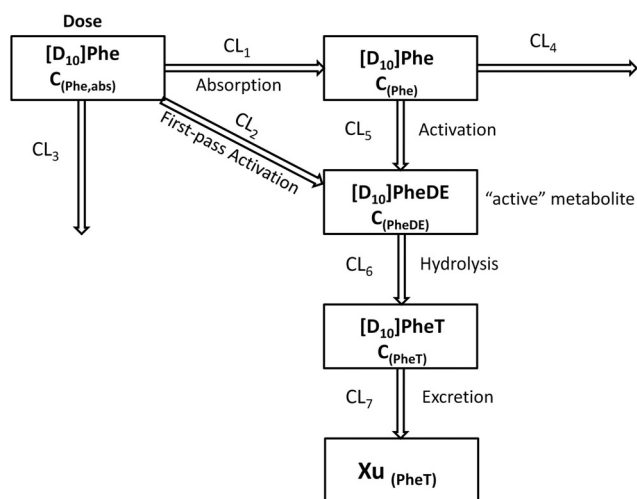


Fig. 2. The PK view of metabolic activation of $[D_{10}]Phe$ after administration (detailed description in Appendix).

The second method was to calculate the amount of [D₁₀]PheT collected in urine up to 48 h ($Xu_{(PheT),t=48}$):

$$A_{act,urine} = Xu_{(PheT),t=48} \quad (3)$$

The percentage of dose activated (f) was calculated as:

$$f = \frac{A_{act}}{D} \times 100\% \quad (4)$$

where D is the administered dose of [D₁₀]Phe (10 μg or 53.2 nmol).

$A_{act,plasma}$ and $A_{act,urine}$ are two indicators of systemic exposure to [D₁₀]PheT and thus formed the basis of diagnostic plot I (Fig. 3, top).

Differences in A_{act} between smoking and oral dosing were calculated as:

$$A_{act(lung)} = A_{act,smk} - A_{act,oral} \quad (5)$$

where $A_{act,smk}$ and $A_{act,oral}$ are A_{act} after smoking and oral dosing, respectively. $A_{act(lung)}$ is a measure of lung contribution to the formation of [D₁₀]PheT. A positive value of $A_{act(lung)}$ indicates that the lung contributed more to the formation of [D₁₀]PheT than liver, and hence played a major role in metabolic activation. In addition, the larger the value of $A_{act(lung)}$, the larger local (lung) exposure to [D₁₀]PheT.

The relative bioavailability of [D₁₀]PheT after oral dosing com-

pared with the smoking administration was calculated from the ratio of oral/smoking $AUC_{(PheT)}$ values (F_{AUC}) and the ratio of oral/smoking $Xu_{(PheT),t=48}$ values (F_{Xu}). F_{AUC} and F_{Xu} were used as two indicators of lung contribution to activation and formed the basis for diagnostic plot II (Fig. 3, bottom). The combination of plots I and II allowed the identification of subjects with substantial local exposure.

According to eq. A23 in Appendix, the amount of [D₁₀]PheDE formed after oral dosing and smoking could be calculated as

$$A_{act} = [(f_a \times f_m) + f_{act}] \times D \quad (6)$$

where f_a , f_{act} , and f_m are the fraction of the dose absorbed, the fraction of the dose converted to [D₁₀]PheDE during first-pass activation, and the fraction of [D₁₀]Phe converted to [D₁₀]PheDE in the systemic circulation, respectively. Because changes in the route of administration would affect only f_a and f_{act} , but f_m would remain the same, eq. 6 could be rewritten as eqs. 6.1 and 6.2 for the oral and smoking arm, respectively.

$$A_{act(oral)} = [(f_{a(oral)} \times f_m) + f_{act(oral)}] \times D \quad (6.1)$$

$$A_{act(smok)} = [(f_{a(smok)} \times f_m) + f_{act(smok)}] \times D \quad (6.2)$$

$f_{a(oral)}$ and $f_{a(smok)}$ are the fraction of [D₁₀]Phe dose absorbed after oral dosing and smoking, respectively. $f_{act(oral)}$ and $f_{act(smok)}$ are the fraction of [D₁₀]Phe converted to active intermediates during first-pass metabolism after oral dosing and smoking, respectively.

Genotyping. Twelve polymorphisms of metabolizing enzymes of Phe were determined by the BioMedical Genomics Center at the University of Minnesota. Genotyping was performed by using the iPLEX Gold method (Sequenom, San Diego, CA). Similar methods have been reported previously (Hecht et al., 2006). In brief, the method is based on the primer-extension reaction that generates allele-specific products with distinct masses detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The method started with polymerase chain reaction amplification followed by shrimp alkaline phosphatase treatment to remove unincorporated dNTPs. Single-base extension (SBE) was carried out by the addition of SBE primers, iPLEX enzymes, and buffers. SBE products were measured with the MassARRAY system (Sequenom), and mass spectra were analyzed with TYPER software (Sequenom). iPLEX reagents and protocols for multiplex polymerase chain reaction, SBE, and generation of mass spectra were based on the manufacturer's instructions.

The polymorphisms of *CYP1A1* and *CYP1B1* investigated in the study were *CYP1A1MspI*, *CYP1A1I462V*, *CYP1B1R48G*, *CYP1B1I19S*, *CYP1B1L432V*, and *CYP1B1N453S*. In addition, *EPHX1Y113H* and *EPHX1H139R*, two polymorphisms of microsomal epoxide hydrolase 1 (*EPHX1*), were also measured. The polymorphisms of the detoxification enzymes GST P1 (*GSTP1*), T1 (*GSTT1*), and M1 (*GSTM1*) measured in the study were *GSTP1A114V*, *GSTP1I105V*, *GSTT1* null, and *GSTM1* null.

Statistical Analysis. A paired t test was used to compare $t_{1/2}$, CL_7 , and $AUC_{(PheT)}$ between the oral and smoking arm. The measurement of relative bioavailability by two methods (F_{AUC} versus F_{Xu}) was compared by using a paired t test after the logarithmic transformation of the original data. Two-way analysis of variance was used to investigate the influence of data source (plasma versus urinary data) and route of administration (oral versus smoking) on the estimates of systemic exposure to [D₁₀]PheT. One-way analysis of variance was used to identify polymorphisms that might have effects on an individual's capacity to activate PAHs. A p value < 0.05 was considered to be significant.

Results

Table 1 reports the half-life, clearance (CL_7), and $AUC_{(PheT)}$ of [D₁₀]PheT after oral dosing and smoking administration of [D₁₀]Phe. No significant difference was observed

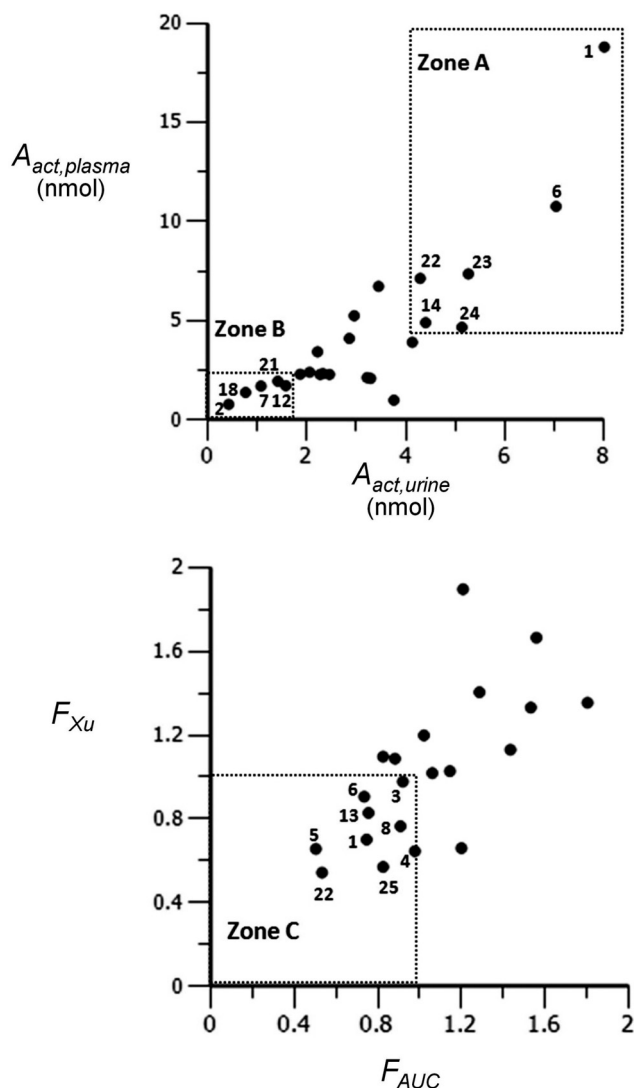


Fig. 3. Diagnostic plots I (top) and II (bottom).

TABLE 1
Half-life, clearance and AUC of [D₁₀]PheT after oral dosing and smoking

Paired *t* test was performed to compare the effects of routes of administration. Results were not significant, *p* > 0.05.

ID	<i>t</i> _{1/2}		<i>CL</i> ₇		<i>AUC</i> _(PheT)	
	Oral	Smoking	Oral	Smoking	Oral	Smoking
	<i>h</i>		<i>l/h</i>		<i>nmol · h/l</i>	
1	7.2	10.9	7.8	13.2	1.07	1.43
2	17.7	8.1	3.1	7.3	0.13	0.11
3	12.6	11.4	5.8	6.4	0.97	1.06
4	11.0	6.7	4.9	6.4	0.37	0.38
5	3.9	4.8	5.5	4.8	0.24	0.48
6	6.3	7.5	4.3	7.3	1.10	1.49
7	12.6	8.1	4.5	13.0	0.97	0.13
8	5.5	5.9	5.4	4.6	0.46	0.51
9	6.4	9.8	3.2	1.3	1.04	0.81
10	6.9	7.5	5.1	7.1	0.55	0.48
11	4.9	4.5	7.5	3.1	0.85	0.70
12	4.4	6.4	3.2	3.6	0.43	0.48
13	7.9	9.9	6.0	7.0	0.58	0.76
14	7.4	7.1	4.0	4.5	1.70	1.11
15	8.8	6.0	4.6	4.0	0.94	0.53
16	5.1	5.2	4.4	4.5	0.93	0.87
17	9.3	7.5	1.9	1.9	1.98	1.27
18	6.1	11.0	2.2	2.2	0.80	0.65
19	5.4	N.A.	1.8	N.A.	0.90	N.A.
20	5.2	6.2	3.7	4.1	1.04	1.02
21	7.4	9.8	3.2	2.9	1.45	0.69
22	5.8	8.9	2.8	4.7	0.82	1.53
23	7.0	6.7	3.0	5.1	2.08	1.45
24	4.8	7.3	5.3	3.0	1.29	1.57
25	6.3	6.9	2.4	2.9	0.65	0.79
Mean	7.4	7.7	4.2	5.2	0.93	0.85
S.D.	3.2	2.0	1.6	3.0	0.49	0.44

N.A., not available because the plasma concentration of [D₁₀]PheT of subject 19 after smoking was below the limit of quantitation.

TABLE 2
A_{act} of oral and smoking arm calculated by two methods

Two-way analysis of variance was performed to evaluate the influence of route of administration and data source (plasma, urine), which was found to be not significant, *p* > 0.05.

ID	Oral Arm				Smoking Arm			
	<i>A_{act,plasma}</i>		<i>A_{act,urine}</i>		<i>A_{act,plasma}</i>		<i>A_{act,urine}</i>	
	<i>nmol</i>	<i>% of dose</i>	<i>nmol</i>	<i>% of dose</i>	<i>nmol</i>	<i>% of dose</i>	<i>nmol</i>	<i>% of dose</i>
1	8.25	15.51	5.62	10.56	18.81	35.36	8.00	15.04
2	0.41	0.77	0.28	0.53	0.79	1.48	0.43	0.81
3	5.57	10.47	3.37	6.33	6.74	12.67	3.45	6.48
4	1.79	3.36	1.33	2.50	2.41	4.53	2.06	3.87
5	1.32	2.48	1.49	2.80	2.30	4.32	2.27	4.27
6	4.62	8.68	6.37	11.97	10.77	20.24	7.03	13.21
7	4.36	8.20	3.29	6.18	1.71	3.21	1.08	2.03
8	2.45	4.61	1.89	3.55	2.30	4.32	2.46	4.62
9	3.24	6.09	5.29	9.94	1.00	1.88	3.76	7.07
10	2.83	5.32	2.28	4.29	3.44	6.47	2.22	4.17
11	6.31	11.86	6.11	11.48	2.14	4.02	3.22	6.05
12	1.36	2.56	1.72	3.23	1.73	3.25	1.58	2.97
13	3.43	6.45	2.45	4.61	5.26	9.89	2.96	5.56
14	6.75	12.69	5.85	11.00	4.91	9.23	4.40	8.27
15	4.3	8.08	4.45	8.36	2.10	3.95	3.29	6.18
16	4.08	7.67	4.20	7.89	3.92	7.37	4.13	7.76
17	3.72	6.99	3.89	7.31	2.34	4.40	2.33	4.38
18	1.74	3.27	1.68	3.16	1.39	2.61	0.77	1.45
19	1.59	2.99	1.31	2.46	N.A.	N.A.	0.26	0.49
20	3.83	7.20	3.43	6.45	4.11	7.73	2.86	5.38
21	4.6	8.65	2.66	5.00	1.96	3.68	1.42	2.67
22	2.29	4.30	2.33	4.38	7.14	13.42	4.29	8.06
23	6.24	11.73	5.95	11.18	7.37	13.85	5.26	9.89
24	6.77	12.73	5.63	10.58	4.68	8.80	5.13	9.64
25	1.57	2.95	1.07	2.01	2.31	4.34	1.87	3.52
Mean	3.74	7.02	3.36	6.31	4.23	7.96	3.06	5.75
S.D.	2.06	3.87	1.86	3.50	3.94	7.41	1.91	3.60

N.A., not available because plasma concentration of [D₁₀]PheT after smoking was below the limit of quantitation.

in *t*_{1/2}, *CL*₇, or *AUC*_(PheT) between the oral and smoking arms of the study, consistent with the results reported previously for 16 subjects (Zhong et al., 2011b).

A_{act} is an estimate of the systemic exposure to [D₁₀]PheT and was calculated by two methods: use of plasma data (*A_{act,plasma}*) and use of urinary data (*A_{act,urine}*). As shown in Table 2, *A_{act,plasma}* and *A_{act,urine}* were 4.23 ± 3.94 and 3.06 ± 1.91 nmol, respectively, in the smoking arm. The percentage of the [D₁₀]Phe dose activated was calculated as 7.96 ± 7.41 and 5.75 ± 3.60 based on plasma and urinary data, respectively. Similar results were obtained from the oral arm. Route of administration (smoking versus oral) or source of data (plasma versus urine) did not have a significant impact on the estimate of *A_{act}*.

Although at the group level no difference was observed in the means of *A_{act}*, a large intersubject variability (>20-fold) in *A_{act}* was observed in both the smoking and oral arms (Table 2). One purpose of this study was to identify people with a potentially increased susceptibility to carcinogenesis by PAH because of their ability to carry out the bay region diol epoxide pathway. Because the route of administration of [D₁₀]Phe in the smoking arm mimicked the uptake of carcinogens by cigarette smokers, plasma and urinary data of the smoking arm were used to identify subjects with a large systemic exposure to [D₁₀]PheT, as shown in diagnostic plot I (Fig. 3, top). Six subjects (1, 6, 14, 22, 23, and 24) whose estimates of systemic exposure (*A_{act}*) were in the top 30% of the population as measured by plasma data (≥4.68 nmol by eq. 2) and urine data (≥4.29 nmol by eq. 3) fell into zone A (large systemic exposure zone). Likewise, five subjects (2, 7, 12, 18, and 21) whose estimates of systemic exposure were in

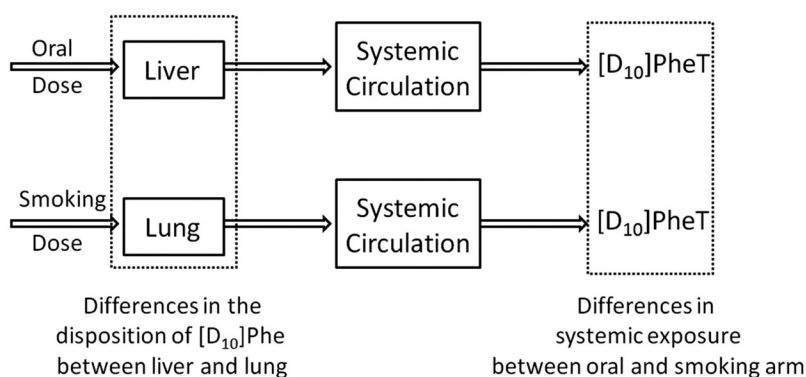


Fig. 4. Comparison of metabolic activation of $[D_{10}]Phe$ in the oral and smoking arm.

the lowest 30% of the population as measured by plasma data (≤ 1.96 nmol by eq. 2) and urine data (≤ 1.58 nmol by eq. 3), fell into zone B (low systemic exposure zone).

The relative bioavailability of $[D_{10}]PheT$ after oral dosing compared with the smoking administration was calculated from the ratio of oral/smoking $AUC_{(PheT)}$ values (F_{AUC}) and the ratio of oral/smoking $Xu_{(PheT),t=48}$ values (F_{Xu}). F_{AUC} and F_{Xu} were used as two indicators of lung contribution to metabolic activation. As shown in Fig. 4, after the administration of $[D_{10}]Phe$ by smoking, the parent molecule $[D_{10}]Phe$ passes through the lung before it reaches the systemic circulation. After oral dosing the parent molecule passes through the liver before it reaches the systemic circulation. Therefore, if F_{AUC} and F_{Xu} are less than 1, the lung has a greater contribution to the formation of $[D_{10}]PheT$. As shown in Table 3, the relative bioavailability measured by F_{AUC} and

F_{Xu} was 1.35 ± 1.33 and 1.35 ± 0.96 , respectively. No significant difference was observed in relative bioavailability measured by the two methods. At the group level no significant difference was observed in the disposition of parent molecule by lung and liver. However, a large intersubject variability was observed (Table 3), illustrating a more than 9-fold range in both F_{AUC} and F_{Xu} . Diagnostic plot II was then developed to identify subjects with significant lung contribution to metabolic activation. Nine subjects (1, 3, 4, 5, 6, 8, 13, 22, and 25) with both F_{AUC} and F_{Xu} values of less than 1 were identified in zone C (Fig. 3, bottom).

The combination of zones A (diagnostic plot I) and C (diagnostic plot II) led to the identification of three subjects (1, 6, and 22) with both a large systemic exposure and a significant lung contribution to activation. These three subjects formed much more $[D_{10}]PheT$ after smoking than oral dosing (Table 4). In addition, $A_{act(lung)}$ of these three subjects was between 0.66 and 10.56 nmol. This amount of $[D_{10}]PheT$ indicated substantial local exposure, especially considering that the total exposure to $[D_{10}]PheT$ in a typical subject after smoking ranged from 3.06 to 4.23 nmol.

Table 5 shows the effects of metabolic enzyme polymorphisms on an individual's capacity to activate PAHs as measured by estimates of A_{act} (eqs. 2 and 3) of both the oral and smoking arms. Among 12 polymorphisms tested only the *GSTM1* polymorphism was associated with a difference in systemic exposure. Figure 5, a (plasma data) and b (urine data), shows the maximum, minimum, and median of A_{act} in *GSTM1*-negative and -positive subjects after smoking. A more than 2-fold difference was observed in A_{act} between *GSTM1*-negative subjects ($A_{act,plasma}: 5.87 \pm 4.85$; $A_{act,urine}: 4.16 \pm 2.00$ nmol; $n = 12$) and *GSTM1*-positive subjects ($A_{act,plasma}: 2.60 \pm 1.76$; $A_{act,urine}: 2.05 \pm 1.16$ nmol; $n = 13$)

TABLE 3

Relative bioavailability by two methods (F_{AUC} and F_{Xu})

Ninety percent confidence interval (90% CI) is of geometric means. Paired t test was performed to compare two methods of calculating relative bioavailability, and results were not significant, $p > 0.05$.

ID	Relative Bioavailability	
	F_{AUC}	F_{Xu}
1	0.75	0.70
2	1.20	0.66
3	0.92	0.98
4	0.98	0.65
5	0.50	0.66
6	0.73	0.91
7	7.32	3.05
8	0.91	0.77
9	1.29	1.41
10	1.14	1.03
11	1.21	1.90
12	0.88	1.09
13	0.76	0.83
14	1.53	1.33
15	1.80	1.36
16	1.06	1.02
17	1.56	1.67
18	1.22	2.19
19	N.A.	5.01
20	1.02	1.20
21	2.11	1.87
22	0.53	0.54
23	1.43	1.13
24	0.82	1.10
25	0.83	0.57
Mean	1.35	1.35
SD	1.33	0.96
90% CI	0.93 – 1.35	0.95 – 1.38

N.A., not available because the plasma concentration of $[D_{10}]PheT$ after smoking was below the limit of quantitation.

TABLE 4

Significant lung contribution to metabolic activation in subjects 1, 6, and 22

Data of a typical subject were based on the group mean ($n = 25$).

ID	$A_{act,plasma}$		$A_{act(lung)}^a$	$A_{act,urine}$		$A_{act(lung)}^b$
	Smoking	Oral		Smoking	Oral	
	nmol					
1	18.81	8.25	10.56	8.00	5.62	2.38
6	10.77	4.62	6.15	7.03	6.37	0.66
22	7.14	2.29	4.85	4.29	2.33	1.96
A typical subject	4.23	3.74		3.06	3.36	

^a The difference of $A_{act,plasma}$ between smoking and oral dosing.

^b The difference of $A_{act,urine}$ between smoking and oral dosing

TABLE 5
Effects of polymorphisms on an individual's capacity to activate PAHs

Gene Polymorphism	Occurrence			<i>p</i> values ^a				
	Normal	Heterozygotes	Homozygotes	Oral		Smoking		
				<i>A_{act,plasma}</i>	<i>A_{act,urine}</i>	<i>A_{act,plasma}</i>	<i>A_{act,urine}</i>	
		%						
<i>CYP1A1 MspI</i> (rs4646903)	64	36	0	N.S.	N.S.	N.S.	N.S.	
<i>CYP1A1 I462V</i> (rs1048943)	88	12	0	N.S.	N.S.	N.S.	N.S.	
<i>CYP1B1 R48G</i> (rs10012)	32	60	8	N.S.	N.S.	N.S.	N.S.	
<i>CYP1B1 A119S</i> (rs1056827)	8	92	0	N.S.	N.S.	N.S.	N.S.	
<i>CYP1B1 L432V</i> (rs1056836)	20	52	28	N.S.	N.S.	N.S.	N.S.	
<i>CYP1B1 N453S</i> (rs1800440)	84	16	0	N.S.	N.S.	N.S.	N.S.	
<i>GSTP1 I105V</i> (rs1695)	36	52	12	N.S.	N.S.	N.S.	N.S.	
<i>GSTP1 A114V</i> (rs1138272)	84	16	0	N.S.	N.S.	N.S.	N.S.	
<i>EPHX1 Y113H</i> (rs1051740)	32	68	0	N.S.	N.S.	N.S.	N.S.	
<i>EPHX1 H139R</i> (rs2234922)	64	28	8	N.S.	N.S.	N.S.	N.S.	
<i>GSTT1</i>	12 (presence)		88 (null)	N.S.	N.S.	N.S.	N.S.	
<i>GSTM1</i>	52 (presence)		48 (null)	*	*	*	**	

N.S., not significant $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

^a One-way analysis of variance was performed to determine the influence of genotype on A_{act} .

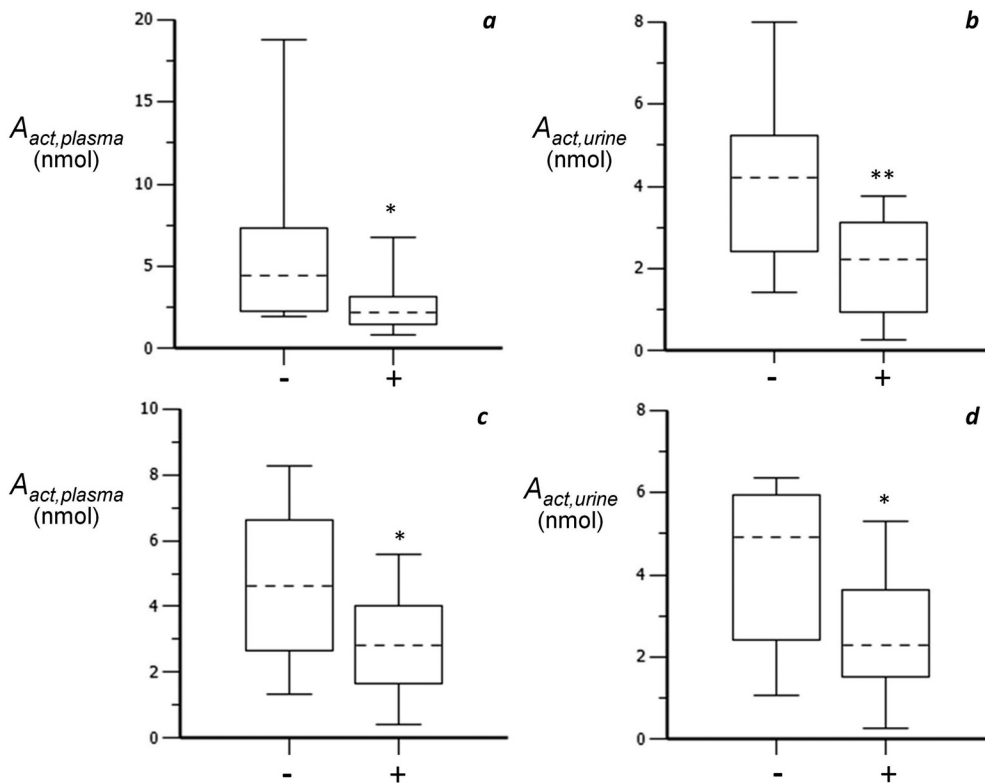


Fig. 5. Comparison of *GSTM1*-negative (-) and -positive (+) subjects after smoking (a and b) and oral dosing (c and d). Dotted lines indicate median. *, $p < 0.05$; **, $p < 0.01$.

after smoking ($p < 0.05$). To further confirm the effects of *GSTM1* on the metabolic activation, estimates of A_{act} from the oral arm were investigated (Fig. 5, c and d), and similar results were observed.

Figure 6 integrates diagnostic plot I and *GSTM1* genotype information and reveals an interesting correlation between genotyping and phenotyping results. The occurrence of the *GSTM1*-negative genotype was 100% ($n = 6$) in zone A compared with 48% in the group as a whole ($n = 25$). In contrast, the occurrence of the *GSTM1*-negative genotype was only 20% ($n = 5$) in zone B. High occurrence of the *GSTM1*-negative genotype in subjects with a large systemic exposure to [D₁₀]PheT, and low occurrence of the *GSTM1*-negative genotype in subjects with a low systemic exposure to [D₁₀]PheT, clearly suggests a correlation between *GSTM1*

genotype and an individual's capacity to activate PAHs. It is of interest to note that the three subjects (1, 6, and 22) with both high systemic exposure and significant lung contribution to activation all were *GSTM1*-negative and African American.

Discussion

Because lung cancer treatment is not particularly effective (5-year survival rate $< 16\%$), prevention is an important alternative, especially considering that tobacco smoking accounts for 90% of cases of the disease in the United States (International Agency for Research on Cancer, 2004; Spiro and Silvestri, 2005). The outcome of smoking cessation, a major prevention strategy, depends on the intensity of inter-

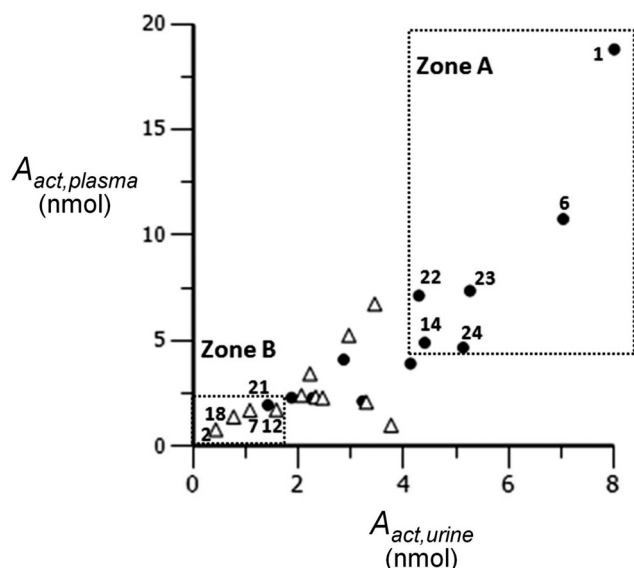


Fig. 6. Correlation between genotyping and phenotyping (Δ , *GSTM1*-positive; \bullet , *GSTM1*-negative).

vention. The rate of successful smoking cessation at 1 year is 3 to 5% when smokers simply try to stop, 7 to 16% when behavioral intervention is provided, and up to 24% when smokers receive both pharmacological treatment and behavioral support (Laniado-Laborin, 2010). Therefore, the successful identification of susceptible individuals could lead to increasing the intensity of intervention for these individuals. This would potentially improve the outcome of smoking cessation interventions. The current study aimed to quantitate an individual's capacity to metabolically activate PAHs (one group of carcinogens involved in lung cancer) and identify subjects with extensive activation and presumably higher lung cancer risk.

The large intersubject variability (>20-fold) in the capacity to activate $[D_{10}]Phe$ was consistent with the large intersubject variability in the activation of PAHs reported in the literature (Cohen et al., 1979) and further justified the use of a PK approach to identify highly susceptible individuals. In our PK study of 25 subjects, the fraction of $[D_{10}]Phe$ converted to $[D_{10}]PheT$ ranged from 0.49 to 15.04% ($A_{act,urine}$) in the smoking arm. Even though only a relatively small fraction of a PAH, as represented by $[D_{10}]Phe$, is metabolically activated in humans, it is believed to be critical in the initiation of carcinogenesis (Gelboin, 1980; Conney, 1982; Cooper et al., 1983; Dipple et al., 1984; Geacintov et al., 1997; Szeliga and Dipple, 1998). Metabolites not quantified in this study include phenols and dihydrodiols as well as unidentified material.

The quantitation of an individual's capacity to activate PAHs was the primary objective of the current study. The unique PK approach used here quantitated both systemic and local exposure to $[D_{10}]PheT$. There are no other published studies on PAH PK in humans to our knowledge. Although the measurement of systemic exposure is important in the identification of susceptible subjects, local (lung) exposure is a physiologically more relevant measurement for two reasons: 1) it captures the magnitude of exposure to carcinogenic intermediates at the specific site where carcinogenesis occurs; and 2) $[D_{10}]Phe$ was used as a surrogate of

BaP and other related PAHs, which generally have their strongest carcinogenic effects at the site of application, although there are exceptions (International Agency for Research on Cancer, 1983, 2010; Hecht, 2003). For locally acting carcinogens, lung exposure is much more relevant to tobacco smoke carcinogenesis of lung cancer than systemic exposure. Despite its crucial role in carcinogenesis by PAHs, local exposure is usually very difficult to measure in clinical trials. In the present study the integration of systemic exposure and relative bioavailability revealed important information regarding local exposure. The concept is that subjects with both large systemic exposure (Fig. 3, top, zone A of plot I) and significant lung contribution to activation (Fig. 3, bottom, zone C of plot II) had substantial local (lung) exposure. As shown in Fig. 4, for a given individual, the difference in systemic exposure after oral dosing and smoking is caused by the different pathways that $[D_{10}]Phe$ takes before reaching the systemic circulation, i.e., one single pass of parent molecule through the liver or lung. In other words, if more $[D_{10}]Phe$ were activated after smoking than oral dosing ($F_{AUC} < 1$ or $F_{Xu} < 1$), then this subject's lung contributed more to the activation than did liver. Therefore, subjects with relative bioavailability less than 1 and subjects with large systemic exposure were of interest.

If relative bioavailability is less than 1, then by definition $A_{act(oral)} < A_{act(smok)}$. According to eqs. 6.1 and 6.2, there are three scenarios when $A_{act(oral)} < A_{act(smok)}$: 1) $f_{a(oral)} < f_{a(smok)}$ and $f_{act(oral)} < f_{act(smok)}$; 2) $f_{a(oral)} > f_{a(smok)}$ and $f_{act(oral)} < f_{act(smok)}$; and 3) $f_{a(oral)} < f_{a(smok)}$ and $f_{act(oral)} > f_{act(smok)}$.

In the first case, because $f_{a(oral)} < f_{a(smok)}$, the lung allowed a larger fraction of $[D_{10}]Phe$ to enter systemic circulation than did the liver, indicating a larger systemic exposure after smoking than oral dosing. In addition, because $f_{act(oral)} < f_{act(smok)}$, more active intermediates would be produced in lung than liver, indicating substantial local exposure. Therefore, case 1 was large systemic exposure, large local exposure. Likewise, cases 2 and 3 would be considered low systemic exposure, large local exposure and large systemic exposure, low local exposure, respectively. Although in case 3 lung may receive lower exposure to the active intermediates than does the liver, given the substantial amount of active intermediates in the circulation, the attack of electrophilic intermediates against lung DNA would still probably be substantial. Therefore, for all the cases related to a relative bioavailability of less than 1, potentially negative health consequences caused by substantial local exposure may be likely. As such, diagnostic plot II was developed to identify subjects with a significant lung contribution to metabolic activation.

As shown in plot I (Fig. 3, top), systemic exposure was calculated by two methods (eqs. 2 and 3). Likewise, in plot II (Fig. 3, bottom) the relative bioavailability was estimated by two methods (F_{AUC} and F_{Xu}) to confirm the identification of subjects with significant lung contribution to activation. The combination of plots I and II led to the identification of three subjects. In these three subjects one single pass through lung produced the same level of $[D_{10}]PheT$ as total exposure in a typical subject. This suggests that these subjects may have higher susceptibility than the rest of the study group.

There was a statistically significant difference in systemic exposure to $[D_{10}]PheT$ between *GSTM1*-negative and -positive subjects. Because *GSTM1* is a detoxification enzyme that catalyzes the conjugation of electrophilic intermediates with

glutathione, the deletion of the *GSTM1* gene reduces the efficiency of the detoxification pathways. As a result, more [D₁₀]Phe may go through the activation pathway, and more end product of the activation pathway was observed in *GSTM1*-negative subjects. Furthermore, approximately a 2-fold difference in activation caused by *GSTM1* might lead to clinically different outcomes considering the important role of PAHs in lung carcinogenesis and decades of uptake of PAHs from cigarette smoking. It has been reported that *GSTM1*-negative subjects have higher PAH-DNA adducts in the lung than *GSTM1*-positive subjects (Rojas et al., 1998, 2000). It is worth noting that the three subjects identified by diagnostic plots I and II all were *GSTM1*-negative. In addition, all three subjects were African American. The development of a population PK model is in progress that will estimate the population mean and intersubject variability of the efficiency of the activation pathway. Age, gender, weight, race, renal function, genotype, and smoking history will be incorporated in the population PK analysis to examine their influence on lung cancer susceptibility.

In other studies, we have found that conjugation of Phe diol epoxides by glutathione is a relatively minor pathway, at least based on the amount of the appropriate *N*-acetylcysteine conjugate found in urine (Hecht et al., 2008). The amounts seem to be too low to account for the decreased levels of [D₁₀]PheT in the *GSTM1*-competent versus *GSTM1*-null individuals observed here. It is possible that glutathione conjugation occurs earlier in the pathway that produces [D₁₀]PheT, for example, by reaction with Phe-1,2-epoxide (Fig. 1).

Although no effects of other polymorphisms on systemic exposure were observed, it is likely that multiple genes are involved in the metabolic activation of PAHs (Shimada, 2006; Hecht, 2010). The low frequency of minor alleles and relatively small number of subjects in the present study may explain the failure to detect an association of these polymorphisms with our measures of exposure.

One limitation of the present study was the lack of a PK profile of the parent compound [D₁₀]Phe caused by its extremely low concentrations in plasma (< 25 pg/ml) and urine (< 5 pg/ml) after the 10-μg dose of [D₁₀]Phe (Zhong et al., 2011b). Animal PK studies have been reported using a dose of 4.5 to 10 mg/kg, which was at least 30,000-fold higher than the dose used in this study (Chu et al., 1992; Schober et al., 2010). The half-life and oral clearance of phenanthrene in a mouse study with an oral dose of 4.5 mg/kg were reported to be 0.32 h and 2.18 liter/h · kg, respectively. Another study in rat and guinea pig indicated that more than 90% of the parent compound was converted to metabolites after oral administration of 10 mg/kg ¹⁴C-labeled Phe. The distribution of Phe to tissues was not well characterized, but Phe is probably extensively distributed to tissues because of its lipophilicity. Collectively, the low dose of [D₁₀]Phe in human study, extensive metabolism, and rapid distribution are likely to contribute to the low concentration of [D₁₀]Phe in plasma and urine.

In summary, in the present study the metabolic activation of Phe in humans has been investigated. Plasma and urinary data of the smoking arm indicated a more than 20-fold difference in an individual's capacity to activate PAHs and formed the basis of diagnostic plot I to identify subjects with large systemic exposure. The relative bioavailability between

oral dosing and smoking also showed a large intersubject variability (> 9-fold), and formed the basis of diagnostic plot II to identify subjects with significant lung contribution to activation. The combination of plots I and II led to the identification of subjects with substantial lung exposure. This approach may have significant potential in the prediction of lung cancer risk. The integration of phenotyping and genotyping results indicated that *GSTM1* played an important role in the detoxification of Phe.

Appendix

In the PK approach to identify susceptible subjects, one primary objective was to quantitate the [D₁₀]PheT formed and use it as a surrogate for the amount of "active" metabolite [D₁₀]PheDE formed during activation (*A_{act}*). This is an indicator of systemic exposure. As shown in Fig. 1, [D₁₀]PheDE is the surrogate for the active intermediate BPDE, which binds to DNA and initiates carcinogenesis. However, direct measurement of [D₁₀]PheDE was very difficult because of the poor stability of this intermediate. Our investigation turned to [D₁₀]PheT, the end product of activation pathway. Based on our understanding of the metabolic activation of Phe as shown in Fig. 2, *A_{act}* could be estimated by quantitating [D₁₀]PheT in plasma or urine.

The absorption and disposition of [D₁₀]Phe after extravascular administration (oral or smoking) could be summarized in four steps (Fig. 2). Step 1, after delivery to the site of absorption, [D₁₀]Phe was transferred to systemic circulation intact (absorption), in the form of [D₁₀]PheT (first-pass activation) and other metabolites; the clearances of these three pathways of step 1 were *CL*₁, *CL*₂, and *CL*₃, respectively. Step 2, after entering the systemic circulation, a small fraction of [D₁₀]Phe was converted to active metabolite [D₁₀]PheDE (activation), and the majority of [D₁₀]Phe was transformed to other metabolites; the clearances of these two pathways of step 2 were *CL*₅ and *CL*₄, respectively. Step 3, [D₁₀]PheDE was converted to [D₁₀]PheT (hydrolysis) and the clearance of this process was *CL*₆. Step 4, [D₁₀]PheT was further converted to sulfate or glucuronide conjugates followed by renal excretion. The clearance of the process that eliminates [D₁₀]PheT from the body was *CL*₇.

Several assumptions were made in steps 3 and 4. The first assumption is that in step 3 all [D₁₀]PheDE was converted to [D₁₀]PheT. The second assumption is that in step 4 all of the [D₁₀]PheT formed was excreted in urine in 48 h.

As such, the following differential equations were derived:

$$\frac{dA_{(Phe,abs)}}{dt} = -(CL_1 + CL_2 + CL_3) \times C_{(Phe,abs)} \quad (A1)$$

$$\frac{dA_{(Phe)}}{dt} = CL_1 \times C_{(Phe,abs)} - (CL_4 + CL_5) \times C_{(Phe)} \quad (A2)$$

$$\frac{dA_{(PheDE)}}{dt} = CL_2 \times C_{(Phe,abs)} + CL_5 \times C_{(Phe)} - CL_6 \times C_{(PheDE)} \quad (A3)$$

$$\frac{dA_{(PheT)}}{dt} = CL_6 \times C_{(PheDE)} - CL_7 \times C_{(PheT)} \quad (A4)$$

$$\frac{dXu_{(PheT)}}{dt} = CL_7 \times C_{(PheT)} \quad (A5)$$

Equation A1 could also be written as:

$$dA_{(Phe,abs)} = -(CL_1 + CL_2 + CL_3) \times C_{(Phe,abs)} dt \quad (A6)$$

The integration of eq. A6 with regard to time from $t = 0$ to $t = \infty$ provides the following expression:

$$\int_{A_{(Phe,abs),t=0}}^{A_{(Phe,abs),t=\infty}} dA_{(Phe,abs)} = -(CL_1 + CL_2 + CL_3) \int_0^{\infty} C_{(Phe,abs)} dt$$

$A_{(Phe,abs)}$ integrated between $t = 0$ and $t = \infty$ is equal to the dose of $[D_{10}]Phe$ (D).

Therefore, $D = (CL_1 + CL_2 + CL_3) \times AUC_{(Phe,abs)}$.

$$AUC_{(Phe,abs)} = \frac{D}{CL_1 + CL_2 + CL_3} \quad (A7)$$

Likewise, the integration of eqs. A2, A3, A4, and A5 from $t = 0$ to $t = \infty$ provides the following expressions:

$$CL_1 \times AUC_{(Phe,abs)} = (CL_4 + CL_5) \times AUC_{(Phe)} \quad (A8)$$

$$CL_2 \times AUC_{(Phe,abs)} + CL_5 \times AUC_{(Phe)} = CL_6 \times AUC_{(PheDE)} \quad (A9)$$

$$CL_6 \times AUC_{(PheDE)} = CL_7 \times AUC_{(PheT)} \quad (A10)$$

$$Xu_{(PheT),t=\infty} = CL_7 \times AUC_{(PheT)} \quad (A11)$$

It is assumed that $Xu_{(PheT),t=\infty} = Xu_{(PheT),t=48}$; that is, almost all of the $[D_{10}]PheT$ formed is excreted into the urine in 48 h.

The integration of eq. A3 from $t = 0$ to $t = T$ ($0 < T < \infty$) provides the following expression:

$$A_{(PheDE),t=T} - A_{(PheDE),t=0} = CL_2 \times AUC_{0(Phe,abs)}^T + CL_5 \times AUC_{0(Phe)}^T - CL_6 \times AUC_{0(PheDE)}^T$$

Because $A_{(PheDE),t=0}$ equals 0, then

$$A_{(PheDE),t=T} = CL_2 \times AUC_{0(Phe,abs)}^T + CL_5 \times AUC_{0(Phe)}^T - CL_6 \times AUC_{0(PheDE)}^T \quad (A12)$$

Likewise, the integration of eqs. A4 and A5 from $t = 0$ to $t = T$ could result in eqs. A13 and A14:

$$A_{(PheT),t=T} = CL_6 \times AUC_{0(PheDE)}^T - CL_7 \times AUC_{0(PheT)}^T \quad (A13)$$

$$Xu_{(PheT),t=T} = CL_7 \times AUC_{0(PheT)}^T \quad (A14)$$

At time T , the $[D_{10}]PheDE$ that has been produced from activation can exist in three different forms: $[D_{10}]PheDE$ in the body, $[D_{10}]PheT$ in the body, and $[D_{10}]PheT$ in urine. Therefore, the amount of $[D_{10}]PheDE$ formed at time T is the sum of $A_{(PheDE),t=T}$, $A_{(PheT),t=T}$, and $Xu_{(PheT),t=T}$.

Combining eqs. A12, A13, and A14:

$$A_{(PheDE),t=T} + A_{(PheT),t=T} + Xu_{(PheT),t=T} = CL_2 \times AUC_{0(Phe,abs)}^T + CL_5 \times AUC_{0(Phe)}^T \quad (A15)$$

When time T approaches infinity, eq. A15 can be written as:

$$A_{(PheDE),t=\infty} + A_{(PheT),t=\infty} + Xu_{(PheT),t=\infty} = CL_2 \times AUC_{(Phe,abs)} + CL_5 \times AUC_{(Phe)} \quad (A16)$$

By definition, the left side of eq. A16 is equal to A_{act} :

$$A_{act} = A_{(PheDE),t=\infty} + A_{(PheT),t=\infty} + Xu_{(PheT),t=\infty} \quad (A17)$$

Combining eqs. A16 and A17 to obtain eq. A18:

$$A_{act} = CL_2 \times AUC_{(Phe,abs)} + CL_5 \times AUC_{(Phe)} \quad (A18)$$

Substituting eqs. A7 and A8 into A18 to obtain eq. A19:

$$A_{act} = CL_2 \times \frac{D}{CL_1 + CL_2 + CL_3} + CL_5 \times \frac{CL_1}{CL_4 + CL_5} \times \frac{D}{CL_1 + CL_2 + CL_3} \quad (A19)$$

If f_a , f_{act} , and f_m are defined as the following:

$$f_a = \frac{CL_1}{CL_1 + CL_2 + CL_3} \quad (A20)$$

$$f_{act} = \frac{CL_2}{CL_1 + CL_2 + CL_3} \quad (A21)$$

$$f_m = \frac{CL_5}{CL_4 + CL_5} \quad (A22)$$

then

$$A_{act} = [(f_a \times f_m) + f_{act}] \times D \quad (A23)$$

where f_a , f_{act} , and f_m are the fraction of the dose absorbed, the fraction of the dose converted to $[D_{10}]PheDE$ during first-pass activation, and the fraction of $[D_{10}]Phe$ converted to $[D_{10}]PheDE$ in the systemic circulation, respectively.

Because $A_{(PheDE),t=\infty}$ and $A_{(PheT),t=\infty}$ both equal 0, eq. A17 could be reduced to:

$$A_{act} = Xu_{(PheT),t=\infty} \quad (A24)$$

In addition, it is assumed that all the $[D_{10}]PheT$ formed was excreted in urine in 48 h. Therefore,

$$A_{act} = Xu_{(PheT),t=48} \quad (A25)$$

The combination of eqs. A24 and A11 leads to eq. A26:

$$A_{act} = AUC_{(PheT)} \times CL_7 \quad (A26)$$

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Authorship Contributions

Participated in research design: Hatsukami, Hecht, and Zimmerman.

Conducted experiments: Wang, Zhong, Hochalter, Rauch, Oliver, and Jensen.

Contributed new reagents or analytic tools: Carmella and Upadhyaya.

Performed data analysis: Wang and Zimmerman.

Wrote or contributed to the writing of the manuscript: Wang and Zimmerman.

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Address correspondence to: Dr. Cheryl L. Zimmerman, College of Pharmacy, University of Minnesota, 308 Harvard St. S.E., Minneapolis, MN 55455. E-mail: zimme005@umn.edu