

# Site-Specific Metabolism of Naphthalene and 1-Nitronaphthalene in Dissected Airways of Rhesus Macaques

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## ABSTRACT

Studies in rodents have demonstrated the importance of cytochrome P450 monooxygenases in generating reactive metabolites that produce Clara cell injury. Pulmonary P450 activities in rodents are much higher than those in primates, raising the issue of relevance of rodent data to primates. Few studies on P450-catalyzed activation of cytotoxicants in subcompartments of primate lung have been reported. Accordingly, infant monkey airway subcompartments, including trachea, proximal, midlevel, distal airways, and parenchyma, were incubated with naphthalene or 1-nitronaphthalene to define metabolism at both high (500  $\mu\text{M}$ ) and low (50  $\mu\text{M}$ ) substrate concentrations. There was a relatively even distribution of metabolizing activities for naphthalene across subcompartments, but at high concentrations of 1-nitronaphthalene, lower airways (midlevel airway through parenchyma) showed higher bioactivation than upper airways. Dihydrodiol was the predominant water-soluble

metabolite of naphthalene generated by all subcompartments, whereas covalently bound metabolites accounted for the greatest percentage of 1-nitronaphthalene metabolites, especially in lower airways. As anticipated, the amounts of metabolite covalently bound as a percentage of total metabolite formed increased dramatically with the 10-fold increase in substrate concentration. With both substrates, the formation of water-soluble metabolites was approximately 100 times less than observed previously in rodents. We conclude that 1) there are significant quantitative differences between rhesus and rodents in substrate bioactivation; 2) the distribution of metabolizing activities for naphthalene but not 1-nitronaphthalene is significantly different for rodents and primates; and 3) a very high percentage of the metabolites generated, particularly for 1-nitronaphthalene, is bound covalently to cellular proteins.

Naphthalene is a polyaromatic hydrocarbon generated as a by-product of various combustion processes, including gasoline use and cigarette smoking (IARC, 2002). Nitration of naphthalene and its methylated congeners in the gas phase forms 1-nitronaphthalene and corresponding methylnitro derivatives. A significant fraction of the total mutagenicity associated with ambient particulates in southern California has been attributed to the presence of these compounds (Gupta et al., 1996).

Naphthalene and 1-nitronaphthalene undergo metabolic

activation catalyzed by the cytochrome P450 monooxygenases to form electrophilic intermediates that seem to be linked to the cytotoxic injury observed in the respiratory airway epithelial cells of rodents treated with these compounds (Verschoyle et al., 1993; for review, see Buckpitt et al., 2002). Previous studies correlated the species- and site-selective susceptibility to naphthalene injury with the rate of naphthalene metabolism and total protein-bound metabolites in different airway subcompartments from rodent lungs (Buckpitt et al., 1995; Cho et al., 1995). 1-Nitronaphthalene cytotoxicity also showed airway selectivity (Paige et al., 1997). In addition, the rate of formation of regioisomeric epoxides from 1-nitronaphthalene differed substantially in target and nontarget tissues (Watt and Buckpitt, 2000). This suggests that site-selective toxicity associated with naphthalene and 1-nitronaphthalene exposure is, in part, related to P450 activity.

A number of cytochrome P450 monooxygenases have been identified in the lung (Willey et al., 1996), and catalytic

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**ABBREVIATIONS:** P450, cytochrome P450; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance.

activities of a few of these enzymes have been reported using either purified proteins or recombinant enzymes (Lanza et al., 1999; Shultz et al., 2001). In general, the amount of P450 protein present and the P450 activity of both microsomal and intact airway preparations from lungs of nonhuman primates or humans are 10- to 100-fold or more lower than those observed in rodents (for review, see Hukkanen et al., 2002). For example, recent comparative metabolic studies of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone showed that the rate of  $\alpha$ -hydroxylation was  $>160$  pmol/min/mg in mouse lung microsomal incubations (Jalas et al., 2003), whereas the highest rate in human lung was 0.057 pmol/min/mg (Smith et al., 2003). An exception to the striking difference in metabolic rates between rodents and humans has been reported with dichloroethylene; biotransformation rates in lung microsomes from humans were only 2- to 3-fold lower than those from mice (Dowsley et al., 1995). The high degree of localization of cytochrome P450 monooxygenases in airway epithelial cells in rodents suggests that rodent-primate differences in xenobiotic metabolism might be due to the fact that comparisons of P450-dependent metabolism were done using microsomes from whole lung rather than airway subcompartments. When comparing P450 activities in microsomal incubations prepared from specific subcompartments of the lungs of rat and rhesus macaque, the rhesus macaque preparations yielded lower activities in all cases, but most of the differences were 2- to 3-fold, not 10- to 100-fold (Lee et al., 1998). Moreover, the rat and the rhesus monkey had a different distribution of P450 isoform activities. The distribution of CYP1A1 and 2B1 activities was more homogenous in rhesus macaques, whereas CYP2E1 activity was higher in the distal bronchiole and parenchyma than in the proximal airways (Lee et al., 1998). In rats, CYP1A1 and CYP2B activities were highest in the parenchyma, whereas CYP2E1 activity was highest in the airways. Whether the distribution of P450 activities for isoform-selective substrates correlates with the distribution of metabolic activity with pulmonary cytotoxicants is unknown.

The studies described in this report provide a detailed assessment of the formation and nature of metabolites from two P450-activated pulmonary cytotoxicants, naphthalene and 1-nitronaphthalene, in lung subcompartments of infant rhesus macaques, a species anatomically and physiologically similar to the human. The studies show a much lower substrate bioactivation than in rodents yet a similar formation of reactive metabolites.

## Materials and Methods

**Animals and General Experimental Protocol.** Male rhesus macaque (*Macaca mulatta*) monkeys used for this study were colony-born at the California National Primate Research Center located on the University of California, Davis, campus. Care of these animals complied with the provisions of the Institute of Laboratory Animal Resources (NRC, 1996) and conformed to practices established by the American Association for Accreditation of Laboratory Animal Care. Six male infant rhesus monkeys were housed from birth until necropsy at 6 months of age in social groups in large 4.2-m<sup>3</sup> stainless steel and glass inhalation exposure chambers updated from those described previously by Hinners et al. (1968). The chambers were prefiltered with high-efficiency particulate-filtered air and activated charcoal adsorber to eliminate most air pollutants from the cham-

bers. A central air handling system supplied chemical, bacteriological, and radiological filtered air at a 2.1 m<sup>3</sup>/min flow rate for a complete air change every 2 min. Before necropsy, monkeys were euthanized with an overdose of pentobarbital after being sedated with Telazol (8 mg/kg i.m.) and anesthetized with Diprivan (0.1–0.2 mg/kg/min i.v.). The animals were then necropsied after exsanguination via the systemic aorta.

**Chemicals and Reagents.** Deficient Waymouth's MB 752/1 medium (without glutathione, L-cysteine, L-cystine, L-glutamine, and L-methionine) was purchased from Invitrogen (Carlsbad, CA). Waymouth's MB 752/1 medium with L-glutamine was purchased from Sigma-Aldrich (St. Louis, MO). Safety-Solve liquid scintillation fluid was purchased from Research Products International Corp. (Mt. Prospect, IL). Thioether conjugate standards of naphthalene (glutathione, cysteine, and cysteinyl-glycine) were prepared by reacting naphthalene oxide with the appropriate thiol as described previously (Buonarati et al., 1990). 1,2-Dihydroxy-1,2-dihydronaphthalene was purchased from Aldrich Chemical Co. (Milwaukee, WI). Glutathione conjugate standards for 1-nitronaphthalene were generated in liver microsomal incubations, and their identities were established by coelution with previously characterized metabolites (Watt et al., 1999). All other chemicals purchased from a commercial vendor were reagent grade or better.

**Radioactive Chemicals.** [1-<sup>14</sup>C]Naphthalene (specific activity 52 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [<sup>14</sup>C]1-Nitronaphthalene was prepared in the laboratory by the nitration of [1-<sup>14</sup>C]naphthalene with a stoichiometric amount of ammonium nitrate and excess trifluoroacetic anhydride in chloroform. The product was purified using normal-phase chromatography. The radiochemical purity of the final product was tested by reverse-phase HPLC and shown to be  $>99\%$  pure. Unlabeled naphthalene or 1-nitronaphthalene was added to achieve final specific activities of 5 and 10 dpm/pmol at 500 and 50  $\mu$ M substrate concentrations, respectively.

**Tissue Preparation, Incubation, and Preparation of Samples for Analysis.** Airway subcompartments (trachea, proximal, midlevel, distal airways, and parenchyma) were obtained as described previously (Plopper et al., 1991) and were placed in deficient Waymouth's medium at 4°C. A small piece of each airway subcompartment was placed in 200 mM methane sulfonic acid, 5 mM diethylenepentaacetic acid for glutathione determination (Lakritz et al., 1997). The remaining dissected airway subcompartments were placed into separate silanized vials containing 1 ml of oxygenated Waymouth's medium (Sigma-Aldrich) with 27 mM sodium bicarbonate, 15 mM HEPES, and the pH was adjusted between 7.4 and 7.5 by dropwise addition of 1 N NaOH for incubation with either naphthalene or 1-nitronaphthalene. The individual airways were then incubated with either 500  $\mu$ M <sup>14</sup>C-labeled or 50  $\mu$ M <sup>14</sup>C-labeled naphthalene or 1-nitronaphthalene, added in 10  $\mu$ l of methanol, for 4 h at 37°C in a shaking water bath. After incubations, samples were stored in a  $-20^{\circ}\text{C}$  freezer until sample preparation. The contents of the incubation vial were extracted twice with an equal volume of hexane to remove nonmetabolized, volatile substrate. Methanol (2 ml) was added to each sample and mixed on a vortex mixer before storing overnight at  $-20^{\circ}\text{C}$ . Samples were centrifuged at 9000g for 15 min at 0°C to remove the protein. The protein pellet was saved for protein covalent binding analysis, and the supernatant was evaporated to dryness and saved for HPLC analysis of water-soluble metabolites.

**HPLC Analysis of Water-Soluble Metabolites of Naphthalene and 1-Nitronaphthalene.** Metabolites generated in incubations of airway subcompartments were separated by methods described previously in Buckpitt et al. 1995 (naphthalene) and Watt et al. (1999) (1-nitronaphthalene). Briefly, the final dried residue of the methanol/medium extract from the incubation was reconstituted in water, and aliquots were injected onto a Spherisorb ODS-2 column (25 cm  $\times$  4.6 mm i.d.; 5- $\mu$ m particle) (Waters, Milford, MA). The mobile phase consisted of 0.06% triethylamine adjusted to pH 3.1

(with phosphoric acid) and acetonitrile. Solvent composition was adjusted from 5 to 7% acetonitrile at time 0 to 7.5 to 13.2% acetonitrile over the first 75 min of a 100-min run at a flow rate of 1 ml/min to separate naphthalene and 1-nitronaphthalene metabolites. Naphthalene metabolite standards (1,2-dihydroxy-1,2-dihydronaphthalene, glutathione conjugates, and cysteinyl-glycine conjugates) and 1-nitronaphthalene metabolite standards (glutathione conjugates derived from the C<sub>5</sub>,C<sub>6</sub>- and C<sub>7</sub>,C<sub>8</sub>-epoxides) were run on the same gradient and spiked into various samples for comparison. The column eluate was monitored with a Hewlett Packard series 1100 UV detector at 256 nm, and 1-min fractions were collected and counted for 5 or 10 min each in a liquid scintillation counter. Quantitative measurements of metabolite formation were calculated based on <sup>14</sup>C elution from the column.

**Protein Covalent Binding.** Protein pellets saved after centrifugation of the sample were washed multiple times with 5 volumes of methanol until an aliquot contained less than 100 dpm/ml. Proteins were dissolved in 1 N NaOH, and protein content was measured using a modification of the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard.

**HPLC Analysis of Reduced Glutathione.** Measurements of reduced glutathione content were conducted using HPLC with electrochemical detection as described by Lakritz et al. (1997). Individual airway tissues were homogenized in 200  $\mu$ l of 200 mM methane sulfonic acid/5 mM diethylenetriaminepentaacetic acid, samples were centrifuged (12,000g, 20 min), and aliquots of the supernatant were used for analysis. The pellets were redissolved in 1 N NaOH, and an aliquot was taken for protein determination as described above.

**Statistical Analysis.** When water-soluble metabolites of naphthalene and 1-nitronaphthalene were below our limit of detection, 0.001 pmol/mg protein was used for those samples because this number is below the detection limits of both naphthalene (limit of detection 0.008 pmol/mg protein) and 1-nitronaphthalene (limit of detection 0.016 pmol/mg protein) metabolites. Data were analyzed using SigmaStat (SPSS Inc., Chicago, IL) for one-way analysis of variance (ANOVA). Once a statistically significant difference was identified among groups ( $p < 0.05$ ), all pairwise multiple comparison procedures (Bonferroni  $t$  test) were done to determine statistical differences between groups ( $p < 0.05$ ).

## Results

**Formation of Water-Soluble and Covalently Bound Metabolites of Naphthalene in Airway Incubations.** Earlier in vitro and in vivo work in rodents has shown that naphthalene is metabolized to a 1,2-epoxide that is subsequently converted to 1,2-dihydroxy-1,2-dihydronaphthalene (diol), 1-naphthol, and a number of thioether conjugates (Fig. 1A).

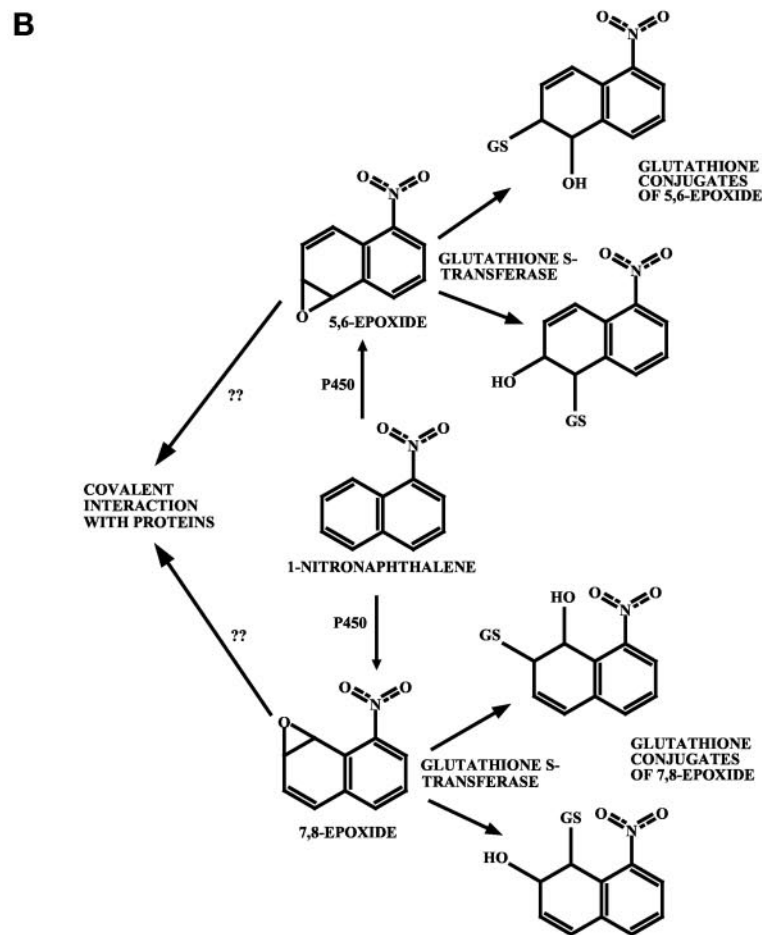
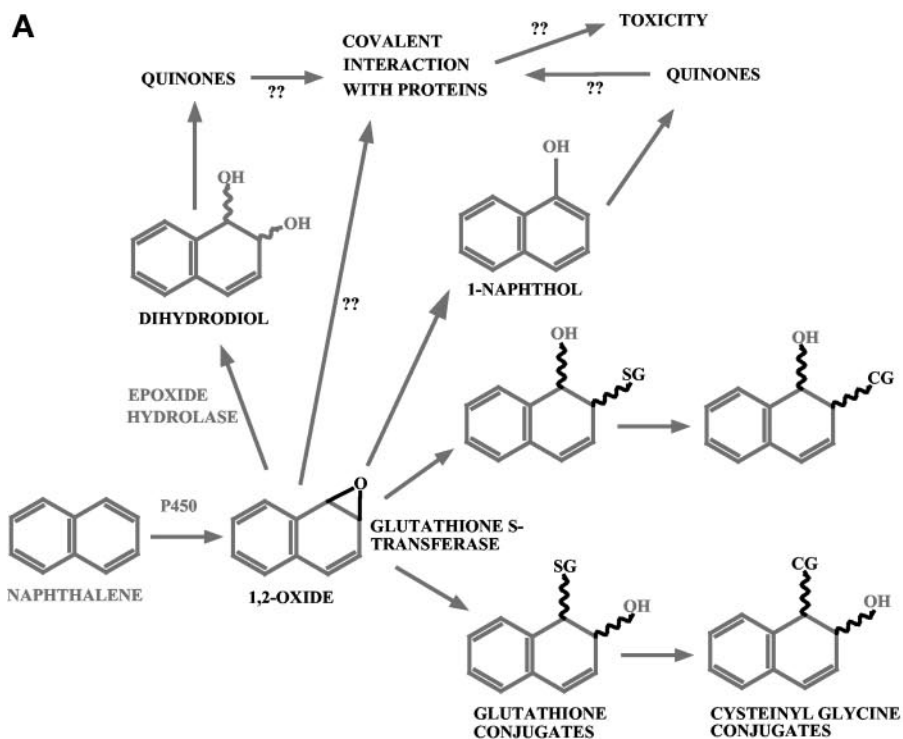
Figure 2, A and B, summarizes the findings of naphthalene metabolism at a 50  $\mu$ M substrate concentration by microdissected airway explants of monkey lung. In addition to those metabolites specifically identified in the radiochromatographic profiles, a number of less abundant, unidentified metabolites were measured and summed to provide an assessment of the total metabolism. In the parenchyma, the dihydrodiol content was 0.115 nmol/mg protein, significantly higher ( $p < 0.05$ ) than that in the proximal and distal airways (Fig. 2A). Total naphthalene metabolism was highest in the midlevel airway (0.88 nmol/mg protein) and lowest in the distal airway (0.13 nmol/mg protein) (Fig. 2B). Naphthalene metabolites that covalently bound to proteins accounted for 26% of the total metabolites in the distal airway and 11 to 15% of the total metabolites produced in incubations of other airway subcompartments. Cysteinyl-glycine conjugates

accounted for between 0 and 5% of the total metabolites, depending upon the airway subcompartment. The total formation of thioether conjugates (glutathione plus cysteinyl-glycine) accounted for about 9% of the total metabolites produced. The amounts of diol generated were variable and represented 41% of the total metabolites in the parenchyma but only 5.7% in the midlevel airways.

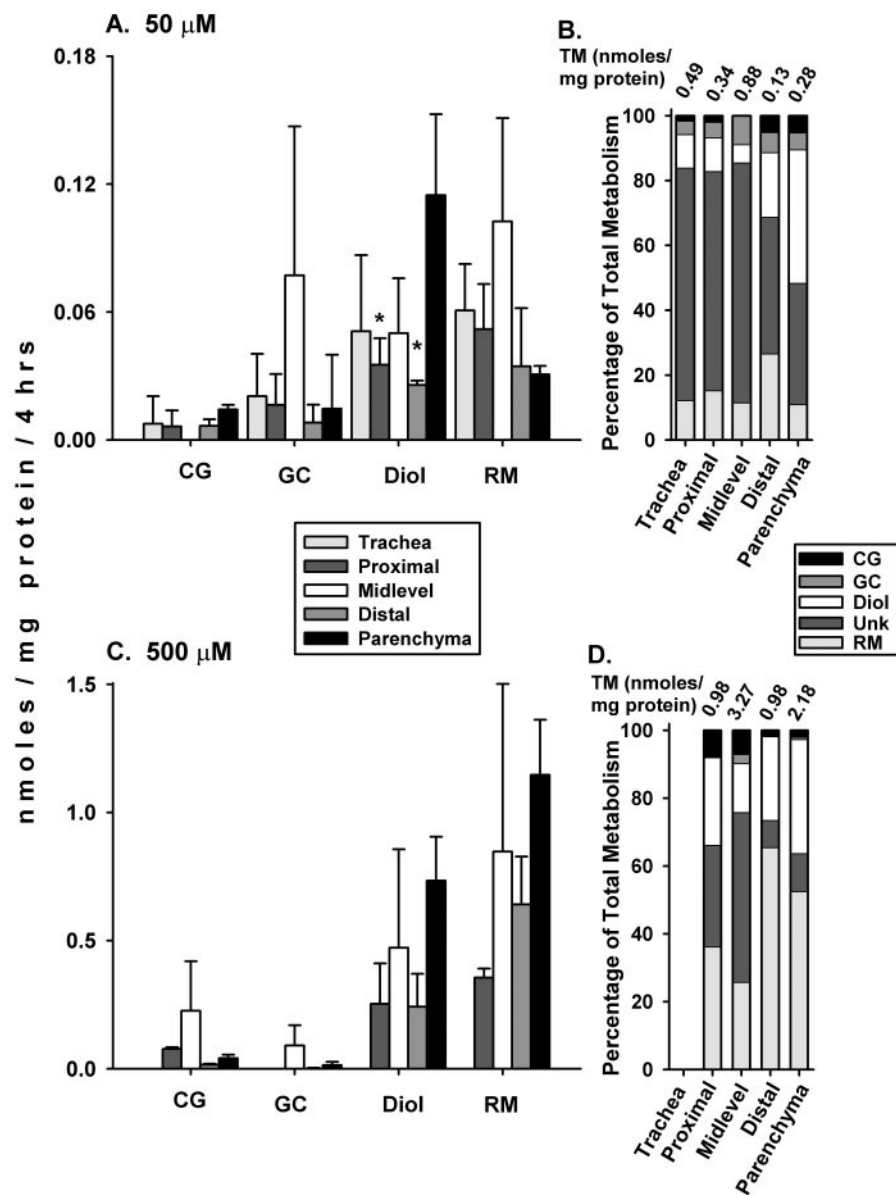
Increasing the substrate concentrations in the incubation to 500  $\mu$ M resulted in a corresponding increase in product formation but also in a striking alteration in the profile of metabolic products generated (Fig. 2, C and D). There was an  $n$  of 3 for each airway subcompartment; however, no trachea samples were obtained. Total naphthalene metabolism was highest in the midlevel airway (3.27 nmol/mg protein) and lowest in both proximal and distal airways (0.98 nmol/mg protein) (Fig. 2D). Total reactive naphthalene metabolites bound covalently to proteins varied from 25 to 65% of total metabolites in midlevel and distal airways, respectively. As a percentage of the total metabolites, cysteinyl-glycine conjugates were highest in the proximal (7.9%) and lowest in the distal (1.6%) airway incubations. However, the amounts of glutathione conjugates generated were lower than the detection limit in the proximal airway and below 3.0% of the total metabolism in all airway levels. The relatively small amounts of glutathione conjugates did not seem to result from lack of intracellular glutathione in subcompartments from juvenile monkeys (Table 1). Diol varied from 14% of the total metabolites in the midlevel airway to 34% in parenchyma.

A comparison of the formation of protein-bound reactive and total metabolites at two different naphthalene concentrations (50 or 500  $\mu$ M) in dissected airways is shown in Fig. 3A. The quantities of total naphthalene metabolites (water-soluble plus covalently bound) generated at 500  $\mu$ M compared with 50  $\mu$ M varied from 2.9-fold higher in the proximal airway to 7.7-fold higher in both distal airway and parenchyma. Similarly, the formation of reactive naphthalene metabolites bound covalently at 500  $\mu$ M varied from 6.9 to 18.6 to 37.4-fold that observed at 50  $\mu$ M substrate concentration in proximal, distal airway, and parenchyma, respectively. The ratio of reactive to total metabolite formation at both substrate concentrations is lowest in the midlevel airway and highest in the distal airway (Fig. 3A).

**Formation of Water-Soluble and Covalently Bound Metabolites of 1-Nitronaphthalene in Airway Incubations.** A number of water-soluble metabolites of 1-nitronaphthalene generated by lung tissue have been identified and characterized in previous studies conducted in rats (Watt et al., 1999). These metabolites include glutathione conjugates derived from the C<sub>5</sub>,C<sub>6</sub>- and C<sub>7</sub>,C<sub>8</sub>-epoxides (Fig. 1B). In addition to the metabolites specifically identified by chromatography with the reference standards, several unidentified metabolites were measured and summed to assess the total metabolites in incubations of monkey lung subcompartments with 1-nitronaphthalene (50 or 500  $\mu$ M). The data showing the formation of water-soluble metabolites, covalently bound reactive metabolites, and the percentage of each class of metabolites to total formed in airway subcompartments incubated with a 50  $\mu$ M concentration of 1-nitronaphthalene is presented in Fig. 4, A and B. Total 1-nitronaphthalene metabolism varied from 0.11 nmol/mg protein in the proximal airway to 0.35 nmol/mg protein in the



**Fig. 1.** Pathways of naphthalene (A) and 1-nitronaphthalene (B) metabolism.



**Fig. 2.** Naphthalene metabolite formation (A and C) and percentage of total metabolites (B and D): cysteinyl-glycine (CG), glutathione conjugates (GC), dihydrodiol (Diol), unidentified water-soluble metabolites (Unk), reactive protein-bound metabolites (RM), and total metabolites (TM) during incubation of airway subcompartments (trachea, proximal, midlevel, distal airways, and parenchyma) with 50  $\mu$ M (A and B) and 500  $\mu$ M (C and D) [ $^{14}$ C]naphthalene. Values are the mean  $\pm$  standard deviation for incubations conducted with subcompartments from three separate animals, except for trachea, where  $n = 0$  at the 500  $\mu$ M substrate concentration. An \* denotes a value significantly different from that in the parenchyma according to one-way ANOVA ( $p < 0.05$ ).

parenchyma (Fig. 4B). Reactive metabolites covalently bound to proteins accounted for more than 30% of the total metabolites at all airway levels, with the distal airway having the highest percentage at 55%. No C<sub>5</sub>,C<sub>6</sub>- and C<sub>7</sub>,C<sub>8</sub>-epoxide-derived glutathione adducts were detected in either proximal or midlevel airways. However, in other airway subcompartments, glutathione adducts from C<sub>5</sub>,C<sub>6</sub>- and C<sub>7</sub>,C<sub>8</sub>-epoxides accounted for 5 to 12% of the total metabolites formed.

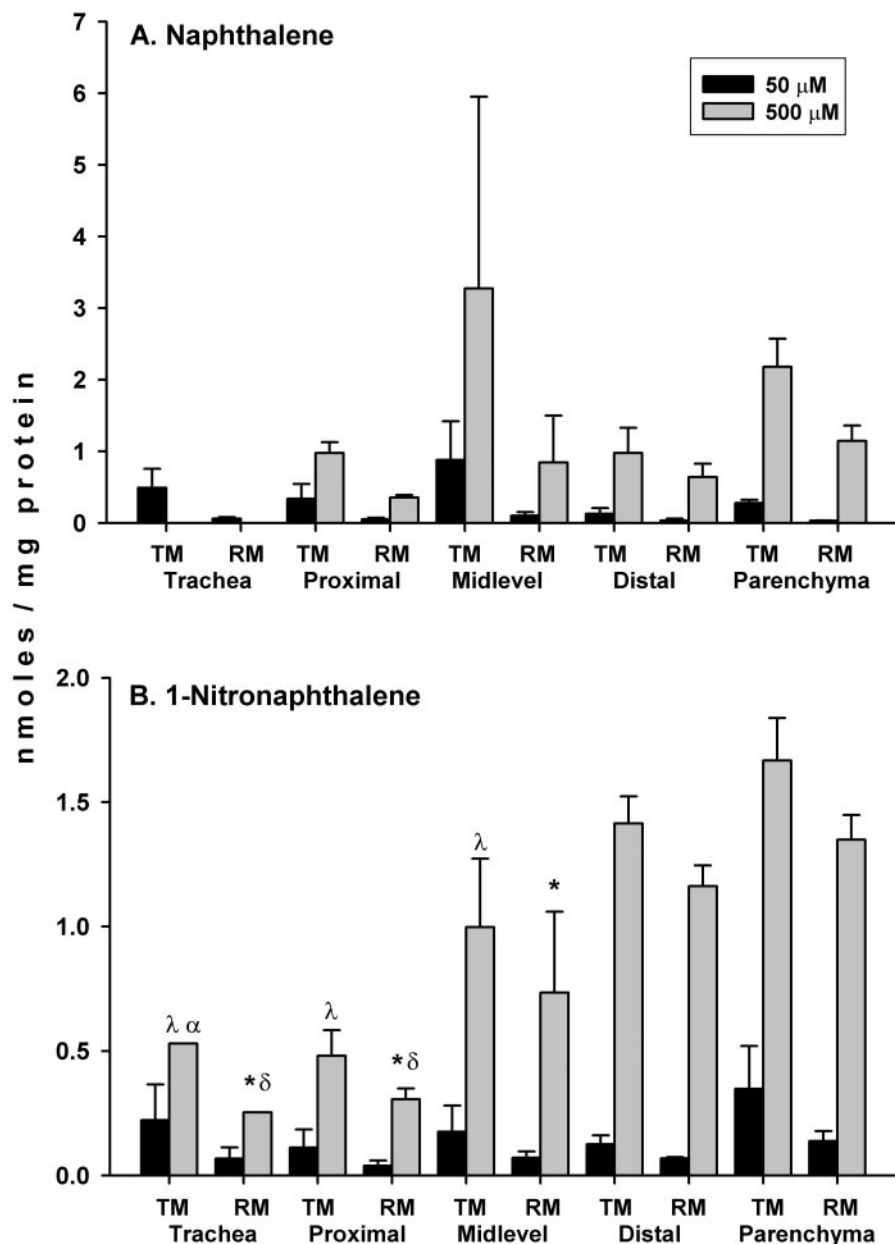
**TABLE 1**  
Reduced glutathione levels in airway subcompartments of rhesus monkeys  
Values are the mean  $\pm$  standard deviation.

Airway Subcompartment	GSH Level <i>nmol / mg protein</i>	<i>n</i>
Trachea	1.6 $\pm$ 0.9	4
Proximal airway	1.8 $\pm$ 1.4	5
Midlevel airway	1.5 $\pm$ 1.4	6
Distal airway	4.6 $\pm$ 2.2	6
Parenchyma	10.9 $\pm$ 11.4	6

GSH, glutathione.

At 500  $\mu$ M 1-nitronaphthalene, no differences in the amounts of water-soluble metabolites were observed in different airway levels. In contrast, reactive metabolite formation was significantly higher ( $p < 0.05$ ) in the parenchyma (1.35 nmol/mg protein) in comparison with the trachea, proximal, and midlevel airways with respective values of 0.25, 0.31, and 0.74 nmol/mg protein (Fig. 4C). In addition, the formation of reactive, covalently bound metabolites in the distal airway (1.16 nmol/mg protein) was significantly higher than that in the trachea and proximal airways ( $p < 0.05$ ).

Total substrate metabolism was lowest (0.48 nmol/mg protein) in the proximal airway and highest (1.67 nmol/mg protein) in the parenchyma at the 500  $\mu$ M substrate concentration (Fig. 4D). In the parenchyma and distal airway, substrate metabolism was higher than that in the trachea, proximal, and midlevel airways ( $p < 0.05$ ) (Fig. 3B). In midlevel airway, substrate metabolism was higher than that in the trachea ( $p < 0.05$ ). Reactive metabolite-protein adducts accounted for 48% of the total metabolites in the trachea and 82% of the total metabolites in the distal airway.



**Fig. 3.** Summary of the formation of total metabolites (TM) and reactive protein bound (RM) metabolites generated in incubations of dissected rhesus monkey airway subcompartments with [ $^{14}\text{C}$ ]naphthalene (A) and [ $^{14}\text{C}$ ]1-nitronaphthalene (B) at either 50 or 500  $\mu\text{M}$  substrate concentrations. Values are the means  $\pm$  standard deviation for incubations conducted with subcompartments from three separate animals, except where  $n = 0$  in the trachea incubated with 500  $\mu\text{M}$  [ $^{14}\text{C}$ ]naphthalene and  $n = 1$  in the trachea incubated with 500  $\mu\text{M}$  [ $^{14}\text{C}$ ]1-nitronaphthalene. For total metabolites generated,  $\alpha$  denotes values significantly different from that in the midlevel airway,  $\lambda$  from both parenchyma and distal airway, according to one-way ANOVA ( $p < 0.05$ ). For reactive protein-bound metabolites, an \* or  $\delta$  denotes a value significantly different from that in the parenchyma or distal airway, respectively, according to one-way ANOVA ( $p < 0.05$ ).

$\text{C}_5$ ,  $\text{C}_6$ - or  $\text{C}_7$ ,  $\text{C}_8$ -epoxide glutathione conjugates detected were below 2% of the total metabolites in all airway levels (Fig. 4D).

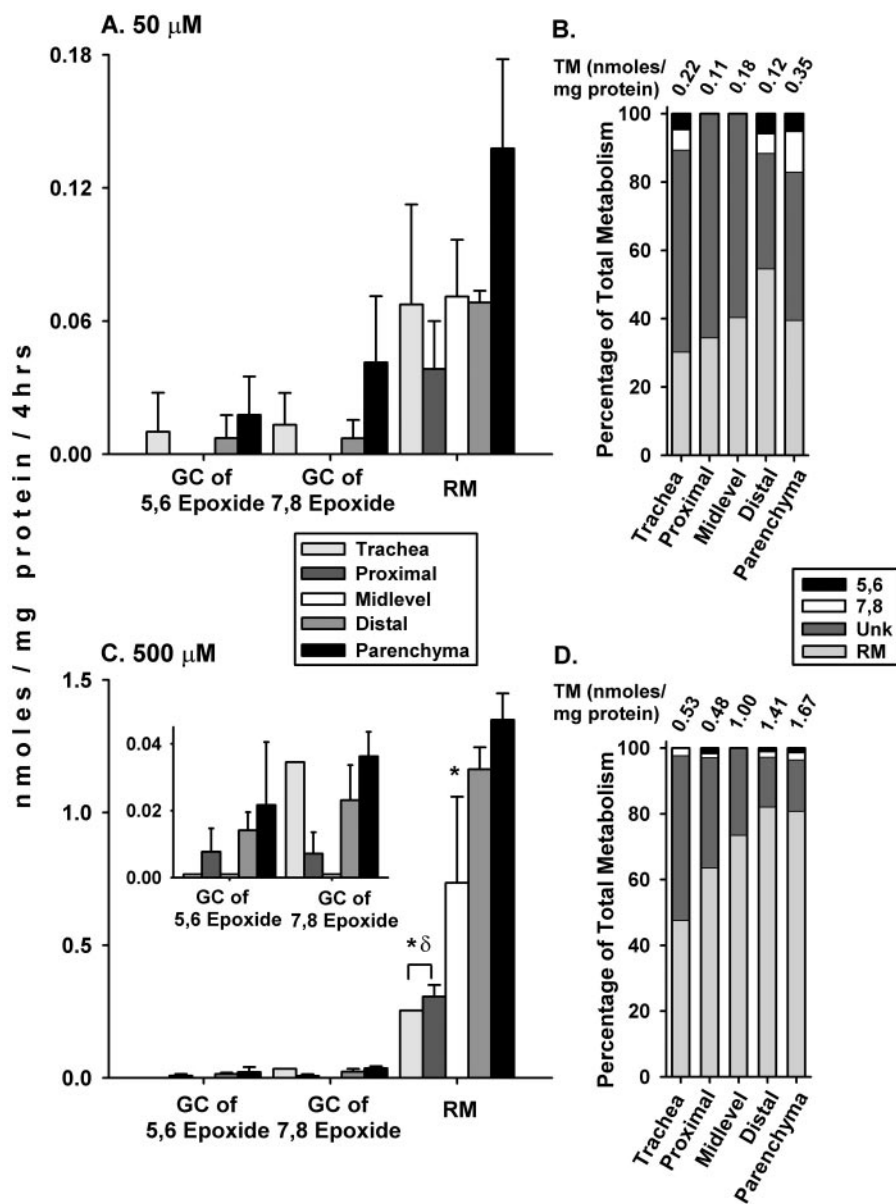
Increasing substrate concentration from 50 to 500  $\mu\text{M}$  increased total metabolism from 2.4-fold in the trachea to 11.3-fold in the distal airway (Fig. 3B). Similarly, the 10-fold higher substrate concentration led to a 3.8-fold increase in reactive metabolite covalent binding in the trachea and to a 17-fold increase in the distal airway. The percentage of bound 1-nitronaphthalene metabolites to total metabolites was lowest in the trachea and highest in the distal airway at both substrate concentrations.

## Discussion

A number of cytochrome P450 monooxygenases have been detected either at the transcript or protein level in lungs of rodents and primates (for review, see Ding and Kaminsky,

2003). In rodents, the importance of these P450s in the metabolic activation of a number of inert chemicals to cytotoxic or carcinogenic intermediates is well accepted (for review, see Yost, 2001). In contrast, the significance of these P450s in catalyzing metabolic activation reactions in humans is not well established primarily because of the lack of quantitative data on cellular protein expression and, in some instances, differences in catalytic activities of the P450 orthologs in rodents versus humans. Moreover, when P450 distribution within the airway trees was measured using isoform-selective substrates, there was a significant difference in pulmonary distribution between rats and monkeys (Lee et al., 1998).

In the current study, the distribution of naphthalene metabolism in the lung was relatively homogenous with slightly higher activities noted in the parenchyma and potentially midlevel airways at both substrate concentrations. In comparison, at the 500  $\mu\text{M}$  substrate concentration, 1-nitronaph-



**Fig. 4.** 1-Nitronaphthalene metabolite formation (A and C) and percentage of total metabolites (B and D): glutathione conjugates (GC) of the  $C_5, C_6$ -epoxides (5,6) and  $C_7, C_8$ -epoxides (7,8), unidentified (Unk), and reactive protein-bound (RM) and total metabolites (TM), during incubation of airway subcompartments (trachea, proximal, midlevel, distal airways, and parenchyma) with subcompartments from 50  $\mu$ M (A and B) and 500  $\mu$ M (C and D) [ $^{14}$ C]1-nitronaphthalene. Values are the mean  $\pm$  standard deviation for incubations conducted with three separate animals, except for trachea, where  $n = 1$  at the 500  $\mu$ M substrate concentration. An \* or  $\delta$  denotes a value significantly different from that in the parenchyma or distal airway, respectively, according to one-way ANOVA ( $p < 0.05$ ).

thalene metabolites generated in the monkey parenchyma and distal airways were higher than those observed in other airway levels, which is consistent with the distribution of CYP2E1 activity in the monkey (Lee et al., 1998). In monkeys, CYP1A1 and CYP2B activities (which were 20–50% of those in rodents) were more uniformly distributed throughout the airway tree than in rats, whereas CYP2E1 activity was mainly found toward the end of the airway. In rats, CYP1A1 and CYP2B activities were highest in the parenchyma, whereas CYP2E1 activity was highest in the airways.

The overall amounts of naphthalene dihydrodiol and glutathione conjugates formed in rhesus lung explants were approximately 70 times less than in comparable incubations with mouse airways (Buckpitt et al., 1995), a finding that is consistent with the results of comparative mouse, monkey, and human lung microsomal incubations (Buckpitt and Bahnson, 1986; Buckpitt et al., 1992). In contrast to the dramatic differences observed in the amount of water-soluble naphthalene metabolites formed between rodents and non-human primates, the differences in the levels of reactive

metabolites bound were not as substantial (Cho et al., 1994). Reactive naphthalene metabolite binding varied from slightly less than 1 nmol/mg protein in mouse upper airways to 3 to 4 nmol/mg protein in more distal airways at 500  $\mu$ M substrate (Cho et al., 1994). In the current studies at the same substrate concentrations, the amounts of bound metabolite were slightly less than 1 nmol/mg protein in airways and slightly higher than this in the parenchyma. Thus, although there are differences in the amount of metabolite bound in mouse compared with monkey, these differences were not 10- to 100-fold. In contrast to the small amounts of reactive naphthalene metabolites generated in mouse parenchyma (Cho et al., 1994), monkey parenchyma generated the largest amounts of reactive metabolites at the high substrate concentration.

Dihydrodiol was the predominant water-soluble naphthalene metabolite generated in incubations with both concentrations of naphthalene. This is consistent with previous data in rhesus monkey (Buckpitt et al., 1992) and human (Buckpitt and Bahnson, 1986) lung microsomal incubations. It is

not clear whether metabolism to the dihydrodiol represents a true detoxication reaction because the diol can be further metabolized to the 1,2-quinone by dihydrodiol dehydrogenase (Smithgall et al., 1988), and the redox activity and electrophilic nature of this metabolite make it a potential suspect in toxicity (Penning et al., 1999). Moreover, evidence has been presented for a trihydroxymercapturic acid metabolite in the urine of mice treated with high doses of naphthalene, a finding that supports the *in vivo* formation of a diol epoxide intermediate (Pakenham et al., 2002).

Both C<sub>5</sub>,C<sub>6</sub>- and C<sub>7</sub>,C<sub>8</sub>-epoxides of 1-nitronaphthalene are generated in rodent microsomal incubations and C<sub>7</sub>,C<sub>8</sub>-epoxides predominate in the lung (Watt et al., 1999). Moreover, CYP2F2 shows regioselectivity for epoxide formation at C<sub>7</sub>,C<sub>8</sub> (Shultz et al., 2001). The regioselectivity noted in rodent lungs was not observed in monkeys, and this is consistent with the fact that CYP2F is undetectable in rhesus macaque airways (Baldwin et al., 2004). Although it is not possible to directly compare the metabolism of 1-nitronaphthalene to water-soluble metabolites in rhesus and rodent because different experimental approaches were used (rodent metabolism studies were conducted using microsomal preparations; Watt et al., 1999), it seems that the amounts of glutathione conjugates generated were considerably lower in rhesus compared with rat lung, with similar levels of GSH or glutathione *S*-transferase in airways of both species (Duan et al., 1993, 1996). Metabolites generated by reductive processes were not detected in previous rodent microsomal incubations or in the current study. This is consistent with *in vivo* studies showing only small amounts of naphthylamine in the urine of 1-nitronaphthalene-treated rats (Halladay et al., 1999).

Interesting differences were observed in the percentages of metabolite bound covalently from naphthalene and 1-nitronaphthalene. At low naphthalene concentrations, less than 27% of the total metabolites were bound covalently, whereas 30 to 55% of the total metabolites generated from 1-nitronaphthalene were bound covalently. As expected, at higher substrate concentrations, larger percentages of the total metabolites became bound covalently to proteins. With both substrates, the amount of metabolite bound covalently accounted for 25 to 65% of the total naphthalene metabolites and 48 to 82% of the total 1-nitronaphthalene metabolites.

Earlier work has provided evidence for the importance of CYP2F in the metabolic activation and subsequent cytotoxicity of naphthalene in rodent models. The kinetics of naphthalene metabolism by heterologously expressed murine CYP2F2 ( $K_m$  and  $k_{cat}$  values of 3  $\mu\text{M}$  and 104<sup>-1</sup>, respectively; Shultz et al., 1999) and excellent correlations between metabolic activities in various lung subcompartments and across species with the amounts of immunoreactive protein present (Baldwin et al., 2004) suggests that this isoform is quantitatively important in the initial step of naphthalene metabolism. The small amounts of naphthalene metabolites generated in dissected airway subcompartments in the present study is fully consistent with recent results showing that although CYP2F isoform is detected at the mRNA level in rhesus (R. M. Baldwin, unpublished data); the protein is not detected in quantitative immunoblotting experiments (Baldwin et al., 2004). Although CYP2F2 also metabolizes 1-nitronaphthalene ( $K_m$  and  $k_{cat}$  of 21  $\mu\text{M}$  and 17.1<sup>-1</sup>), the quantitative contribution of this isozyme to the initial epoxidation

and the toxicity of 1-nitronaphthalene is unclear. Previous work with isozyme-selective P450 inhibitors supported the involvement of CYP2B in the metabolic activation of 1-nitronaphthalene (Verschoyle et al., 1993), and this is consistent with the nearly equal susceptibility of the rat and mouse to 1-nitronaphthalene toxicity.

Overall, the data from the current work have demonstrated dramatic rodent-primate differences in the metabolism of two pulmonary toxicants that require P450 activation to elicit toxicity. Moreover, the distribution of metabolic activation and the profiles of metabolites are very different in primates and rodents. These differences raise important questions about the use of data obtained in rodent bioassays for chemicals such as naphthalene, which seem to be very weak respiratory tract carcinogens. The finding of detectable levels of naphthalene in both fat and breast milk in a significant percentage of humans tested raise legitimate concerns about the potential toxicity of this agent (IARC, 2002). With perhaps a single exception of the data set available on 4-ipomeanol metabolism and toxicity, we lack sufficient information to indicate whether the studies conducted in rodents translate well to the human. 4-Ipomeanol is a bioactivated furan that results in lung-selective toxicity in almost every species tested (rats, mice, rabbits, dogs, and cattle (for review, see Gram, 1997; Dutcher and Boyd, 1979)). Good correlations between the rates of metabolic activation of 4-ipomeanol and target tissue toxicity have been observed. Human lung microsomes and cell lines showed relatively low rates of metabolic activation (McLemore et al., 1990). Subsequent phase I and II clinical trials with 4-ipomeanol produced no evidence for lung toxicity; indeed, doses were limited by evidence of hepatic toxicity (Rowinsky et al., 1993; Lakhanpal et al., 2001).

At face value, our work would suggest that naphthalene poses a very limited hazard to human populations. However, this is based on several assumptions: 1) that rhesus macaques are a good model for the human; 2) that the activities measured here are a result of small amounts of P450 protein spread over many of the cells in the specimens obtained; 3) that the results obtained in juvenile animals in this study would be similar to results obtained with adult animals; and 4) that overall metabolism is more important than the formation of metabolites that bind covalently to protein. On these latter points, previous work has shown that the levels of P450 activity are lower in early postnatal animals, which are more susceptible to naphthalene toxicity (Fanucchi et al., 1997). As shown in these studies, a high percentage of the overall metabolites generated bind covalently to proteins, and although there are differences in the amounts bound in rodents compared with primates, these are generally less than 2- to 3-fold. Ongoing studies are focused on determining the importance of specific adducts in the mechanisms of cytotoxicity of naphthalene and 1-nitronaphthalene. If such relationships can be established, adduct formation might be used to determine whether exposed human populations are capable of generating sufficient quantities of precursor metabolites to be harmful.

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## References

- Baldwin RM, Jewell WT, Fanucchi MF, Plopper CG, and Buckpitt AR (2004) Comparison of pulmonary/nasal CYP2F expression levels in rodents and rhesus macaque. *J Pharmacol Exp Ther* **309**:127–136.
- Buckpitt A, Boland B, Isbell M, Morin D, Shultz M, Baldwin R, Chan K, Karlsson A, Lin C, Taff A, et al. (2002) Naphthalene-induced respiratory tract toxicity: metabolic mechanisms of toxicity. *Drug Metab Rev* **34**:791–820.
- Buckpitt A, Buonarati M, Avey LB, Chang AM, Morin D, and Plopper CG (1992) Relationship of cytochrome P450 activity to Clara cell cytotoxicity. II. Comparison of stereoselectivity of naphthalene epoxidation in lung and nasal mucosa of mouse, hamster, rat and rhesus monkey. *J Pharmacol Exp Ther* **261**:364–372.
- Buckpitt A, Chang AM, Weir A, Van Winkle L, Duan X, Philpot R, and Plopper C (1995) Relationship of cytochrome P450 activity to Clara cell cytotoxicity. IV. Metabolism of naphthalene and naphthalene oxide in microdissected airways from mice, rats and hamsters. *Mol Pharmacol* **47**:74–81.
- Buckpitt AR and Bahnson LS (1986) Naphthalene metabolism by human lung microsomal enzymes. *Toxicology* **41**:333–341.
- Buonarati M, Jones AD, and Buckpitt A (1990) In vivo metabolism of isomeric naphthalene oxide glutathione conjugates. *Drug Metab Dispos* **18**:183–189.
- Cho M, Chichester C, Morin D, Plopper C, and Buckpitt A (1994) Covalent interactions of reactive naphthalene metabolites with proteins. *J Pharmacol Exp Ther* **269**:881–889.
- Cho M, Chichester C, Plopper C, and Buckpitt A (1995) Biochemical factors important in Clara cell selective toxicity in the lung. *Drug Metab Rev* **27**:369–386.
- Ding X and Kaminsky LS (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol* **43**:149–173.
- Dowsley TF, Forkert PG, Benesch LA, and Bolton JL (1995) Reaction of glutathione with the electrophilic metabolites of 1,1-dichloroethylene. *Chem Biol Interact* **95**:227–244.
- Duan X, Buckpitt AR, Pinkerton KE, Ji C, and Plopper CG (1996) Ozone-induced alterations in glutathione in lung subcompartments of rats and monkeys. *Am J Respir Cell Mol Biol* **14**:70–75.
- Duan X, Buckpitt AR, and Plopper CG (1993) Variation in antioxidant enzyme activities in anatomic subcompartments within rat and rhesus monkey lung. *Toxicol Appl Pharmacol* **123**:73–82.
- Dutcher JS and Boyd MR (1979) Species and strain differences in target organ alkylation and toxicity by 4-ipomeanol. Predictive value of covalent binding in studies of target organ toxicities by reactive metabolites. *Biochem Pharmacol* **28**:3367–3372.
- Fanucchi MV, Buckpitt AR, Murphy ME, and Plopper CG (1997) Naphthalene cytotoxicity of differentiating Clara cells in neonatal mice. *Toxicol Appl Pharmacol* **144**:96–104.
- Gram TE (1997) Chemically reactive intermediates and pulmonary xenobiotic toxicity. *Pharmacol Rev* **49**:297–341.
- Gupta R, Harger W, and Arey J (1996) The contribution of nitro and methylnitronaphthalenes to the vapor phase mutagenicity of ambient air samples. *Atmos Environ* **30**:3157–3166.
- Halladay JS, Sauer JM, and Sipes IG (1999) Metabolism and disposition of [(14)C]1-nitronaphthalene in male Sprague-Dawley rats. *Drug Metab Dispos* **27**:1456–1465.
- Hinners RG, Burkhardt JK, and Punte CL (1968) Animals inhalation exposure chambers. *Arch Environ Health* **16**:194–206.
- Hukkanen J, Pelkonen O, Hakkola J, and Raunio H (2002) Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. *Crit Rev Toxicol* **32**:391–411.
- International Agency for Research on Cancer (IARC) (2002) IARC monographs on the evaluation of carcinogenic risks to humans, in *Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene*, vol 82, pp 367–435, IRAC, Lyon, France.
- Jalas JR, Ding X, and Murphy SE (2003) Comparative metabolism of the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol by rat cytochrome p450 2A3 and human cytochrome p450 2a13. *Drug Metab Dispos* **31**:1199–1202.
- Lakhanpal S, Donehower RC, and Rowinsky EK (2001) Phase II study of 4-ipomeanol, a naturally occurring alkylating furan, in patients with advanced hepatocellular carcinoma. *Investig New Drugs* **19**:69–76.
- Lakritz J, Plopper CG, and Buckpitt AR (1997) Validated high-performance liquid chromatography-electrochemical method for determination of glutathione and glutathione disulfide in small tissue samples. *Anal Biochem* **247**:63–68.
- Lanza DL, Code E, Crespi CL, Gonzalez FJ, and Yost GS (1999) Specific dehydrogenation of 3-methylindole and epoxidation of naphthalene by recombinant human CYP2F1 expressed in lymphoblastoid cells. *Drug Metab Dispos* **27**:798–803.
- Lee C, Watt KC, Chang AM, Plopper CG, Buckpitt AR, and Pinkerton KE (1998) Site-selective differences in cytochrome P450 isoform activities. Comparison of expression in rat and rhesus monkey lung and induction in rats. *Drug Metab Dispos* **26**:396–400.
- Lowry OH, Rosebrough NJ, Farr AL, and Randell RJ (1951) Protein measurement with Folin phenol reagent. *J Biol Chem* **193**:265–275.
- McLemore TL, Litterst CL, Coudert BP, Liu MC, Hubbard WC, Adelberg S, Czerwinski M, McMahon NA, Eggleston JC and Boyd MR (1990) Metabolic activation of 4-ipomeanol in human lung, primary pulmonary carcinomas and established human pulmonary carcinoma cell lines. *J Natl Cancer Inst* **82**:1420–1426.
- Paige R, Wong V, and Plopper C (1997) Dose-related airway-selective epithelial toxicity of 1-nitronaphthalene in rats. *Toxicol Appl Pharmacol* **147**:224–233.
- Pakenham G, Lango J, Buonarati M, Morin D, and Buckpitt A (2002) Urinary naphthalene mercapturates as biomarkers of exposure and stereoselectivity of naphthalene epoxidation. *Drug Metab Dispos* **30**:247–253.
- Penning TM, Burczynski ME, Hung CF, McCoull KD, Palackal NT, and Tsuruda LS (1999) Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active o-quinones. *Chem Res Toxicol* **12**:1–18.
- Plopper CG, Chang AM, Pang A, and Buckpitt AR (1991) Use of microdissected airways to define metabolism and cytotoxicity in murine bronchiolar epithelium. *Exp Lung Res* **17**:197–212.
- Rowinsky EK, Noe DA, Ettinger DS, Christian MC, Lubejko BG, Fishman EK, Sartorius SE, Boyd MR, and Donehower RC (1993) Phase I and pharmacological study of the pulmonary cytotoxin 4-ipomeanol on a single dose schedule in lung cancer patients: hepatotoxicity is dose limiting in humans. *Cancer Res* **53**:1794–1801.
- Shultz MA, Choudary PV, and Buckpitt AR (1999) Role of murine cytochrome P-450 2F2 in metabolic activation of naphthalene and metabolism of other xenobiotics. *J Pharmacol Exp Ther* **290**:281–288.
- Shultz MA, Morin D, Chang AM, and Buckpitt A (2001) Metabolic capabilities of CYP2F2 with various pulmonary toxicants and its relative abundance in mouse lung subcompartments. *J Pharmacol Exp Ther* **296**:510–519.
- Smith GB, Bend JR, Bedard LL, Reid KR, Petsikas D, and Massey TE (2003) Biotransformation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in peripheral human lung microsomes. *Drug Metab Dispos* **31**:1134–1141.
- Smithgall TE, Harvey RG, and Penning TM (1988) Spectroscopic identification of ortho-quinones as the products of polycyclic aromatic trans-dihydrodiol oxidation catalyzed by dihydrodiol dehydrogenase. A potential route of proximate carcinogen metabolism. *J Biol Chem* **263**:1814–1820.
- Verschoye RD, Carthew P, Wolf CR, and Dinsdale D (1993) 1-Nitronaphthalene toxicity in rat lung and liver: effects of inhibiting and inducing cytochrome P450 activity. *Toxicol Appl Pharmacol* **122**:208–213.
- Watt KC and Buckpitt AR (2000) Species differences in the regio- and stereoselectivity of 1-nitronaphthalene metabolism. *Drug Metab Dispos* **28**:376–378.
- Watt KC, Morin DM, Kurth MJ, Mercer RS, Plopper CG, and Buckpitt AR (1999) Glutathione conjugation of electrophilic metabolites of 1-nitronaphthalene in rat tracheobronchial airways and liver: identification by mass spectrometry and proton nuclear magnetic resonance spectroscopy. *Chem Res Toxicol* **12**:831–839.
- Wiley JC, Coy E, Brolly C, Utell MJ, Frampton MW, Hammersley J, Thilly WG, Olson D, and Cairns K (1996) Xenobiotic metabolism enzyme gene expression in human bronchial epithelial and alveolar macrophage cells. *Am J Respir Cell Mol Biol* **14**:262–271.
- Yost GS (2001) Bioactivation of toxicants by cytochrome p450-mediated dehydrogenation mechanisms. *Adv Exp Med Biol* **500**:53–62.

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