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Structure–Function Studies of Naphthalene, Phenanthrene, Biphenyl, and Their Derivatives in Interaction with and Oxidation by Cytochromes P450 2A13 and 2A6

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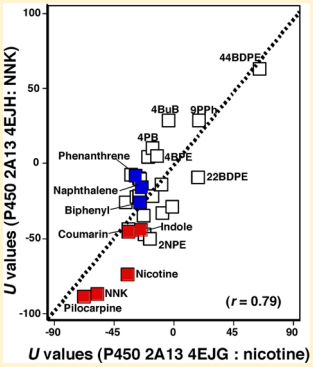
Supporting Information

ABSTRACT: Naphthalene, phenanthrene, biphenyl, and their derivatives having different ethynyl, propynyl, butynyl, and propargyl ether substitutions were examined for their interaction with and oxidation by cytochromes P450 (P450) 2A13 and 2A6. Spectral interaction studies suggested that most of these chemicals interacted with P450 2A13 to induce Type I binding spectra more readily than with P450 2A6. Among the various substituted derivatives examined, 2-ethynylnaphthalene, 2-naphthalene propargyl ether, 3-ethynylphenanthrene, and 4-biphenyl propargyl ether had larger $\Delta A_{max}/K_s$ values in inducing Type I binding spectra with P450 2A13 than their parent compounds. P450 2A13 was found to oxidize naphthalene, phenanthrene, and biphenyl to 1-naphthol, 9-hydroxyphenanthrene, and 2- and/or 4-hydroxybiphenyl, respectively, at much higher rates than P450 2A6. Other human P450 enzymes including P450s 1A1, 1A2, 1B1, 2C9, and 3A4 had lower rates of oxidation of naphthalene, phenanthrene, and biphenyl than P450s 2A13 and 2A6. Those alkynylated derivatives that strongly induced Type I binding spectra with P450s 2A13 and 2A6 were extensively oxidized by these enzymes upon analysis with HPLC. Molecular docking studies supported the hypothesis that ligand-interaction energies (*U* values) obtained with reported crystal structures of P450 2A13 and 2A6 bound to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, indole, pilocarpine, nicotine, and coumarin are of use in understanding the basis of possible molecular interactions of these xenobiotic chemicals with the active sites of P450 2A13 and 2A6 enzymes. In fact, the ligand-interaction energies with P450 2A13 4EJG bound to these chemicals were found to relate to their induction of Type I binding spectra.



INTRODUCTION

Naphthalene, phenanthrene, and biphenyl are aromatic hydrocarbon contaminants found in the environment, and the former two chemicals belong to the class of polycyclic aromatic hydrocarbons (PAHs) that includes toxic and carcinogenic compounds such as benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]-anthracene, and benzo[*c*]phenanthrene.[1−5] These carcinogenic PAHs have been shown to require metabolic activation by so-called xenobiotic-metabolizing enzymes, such as cytochromes P450 (P450), to evoke their toxic and carcinogenic responses in laboratory animals as well as in humans.[6−8]

Naphthalene has been shown to cause toxic responses in several animal species and is known to require bioactivation by P450 2F2 in mice to elicit toxicity.[9,10] Hu et al.[11] also reported that P450 2A5 plays an important role in olfactory mucosal toxicity in mice. Metabolism of naphthalene by different P450 enzymes has been reported in humans[12,13] and also in Rhesus monkeys.[14] Metabolism of phenanthrene and biphenyl has also been reported in various laboratory animals;[15−22] however, detailed studies on the roles of human P450s 2A13 and 2A6 in the metabolism of naphthalene, phenanthrene, and biphenyl have not been reported, except that Fukami et al. showed that P450 2A13 can catalyze the oxidation of naphthalene in humans.[13]

Our previous studies have shown that naphthalene, phenanthrene, biphenyl, and their substituted derivatives induce Type I binding spectra with P450s 2A13 and 2A6, suggesting that these chemicals may be oxidized by these P450 enzymes.[23] In this study, we studied how these chemicals are

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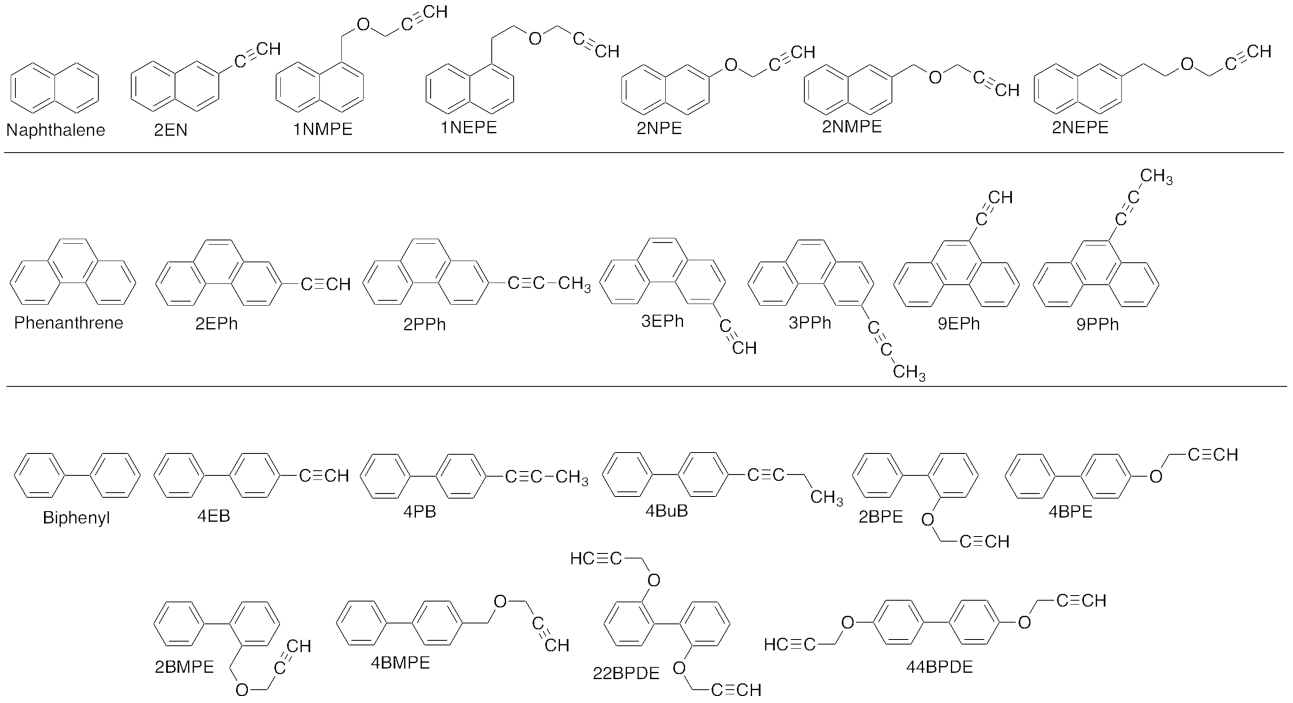

Figure 1. Structures of chemicals used in this study.

oxidized by P450s 2A13 and 2A6 and whether there are structure−function relationships for interacting with and biotransformation by these enzymes, based on analysis with HPLC. We used naphthalene and six substituted derivatives, phenanthrene and six substituted derivatives, and biphenyl and nine substituted derivatives for spectral interaction with and metabolism by P450s 2A13 and 2A6. Other P450 enzymes—human 1A1, 1A2, 1B1, 2C9, and 3A4—were also used to study the oxidation of parent compounds naphthalene, phenanthrene, and biphenyl. Docking simulations of the interactions of P450s 2A13 and 2A6 and these chemicals were also examined to visualize how these chemicals interact with these P450 enzymes.

EXPERIMENTAL PROCEDURES

Chemicals. Naphthalene, phenanthrene, and biphenyl were obtained from Sigma-Aldrich (St. Louis, MO) or Wako Pure Chemical (Osaka, Japan) (Figure 1). 1-Naphthol, 4-naphthol, 9-hydroxyphenanthrene, and 2- and 4-hydroxybiphenyl were also obtained from Wako Pure Chemical. Acetylenic PAHs and biphenyl derivatives, including 2-ethynylnaphthalene (2EN), 1-naphthalene methyl propargyl ether (1NMPE), 1-naphthalene ethyl propargyl ether (1NEPE), 2-naphthalene propargyl ether (2NPE), 2-naphthalene methyl propargyl ether (2NMPE), 2-naphthalene ethyl propargyl ether (2NEPE), 2-ethynylphenanthrene (2EPh), 3-ethynylphenanthrene (3EPh), 9-ethynylphenanthrene (9EPh), 2-(1-propynyl)phenanthrene (2PPh), 3-(1-propynyl)phenanthrene (3PPh), 9-(1-propynyl)phenanthrene (9PPh), 4-ethynylbiphenyl (4EB), 4-propynylbiphenyl (4PB), 4-butynylbiphenyl (4BuB), 2-biphenyl propargyl ether (2BPE), 4-biphenyl propargyl ether (4BPE), 2-biphenyl methyl propargyl ether (2BMPE), 4-biphenyl methyl propargyl ether (4BMPE), 2,2′-biphenyl dipropargyl ether (22BDPE), and 4,4′-biphenyl dipropargyl ether (44BDPE) (Figure 1), were synthesized as described previously.[23−30] These substituted chemicals have been used to study the mechanisms of inhibition of human P450 enzymes, including P450s 2A13 and 2A6.[23−30]

Other chemicals and reagents used in this study were obtained from the sources described previously and were of the highest quality commercially available.[23−25]

Enzymes. Human P450s, NADPH-P450 reductase, and cytochrome b_5 enzymes used in this study were expressed in *Escherichia coli* as described previously.[23−25] Bacterial bicistronic P450s 1A2, 1B1 (1B1.3, L432 V), 2A6, 2A13, 2C9, and 3A4 coexpressing human NADPH-P450 reductase were also prepared, and *E. coli* membranes were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v). Human P450s 1A1, 2A6, and 2A13 and NADPH-P450 reductase were purified from membranes of recombinant *E. coli* as described elsewhere.[23−25] Catalytic activities (nmol of product formed/min/nmol P450) for these eight P450 enzymes were determined using typical model substrates and were found to be 35, 3.2, and 14 min^{-1} for 7-ethoxyresorufin O-deethylation by P450s 1A1, 1A2, and 1B1, respectively; 3.3 and 1.4 min^{-1} for coumarin 7-hydroxylation by P450s 2A6 and 2A13, respectively; 3.5 min^{-1} for flurbiprofen 4′-hydroxylation by P450 2C9; and 3.2 and 6.5 min^{-1} for midazolam 1′- and 4′-hydroxylation, respectively, by P450 3A4.

Spectral Binding Titrations. Purified P450s 2A13 and 2A6 were diluted to 1.0 μM in 0.10 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), and binding spectra were recorded with subsequent additions of chemicals in a JASCO V-550 or OLIS-Aminco DW2a spectrophotometer (Online Instrument Systems, Bogart, GA) as described previously.[23−25] Briefly, the chemicals were added to the buffer with or without the P450, and the spectra were recorded between 350 and 500 (or 700) nm. The substrate binding spectra were obtained by subtracting the blank spectra (in the absence of the P450) from the P450 spectra (in the presence of the P450). Spectral dissociation constants (K_s) were estimated using GraphPad Prism software (GraphPad Software, San Diego, CA), using either hyperbolic plots or quadratic fits in cases of tight binding.

Oxidation of Naphthalene. Oxidative metabolism of naphthalene by P450s 1A2, 1B1, 2A6, 2A13, 2C9, and 3A4 was determined in a standard incubation mixture (0.25 mL) containing 25 pmol of P450 (in bicistronic membranes), 50 μM naphthalene, and an NADPH-generating system according to the method described by Fukami et al.[13] (The chemicals were dissolved in $(CH_3)_2SO$ as 10 mM stock solutions and diluted, with the organic solvent concentration ≤ 0.5%, v/v.). In reconstitution experiments, P450 membranes were replaced by purified P450 1A1 (25 pmol), NADPH-P450 reductase (50 pmol), and L-α-dilauroyl-*syn*-glycero-3-phosphocholine (50 μg) as described previously.[23−25] Incubation was carried out at 37 °C for 20 min,

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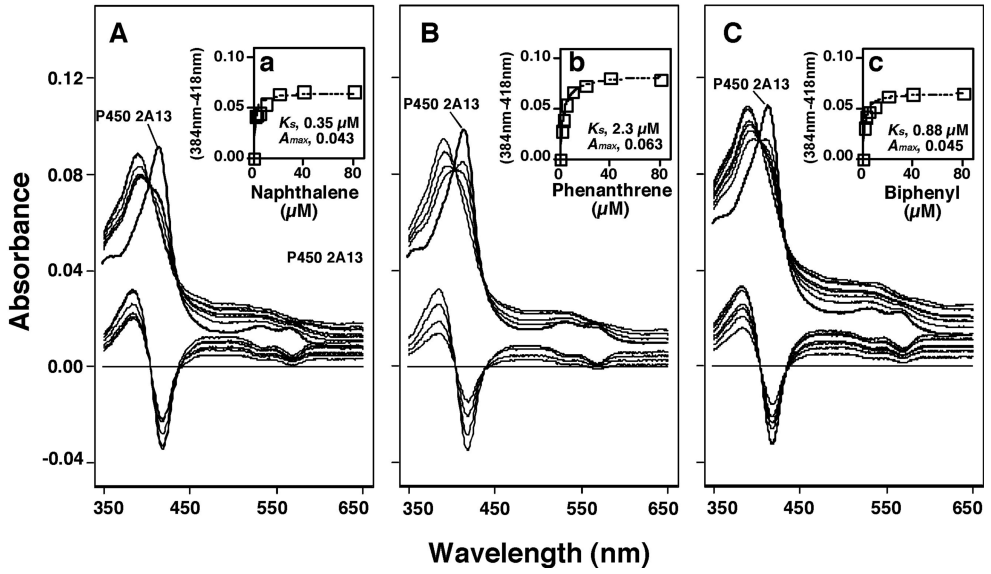

Figure 2. Spectral changes produced by the interaction of naphthalene (A), phenanthrene (B), and biphenyl (C) with P450 2A13. The lower portion of each figure indicates the difference spectra obtained.

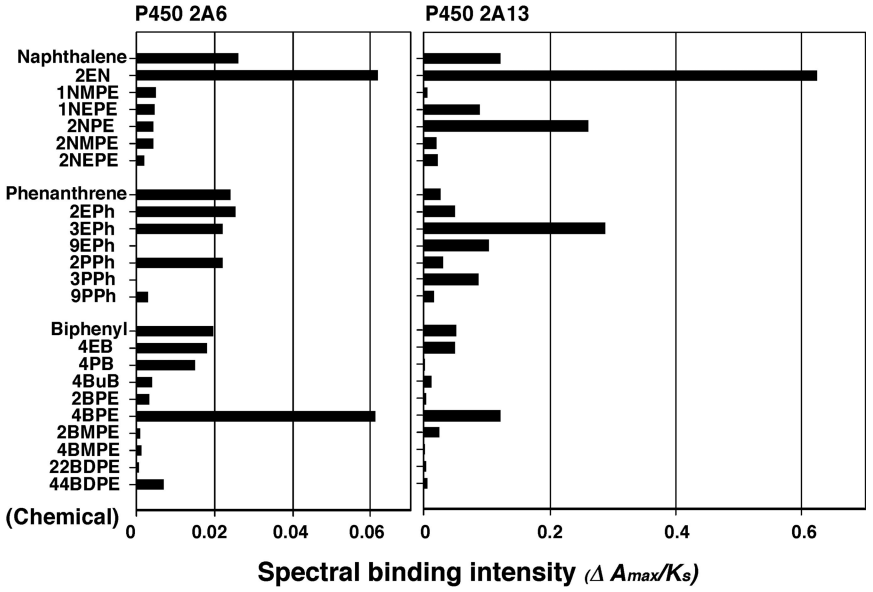

Figure 3. Comparison of spectral changes (spectral binding efficiency, $\Delta A_{max}/K_s$ ratio) from the interaction of various chemicals with P450s 2A6 and 2A13.

following a preincubation time of 1 min. Reactions were terminated by adding 100 μL of CH_3CN, and following centrifugation, the upper layer was subjected to HPLC using a Mightsil RP-18 C18 GP column. (Note that naphthalene and its metabolites are volatile under a nitrogen stream after these chemicals are extracted with organic solvents.) The mobile phase used was 30% CH_3CN (v/v, in H_2O) containing 0.01% H_3PO_4 (w/v).

Oxidation of Phenanthrene and Biphenyl and Their Derivatives and Naphthalene Derivatives. Oxidative metabolism of phenanthrene and biphenyl and their derivatives (and also naphthalene derivatives) was determined in a standard reaction mixture (final volume of 0.25 mL) as described above. After incubation at 37 °C for 20 min, extraction was done by adding 0.25 mL of cold CH_3OH and then 0.5 mL of a mixture of $CHCl_3$ and ethyl acetate (1:1, v/v). After centrifugation, the lower organic layer was recovered and the extraction was repeated. The extracts were combined and concentrated under a nitrogen stream. HPLC separation was done with a JASCO system (Tokyo, Japan) equipped with a Wakopack Navi C18-5 octadecylsilane column (2.0 mm × 150 mm) (Wako Pure Chemical) with UV detection at 254 nm and fluorescence detection with an excitation wavelength of 242 nm and emission wavelength of 380 nm. Elution of chemicals and their metabolites utilized a linear gradient from 20 to 100% CH_3OH (v/v, in H_2O) for 25 min, followed by holding at 100% CH_3OH for 5 min, with a flow rate of 0.2 mL/min.

Other Enzyme Assays. Coumarin 7-hydroxylation, 7-ethoxyresorufin O-deethylation, flurbiprofen 4-hydroxylation, and midazolam 1′- and 4′-hydroxylation activities were determined using bicistronic bacterial membrane or reconstituted monooxygenase systems as described previously.[23−25,31−33]

Docking Simulations into Human P450 Enzymes. Crystal structures of P450 2A13 bound to NNK (PDB 4EJH),[34] indole (PDB 2P85),[35] pilocarpine (PDB 3T3S),[36] and nicotine (PDB 4EJG)[34] have been reported and were used for these studies. Structures of P450 2A6 bound to coumarin (PDB 1Z10),[37] pilocarpine (PDB 3T3R),[36] and nicotine (PDB 4EJJ)[34] have also been reported. Simulations were carried out after removing each ligand from these P450 structures

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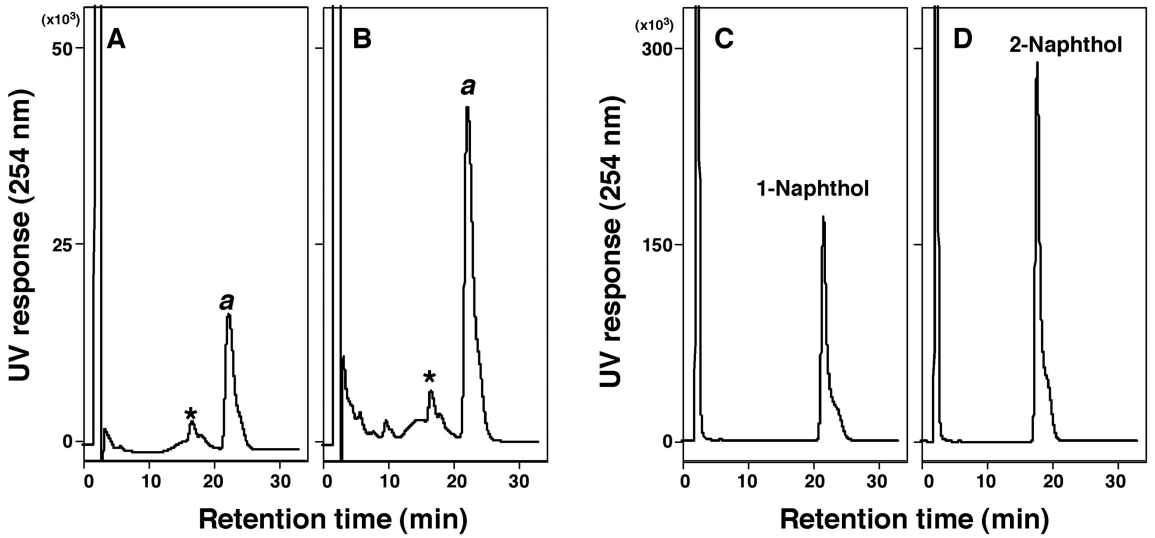

Figure 4. Oxidation of naphthalene by P450s 2A6 (A) and 2A13 (B) by HPLC analysis. Standards 1-naphthol (C) and 2-naphthol (D) were also analyzed. Formation of 1-naphthol from naphthalene by P450s 2A6 and 2A13 is shown as "*a*". An asterisk (*) indicates minor peaks for products that were not present in the absence of an NADPH-generating system.

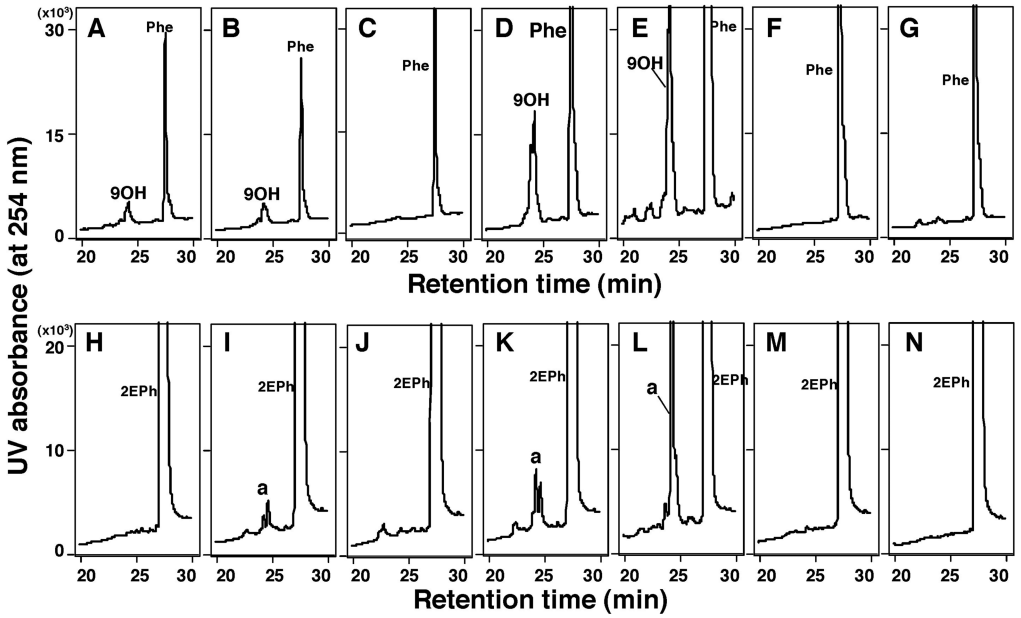

Figure 5. Oxidation of phenanthrene (Figure 6A–G) and 2EPh (Figure 6H–N) by P450s 1A1 (A, H), 1A2 (B, I), 1B1 (C, J), 2A6 (D, K), 2A13 (E, L), 2C9 (F, M), and 3A4 (G, N). Standard incubation conditions were used for analysis by HPLC, and duplicate determinations for each incubation showed that very similar products were formed for these chemicals.

using the MMFF94x force field described in MOE software (ver. 2015.10, Chemical Computing Group, Montreal, Canada).[23−25] Ligand-interaction energies (*U* values) were obtained by use of the ASEdock program in MOE. Lower *U* values indicate a stronger interaction between a chemical and the enzyme.

Kinetic Analysis. Kinetic parameters were estimated by nonlinear regression analysis using KaleidaGraph (Synergy Software, Reading, PA) or GraphPad Prism (GraphPad Software, La Jolla, CA).

RESULTS

Spectral Interactions of Naphthalene, Phenanthrene, Biphenyl, and Their Derivatives with P450s 2A13 and 2A6. As has been reported previously,[23] naphthalene, phenanthrene, and biphenyl interact with P450 2A13, inducing Type I binding spectral changes (Figure 2). The K_s values obtained with these three chemicals were determined to be 0.35, 2.3, and 0.88 μM, respectively, and the ΔA_{max} values obtained were 0.043, 0.063, and 0.045, respectively (Figure 2).

Naphthalene and its six derivatives, phenanthrene and its six derivatives, and biphenyl and its nine derivatives were also compared for their abilities ($\Delta A_{max}/K_s$ ratio) to induce spectral changes with P450 2A6 as well as P450 2A13 (Figure 3). It should be pointed out that the scale for the $\Delta A_{max}/K_s$ ratio on the *x* axis is different for P450s 2A13 and 2A6, indicating that the former enzyme showed higher spectral changes than the latter. 2EN induced Type I binding spectra with P450 2A13 to a much greater extent than the parent naphthalene, and the $\Delta A_{max}/K_s$ ratio for the interaction of 2EN with P450 2A13 was about 10-fold greater than that with P450 2A6 (Figure 3). 2NPE, 3EPh, and 4BPE were also found to show higher

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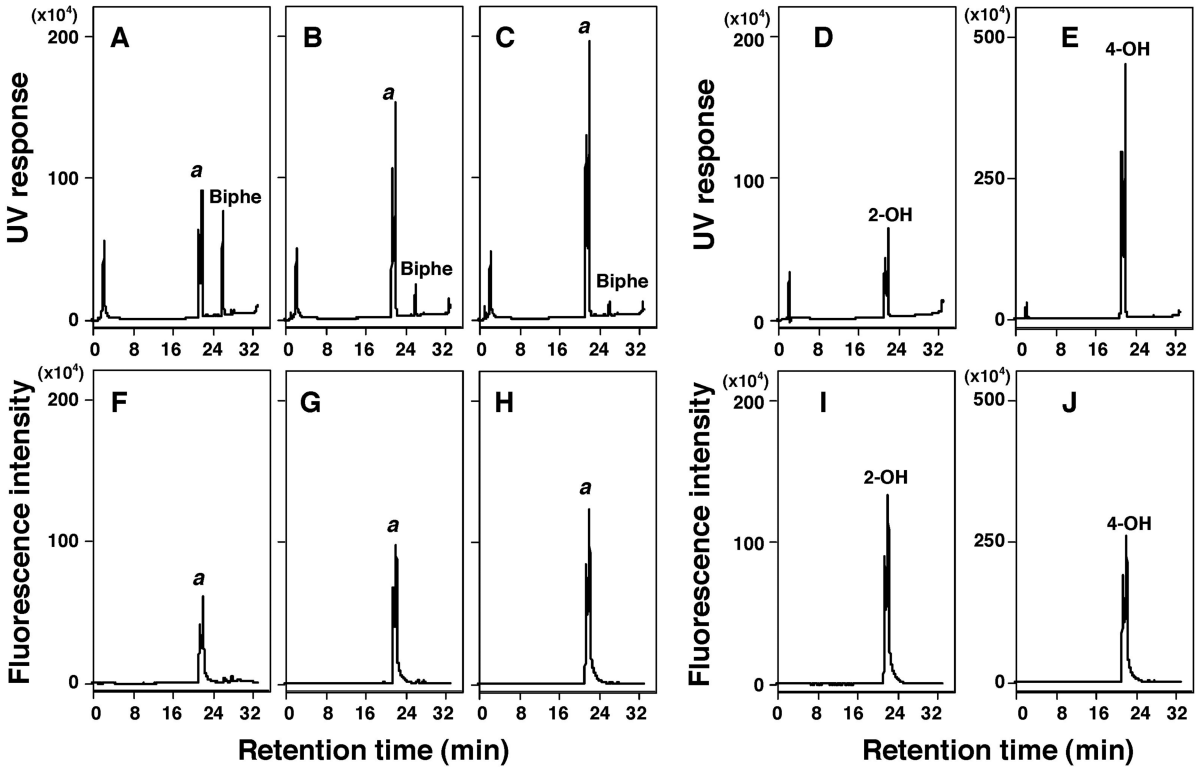

Figure 6. Oxidation of biphenyl by P450 2A13 by HPLC analysis with UV detection (A–C) and fluorescence detection (F–H). HPLC chromatograms of incubations of biphenyl (50 μM) with P450 2A13 at 0.025 μM (A, F), 0.05 μM (B, G), and 0.1 μM (C, H) and chromatograms of standard 2-hydroxybiphenyl (2.5 nmol; D, I) and standard 4-hydroxybiphenyl (E, J). Detection was by UV absorbance (A–E) or fluorescence intensity (F–J).

intensities of interaction with P450 2A13 than parent compounds naphthalene, phenanthrene, and biphenyl. 4BPE was also found to induce spectral changes with P450 2A6 at a higher level than biphenyl.

Oxidation of Naphthalene, Phenanthrene, and Biphenyl by Human P450 Enzymes. Oxidation of naphthalene was determined with P450s 2A13 and 2A6 according to the method of Fukami et al.[13] P450s 2A13 and 2A6 produced one major peak that corresponded to the 1-naphthol standard, but not 2-naphthol, in the retention time on HPLC chromatograms, with P450 2A13 showing more activity than P450 2A6 (Figure 4). Since we did not extract naphthalene and its metabolites and concentrate them for HPLC analysis due to their volatile nature, it is not known whether the small peaks seen in the chromatograms are due to 2-naphthol and other metabolites.

P450 2A13 was also active in oxidizing phenanthrene to 9-hydroxyphenanthrene (Supporting Information Figure S1). The 9-hydroxyphenanthrene standard and a metabolite produced by P450 2A13 had similar, profiles showing two peaks on HPLC chromatograms (it is not known at present why two peaks appear for 9-hydroxyphenanthrene or if it arises by an experimental artifact possible to detector saturation). When zooming in on the *x*-axis, several minor peaks appeared only in the presence of an NADPH-generating system with P450 2A13 (Supporting Information Figure S1b). We also found very similar, but different, chromatographic profiles of product formation for the metabolism of 2EPh and phenanthrene by P450 2A13 (Supporting Information Figure S1C,D,c,d). We do not have a standard 2EPh metabolite, but the major peak (*) seems to be similar in nature to 9-hydroxyphenanthrene.

In order to compare the products formed for the metabolism of phenanthrene and 2EPh, we determined their activities with seven human P450 enzymes, P450s 1A1, 1A2, 1B1, 2A6, 2A13, 2C9, and 3A4 (Figure 5). The results show that P450 2A13 is the most active at oxidizing phenanthrene (Figure 5E) and 2EPh (Figure 5L) to 9-hydroxyphenanthrene and metabolite *a*, respectively, followed by P450 2A6 (Figure 5D,K). Other P450 enzymes examined—P450s 1A1, 1A2, and 1B1.3—were active at producing 9-hydroxyphenanthrene from phenanthrene, and P450s 1A2 and 1B1 oxidized 2EPh at lower levels than P450s 2A13 and 2A6.

P450 2A13 oxidized biphenyl to 2- and/or 4-hydroxybiphenyl on analysis with HPLC (Figure 6). Our HPLC assay did not separate 2- and 4-hydroxylated biphenyl products. It should also be noted that both standards 2- and 4-hydroxybiphenyl produced two peaks, as in the case of 9-hydroxyphenanthrene as described above. Increasing the P450 concentration from 0.025 to 0.1 μM caused an increase in the formation of metabolite(s) (both with UV and fluorescence detection).

P450s 1A1, 1A2, 1B1, 2A6, 2A13, 2C9, and 3A4 were compared for their ability to oxidize naphthalene, phenanthrene, and biphenyl (P450 1A1 was measured in reconstituted monooxygenase systems containing L-α-dilauroyl-*sn*-glycero-3-phosphocholine and NADPH-P450 reductase as described in the Experimental Procedures). P450 2A13 was found to be the most active enzyme at oxidizing these chemicals, followed by P450 2A6 (Table 1). With the other P450s examined, naphthalene hydroxylation activity was detected with P450s 1A2, 1B1, and 3A4, in that order, but it was not detected with

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Table 1. Oxidation of Naphthalene, Phenanthrene, and Biphenyl by Human P450 Enzymes[a]

P450	naphthalene[b] 1-naphthol[c]	phenanthrene[b] 9-OH-phenanthrene[c]	biphenyl[b] 2- and 4-OH-biphenyl[c]
	(nmol/min/nmol P450)		
1A1	<0.01	0.33 ± 0.034	0.29 ± 0.033
1A2	0.91 ± 0.11	0.23 ± 0.024	0.24 ± 0.027
1B1	0.41 ± 0.062	0.02 ± 0.01	<0.1
2A6	2.6 ± 0.44	1.69 ± 0.22	2.3 ± 0.31
2A13	6.1 ± 0.88	3.14 ± 0.35	3.1 ± 0.19
2C9	<0.01	<0.01	<0.1
3A4	0.04 ± 0.013	0.05 ± 0.02	<0.1

[a]Oxidations of naphthalene, phenanthrene, and biphenyl by P450 enzymes were determined as described in the Experimental Procedures. Data shown are the mean of three or four samples determined ± SD. [b]Substrate used. [c]Product measured.

P450s 1A1 and 2C9. Phenanthrene 9-hydroxylation activity was detected with P450s 1A1, 1A2, 3A4, and 1B1.3 but not P450 2C9. Oxidation of biphenyl was detected with P450s 1A1 and 1A2 but not with P450s 1B1, 2C9, and 3A4.

Oxidation of Various Substituted Derivatives of Naphthalene, Phenanthrene, and Biphenyl by P450s 2A13 and 2A6. Since our results suggested that P450s 2A13 and 2A6 are the major enzymes involved in the oxidation of naphthalene, phenanthrene, and biphenyl, we extended our study to include the metabolism of a number of substituted derivatives of these three compounds by these enzymes. We first evaluated the oxidation of six acetylenic derivatives, 2EN, 2NPE, 3EPh, 2PPh, 3PPh, and 4BMPE, by P450 2A13 in the absence and presence of an NADPH-generating system (Figure 7). These six chemicals were found to be metabolized by P450 2A13 to product(s) that were not present in the absence of an NADPH-generating system in the reaction mixture, although detailed examinations to identify these products were not done (Figure 7). LC-MS analysis suggested the formation of oxygenated products of 2EPh, 2NPE, and 3EPh (results not shown).

We further examined the oxidation of 21 substituted derivatives, as well as naphthalene, phenanthrene, and biphenyl, by P450 2A13 and compared the spectral binding constants (K_s) of these chemicals with the enzyme (Table 2). P450 2A6 was also used to determine the oxidation of some of the derivatives as well as for a comparison of its spectral binding constants with these chemicals. P450 2A13 oxidized 2EN, 1NEPE, 2NPE, 2EPh, 3EPh, 2PPh, 3PPh, 2BPE, and 4BPE as well as the three parent compounds. Product formation was not detected for the other chemicals with P450 2A13 in our HPLC assay. P450 2A6 oxidized 2EN, 2EPh, 3EPh, and 4BPE as well as the parent compounds. However, product formation (peak height of the products) was always lower in the case of P450 2A6 compared to that with P450 2A13 (results not shown).

Molecular Interactions of the Chemicals Studied with P450s 2A13 and 2A6. Molecular docking analyses of these chemicals with P450 2A13 were performed using reported structures of P450 2A13 (4EJH),[34] 2A13 (2P85),[35] 2A13 (3T3S),[36] and 2A13 (4EJG)[34] bound to NNK, indole, pilocarpine, and nicotine, respectively. Since all biological units in the crystal structures of P450 2A13 4EJH, 2P85, and 4EJG are reported as octamers and P450 2A13 3T3S, as a hexamer, we selected one default biological unit for docking analysis. Docking analysis was also carried out with reported structures of P450 2A6 (1Z10),[36] 2A6 (3T3R),[37] and 2A6 (4EJJ).[34] Before performing simulation studies, the ligands were removed from the monomer structures of P450 2A13, and these ligand-free structures were used for docking analysis (Supporting Information Figure S2). We first determined

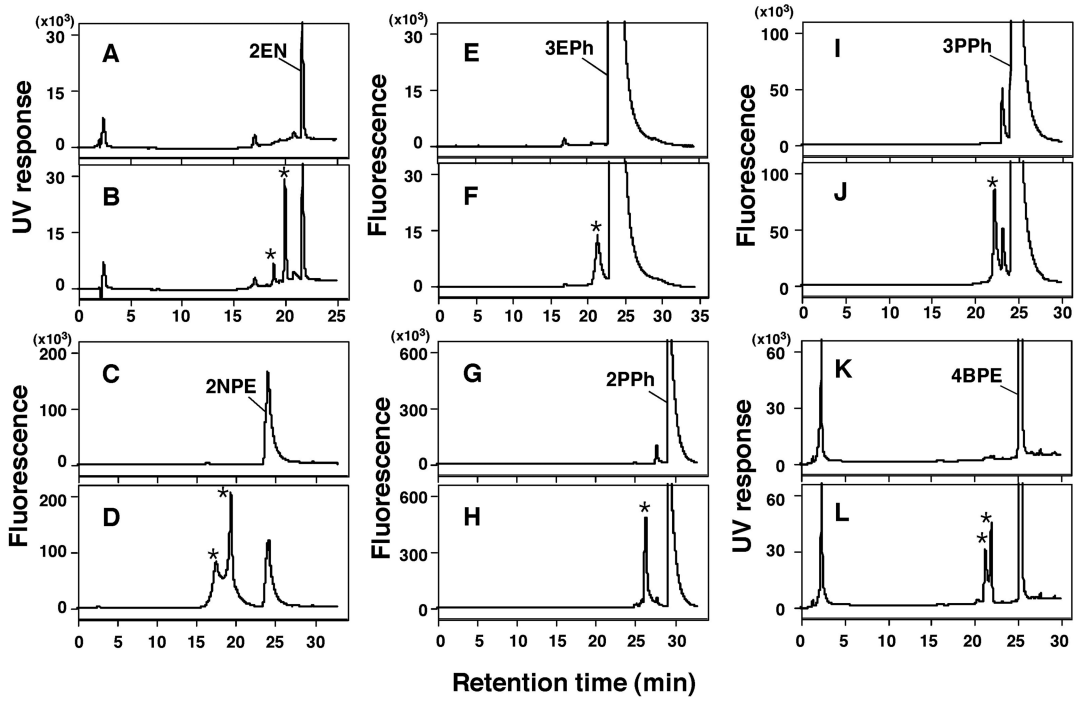

Figure 7. Metabolism of 2EN (A, B), 2NPE (C, D), 3EPh (E, F), 2PPh (G, H), 3PPh (I, J), and 4BPE (K, L) by P450 2A13 in the absence (A, C, E, G, I, and K) and presence (B, D, F, H, J, and L) of an NADPH-generating system, using HPLC analysis. Possible formation of metabolite(s) from these chemicals is denoted with an asterisk (*) in the figure.

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Table 2. Spectral Binding Constants and Oxidation Results Observed for the Studied Chemicals with P450s 2A13 and 2A6

	P450 2A13		P450 2A6	
chemical	Type I spectra[a] K_s (μM)	metabolism[b] (HPLC)	Type I spectra[a] K_s (μM)	metabolism[b] (HPLC)
naphthalene	0.35 ± 0.02	yes	2.9 ± 0.4	yes
2EN	0.080 ± 0.04	yes	1.0 ± 0.1	yes
1NMPE	13.3 ± 3.5	not detected	9.3 ± 1.8	not done
1NEPE	1.1 ± 0.1	yes	11.5 ± 2.3	not done
2NPE	0.42 ± 0.10	yes	33 ± 9	not done
2NMPE	4.9 ± 0.6	not detected	21 ± 0	not done
2NEPE	2.7 ± 0.6	not detected	19 ± 6	not done
phenanthrene	2.3 ± 0.2	yes	2.8 ± 0.4	yes
2EPh	1.1 ± 0.3	yes	3.3 ± 0.4	yes
3EPh	0.45 ± 0.08	yes	3.3 ± 0.9	yes
9EPh	0.95 ± 0.08	not detected	ND (10 μM)	not done
2PPh	3.9 ± 1.2	yes	1.6 ± 0.2	yes
3PPh	1.2 ± 0.4	yes	ND (10 μM)	not done
9PPh	6.4 ± 1.0	not detected	13 ± 3	not done
biphenyl	0.88 ± 0.16	yes	2.7 ± 0.5	yes
4EB	1.9 ± 0.2	not detected	5.4 ± 0.5	not detected
4PB	28 ± 12	not detected	7.0 ± 1.9	not done
4BuB	3.4 ± 1.1	not detected	12 ± 2	not done
2BPE	15.4 ± 1.7	yes	15 ± 2	not done
4BPE	0.48 ± 0.06	yes	0.75 ± 0.16	yes
2BMPE	1.7 ± 0.3	not detected	17 ± 3	not done
4BMPE	66 ± 19	not detected	43 ± 33	not done
22BDPE	11 ± 6	not detected	32 ± 9	not done
44BDPE	6.5 ± 1.6	not detected	2.6 ± 0.8	not detected

[a]Data reported are the mean with SE. [b]HPLC analysis was used for monitoring metabolism. When peak(s) were detected only in the presence of an NADPH-generating system as described in Figure 8, metabolism results were marked as "yes". When no metabolite peaks were detected under these assay conditions, results were marked as "not detected". Several of the chemicals were not examined with P450 2A6 (marked as "not done").

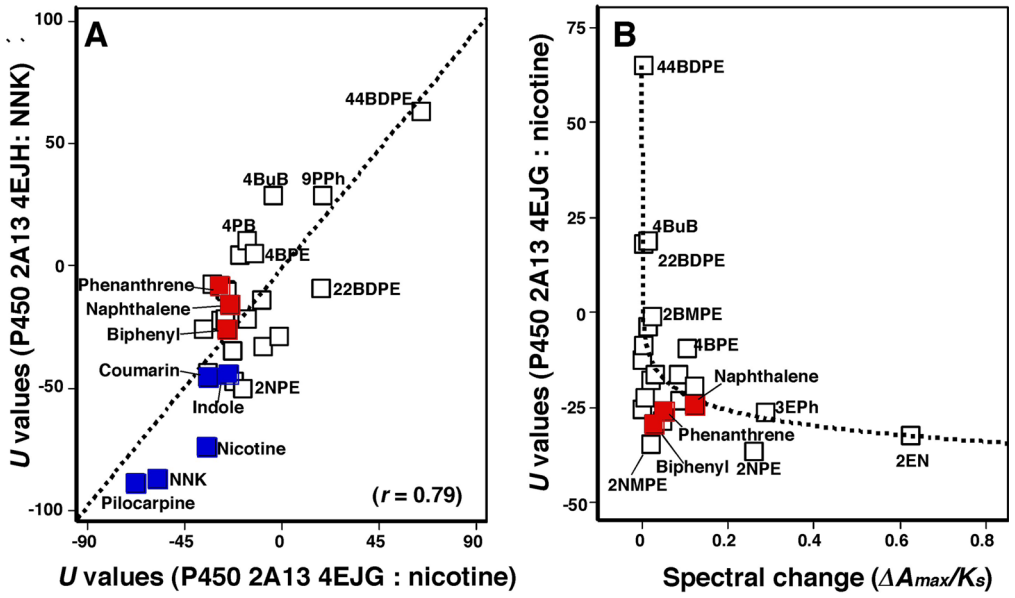

Figure 8. Correlations of ligand-interaction energies (U values) using molecular docking with P450s 2A13 4EJG (nicotine-type) and 4EJH (NNK-type) with 24 chemicals used in this study and NNK, indole, pilocarpine, nicotine, and coumarin. The latter five chemicals are indicated with blue squares, and naphthalene, phenanthrene, and biphenyl are marked with red squares (A). Effects of spectral binding intensities ($\Delta A_{max}/K_s$ ratio) on ligand binding energies (U values) using P450 2A13 4EJG (nicotine-type) (B).

optimal U values (ligand-interaction energy) (on analysis with MMFF94x force field) using P450 4EJG and found that U values for nicotine, naphthalene, phenanthrene, and biphenyl were −35.2, −24.3, −29.2, and −25.7, respectively (Supporting Information Figure S2), and that of coumarin was −34.4 (results not shown).

There was a good relationship between the U values of P450 2A13 4EJG (nicotine-type) and 4EJH (NNK-type) with these chemicals (r = 0.79, p < 0.01), and we found that the parent

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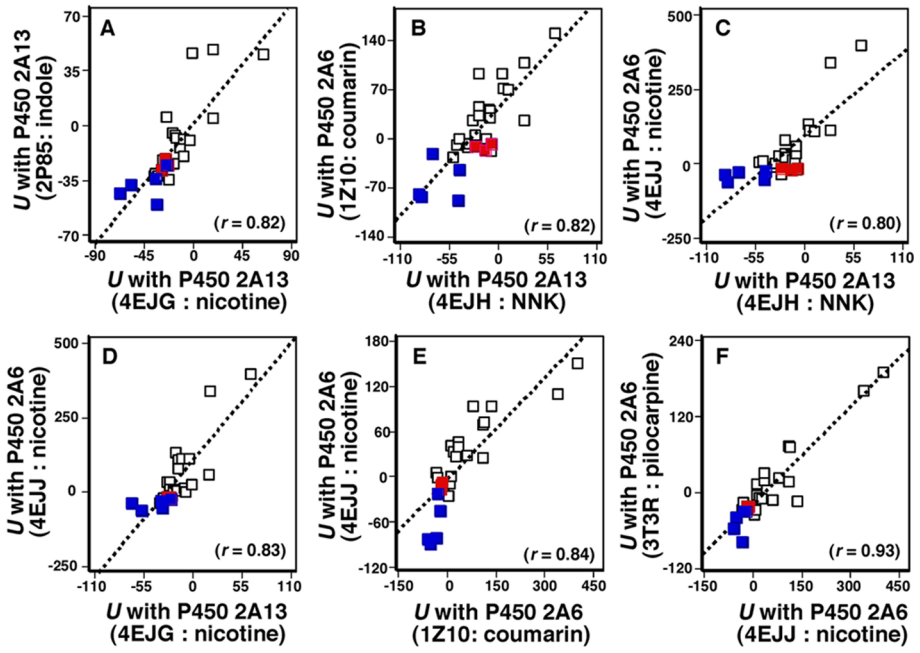

Figure 9. Correlation of ligand-interaction energy obtained with P450 2A13 4EJG (nicotine-type) and P450 2A13 2P85 (indole-type) (A), P450 2A13 4EJH (NNK-type) and P450 2A6 1Z10 (coumarin-type) (B), P450 2A13 4EJH (NNK-type) and P450 2A6 4EJJ (nicotine-type) (C), P450 2A13 4EJG (nicotine-type) and P450 2A6 4EJJ (nicotine-type) (D), P450 2A6 1Z10 (coumarin-type) and P450 2A6 4EJJ (nicotine-type) (E), and P450 2A6 4EJJ (nicotine-type) and P450 2A6 3T3R (pilocarpine-type) (F).

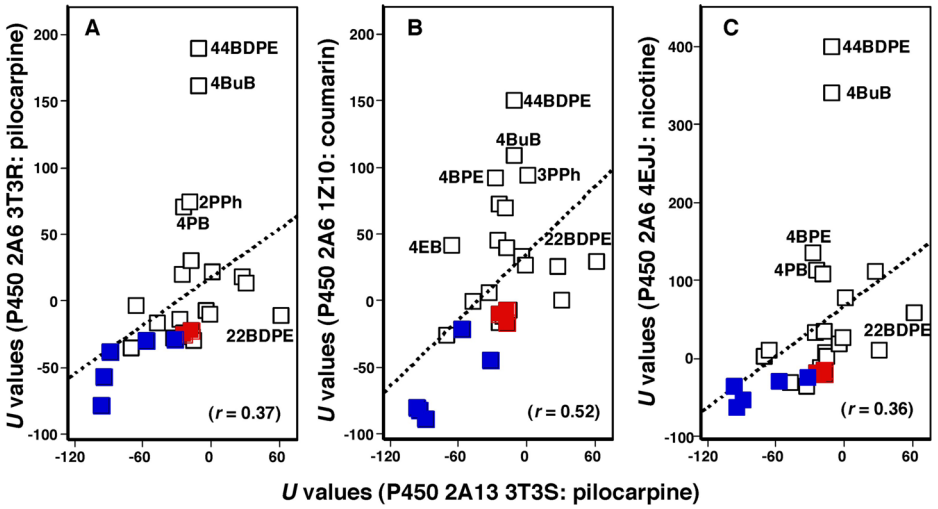

Figure 10. Correlation of ligand-interaction energy obtained between P450 2A13 3T3S (pilocarpine-type) and P450 2A6 3T3R (pilocarpine-type) (A), P450 2A6 1Z10 (coumarin-type) (B), and P450 2A6 4EJJ (nicotine-type) (C).

compounds naphthalene, phenanthrene, biphenyl, and coumarin had *U* values comparable to those of NNK, indole, pilocarpine, nicotine, and coumarin (Figure 8). Compounds that were oxidized by P450 2A13 (indicated by HPLC analysis) had small *U* values under these assay conditions (Figure 8A). When spectral changes ($\Delta A_{max}/K_s$ values) and *U* values (using P450 2A13 4EJG for docking) were compared, some relationship was found between these values when they were analyzed with logarithmic curve fitting (Cricket Graph) (Figure 8B). However, we did not find any positive correlation when *U* values (with P450 2A13 4EJG) and spectral binding constants (K_s values) were compared.

A correlation of the *U* values using P450 2A13 4EJH, 2P85, 3T3S, and 4EJG bound to NNK, indole, pilocarpine, and nicotine, respectively, and P450 2A6 3T3R, 4EJJ, and 1Z10 bound to pilocarpine, nicotine, and coumarin, respectively, was assessed with all of the chemicals used in this study as well as with NNK, indole, pilocarpine, nicotine, and coumarin (Supporting Information Table S1). As in the comparison between the *U* values of P450 2A13 4EJG (nicotine-type) and 4EJH (NNK-type) shown above, there were also good corralations for combinations of the *U* values obtained with these chemicals and the parent compounds, naphthalene, phenanthrene, and biphenyl, and coumarin (marked in red) had similar *U* values comparable to those of NNK, indole, pilocarpine, nicotine, and coumarin (marked in blue) (Figure 9). However, we found relatively low correlation coefficients for the comparison of *U* values of P450 2A13 3T3S bound to pilocarpine and P450 2A6 3T3R (r = 0.37), 1Z10 (r = 0.52), and 4EJJ (r = 0.36) bound to pilocarpine, coumarin, and

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nicotine (Figure 10), indicating that the crystal structure of P450 2A13 with pilocarpine may be different from that with other compounds. 44BDPE and 4BuB and some other chemicals in structures with P450 2A6 were far from the regression line.

DISCUSSION

The present studies show that P450 2A13 plays very important roles in oxidizing naphthalene, phenanthrene, and biphenyl and several of their substituted derivatives in humans and that P450 2A6 also participates in metabolizing several of these chemicals. Other human P450s, including P450s 1A1, 1A2, 1B1, 2C9, and 3A4, used in this study had lower activities at oxidizing naphthalene, phenanthrene, and biphenyl than P450s 2A13 and 2A6. Turnover rates (nmol of product formed/min/nmol P450) of the oxidation of naphthalene, phenanthrene, and biphenyl to 1-naphthol, 9-hydroxyphenanthrene, and 2- and/or 4-hydroxybiphenyl by P450 2A13 were 6.1, 3.1, and 3.2 min^{-1}, respectively, and catalytic activities by P450 2A6 were always lower than those by P450 2A13. Naphthalene, phenanthrene, and biphenyl induced Type I binding spectra with P450 2A13, with K_s values of 0.35, 2.3, and 0.88 μM, respectively, and these K_s values were lower than those (2.9, 2.8, and 2.7 μM, respectively) with P450 2A6, suggesting possible relationships between the spectral binding intensities and metabolism rates of these chemicals by P450s 2A13 and 2A6. It should be mentioned that we did not determine the formation of epoxide intermediates during the metabolism of the above three chemicals by P450 enzymes in this study, although there are reports suggesting the production of epoxides from these chemicals by P450 enzymes in laboratory animals.[10,12,16,20,21]

Other naphthalene, phenanthrene, and biphenyl derivatives were also analyzed for their spectral binding abilities with P450s 2A13 and 2A6 as well as for their oxidation by these enzymes. The results showed that compounds such as 2EN, 1NEPE, 2NPE, 2EPh, 3EPh, 2PPh, 3PPh, and 4BPE, whose spectral binding constants (K_s value) and intensities ($\Delta A_{max}/K_s$ ratio) with P450 2A13 were comparable with those of parent compounds naphthalene, phenanthrene, and biphenyl, were found to be oxidized by this enzyme similarly in the presence and absence of an NADPH-generating system. We also found the formation of oxygenated products of 2EPh, 1NEPE, 2NPE, and 3EPh by P450 2A13 on analysis with LC-MS. In a similar way, 2EN, 2EPh, 3EPh, 2PPh, and 4BPE (having spectral binding intensities with P450 2A6 comparable to those of the parent compounds) were oxidized by this enzyme, although product formation (peak height of the products) was always lower for P450 2A6 than for P450 2A13, as in the cases of naphthalene, phenanthrene, and biphenyl. P450 2A13 has 94% amino acid sequence similarity to P450 2A6, and the volume of the active site cavity of P450 2A13 (307 $Å^3$) is known to be larger than that of P450 2A6 (260 $Å^3$).[34−37] The higher catalytic activity of P450 2A13 compared to that of P450 2A6 toward these chemicals may be due to the size and shape of the substrate binding pocket in the active sites of these P450s.

In this study, it was also found that there were exceptions to the relationships observed between the spectral binding intensities and catalytic activities for several chemicals with P450s 2A13 and 2A6 (Table 2). For example, 2NMPE, 2NEPE, 9EPh, 9PPh, 4EB, 4BuB, 2BMPE, and 44BDPE (for P450 2A13) and 4EB and 44BDPE (for P450 2A6), whose K_s values were $\leq$10 μM, were not found to be oxidized by these P450 enzymes under our standard assay conditions. Most of these acetylenic derivatives have been previously synthesized to study the mechanisms of the inhibition of P450 enzymes, particularly P450 1 and 2 family enzymes.[27−31,38] Our previous studies indicated that most of these substituted chemicals, as well as their parent compounds naphthalene, phenanthrene, and biphenyl, are able to inhibit 7-ethoxyresorufin O-deethylation catalyzed by P450 1B1 and coumarin 7-hydroxylation by P450s 2A13 and 2A6.[23,26] In addition, 2EN has been reported to be a mechanism-based inhibitor of P450s 2B1, 2B4, 2B6, and 2B11,[39−41] and our previous findings indicated that 2EN inhibited P450 2A13- and 2A6-catalyzed coumarin 7-hydroxylation with IC_{50} values of 1.8 and 8.8 μM, respectively. These results suggest that the ability of some of these compounds to be metabolized by P450 enzymes may be influenced by their inhibition of the enzymes.

Our molecular docking simulation studies indicated that the ligand-binding energies (U values) are correlated for all of the 24 chemicals examined when comparing the crystal structures of P450 2A13 4EJH (NNK ligand) and P450 2A13 4EJG (nicotine ligand) (r = 0.79). Interestingly, the plotted positions in Figure 8A for naphthalene, phenanthrene, and biphenyl were at relatively similar locations to those of NNK, indole, pilocarpine, nicotine, and coumarin, which are typical ligands for P450 2A13.[34−37] Good correlations were also found when U values were compared using P450 2A13 4EJH (NNK-type), 2P85 (indole-type), 3T3S (pilocarpine-type), and 4EJG (nicotine-type), P450 2A6 3T3R (pilocarpine-type), 4EJJ (nicotine-type), and 1A10 (coumarin-type) with the chemicals used in this study. These results indicated that the structure of the substrate binding pocket of P450 2A6 (reported as cocrystals with coumarin, nicotine, or pilocarpine) is almost the same as that of P450 2A13 with NNK, nicotine, or indole. However, there were some exceptions, with relatively low correlation coefficients when comparing U values of P450 2A13 3T3S bound to pilocarpine and P450 2A6 3T3R (r = 0.37), 1Z10 (r = 0.52), and 4EJJ (r = 0.36) bound to pilocarpine, coumarin, and nicotine (Figure 10). Several chemicals (e.g., 44BDPE, 4BuB, and others) that were not good ligands for interacting with P450s 2A13 and 2A6 and being substrates for oxidation by these enzymes are likely to cause such weak correlations. In addition, some other compounds (e.g 4BuB, 2BMPE, 22BDPE, and 44BDPE) that were not oxidized by P450 2A13 showed higher ligand-binding energies on analysis using reported P450 2A13 4EJH, 2P85, 3T3S, and 4EJG structures bound to NNK, indole, pilocarpine, and nicotine. These results indicate the usefulness of molecular docking analysis in predicting possible relationships between the interaction of chemicals with the active sites of P450 enzymes and their susceptibilities to be metabolized by these enzymes. Comparison of ligand-binding energies (using P450 2A13 4EJG) and spectral binding intensities with these 24 chemicals is of interest because correlations were observed in these cases when logarithmic extrapolation was employed (Figure 8B). Those compounds having higher spectral binding intensities (e.g., 2EN, 3EPh, and 2NPE) showed lower U values, whereas compounds such as 44BDPE, 4BuB, 22BDPE, and 2BPE—which showed lower spectral binding intensities—had higher U values. However, it should be noted that we could not detect a positive correlation when spectral binding constants (K_s) were used for comparisons with U values. It is necessary to perform more studies to establish the relationships between biological responses and molecular docking results to understand the selectivity of P450 reactions.

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Human P450s 2A13 and 2A6 are known to catalyze the activation and detoxication of environmental carcinogens such as tobacco-related nitrosamines (e.g., NNK and *N*-nitrosonornicotine) and to metabolize different kinds of chemicals including coumarin, nicotine, phenacetin, naphthalene, 4-aminobiphenyl, and styrene.[13,35,42−46] P450 2A13 is expressed mainly in the respiratory tract, whereas P450 2A6 is found primarily in the liver.[47−49] We have previously shown that P450 2A13 plays a more significant role than P450 2A6 in interacting with and metabolizing diverse environmental chemicals including PAHs.[23,25] Our past and present studies also support the importance of P450s 2A13 and 2A6 in metabolizing acenaphthene and acenaphthylene[40] as well as pyrene, 1-hydroxypyrene, 1-nitropyrene, and 1-acethylpyrene,[50,51] and in the present study, their importance was also shown for metabolizing naphthalene, phenanthrene, biphenyl, and their alkynyl derivatives.

A role for P450 2A13 in the hydroxylation of naphthalene was reported previously by Fukami et al.,[13] who showed that naphthalene is oxidized by this enzyme to form a major metabolite, 1-naphthol, and a minor metabolite, 2-naphthol, through the suggested formation of naphthalene 1,2-epoxide. They also showed that these metabolic activities of P450 2A13 were higher than those observed with P450s 1A1 and 1A2. Cho et al.[12] also reported the *in vitro* metabolism of naphthalene by human liver microsomal P450 enzymes and showed that several P450 enzymes—including P450s 1A2, 2A6, and 2B6—catalyze the formation of 1-naphthol and 2-naphthol on analysis with HPLC, but they did not examine metabolism by P450 2A13. Naphthalene has been shown to be bioactivated by mouse P450 2F2, goat P450 2F3, and rat P450 2F4 to lung toxicants in these species;[9,11] however, the roles of human P450 2F1 (which is reported to be present in the lung and other tissues) in the metabolic activation of naphthalene and other chemicals including styrene, 3-methylindole, benzene, and trichloroethylene are not known at present due to difficulties with expressing P450 2F1 in heterogeneous systems such as *E. coli*.[52] Mouse P450 2A5 has been reported to play important roles in olfactory mucosal toxicity but not in lung toxicity.[11]

Urinary hydroxylated metabolites of phenanthrene have been used as biomarkers to determine exposure of humans to environmental PAHs in gasoline, diesel fuel, tobacco smoke, and diet.[53−55] Several metabolites—including 1-, 4-, and 9-hydroxyphenanthrene—have been identified in human urine,[53−55] and *in vitro* studies have suggested that a number of P450 enzymes are involved in the formation of several hydroxylated and dihydrodiol metabolites of phenanthrene in humans.[18,56] Our present results support the conclusion that 9-hydroxyphenanthrene is a major metabolite resulting from the incubation of phenanthrene with P450s 2A13 and 2A6.

Little is known about the metabolism of biphenyl by human P450 enzymes. Creaven et al.[19] reported that liver microsomes from several animal species including rats, mice, rabbits, and other species produce more 4-hydroxybiphenyl than 2-hydroxybiphenyl from biphenyl. Other studies have suggested that 3-hydroxybiphenyl (as well as 2- and 4-hydroxybiphenyl) is formed with liver microsomes from rats, hamsters, mice, and rabbits and that the major metabolite is 4-hydroxybiphenyl in all animal species examined.[20,21] Our present studies identified 2- and/or 4-hydroxybiphenyl in incubations of human P450s 2A13 and 2A6 with biphenyl, but the two products were not separated in this study. Human P450s 1A1 and 1A2 produced biphenyl metabolite(s), but P450s 1B1.1, 1B1.3, 2C9, and 3A4 did not.

In conclusion, our present study shows that P450s 2A13 and 2A6 are important enzymes at oxidizing naphthalene, phenanthrene, biphenyl, and several of their alkynyl derivatives. Other human P450 enzymes—including P450s 1A1, 1A2, 1B1, 2C9, and 3A4—had some role in several oxidation pathways of these chemicals. Molecular docking analysis showed correlations between ligand-interaction energies (*U* values) for these 24 chemicals with the active sites of P450s 2A13 and 2A6 and the susceptibilities of these chemicals to be oxidized by the enzymes. The results also support the usefulness of molecular docking analysis in understanding the basis of molecular interaction of xenobiotic chemicals with active sites of P450 proteins and possibly other enzymes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00083.

Correlation coefficients (*r* values) in ligand-interaction energies (*U* values) using reported structures of P450 2A13 and 2A6; oxidation of phenanthrene and 2EPh by P450 2A13 in the absence and presence of an NADPH-generating system (HPLC with UV detection); and docking simulation of the interactions of nicotine, naphthalene, phenanthrene, and biphenyl with P450 2A13 (4EJG) (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

P450, cytochrome P450; PAHs, polycyclic aromatic hydrocarbons; 2EN, 2-ethynylnaphthalene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 1NMPE, 1-naphthalene methyl propargyl ether; 1NEPE, 1-naphthalene ethyl propargyl ether; 2NPE, 2-naphthalene propargyl ether; 2NMPE, 2-naphthalene methyl propargyl ether; 2NEPE, 2-naphthalene ethyl propargyl ether; 2EPh, 2-ethynylphenanthrene; 3EPh, 3-ethynylphenanthrene; 9EPh, 9-ethynylphenanthrene; 2PPh, 2-(1-propynyl)phenanthrene; 3PPh, 3-(1-propynyl)-

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phenanthrene; 9PPh, 9-(1-propynyl)phenanthrene; 4EB, 4-ethynylbiphenyl; 4PB, 4-propynylbiphenyl; 4BuB, 4-butynylbiphenyl; 2BPE, 2-biphenyl propargyl ether; 4BPE, 4-biphenyl propargyl ether; 2BMPE, 2-biphenyl methyl propargyl ether; 4BMPE, 4-biphenyl methyl propargyl ether; 22BDPE, 2,2′-biphenyl dipropargyl ether; 44BDPE, 4,4′-biphenyl dipropargyl ether

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