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Mechanism-Based Inhibition of Mouse P4502b-10 by Selected Arylalkynes*

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ABSTRACT. Suicide inhibitors of cytochrome P450 families are excellent tools to predict which isoforms mediate the metabolism/activation of a variety of chemical agents. We compared the inhibitory effects of several arylalkynes on mouse cytochromes P450 with published data for the rat model. The inhibition of P4502b specific dealkylation of benzyloxyresorufin by 2-ethynylnaphthalene (2-EN), 5-phenyl-1-pentyne (PPY), 4-phenyl-1-butyne (PBY), and 9-ethynylphenanthrene (9-EPh) was measured in hepatic microsomes from male mice treated with 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) to induce cytochrome P4502b. Pulmonary microsomes were prepared from untreated mice. 9-EPh, 2-EN, and PPY caused a time-, concentration-, and NADPH-dependent loss in P4502b activity in both tissues. PBY, however, demonstrated this type of inhibition only in liver microsomes. The IC_{50} was calculated for both liver and lung microsomes and compared with published K_i (concentration required for half-maximal inhibition) or K_I (concentration required for half-maximal inactivation) values for the rat. PPY, PBY, and 9-EPh were equally effective inhibitors of mouse P4502b and rat P4502B1. 2-EN was a 5- to 10-fold less potent inhibitor of mouse P4502b, as compared with the rat, even though it was shown to bind to the active site of the mouse isoform as demonstrated by its metabolism to 2-naphthylacetic acid. These data suggest that the active site of the mouse P4502b enzyme is functionally similar to the rat P4502B isoform, with the exception of the disparity in its susceptibility to inactivation by 2-EN as measured by the K_i values. BIOCHEM PHARMACOL 52;10:1507–1513, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. cytochrome P450; suicide inhibition; arylalkynes; mouse; liver; lung

The P450‡ family of cellular proteins has been investigated extensively for its role in the metabolism of endogenous compounds and the activation and detoxification of environmental agents. Different P450 families exhibit different affinities for certain chemical compounds that share similar structural characteristics, and, therefore, potential risk from exposure to environmental agents may correlate to P450 isoform profiles in an affected population. The development of specific substrates, as well as inhibitors, for several of the P450 families has aided in the characterization of isozyme profiles in rodent models of metabolism.

Suicide inhibitors of P450 mediate the destruction of the hemoprotein by their reversible binding and subsequent metabolism to a reactive intermediate, which irreversibly binds to the P450 protein. The observation that certain acetylene-containing compounds produce destruction of the heme group of P450 led to the development of a family of arylalkynes that have been utilized as tools to investigate the suicide inhibition of the P4501A and P4502B families, primarily in the rat [1]. While it has been determined that both the size of the aromatic ring system and the placement of the carbon-carbon triple bond contribute to selectivity for certain P450s in the rat [2], no direct comparison of the potencies of these compounds in the mouse model has been described. Confirmation that they are effective and selective inhibitors of the homologous P450s in mouse is important, because these compounds have been utilized as tumor modulatory agents in response to certain nitrosamines in the mouse lung tumor model [3] and in mouse skin tumors initiated by 7,12-dimethylbenz[*a*]anthracene and benzo [*a*]pyrene [4]. These studies describe the inhibitory properties of selected arylalkynes for cytochrome P4502b-10 in the male Swiss mouse, and the results suggest that, with the

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‡ *Abbreviations:* 2-EN, 2-ethynylnaphthalene; PPY, 5-phenyl-1-pentyne; PBY, 4-phenyl-1-butyne; 9-EPh, 9-ethynylphenanthrene; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; P450, cytochrome P450; P4502B, rat cytochrome P450 isozyme which is considered to be phenobarbital-inducible; P4502b-10, mouse cytochrome P450 which is homologous to the rat P4502B; and 1- and 2-NA, 1- and 2-naphthylacetic acid.

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TABLE 1. Comparison of IC_{50} values obtained for mouse cytochrome P4502b-10 in liver and lung microsomes following a 10-min incubation at 37°

Inhibitor	IC_{50} (liver)* (μM)	IC_{50} (lung)† (μM)
2-EN	0.84	0.94
9-EPh	0.15	0.06
PBY	5.9	4.9
PPY	0.81	0.38

* TCPOBOP-induced liver microsomes.
† Untreated or oil-treated lung microsomes.

exception of 2-EN, the compounds exhibit inhibitory potencies for P450 enzyme activities similar to those reported for the rat model.

MATERIALS AND METHODS

Chemicals

2-EN and 9-EPh were synthesized as described [5]. PBY and PPY were purchased from Farchan Laboratories (Gainesville, FL). 1- and 2-NA were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Benzyloxyresorufin and resorufin (sodium salt) were purchased from Molecular Probes (Eugene, OR). TCPOBOP was synthesized from 2,3,5-trichloropyridine [6] in the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD. All other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Animals and Treatment

Male Swiss mice (NIH:Cr) were utilized as the source of microsomes for all experiments. Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, 1985). Liver microsomes were prepared [7] from mice treated with TCPOBOP (1 μmol/kg) to induce cytochrome P4502b fully and killed 96 hr following injection. Pooled lung microsomes were prepared [8] from untreated or olive oil-treated animals (at least three mice/pool).

Enzyme Inhibition and Fluorimetric Assays

Microsomes from liver or lung (100–200 μg protein) were incubated at 37° in 50 mM Tris/25 mM $MgCl_2$ with the indicated concentration of inhibitor, and NADPH (60 mM) for 10 min. Benzyloxyresorufin (5 μM) was added to initiate the reaction for P4502b-specific dealkylase activity, which was followed over a 10-min time course at 37°. The accumulation of resorufin was monitored fluorimetrically at an excitation wavelength of 522 nm and an emission wavelength of 586 nm [9], and the increase in fluorescence with time was linear for all samples assayed in these experiments. Resorufin standards were assayed daily to determine specific activity of the P450s. The data were plotted as the percent of activity observed in the DMSO control versus concentration of each inhibitor, and linear regression analysis was performed to yield an extrapolated IC_{50} value.

To determine K_i or K_I and limiting $T_{1/2}$ values for each inhibitor, time–course experiments were conducted in which microsomes were preincubated with the inhibitors in the presence or absence of NADPH. Reactions were initiated at the end of the preincubation time by the addition of benzyloxyresorufin substrate, and P4502b activity was monitored at 37° over a 10-minute time course. The fluorimetric data were fit to a linear equation using Sigmaplot (Jandel Scientific) and plotted with respect to preincubation time for each inhibitor concentration. Values of k_{obs} were generated utilizing the slopes of regression line for each concentration. A plot of the $1/k_{obs}$ values versus the inverse of the inhibitor concentration yielded an approximate K_i and $T_{1/2}$ for the inactivation process.

For those compounds that did not appear to demonstrate NADPH-dependent loss of P4502b activity with preincubation, a Dixon plot of the activity in the presence of several inhibitor concentrations without preincubation was generated, and K_i values were determined from the x intercept.

Metabolism of 2-EN and Identification of 2-NA

The formation of 2-NA was determined as described [10] with the following modifications. Each incubation mixture contained 25 μg (liver) or 50 μg (lung) microsomal protein, 14 μM 2-EN and 0.25 μM 1-NA was added at the

TABLE 2. Summary of K_I and $T_{½}$ values for the inhibition of mouse P4502b-10 activity by selected arylalkynes and comparison with values reported for rat P4502B

	Mouse liver		Mouse lung		Rat liver	
Inhibitor	K_I	$T_{½}$	K_I	$T_{½}$	K_I	$T_{½}$
2-EN	0.89 μM	2.0 min	1.4 μM	1.9 min	0.14 μM	3.0 min*
9-EPh	98 nM	1.8 min	37 nM	1.9 min	70 nM	1.5 min*
PPY	0.26 μM	1.9 min	0.77 μM	2.8 min	0.25 μM†	5 min†
PBY	3.1 μM	11 min	ND‡	ND	3.9 μM†	16 min†

* Ref. 2.
† Allworth *et al.*, manuscripted submitted for publication.
‡ ND: no NADPH-dependent loss detected.

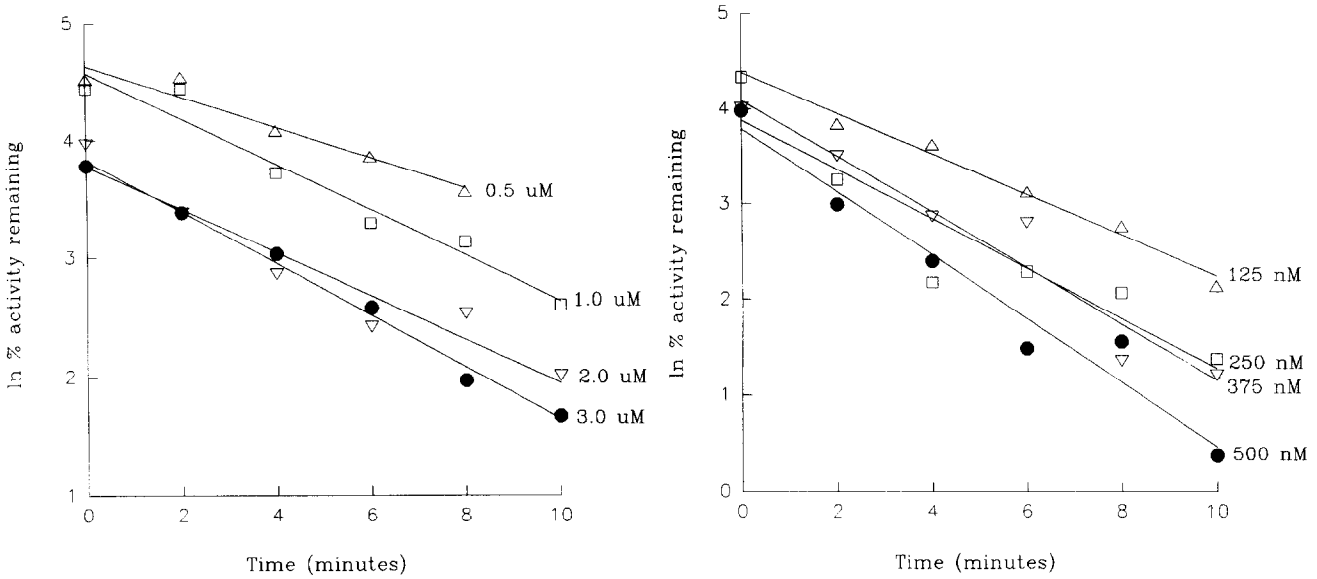

FIG. 1. Benzyloxyresorufin O-dealkylase activity in TCPOBOP-induced mouse liver microsomes versus time of preincubation with NADPH for 2-EN (left) and 9-EPh (right). The k_{obs} for each inhibitor concentration was determined from the slopes.

termination of the reaction as the internal standard. All incubations were performed at 30°.

RESULTS

The basal expression of P4502b-10 is only slightly above the limit of detectability in uninduced mouse liver [11]. We therefore utilized liver microsomes from mice treated with a TCPOBOP, a selective P4502b inducer, as this compound effectively induces mouse liver P4502b approximately 100-fold over control animals at doses as low as 3 mg/kg [11]. By contrast, rat and mouse lungs contain relatively high levels of P4502B, which are considered constitutively expressed and non-inducible by classic inducers of hepatic P4502B [12, 13].

Benzyloxyresorufin O-dealkylase activity in TCPOBOP-induced liver microsomes and in lung microsomes was inhibited significantly by all of the arylalkynes examined, with 9-EPh being the most effective and PBY the least. Linear regression analysis of the best fit line from these inhibition curves yielded extrapolated IC_{50} values that were similar within a factor of 2 to the K_i/K_I values reported for rat liver microsomes (Tables 1 and 2). The exception appeared to be 2-EN, which demonstrated a 6-fold lower inhibitory potency towards the mouse P450 than that reported for the rat (K_I = 0.14 μM).

To discern whether the inhibition observed was due to a reversible or irreversible inactivation process, time–course studies were performed involving preincubation in the presence and absence of NADPH for each inhibitor in both tissues. We observed NADPH- and time-dependent loss of P4502b enzyme activity following preincubation with both 2-EN and 9-EPh in liver and lung microsomes (Figs. 1 and 2). The extrapolated values for K_I were 0.89 and 1.4 μM for 2-EN, respectively (Fig. 3). The approximate $T_{1/2}$ for the inactivation process for liver was 2.0 and 1.9 min for lung. Inactivation by 9-EPh was also apparent in both liver and lung, demonstrating K_I values of 98 and 37 nM, and $T_{1/2}$ values of 1.8 and 1.9 min, respectively (Fig. 3 and Table 2).

PPY caused time- and NADPH-dependent loss of activity in both tissues during preincubation (data not shown), with the K_I value in mouse liver corresponding to the value obtained in the rat liver microsomes [K_I = 0.26 in mouse (Table 2); K_I = 0.25 in rat liver[4]]. By contrast, PBY was only an apparent mechanism-based inhibitor in the liver with a relatively long $T_{1/2}$ (10.5 min). While lung microsomes did not demonstrate clear time- and NADPH-dependence for the inhibition of P4502b activity by PBY, the extrapolated K_i value of 5.4 μM (Fig. 4) agreed well with the value obtained in the liver and that observed in the rat liver [3.1 μM in mouse liver (Table 2); 3.9 μM in rat liver§].

The 5- to 10-fold difference we observed in 2-EN inhibition of mouse liver and lung P450 when compared with rat liver may be due to either differential ability to metabolize the inhibitor, or, alternatively, to failure of the metabolite to bind to the active site of the mouse P450. To address the first possibility, we monitored the metabolism of 2-EN to 2-NA in both the liver and lung microsomal preparations. Both sources of microsomes formed 2-NA in a time- and NADPH-dependent manner (Figs. 5 and 6). Liver microsomes formed approximately 2.5-fold more metabolite than lung microsomes, and about half of the metabolite that phenobarbital-induced rat microsomes produced (Table 3). Therefore, 2-EN appears to be a relatively good substrate for the mouse isoform. The loss of the linear formation of 2-NA (Fig. 6) is consistent with enzyme inactivation, although the deviation from linearity appears to

§ Alworth *et al.*, manuscript submitted for publication.

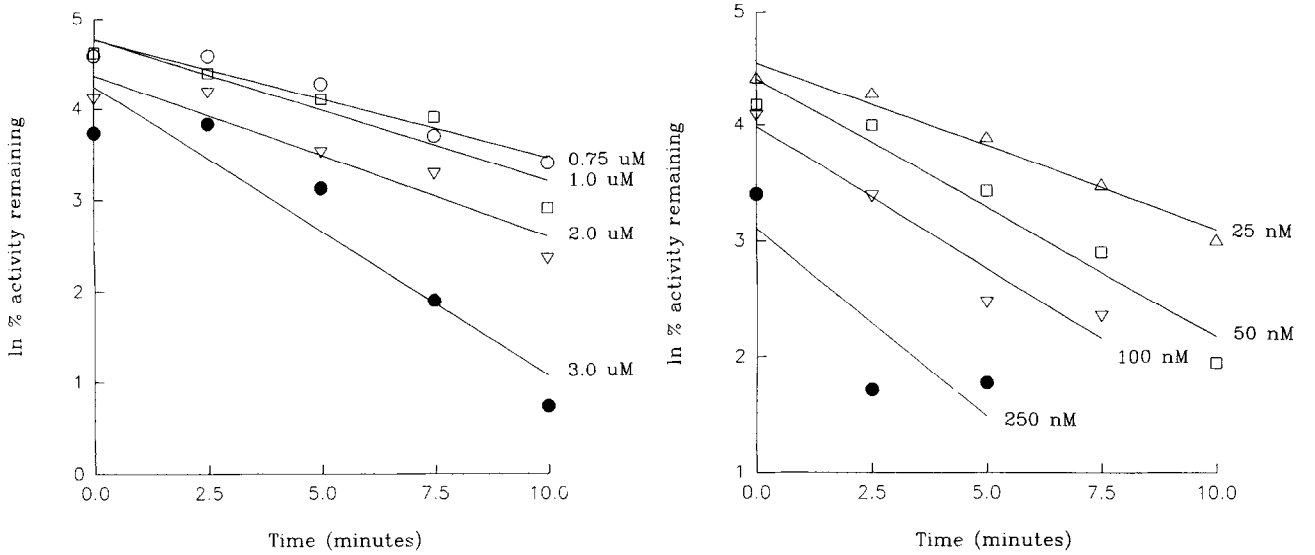

FIG. 2. Benzyloxyresorufin *O*-dealkylase activity in mouse lung microsomes versus time of preincubation with NADPH for 2-EN (left) and 9-EPh (right). The k_{obs} for each inhibitor concentration was determined from the slopes.

require more time than would be predicted based on the extrapolated $T_{1/2}$ values from the preincubation studies (Fig. 6). This is most likely due to the difference in reaction temperatures, which was 37° in the preincubation studies and 30° in the metabolism experiments.

DISCUSSION

The polycyclic aromatic acetylenes 9-EPh and 2-EN, which are both potent suicide inhibitors of rat P4502B, were also inhibitory for the mouse homologue, P4502b-10. Further experiments conducted to determine the nature of this inhibition demonstrated that both 2-EN and 9-EPh were time-, concentration-, and NADPH-dependent inhibitors of the mouse isoform (Figs. 1 and 2). Extrapolation of K_I values (Fig. 3 and Table 2) yielded values that showed good agreement to previously determined K_I values. The K_I values for 9-EPh in mouse liver and lung microsomes were comparable to the K_I of this inhibitor reported for rat liver microsomes.

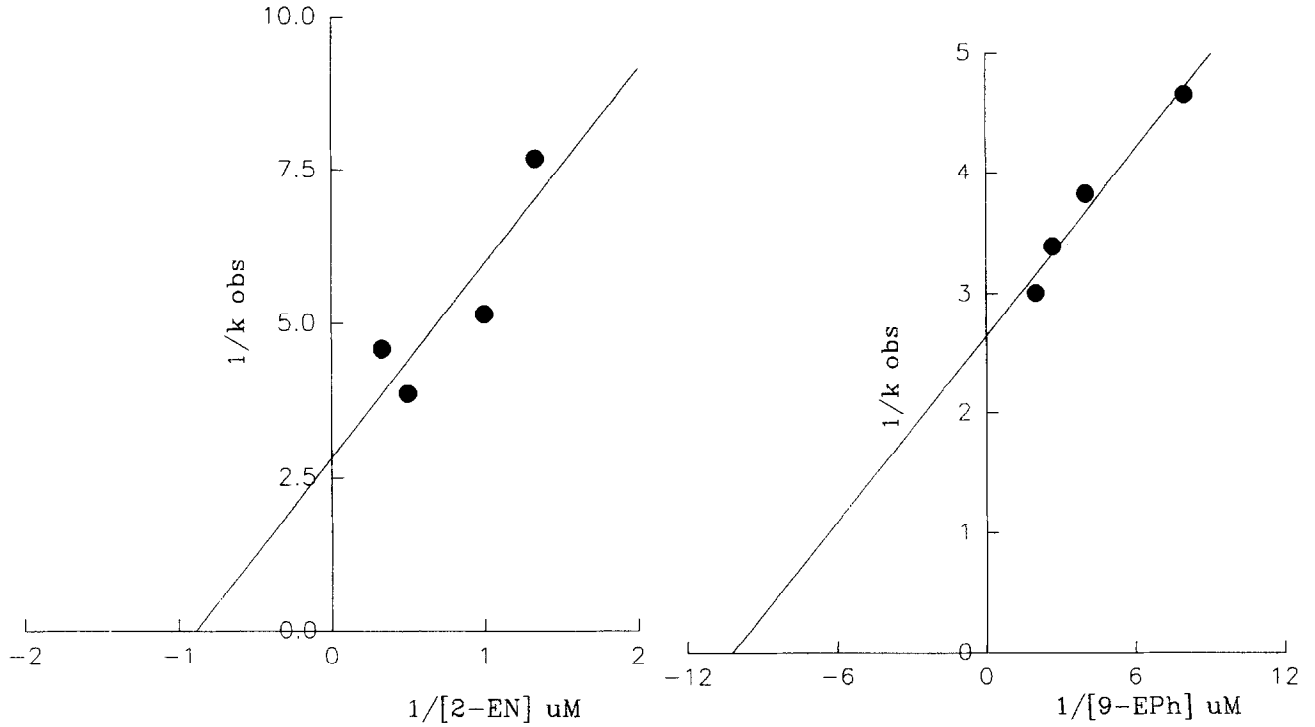

FIG. 3. Double-reciprocal plots for the inhibition of mouse liver P4502b-10 by 2-EN (left) and 9-EPh (right). The rate constant ($k_{inactivation}$) was determined from the y-intercept, and the K_I was determined from the x-intercept.

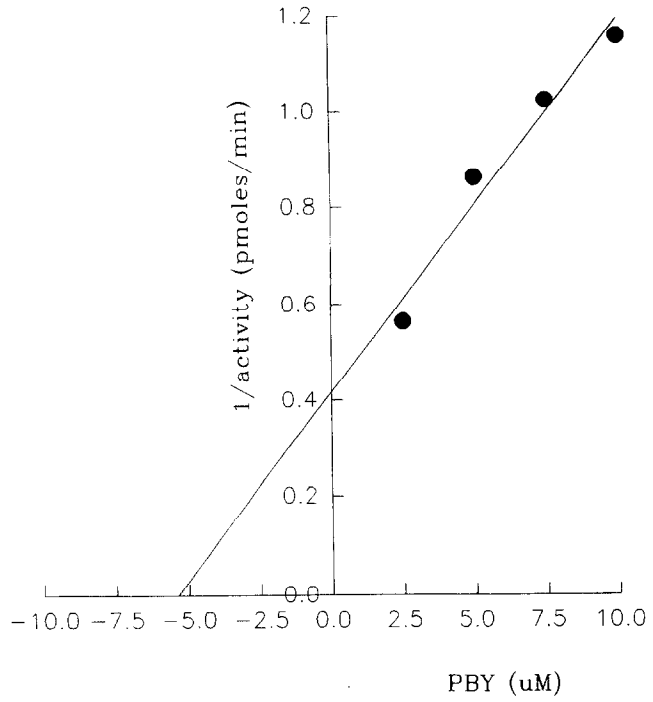

FIG. 4. Dixon plot of the reciprocal of benzyloxyresorufin O-dealkylase activity versus inhibitor concentration for PBY in mouse lung microsomes. Extrapolation to the x-intercept yielded the K_i for the inhibitor.

By contrast, we observed a 5- to 10-fold difference in the inhibitory potency of 2-EN in both liver and lung, as compared to the K_I for the rat isoform (0.14 μM in rat liver, 0.89 μM in mouse liver and 1.4 μM in lung). To address this discrepancy, we conducted experiments to determine whether the mouse isoform was capable of metabolizing the inhibitor. Incubation of microsomes from either liver or lung with 2-EN yielded 2-NA in a time- and NADPH-dependent manner (Figs. 5 and 6), demonstrating that 2-EN is a good substrate for the mouse isoform. The extent of 2-EN metabolism in microsomal fractions (Table 3) correlated to the extrapolated K_I values described here in the mouse and previously reported in the rat liver [2]. Rat microsomes metabolized 2-EN at a rate of 12.7 nmol/10 min/mg protein, which was approximately twice the rate of the mouse liver. Further, mouse lung microsomes metabolized 2-EN at approximately 25% the rate observed in the rat liver. A comparison of 2-EN metabolism to observed K_I values yielded a correlation coefficient of 0.999. Since the $T_{1/2}$ value for the inactivation P4502b-10 was similar to that observed for rat P4502B1, the data suggest that the disparity may be mediated by a differential ability of 2-EN to bind to the active site of the mouse isoform.

To address the possibility that the active sites of the rat and mouse isoforms are different, we have compared the amino acid sequence of the peptide reported to be labeled by 2-EN and 9-EPh at the binding site for rat P4502B [10] with the comparable sequence in mouse P4502b-10. There were four amino acid differences in the mouse P450 over the 24 amino acid peptide sequence. These amino acid

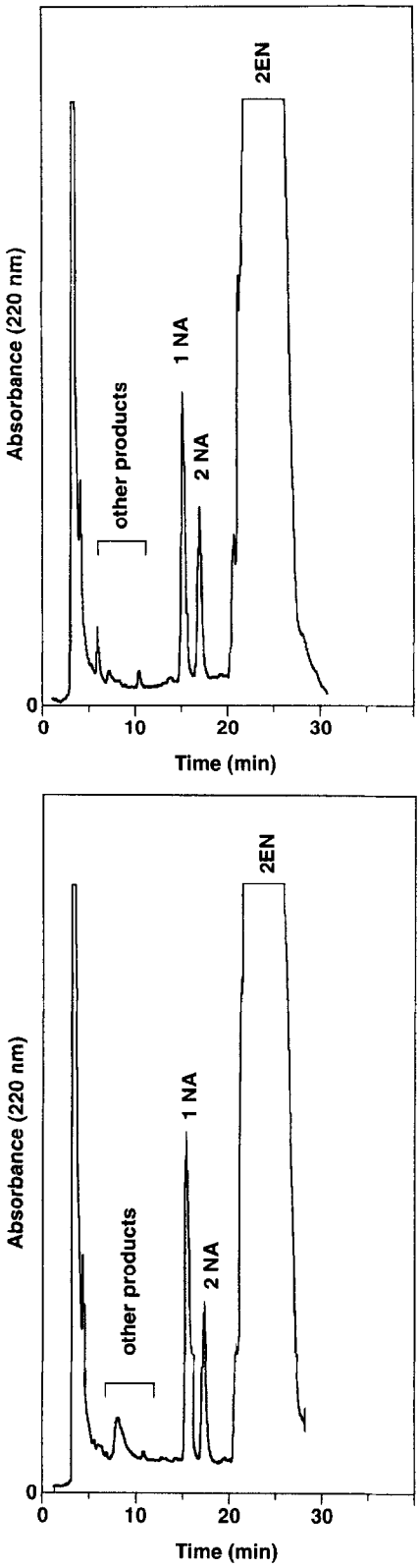

FIG. 5. Representative chromatograms from an HPLC reverse-phase analysis of the metabolites extracted from an incubation mixture containing mouse liver (top) or lung (bottom) microsomes. The incubation and chromatographic conditions for the analysis of 2-NA were as previously described [10] with the following modifications: each incubation mixture contained 25 μg (liver) or 50 μg (lung) microsomal protein, 14 μM 2-EN, and 1-NA as internal standard. All incubations were conducted at 30°.

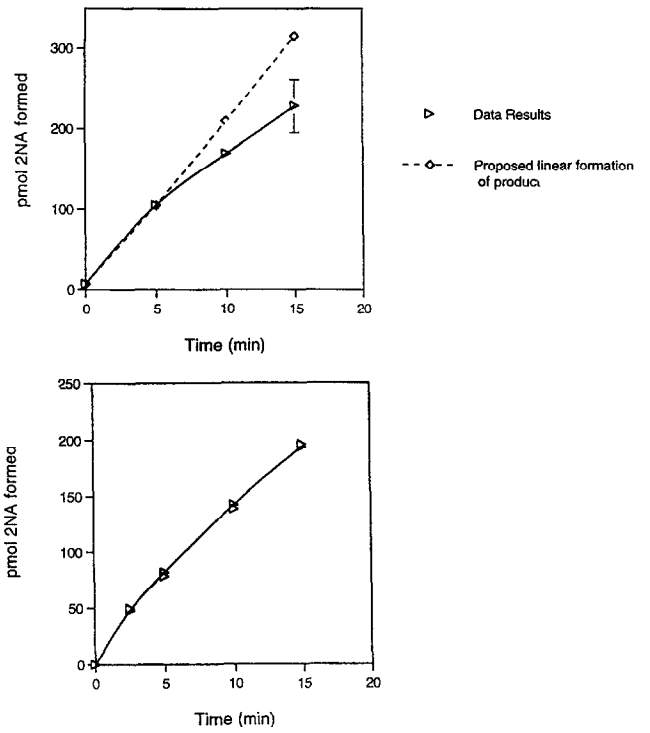

FIG. 6. Time course of 2-EN metabolism to 2-NA in liver (top) and lung (bottom) microsomes. Experimental conditions were as described in the legend of Fig. 5. The reactions were quenched at various time points by the addition of phosphoric acid. Values are means ± range, N = 2.

changes, summarized in Table 4, do not include serine, threonine, or tyrosine, which are the more nucleophilic amino acids in the rat peptide [10, 14]. We hypothesize that in the mouse P450 the reactive ketene (Scheme 1) may have easier access to a water molecule than a nucleophilic amino acid. This may be due, in part, to the difference in amino acid at position 308. In the mouse, the histidine residue is smaller than the arginine present in the rat, which may allow more water molecules into the active site. This hypothesis is supported by the topological analysis of

TABLE 3. Determination of 2-EN metabolism by mouse liver and lung microsomes, and comparison with rat liver microsomes

Microsomal preparation	Activity (nmol 2NA formed/ 10 min/mg protein)
TCPOBOP-induced mouse liver – NADPH	0.13*
TCPOBOP-induced mouse liver	6.62 ± 0.31
Phenobarbital-induced rat liver†	12.7*
Mouse lung	2.77 ± 0.12

The formation of 2-NA was determined as described in Ref. 10, with the following modifications. Each incubation mixture contained 25 μg (liver) or 50 μg (lung) microsomal protein, 14 μM 2-EN, and 1-NA as the internal standard. Values are means ± SD of three preparations, each assayed in duplicate.

* Represents the mean of duplicate determinations.

† 0.1% Phenobarbital in the drinking water for 12 days.

TABLE 4. Comparison of the rat P4502B active site peptide (amino acids 290–314) to the corresponding mouse P4502b-10

Amino acid position	Rat P4502B	Mouse P4502b-10
290	Isoleucine	Methionine
292	Leucine	Valine
298	Alanine	Valine
308	Arginine	Histidine

rat P4502B1 and mouse P4502b-10 [15], suggesting that the latter isoform has a more open active site. The difference we observed in inhibitory potency between mouse liver and lung may also reflect the differences in the relative abundance and proportion of other P450 families between these two tissues. Further experiments would have to be conducted to determine the site of binding of the inhibitor to the mouse P450.

The arylalkynes PBY and PPY both inhibited mouse liver and lung P4502b-10 with approximately the same potency as reported for the rat P4502B [3]. While PPY demonstrated time-, concentration-, and NADPH-dependent loss of P4502b-10 activity in both mouse liver and lung, only liver microsomes responded in this manner following preincubation with PBY. The lack of apparent enzyme inactivation in lung microsomes may be related to both the concentrations of inhibitor required to show inhibition and the relatively long inactivation time of PBY (10.5 min). Studies in lung microsomes with these concentrations demonstrate significant loss of enzyme activity with PBY without preincubation with NADPH, such that a further loss with preincubation time is difficult to observe. The K_i value, however, does agree well with the K_I observed in the rat liver model for PBY (3.9 μM‖). PPY is also considered a mechanism-based inactivator of rat liver P4502B (K_I = 0.25 μM‖), which agrees with our observations in both mouse liver and lung microsomes.

These compounds have also been shown by Alworth and coworkers to be protective against 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-initiated lung tumors in sensitive A/J mice [3]. Interestingly, this same experiment demonstrated that 2-EN was not an effective tumor modulatory agent in the mouse lung model when given at doses that are inhibitory for P4502B in the rat. This supports our results that this compound shows species-specific inhibition of the P4502B family, and suggests that increasing the dose approximately 10-fold may yield the protection against tumorigenesis that was observed with the other arylalkynes. Further, it appears that 9-EPh was the most effective suicide inhibitor of mouse lung P4502b-10, and may be utilized as a potent anti-tumor agent in the mouse lung model.

In summary, we have determined the inhibitory potencies of a series of arylalkynes towards mouse cytochrome P4502b-10 and compared these values to the homologous

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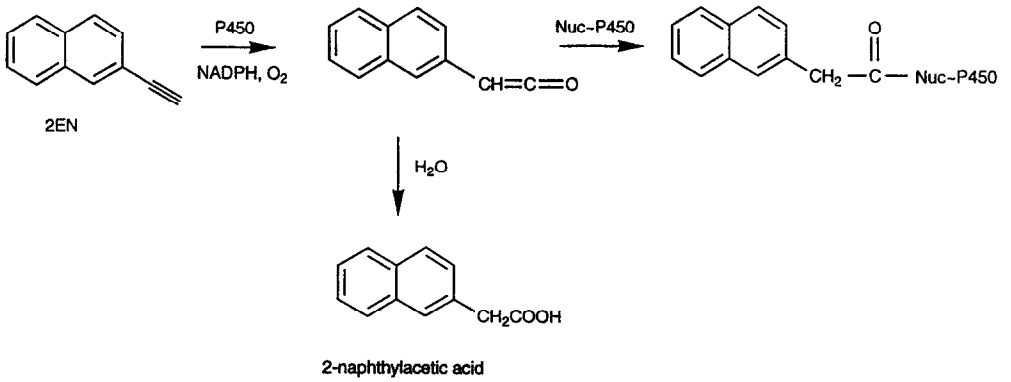

SCHEME 1. Proposed reaction for the formation of 2-NA and labeling of P450 by 2-EN [16].

rat P450 isoform. These data support the hypothesis that the active sites of these detoxifying enzymes are conserved between strains, and that anti-tumorigenesis effects seen in mice were, in fact, due to enzyme inhibition. These findings encourage future experiments to be designed to evaluate the role of specific P450 families in carcinogen metabolism. They will also be valuable in the elucidation of metabolic pathways associated with detoxification of endogenous substances and potentially new compounds of therapeutic benefit.

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