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Binding of Diverse Environmental Chemicals with Human Cytochromes P450 2A13, 2A6, and 1B1 and Enzyme Inhibition

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Abstract

A total of 68 chemicals including derivatives of naphthalene, phenanthrene, fluoranthene, pyrene, biphenyl, and flavone were examined for their abilities to interact with human P450s 2A13 and 2A6. Fifty-one of these 68 chemicals induced stronger Type I binding spectra (iron low- to high-spin state shift) with P450 2A13 than those seen with P450 2A6, i.e. the spectral binding intensities (A_{\max}/K_s ratio) determined with these chemicals were always higher for P450 2A13. In addition, benzo[c]phenanthrene, fluoranthene, 2,3-dihydroxy-2,3-dihydrofluoranthene, pyrene, 1-hydroxypyrene, 1-nitropyrene, 1-acetylpyrene, 2-acetylpyrene, 2,5,2',5'-tetrachlorobiphenyl, 7-hydroxyflavone, chrysin, and galangin were found to induce a Type I spectral change only with P450 2A13. Coumarin 7-hydroxylation, catalyzed by P450 2A13, was strongly inhibited by 2'-methoxy-5,7-dihydroxyflavone, 2-ethynylnaphthalene, 2'-methoxyflavone, 2-naphthalene propargyl ether, acenaphthene, acenaphthylene, naphthalene, 1-acetylpyrene, flavanone, chrysin, 3-ethynylphenanthrene, flavone, and 7-hydroxyflavone; these chemicals induced Type I spectral changes with low K_s values. On the basis of the intensities of the spectral changes and inhibition of P450 2A13, we classified the 68 chemicals into eight groups based on the order of affinities for these chemicals and inhibition of P450 2A13. The metabolism of chemicals by P450 2A13 during the assays explained why some of the chemicals that bound well were poor inhibitors of P450 2A13. Finally, we compared the 68 chemicals for their abilities to induce Type I spectral changes of P450 2A13 with the Reverse Type I binding spectra observed with P450 1B1: 45 chemicals

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interacted with both P450s 2A13 and 1B1, indicating that the two enzymes have some similarity of structural features regarding these chemicals. Molecular docking analyses suggest similarities at the active sites of these P450 enzymes. These results indicate that P450 2A13, as well as Family 1 P450 enzymes, is able to catalyze many detoxication and activation reactions with chemicals of environmental interest.

Introduction

A variety of chemicals cause toxic and/or carcinogenic responses at respiratory organ sites in humans.^{1,2} These chemicals include polycyclic aromatic hydrocarbons (PAHs, e.g. benzo[a]pyrene (B[a]P) and benzo[c]phenanthrene (B[c]Phe)), arylamines (e.g. 2-aminoanthracene and 2-aminofluorene), heterocyclic amines (e.g. 2-amino-3,5-dimethylimidazo[4,5-f]quinoline (MeIQ) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1)), tobacco-related nitrosamines (e.g. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN)), biphenyl and polychlorinated biphenyls, and plant toxins and mycotoxins such as flavonoids and aflatoxins.^{3–5} Most of these chemicals require metabolism to reactive products by enzymes in order to evoke their toxic and/or carcinogenic effects.^{6–10} P450 enzymes have been shown to be the major enzymes involved in these reactions,⁶ and several lines of evidence support the view that the levels of expression of individual forms of P450 enzymes in different organs may define how these chemicals induce toxicity and carcinogenesis in these organs.^{8,9,11,12}

In humans, P450s 2A6 and 2A13 are expressed mainly in the liver and respiratory tract, respectively.^{1,13–17} P450 2A6 is known to be active in catalyzing metabolism of several drugs, e.g. coumarin and phenacetin, and also the metabolic activation of tobacco-related nitrosamines (including NNK and NNN) to carcinogenic metabolites.^{1,18,19} However, P450 2A13 is more active than P450 2A6 in activating NNK and NNN^{13,20} and these findings are of interest because these enzymes are mainly expressed in respiratory organs, the sites of exposure to numerous environmental chemicals.^{1,21–23} The chemicals described above have been shown to be oxidized to chemically inert products or sometimes reactive metabolites, mainly by the Family 1 P450 enzymes.⁶ The roles of P450s 2A13 and 2A6 in these processes have not been well characterized for a large number of compounds, especially carcinogens other than nitrosamines.^{24–26} Interestingly Fukami *et al.*²⁵ have recently reported that the pollutants naphthalene, styrene, and toluene are metabolized more efficiently by P450 2A13 than P450 2A6.

In this study, we examined whether P450 2A13 and P450 2A6 interact with, are inhibited by, and/or metabolize various chemicals, including derivatives of naphthalene, phenanthrene, pyrene, biphenyl, flavone, and several other chemicals that we have previously studied with human Family 1 P450 enzymes, particularly P450 1B1.^{27–30} A total of 68 chemicals were analyzed for spectral binding changes with P450 2A13 and inhibition of coumarin 7-hydroxylation activities catalyzed by this enzyme. Most of these chemicals were also studied with P450 2A6. Interaction with and inhibition of P450 1B1 by several of these chemicals was measured and combined with our previous results^{27–30} in order to compare the similarity of interactions between the human P450 1B1 and 2A enzymes.

Experimental Procedures

Chemicals

Escherichia coli DH5α cells were purchased from Invitrogen (Carlsbad, CA). The carcinogens were obtained from the National Cancer Institute Chemical Carcinogen Repository/Midwest Research Institute (Kansas City, MO) or Toronto Research Chemicals (Toronto, Ontario, Canada).

Acetylenic PAHs and biphenyls, including 1-ethynylpyrene (1EP), 2-ethynylpyrene (2EP), 4-ethynylpyrene (4EP), 1-vinylpyrene (1VP), 1-(1-propynyl)pyrene (1PP), 2-ethynylphenanthrene (2EP), 3-ethynylphenanthrene (3EP), 9-ethynylphenanthrene (9EP), 2-(1-propynyl)phenanthrene (2PPh), 3-(1-propynyl)phenanthrene (3PPh), 9-(1-propynyl)phenanthrene (9PPh), 2-ethynylnaphthalene (2EN), 4-ethynylbiphenyl (4Ebi), and 4-(1-propynyl)biphenyl (4Pbi) were synthesized as described previously.^{28,29,31-33} Benz[a]anthracene (B[a]A), chrysene, 5-methylchrysene (5MeCh), 7,12-dimethylbenz[a]anthracene (7,12-DMBA), B[a]P, benzo[e]pyrene (B[e]P), fluoranthene (FA), and B[b]FA were obtained from SigmaAldrich (St. Louis, MO) or Kanto Kagaku Co. (Tokyo). 7-Ethoxyresorufin and resorufin were purchased from SigmaAldrich. Flavone, pyrene, naphthalene, phenanthrene (Ph), biphenyl, 7-ethoxyresorufin, and resorufin were obtained from SigmaAldrich or Kanto Kagaku Co. (Tokyo). Thirteen methoxylated flavonoids, with or without dihydroxy substitution at the 5,7-positions, were synthesized; the methods for the synthesis of these chemicals have been published.^{30,33} Derivatives of naphthalenes and biphenyls were also synthesized as previously described.^{28-30,34}

α-Naphthoflavone (ANF), β-naphthoflavone (BNF), flavone, flavanone, 3-hydroxyflavone (flavonol) (3HF), 5-hydroxyflavone (5HF), 7-hydroxyflavone (7HF), 5,7-dihydroxyflavone (57DHF, chrysin), 3,5,7-trihydroxyflavone (357THF, galangin), and 4'-methoxy-5,7-dihydroxyflavone (4M57DHF, acacetin) were obtained from SigmaAldrich or Wako Pure Chemical (Osaka) or Kanto Kagaku Co. 2'-Methoxyflavone (2MF), 3'-methoxyflavone (3MF), 4'-methoxyflavone (4MF), 2'-methoxy-5,7-dihydroxyflavone (2M57DHF), 3'-methoxy-5,7-dihydroxyflavone (3M57DHF), and 3'4'-dimethoxy-5,7-dihydroxyflavone (34DM57DHF) were synthesized as described previously.³⁰ All of the flavonoids and substrates for P450 assays were dissolved in (CH₃)₂SO (dimethylsulfoxide) and added directly to the incubation mixtures; the final concentration of organic solvent in the assay was <0.4% (v/v).

2,5,2',5'-, 3,5,3',5'-, 3,4,3',4'- and 2,4,3',4'-tetrachlorobiphenyls (TCB) were kindly donated by Dr. Nobuyuki Koga of Nakamura Gakuen University (Fukuoka, Japan).

Enzymes

Expression and purification of P450 2A6 and 2A13 enzymes were carried out using previously described methods,³⁵ with some modifications.³⁶ The expression vector pKK322-2/2A13 containing CYP2A13 cDNA was kindly provided by Dr. E. E. Scott (Univ. Kansas). The 2A13 insert replaced 2A6 in the "bicistronic" construct (P450 plus human NADPH-P450 reductase).³⁷ Bacterial bicistronic P450 1B1,³⁸ 2A6,³⁵ and 2A13 were prepared and the *E. coli* membranes were suspended in 10 mM Tris-HCl buffer (pH 7.4)

containing 1.0 mM EDTA and 20% glycerol (v/v) as described.³⁸ P450 1B1 was purified as described elsewhere.^{27,32,38}

Enzyme Assays

Coumarin 7-hydroxylation activities of P450s 2A6 and 2A13 were determined using bicistronic bacterial membranes expressing P450 2A13 or 2A6 (together with human NADPH-P450 reductase) as described previously.^{39,40} Each reaction mixture contained 10 pmol of P450 2A13 or 2A6 in 0.50 mL of 100 mM potassium phosphate buffer (pH 7.4) and 10 µM coumarin. An NADPH-generating system⁴¹ was added to start reactions at 37 °C, which were continued for 20 min and terminated by adding 20 µL of 20% Cl₃CCO₂H, (w/v). The oxidized products were extracted with two volumes of CH₂Cl₂, and the resultant organic layer was transferred to a clean test tube and extracted into 1.0 mL of 30% (w/v) sodium borate buffer (pH 9.6). The production of 7-hydroxycoumarin was measured (in a microtiter plate) using a fluorescence plate reader (excitation 350 nm/emission 453 nm, SynergyMx, BioTek, Winooski, VT).

In several cases in which the chemicals contained hydroxyl groups, coumarin 7-hydroxylation activity was determined by HPLC as described previously.⁴² Briefly, incubation mixtures (described above) were mixed with 100 µL of 17% HClO₄ (w/v) to stop the reaction. After centrifugation ($2 \times 10^3 \times g$), the supernatant was used for HPLC analysis as described.⁴²

Spectral Binding Titrations

Purified P450 enzymes were diluted to 1.0 µM in 0.10 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), and the binding spectra were recorded with subsequent additions of chemicals in a JASCO V-550 or OLIS-Aminco DW2a spectrophotometer (On-Line Instrument Systems, Bogart, GA) as described previously.^{29,43,44} Briefly, the chemical inhibitors were added to the buffer with or without P450 and the spectra were recorded between 350 nm and 500 (or 700) nm. The substrate binding spectra were obtained by subtracting the blank spectra (in the absence of P450) from the P450 spectra (in the presence of P450). Spectral dissociation constants (K_s) were estimated using GraphPad Prism software (GraphPad Software, San Diego, CA), either using hyperbolic plots or else quadratic fits for tight binding.

Rates of Oxidation of Chemicals

Disappearance of chemicals upon incubation with a P450 2A13 system was determined in an incubation mixture (0.50 mL) containing 50 pmol of bicistronic P450 membranes, 10 µM chemical, and an NADPH-generating system.⁴¹ (The chemicals were dissolved in CH₃CN as 1 mM stock solutions and diluted 100-fold, with the solvent concentration 0.1%, v/v.) Incubation was carried out at 37 °C for 0, 20, 40, 80, 160 and 240 s, following a preincubation time of 1 min. Reactions were terminated by adding 100 µL of 17% HClO₄ (w/v) and the residual substrate and products were extracted twice with CH₂Cl₂. The HPLC separation system was a Waters Acuity UPLC system (Waters, Milford, MA) utilizing an Acuity UPLC BEH octadecylsilane (C18) column (1.7 µm, 2.1 mm × 100 mm) at 40 °C. The analyses were carried out with a linear gradient increasing from 80% mobile phase A

(100% H₂O) to 100% mobile phase B (100% CH₃CN) over 5 min at a flow rate of 0.4 mL min⁻¹. Each sample (10 µL) was injected using an autosampler system and detection was performed with a Waters photodiode array detector (210–400 nm). The wavelength range-integrated absorbance in the peak (for the parent compound) at time zero was equated to the 10 µM concentration and used to calculate the concentrations remaining at subsequent time points.

Other Assays

P450⁴⁵ and protein⁴⁶ concentrations were estimated by the methods described previously.

Docking Simulations into Human P450 Enzymes

The crystal structures of P450s 1B1,⁴⁷ 2A6,⁴⁸ and 2A13^{49,50} have recently been reported and were used. Simulations were carried out for P450 enzymes using the MMFF94x force field described in the MOE software (ver. 2011.10, Computing Group, Montreal, Canada).^{30,51} P450s 2A13 and 1B1 were superimposed during modeling of the three-dimensional structures. Lower *U* values (ligand-interaction energy) are an indication of higher interaction between a chemical and P450.

Results

Spectral Interactions of Chemicals with P450s 1B1, 2A13, and 2A6

We first examined the spectral interaction of phenanthrene with P450 1B1, 2A13, and 2A6 (Figure 1). Phenanthrene produced Reverse Type I binding spectra with P450 1B1 (Figure 1A) and Type I binding spectra with P450 2A13 (Figure 1B) and 2A6 (Figure 1C) on analysis of spectral shifts at the Soret peaks. The addition of phenanthrene to P450 1B1 also caused increases in intensities in the α and β bands, while these bands of P450s 2A13 and 2A6 coalesced with increasing concentrations of phenanthrene (Figures 1a, 1b, and 1c).

Ten chemicals including 2EN, 2EPh, biphenyl, acenaphthene, acenaphthylene, resveratrol, flavone, 2,5,2',5'-TCB, and ANF (as well as phenanthrene) were found to induce Type I binding spectra with P450 2A13, but the latter two chemicals (2,5,2',5'-TCB and ANF) did not induce spectral changes with P450 2A6 (Figure 2). The spectral intensities, as expressed by the A_{max}/K_s ratios, were compared for 24 chemicals with P450s 2A13 and 2A6. All of these chemicals (except for 3,4,3',4'-, 3,5,3',5'-, and 2,4,3',4'-TCB, for which no spectral binding was observed), had higher affinities with P450 2A13 than with P450 2A6 (Figure 3). Of particular interest was the observation that several chemicals—pyrene, 1-hydroxypyrene, 1-nitropyrene (1NP), 1-acetylpyrene, 2-acetylpyrene, B[c]Phe, FA, 2,3-dihydroxy-2,3-dihydrofluoranthene (FA-2,3-diol), 2,5,2',5'-TCB, and ANF—induced spectral changes with P450 2A13 but not with P450 2A6 (Figure 3). Other compounds that did not induce Type I binding spectra with P450 2A6 are listed in Table 1.

Inhibition of P450 2A13- and 2A6-dependent Coumarin 7-Hydroxylation Activity by 68 chemicals

Inhibition of coumarin 7-hydroxylation catalyzed by P450 2A13 and 2A6 was determined with 68 chemicals (the coumarin 7-hydroxylation turnover number by P450 2A13 (0.6

min^{-1}) was lower than that for P450 2A6 (1.5 min^{-1}). The results with 16 of the 68 chemicals that inhibited coumarin 7-hydroxylation catalyzed by both P450s 2A13 and 2A6 are presented in Figures 4A and 4B. Acenaphthene, acenaphthylene, 2EN, and flavanone were found to be highly inhibitory with P450 2A13; the IC_{50} values obtained were 3.1, 3.3, 1.8, and $4.3 \mu\text{M}$, respectively (Figure 4A). In general, P450 2A13 was more susceptible to inhibition by these chemicals, and only 2EN strongly inhibited P450 2A6 (IC_{50} value of $8.8 \mu\text{M}$) (Figure 4B). The IC_{50} values for inhibition of coumarin 7-hydroxylation obtained with 40 chemicals were compared for P450s 2A13 and 2A6 (Figure 5). Of the 40 chemicals, 33 inhibited P450 2A13 with IC_{50} values $<180 \mu\text{M}$, and eight of these 33 chemicals did not inhibit P450 2A6. Interestingly, both FA-2,3-diol and 1-hydroxypyrene were very inhibitory with P450 2A6, although these chemicals did not induce Type I binding spectra (Figure 5).

Classification of Chemicals into Eight Groups on the Basis of Interaction with and Inhibition of P450 2A13

On basis of the intensities of spectral binding and inhibition of P450 2A13, we tentatively classified the 68 chemicals into eight groups (Table 2). The 13 compounds in Group 1 were highly interactive with (spectrally) and inhibited P450 2A13 (Table 2 and Figure 6). Group 2 (10 chemicals) also induced strong spectral changes but inhibited P450 2A13-dependent coumarin 7-hydroxylation with IC_{50} values between 15 and $80 \mu\text{M}$ (Table 2, Figure 6). Collectively these two groups included eight flavonoid derivatives, five naphthalene derivatives, three pyrenes, three biphenyl derivatives, two phenanthrene derivatives, acenaphthene, and acenaphthylene. Groups 3, 4, and 5 contain chemicals that interacted with P450 2A13 with K_s values $< 10 \mu\text{M}$ but were relatively weak inhibitors of P450 2A13. Group 6 contains chemicals with K_s values $> 10 \mu\text{M}$ but IC_{50} values $< 20 \mu\text{M}$, and Group 7 includes chemicals that interacted with P450 2A13 relatively weakly. The 17 chemicals in Group 8 were essentially negative in interaction with P450 2A13 (these chemicals were also negative in interacting with P450 2A6).

Decreases in Chemical Concentration Due to Oxidation by P450 2A13

In order to determine if these chemicals are metabolized by P450 2A13, we examined time-dependent decreases in their concentrations after incubation with an NADPH-generating system, as described in the Experimental Procedures (Figure 7). In Figure 7, the group numbers of these chemicals are indicated at the top (G1–G8) in each section. Nine of the 13 chemicals in Group 1 were examined, and the results showed that these chemicals were not very rapidly metabolized by P450 2A13, except for 2M57DHF (Figure 7). In contrast, three chemicals examined in Group 2 were found to be oxidized rapidly by P450 2A13, although the oxidations of 9-EPh and pyrene were slow. Naphthalene, 1PP, and 4Ebi (Group 2) were not rapidly oxidized by P450 2A13 (results not shown). Similar results were found with the chemicals in Group 3, in which B[c]Phe and 1-NP were oxidized rapidly by P450 2A13, but not so extensively in the cases of 3PPh and 2EPh (and also 1EP and 2,5,2'-5'-TCB, results not shown). It should be noted that the six chemicals in Group 3 produced spectral changes with but did not inhibit P450 2A13 even at concentrations of $100 \mu\text{M}$. Three chemicals in Group 4 were slowly oxidized by P450 2A13 (phenanthrene and 4-butynylbiphenyl (4Bubi) were rapidly oxidized, results not shown). In Group 5, resveratrol, FA-2,3-diol, and 9-PPh were very rapidly oxidized by P450 2A13, although the other five compounds were slowly

oxidized. 4Pbi and 2-BPE (Group 7) were oxidized by P450 2A13, but the other three chemicals were not.

Similarities of Interaction of 68 Chemicals with P450s 2A13 and 1B1

We previously reported that there are many types of chemicals that induce Reverse Type I binding spectra with P450 1B1 and inhibit EROD catalyzed by that enzyme,^{27–30,38,51} including derivatives of naphthalene, phenanthrene, pyrene, biphenyl, and flavone. Most of these chemicals were examined in the current study with P450s 2A13 and 2A6. In the present study we examined the interaction of P450 1B1 with more chemicals,^{27–30,38} including benzene, phenol, toluene, styrene, acenaphthene, acenaphthylene, anthracene, B[a]A, B[a]A-1,2-diol, B[a]A-3,4-diol, 7,12-DMBA, 7,12-DMBA-3,4-diol, 7,12-DMBA-5,6-diol, B[c]Phe, B[c]Phe-3,4-diol, FA, FA-2,3-diol, B[b]FA, chrysene, chrysene-1,2-diol, B[a]P-4,5-diol, B[a]P-7,8-diol, 1-NP, 1-hydroxypyrene, 1- and 2-acetylpyrene, 4-aminobiphenyl (4-ABP), and 2,5,2',5'-TCB. We compared the results observed for the interactions of P450s 2A13 and 1B1 with the 68 chemicals in their abilities (A_{max}/K_s ratio) to induce Type I binidng and Reverse Type I binding, respectively (Figure 8) (note that the scales in Figures 8A and 8B differ from those in Figures 8C and 8D). The results showed different patterns for these two P450s for the Type I and Reverse Type I binding spectra by these chemicals; however, 45 of the 68 chemicals examined were able to induce spectral changes with both P450s 2A13 and 1B1.

We compared the reported structures of the active sites of P450s 2A13 and 1B1^{47,50} and found similarities in these two P450s (Figure 9). Both Asn297 in P450 2A13 and Asp326 in P450 1B1 were found at similar positions relative to the different substrates studied, and several other similar amino acids surrounded the active sites of P450s 2A13 and 1B1 (Figure 9).

Discussion

At least three types of spectral binding (i.e., Type I binding, Type II binding, and Reverse Type I binding spectra) are known to occur upon binding of numerous substrates and chemical inhibitors with different P450 enzymes.^{43,44,52–54} We have previously shown that a variety of chemical inhibitors—e.g. structural derivatives of pyrene, naphthalene, phenanthrene, biphenyl, and flavone—interact with human P450 1B1 to produce Reverse Type I binding spectra and that the spectral dissociation constants (K_s) and the magnitudes of the binding (A/K_s) of these compounds are generally well correlated with potencies of these chemicals to inhibit P450 1B1, as determined with EROD as a model reaction.^{28,29}

The Type I spectral change is generally agreed to result from the displacement of water as the heme axial ligand and a low-to high-spin iron transition.^{43,44} That is, binding of the substrate in the active site leads to displacement of the water molecule. A Reverse Type I spectral changed is just the opposite, where substrate binding leads to addition of water as the axial ligand. Features of individual substrates that influence P450 binding are recognized in some cases but are generally not well understood.

The localization of P450s 2A6 and 2A13 is of interest in regard to their activities towards carcinogens and to risk assessment. Su et al.¹³ cloned P450 2A13 cDNA from human nasal mucosa and demonstrated mRNA expression in human lung, nasa mucosa, and trachea; expression in the liver was much lower. P450 2A6 mRNA was expressed in the order liver \gg nasal mucosa > trachea > lung.¹³ More recently Chiang et al.¹⁵ used selective antibodies to measure protein expression. P450 2A6 was expressed in human liver but P450 2A13 was not. Roughly equal levels of P450 2A6 and 2A13 protein were expressed in lung (bronchiole and alveolus) but P450 2A13 was not.¹⁵ However, Zhu et al.¹⁶ used an anti-P450 2A13 antibody and reported immunostaining in trachea and bronchiole but not alveolar cells. The difference in the bronchiole data of Chiang et al.¹⁵ are not understood at this time. The P450 2A13 proximal promoter has been reported to interact with members pf the CCAAT/enhancer binding protein (C/EBP) family in the respiratory tract, and silencing by epigenetic mechasnisms has also been reported.^{1,14}

In this study we found that P450 2A13, as well as P450 2A6, has the ability to interact with and be inhibited by diverse environmental chamicals, including a variety of derivatives of naphthalene, phenanthrene, pyrene, biphenyl, flavone, and flavanone, most of which were used in our previous experiments in searching for specific P450 1B1 inhibitors.^{27-30,38} Fifty-one of the 68 chemicals tested showed Type I binding spectra with P450 2A13, and 25 of the 47 chemicals analyzed induced Type I binding spectra with P450 2A6. Among these chemicals tested with P450s 2A13 and 2A6, B[c]Phe, FA, pyrene, 1-hydroxypyrene, 1NP, 1- and 2-acetylpyrene, 2,5,2',5'-TCB, 7HF, 57DHF (chrysin), 357THF (galangin), and ANF did not induce spectral changes with P450 2A6. These chemicals were also found to be non-inhibitory or weak inhibitors of P450 2A6-dependent coumarin 7-hydroxylation activity. Thus, different selectivities of several chemicals in inducing spectral changes with these P450s were found in this study, although it should be noted that 2-EN, naphthalene, 1PP, 1EP, 2EPh, phenanthrene, acenaphthene, acenaphthylene, biphenyl, and resveratrol had relatively similar tendencies to induce spectra with P450s 2A13 and 2A6 (Figure 3). The sequence similarity between the two enzymes is 93.5% (differences in only 32 of their 494 amino acids);^{50,55} however, there are differences in the identities of 10 amino acids surrounding the active sites of these two P450s.^{49,50} Recently, Scott and her associates have reported elegant studies showing that mutations in these “peripheral” amino acids determine the binding selectivities of several substrates, e.g. coumarin, phenacetin, 2'-methoxyacetophenone, and phenethylisothiocyanate.^{49,55,56}

Styrene, toluene, and 4-ABP have been reported to be metabolized efficiently by P450 2A13,²⁵ although our current studies showed that these chemicals did not induce spectral changes with the enzyme nor inhibited coumarin 7-hydroxylation by P450 2A13 (or 2A6). Other compounds that did not interact with P450 2A13 or 2A6 include benzene, toluene, eight PAHs, and four flavonoids (e.g. 4MF, 4M57DHF, 34DM57DHF, and 57DHF). The latter four compounds are of interest in that several other flavonoid derivatives—e.g., flavone, flavanone, 57DHF, 2MF, and 2M57DHF—were found to interact with and inhibit P450 2A13, suggesting the roles of structural details of flavonoids and other chemicals in the interaction with the active sites of these enzymes.^{29,30}

Both acenaphthene and acenaphthylene, two known environmental PAHs found in tobacco smoke and broiled food,^{57,58} were found to interact with P450s 2A13 and 2A6 and induce Type I binding spectra with K_s values <1 μM for P450 2A13 (and 1.2 and 4.4 μM , respectively, for P450 2A6). Acenaphthene and acenaphthylene inhibited P450 2A13-dependent coumarin 7-hydroxylation with IC₅₀ values of 3.1 and 3.3 μM and P450 2A6-dependent activities of 29 and 33 μM , respectively. These two chemicals did not induce spectral changes with P450 1B1 but inhibited EROD with IC₅₀ values of 11 and 12 μM , respectively. It is not presently known how acenaphthene and acenaphthylene are metabolized by P450 enzymes (including P450s 2A13, 2A6, and 1B1) to produce detoxicated products or reactive metabolites, and work towards that goal is underway in this laboratory (reaction products were not identified in Figure 7). In this study, we also found that the two environmental carcinogens FA and B[c]Phe^{59–61} induced Type I binding spectra with P450 2A13, having K_s values of 2.4 and 1.1 μM , respectively. Both chemicals also induced Reverse Type I binding spectra with P450 1B1 (K_s values of 1.6 and 2.5 μM , respectively). IN the paper immediately following this one,³⁶ we found that B[c]Phe and also B[c]Phe-3,4-diol and FA-2,3-diol are activated to genotoxic metabolites by P450s 2A13, 2A6, and 1B1 in the tester strain *Salmonella typhimurium* NM2009, indicating that the former these enzymes may have important roles in metabolic activation of carcinogenic PAHs to reactive metabolites bound to DNA.

On the basis of the results obtained in the spectral titrations and catalytic inhibition experiments, we tentatively classified the 68 chemicals studied here into eight groups. Group 1 (K_s values of 0.4–3 μM and IC₅₀ values of 0.4–10 μM) contains six flavonoid derivatives, three naphthalene derivatives, 3EPh, 1-acetylpyrene, acenaphthene, and acenaphthylene. Among nine chemicals tested in this group for the metabolic disappearance experiments, the concentrations of 2M57DHF, 1-acetylpyrene, 3EPh, and 2NEPE were decreased after incubation with P450 2A13. Six flavonoids (including flavone, flavanone, 7HF, 2MF, 57DHF, and 2M57DHF) were classified in Group 1, while four other similar compounds (3HF, 4MF, 4M57DHF, and 34DM57DHF) were placed in Group 8 (K_s values of >100 μM and IC₅₀ values of >200 μM). It should be mentioned that in some of our previous work²⁵ the latter four flavonoids were highly interactive with and inhibited P450 1B1, suggesting that different orientations exist in P450s 2A13 and 1B1 in their interactions with different flavonoid structures. Another interesting point in the present results is that several chemicals in different groups were rapidly metabolized by P450 2A13, including 3MF, ANF, 1-naphthalene ethyl propargyl ether (1NEPE), and 2BMPE in Group 2, B[c]Phe and 1NP in Group 3, resveratrol, FA-2,3-diol, and 9PPh in Group 5, and 4Pbi and 2BPE in Group 7. Further studies are required to determine structure-function relationships in the interactions of these diverse chemicals with P450 2A13.

Multiple crystal structures are available for both P450 2A6^{48,55,62} and P450 2A13,^{49,50,55} bound to several ligands. These have been considered relatively rigid structures, although recent work by DeVore and Scott⁴⁹ revealed protein movement and flexibility, which may be relevant to ligand movement into and out of the active site. This collection of structures, along with results from site-directed and random mutagenesis,^{52,63–65} have identified key residues involved in substrate recognition and catalysis in these two P450 enzymes, which

differ in only 32 of their 494 amino acids. Critical residues include those at positions 117, 372,⁶³ 297,^{48,64,65} and 208, 300, and 301.^{52,64} Several laboratories have used structures, modeling, and screening to identify competitive inhibitors^{62,66–68} and mechanism-based inactivators^{66,69} that are selective in discriminating between P450 2A6 and 2A13. The crystallography and the studies with various substrates and inhibitors indicate a more spacious active site for P450 2A13 than P450 2A6,⁴⁹ although the subtleties of binding require more sophisticated analysis. Some of the features (of discrimination) were seen in our work, at least in the context of the amino acid residues involved.

We compared the reported structures of active sites of P450s 2A13 and 1B1^{47,50} and found that there were similarities between these two P450s (Figure 9). Both Asn297 in P450 2A13 and Asp326 in P450 1B1 exist in similar positions, and several other similar amino acids surround the active sites of P450s 2A13 and 1B1 (Figure 9). Several of the phenylalanine residues of these two P450s may determine how these chemicals interact with the enzymes.

In conclusion, the present study showed that P450s 2A13 and 2A6 can interact with diverse chemicals including derivatives of naphthalene, phenanthrene, fluoranthene, pyrene, biphenyl, and flavonoids; P450 2A13 interacts more readily with most of these chemicals than does P450 2A6. Aside from any peculiarities of specific bonding interactions, some of these observed interaction may be due in part to the larger active site of P450 2A13.^{48,49,55,64} On analysis of the spectral titrations and inhibition of P450 2A13 activity, the 68 chemicals examined in this study were classified into eight groups, depending on the orders of intensities of interaction with P450 2A13. The results suggest that interactions may occur in both direct (i.e., binding) and metabolism-dependent manners, and further characterization is required to elicit structure-function rules in the interactions of these chemicals with the P450 2A enzymes. Finally, our results suggest that there is some similarity in the molecular structures of the active sites of P450s 2A13 and 1B1. These results, along with those in the accompanying paper,³⁶ indicate that P450 2A13 participates in the metabolism of many chemicals of environmental interest, including PAHs and others.

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Abbreviations

4-ABP	4-aminobiphenyl
ANF	α-naphthoflavone
B[a]A	benz[a]anthracene
B[a]A-1,2-diol	1,2-dihydroxy-1,2-dihydrobenz[a]anthracene

B[a]A 3,4-diol	3,4-dihydroxy-3,4-dihydrobenz[<i>a</i>]anthracene
B[b]FA	benzo[<i>b</i>]fluoranthene
BNF	β -naphthoflavone
B[a]P	benzo[<i>a</i>]pyrene
B[e]P	benzo[<i>e</i>]pyrene
B[a]P-4,5-diol	4,5-dihydroxy-4,5-dihydrobenzo[<i>a</i>]pyrene
B[a]P-7,8-diol	7,8-dihydroxy-7,8-dihydrobenzo[<i>a</i>]pyrene
22BDPE	2,2'-biphenyl dipropargyl ether
B[b]FA	benzo[<i>b</i>]fluoranthene
B[c]Phe	benzo[<i>c</i>]phenanthrene
B[c]Phe-3,4-diol	3,4-dihydroxy-3,4-dihydrobenzo[<i>c</i>]phenanthrene
2BMPE	2-biphenyl methyl propargyl ether
4BMPE	4-biphenyl methyl propargyl ether
2BPE	2-biphenyl propargyl ether
4Bubi	4-butynylbiphenyl
57DHF	5,7-dihydroxyflavone (chrysin)
34DM57DHF	3'4'-dimethoxy-5,7-dihydroxyflavone
7,12-DMBA	7,12-dimethylbenz[<i>a</i>]anthracene
7,12-DMBA-3,4-diol	3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[<i>a</i>]anthracene
7,12-DMBA-5,,6-diol	5,6-dihydroxy-5,6-dihydro-7,12-dimethylbenz[<i>a</i>]anthracene
4Ebi	4-ethynylbiphenyl
2EN	2-ethynylnaphthalene
1EP	1-ethynylpyrene
2EP	2-ethynylpyrene
4EP	4-ethynylpyrene
2EPh	2-ethynylphenanthrene
3EPh	3-ethynylphenanthrene
9EPh	9-ethynylphenanthrene
EROD	7-ethoxyresorufin <i>O</i> -deethylation
FA	fluoranthene
FA-2,3-diol	2,3-dihydroxy-2,3-dihydrofluoranthene
3HF	3-hydroxyflavone

5HF	5-hydroxyflavone
7HF	7-hydroxyflavone
LB	Luria-Bertani
2M57DHF	2'-methoxy-5,7-dihydroxyflavone
3M57DHF	3'-methoxy-5,7-dihydroxyflavone
4M57DHF	4'-methoxy-5,7-dihydroxyflavone (acacetin)
5MeCh	5-methylchrysene
MeIQ	2-amino-3,5-dimethylimidazo[4,5- <i>f</i>]quinoline
2MF	2'-methoxyflavone
3MF	3'-methoxyflavone
4MF	4'-methoxyflavone
1NEPE	1-naphthalene ethyl propargyl ether
2NEPE	2-naphthalene ethyl propargyl ether
1NMPE	1-naphthalene methyl propargyl ether
2NMPE	2-naphthalene methyl propargyl ether
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	<i>N</i> -nitrosonornicotine
1NP	1-nitropyrene
2NPE	2-naphthalene propargyl ether
PAHs	polycyclic aromatic hydrocarbons
4Pbi	4-(1-propynyl)biphenyl
Ph	phenanthrene
1PP	1-(1-propynyl)pyrene
4PP	4-(1-propynyl)pyrene
2PPh	2-(1-propynyl)phenanthrene
3PPh	3-(1-propynyl)phenanthrene
9PPh	9-(1-propynyl)phenanthrene
TB	Terrific Broth
TCB	tetrachlorobiphenyl
357THF	3,5,7-trihydroxyflavone (galangin)
Trp-P-1	3-amino-1,4-dimethyl-5 <i>H</i> -pyrido[4,3- <i>b</i>]indole
1VP	1-vinylpyrene

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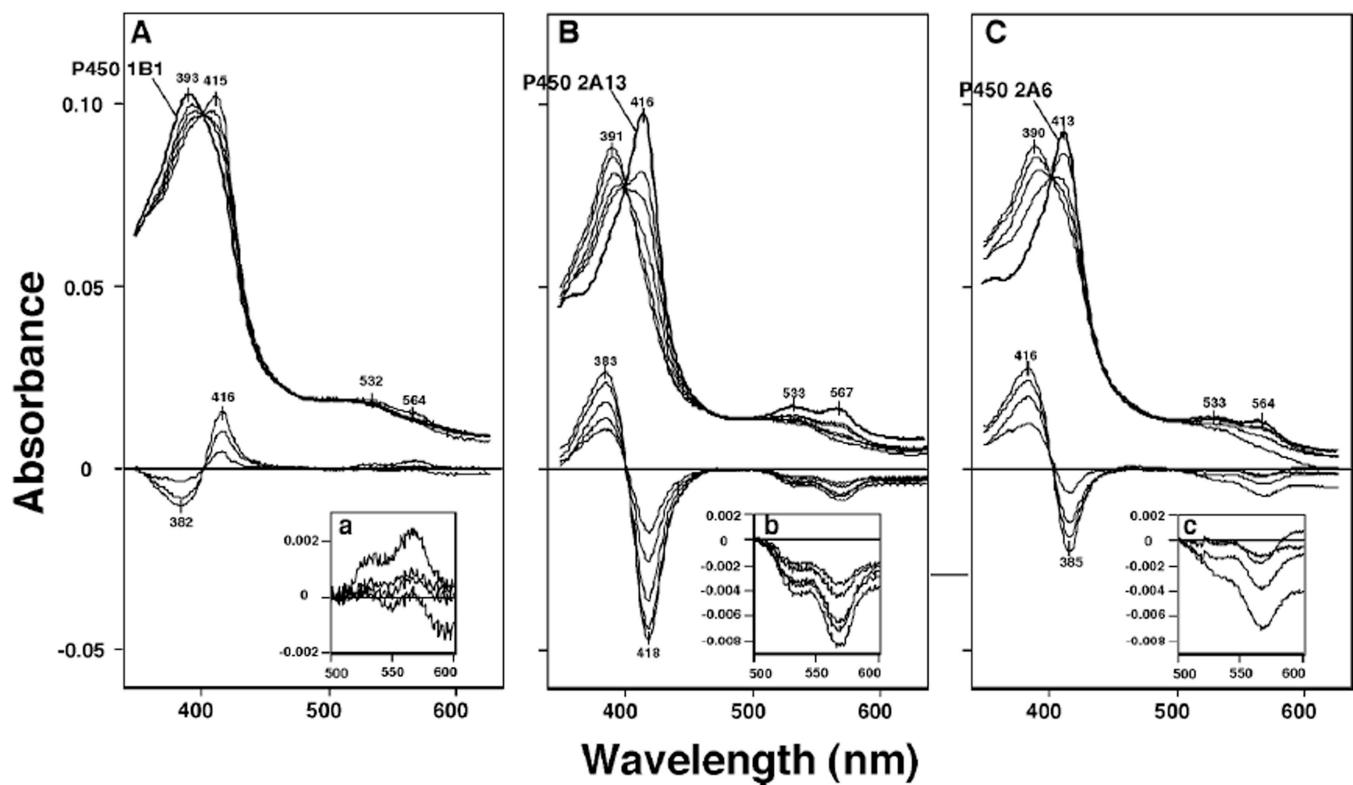
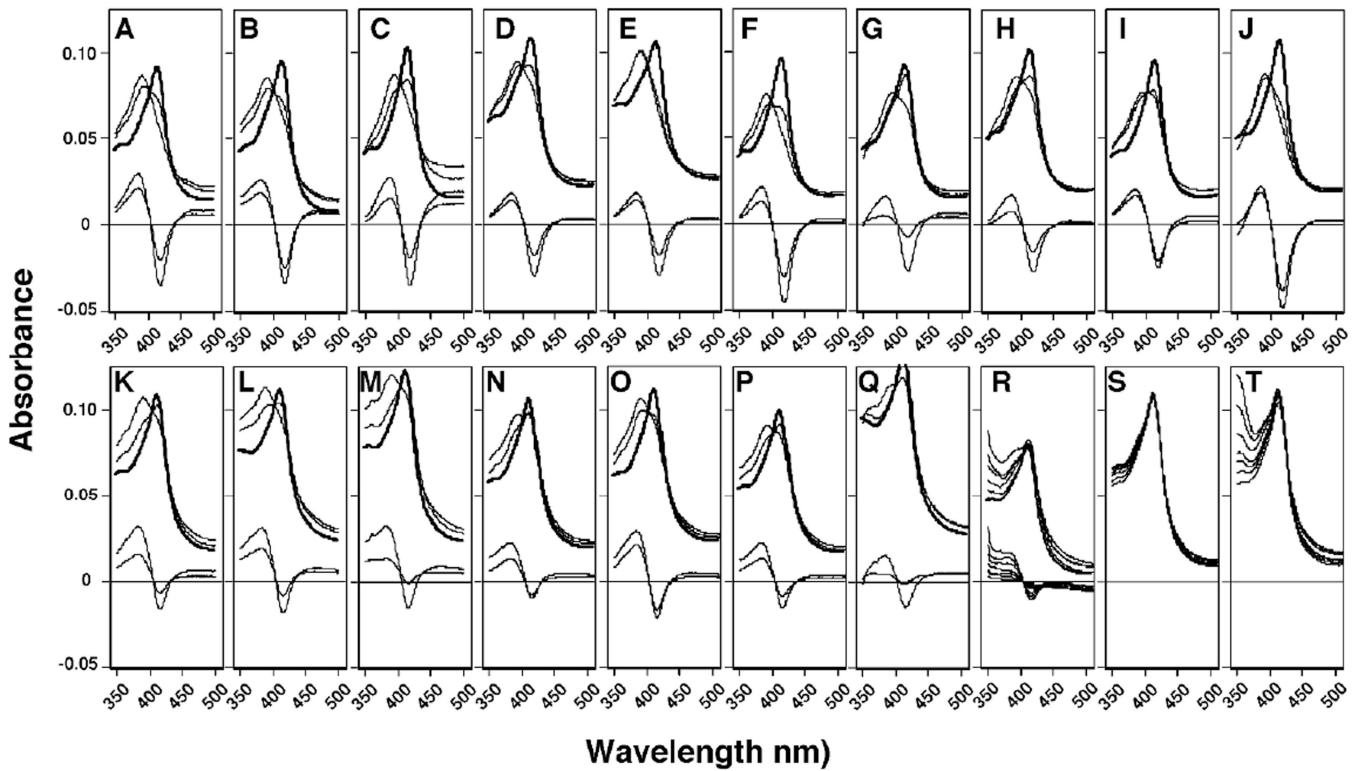
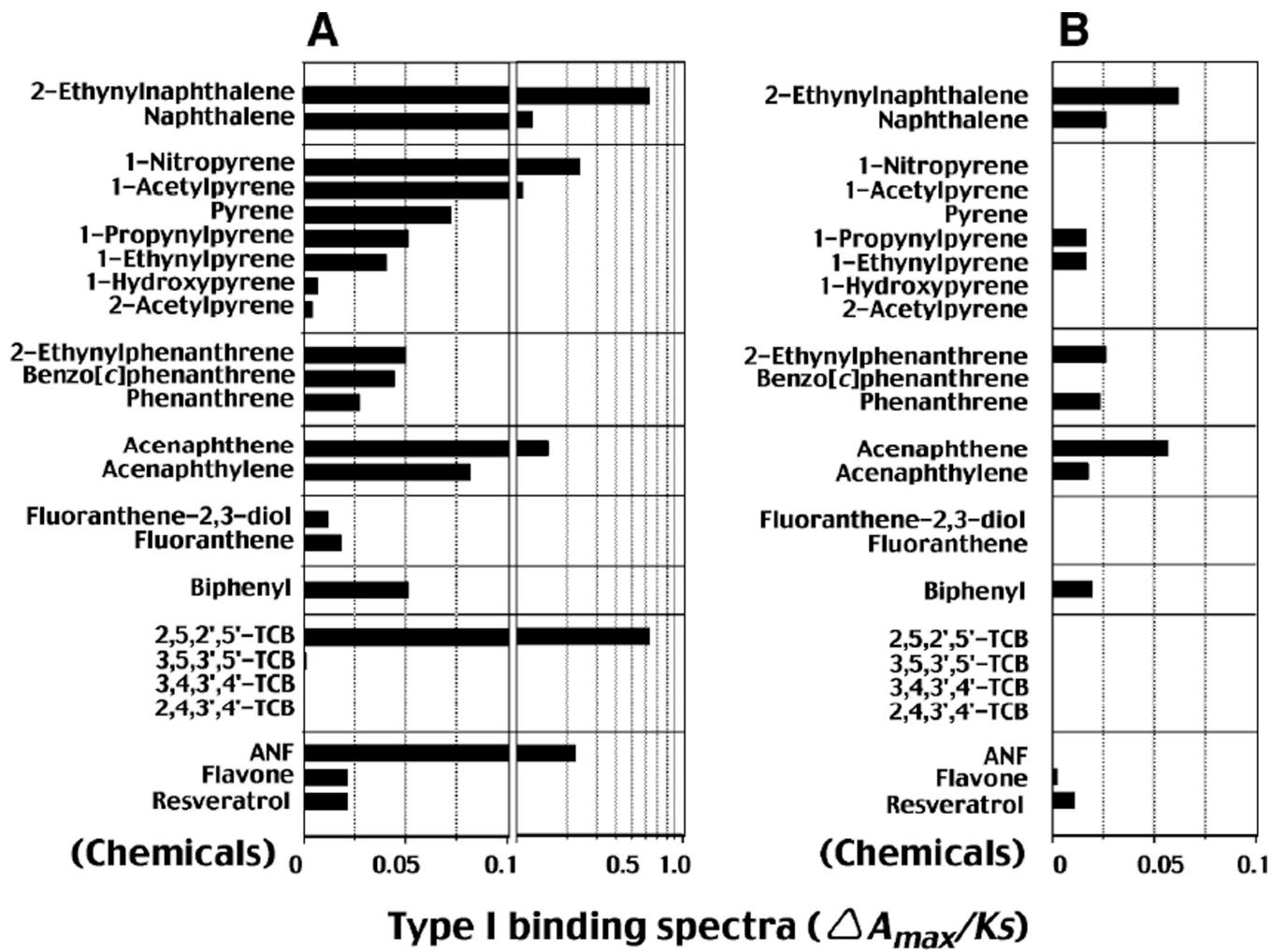


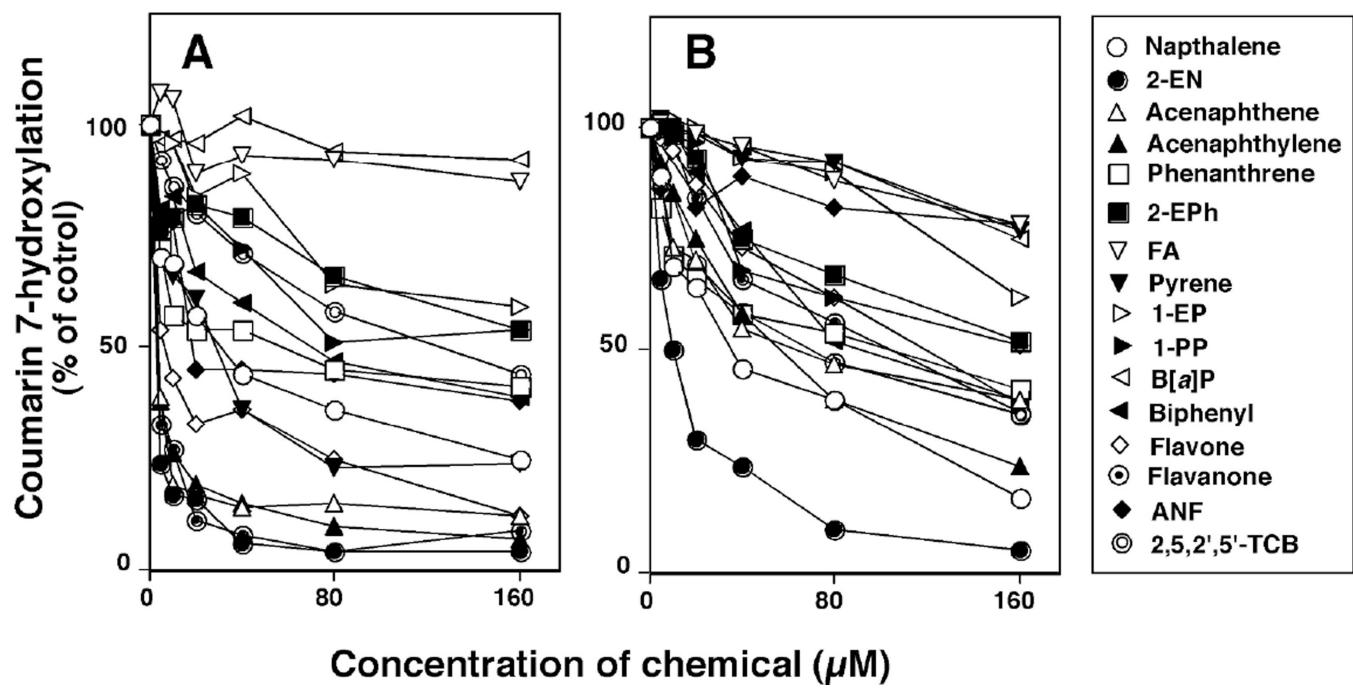
Figure 1.
Reverse Type I binding spectra of P450 1B1 (A and a) and Type I binding spectra of P450 2A13 (B and b) and 2A6 (C and c) with phenanthrene (1.25–20 μ M). The absolute spectra and difference spectra are shown in the upper and lower parts, respectively. Inserts (a, b, c) show the expansion of the P450 α and β bands.

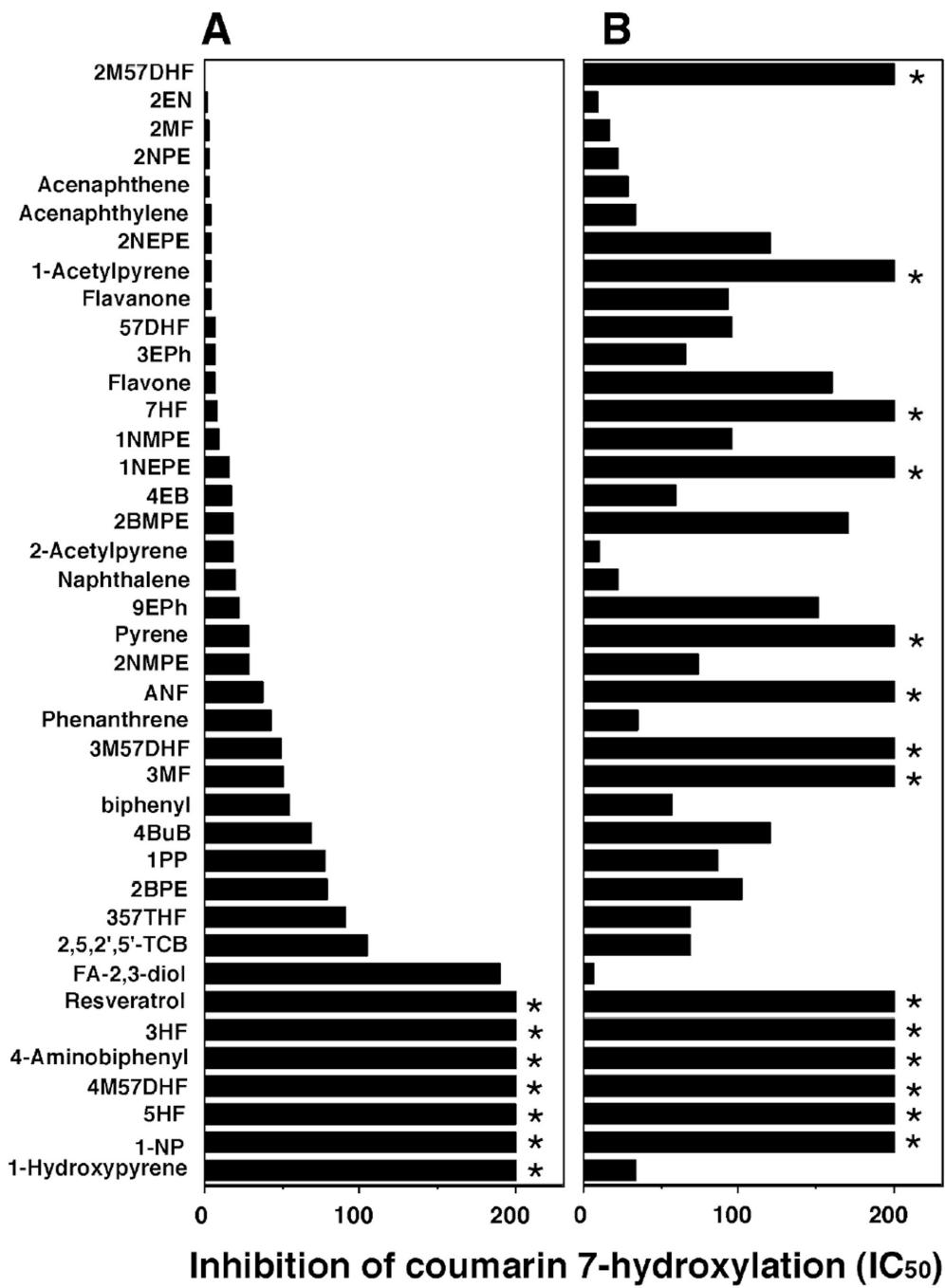
**Figure 2.**

Type 1 binding spectra of P450 2A13 (A–J) and 2A6 (K–T) induced by naphthalene (A and K), 2-EN (B and L), 2EPh (C and M), biphenyl (D and N), acenaphthene (E and O), acenaphthylene (F and P), resveratrol (G and Q), flavone (H and R), 2,5,2',5'-TCB (I and S), and ANF (J and T). The P450 concentration was 1.0 μ M and chemical concentrations used varied between 0.6 and 20 μ M. Each part includes absolute spectra (upper section) and difference spectra (lower section).

**Figure 3.**

Comparison of spectral intensities (A_{max}/K_s ratio) of interaction of chemicals with P450s 2A13 (A) and 2A6 (B).



**Figure 5.**

Comparison of inhibition of coumarin 7-hydroxylation by 50 selected chemicals for P450s 2A13 (A) and 2A6 (B). Asterisks indicate IC₅₀ values >200 μM.

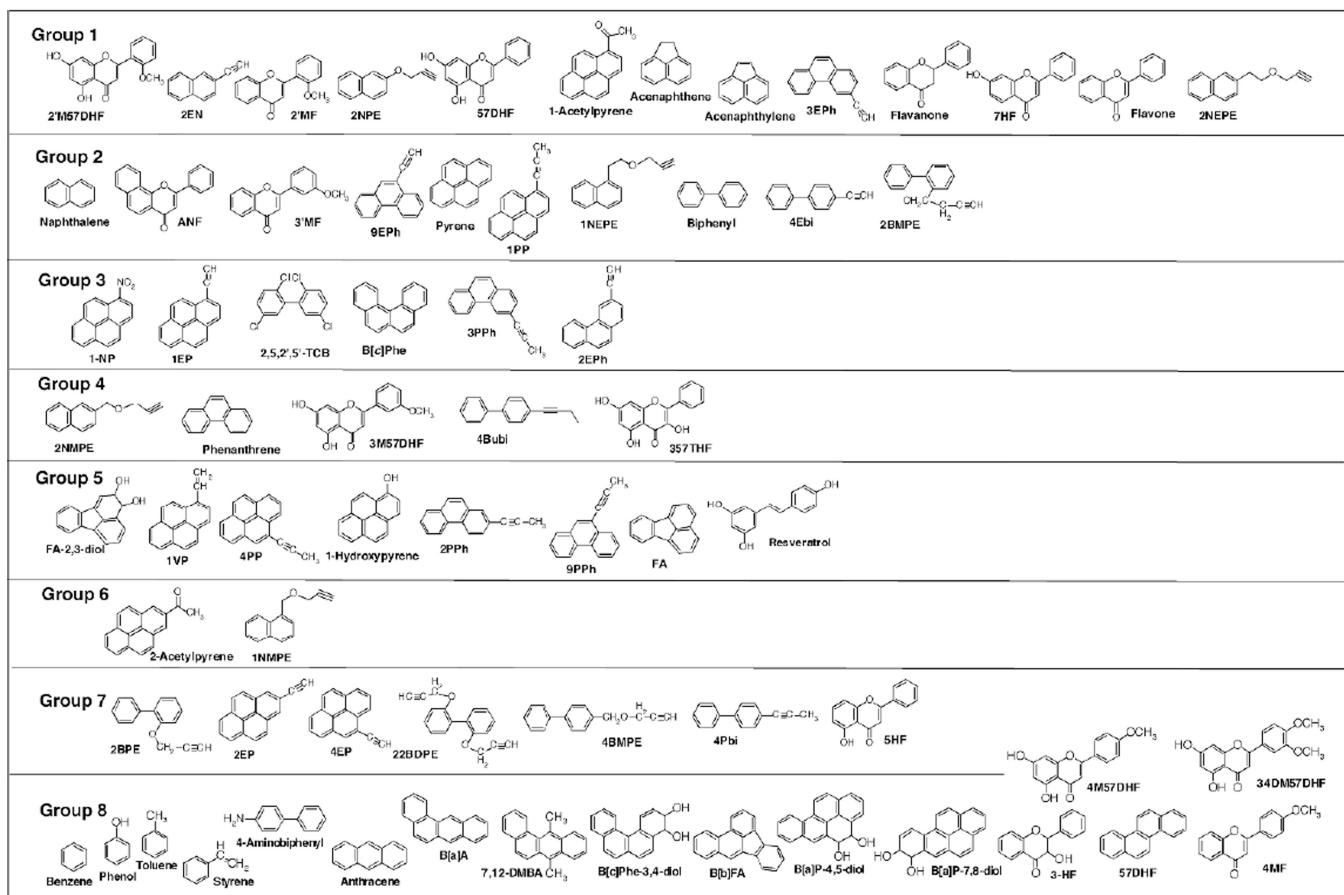
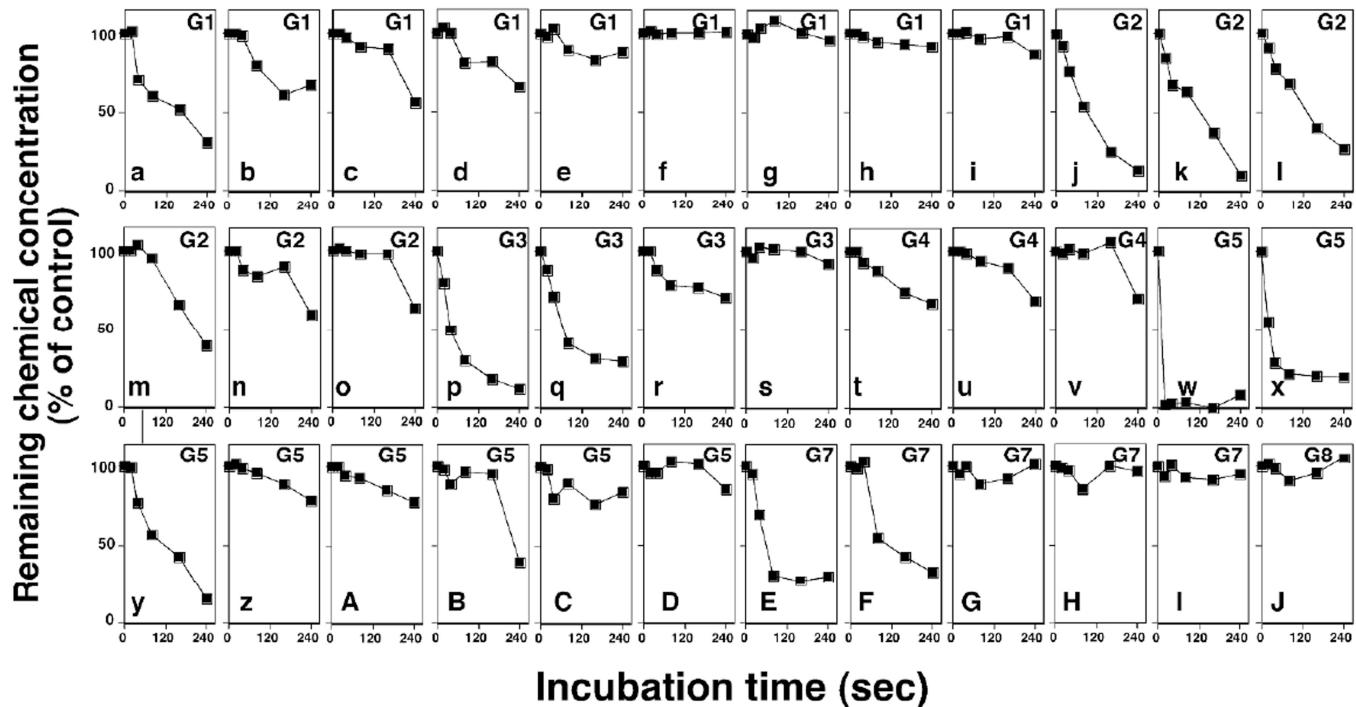
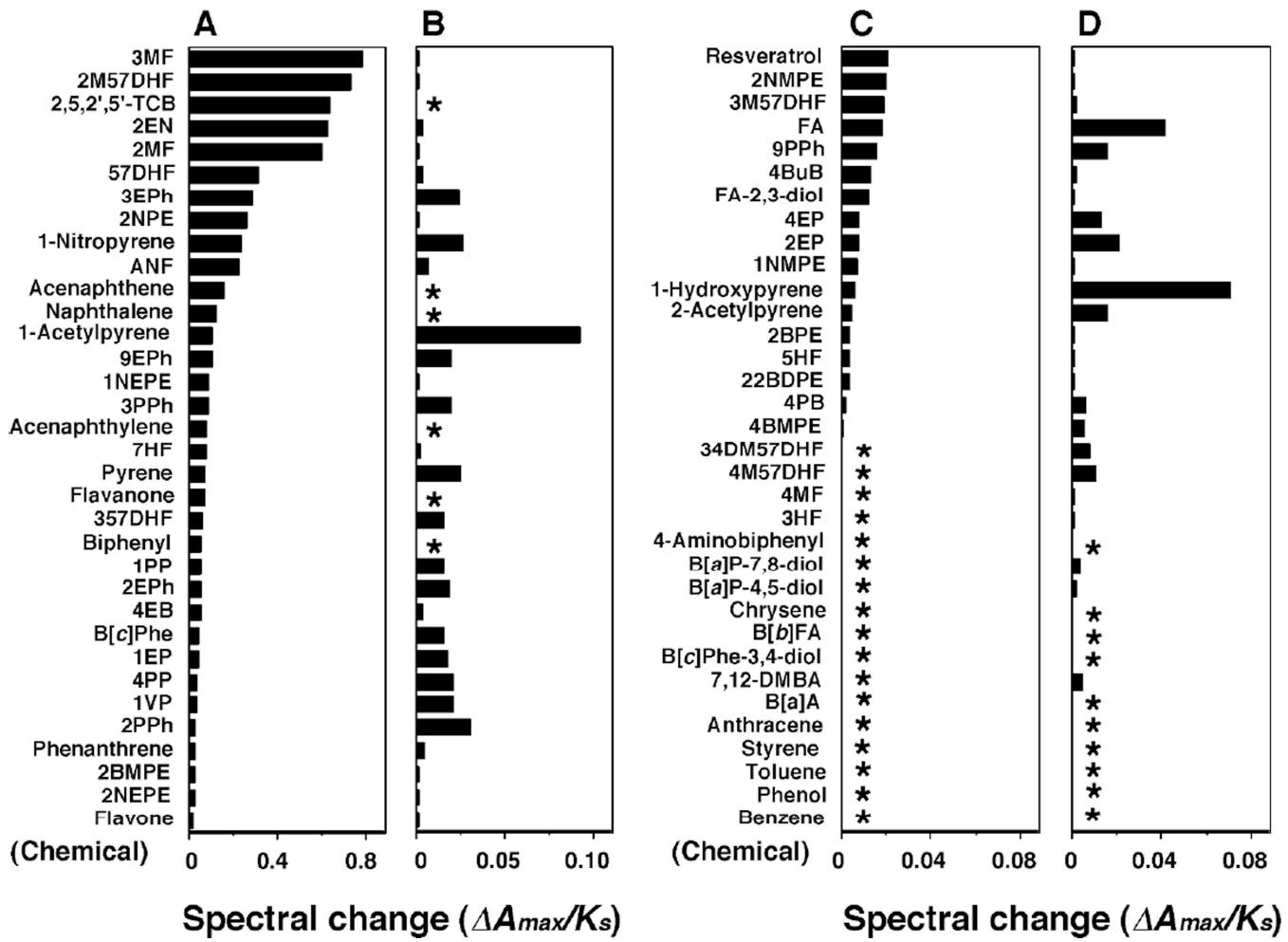


Figure 6.
Classification of compounds into Groups 1–8.

**Figure 7.**

Kinetics of disappearance of chemicals due to catalysis by P450 2A13. Chemicals analyzed were a, 2M57DHF; b, 1-acetylpyrene; c, 3EPh; d, 2NEPE; e, 2MF; f, flavone; g, flavanone; h, 7HF; i, 57DHF; j, 3MF; k, ANF; l, 1NEPE; m, 2BMPE; n, 9EPh; o, pyrene; p, B[c]Phe; q, 1NP; r, 3PPh; s, 2EPh; t, 2NMPE; u, 357THF; v, 3M57DHF; w, resveratrol; x, FA-2,3-diol; y, 9PPh; z, 4PP; A, 1VP; B, 1-hydroxypyrene; C, 2PPh; D, FA; E, 4Pbi; F, 2BPE; G, 4BMPE; H, 22BDPE; I, 5HF; J, B[c]Phe-3,4-diol. G1–G8 represent the Group numbers of chemicals (classification of Figure 6).

**Figure 8.**

Comparison of spectral intensities (A_{max}/K_s ratio) of Type I binding (A and C) and Reverse Type I binding (B and D) for P450s 2A13 and 1B1, respectively, measured with 68 chemicals. Of the 68 chemicals examined, 45 induced Type I spectra with P450 2A13 and reverse Type I spectra with P450 1B1. Asterisks indicate that no spectral changes were detected.

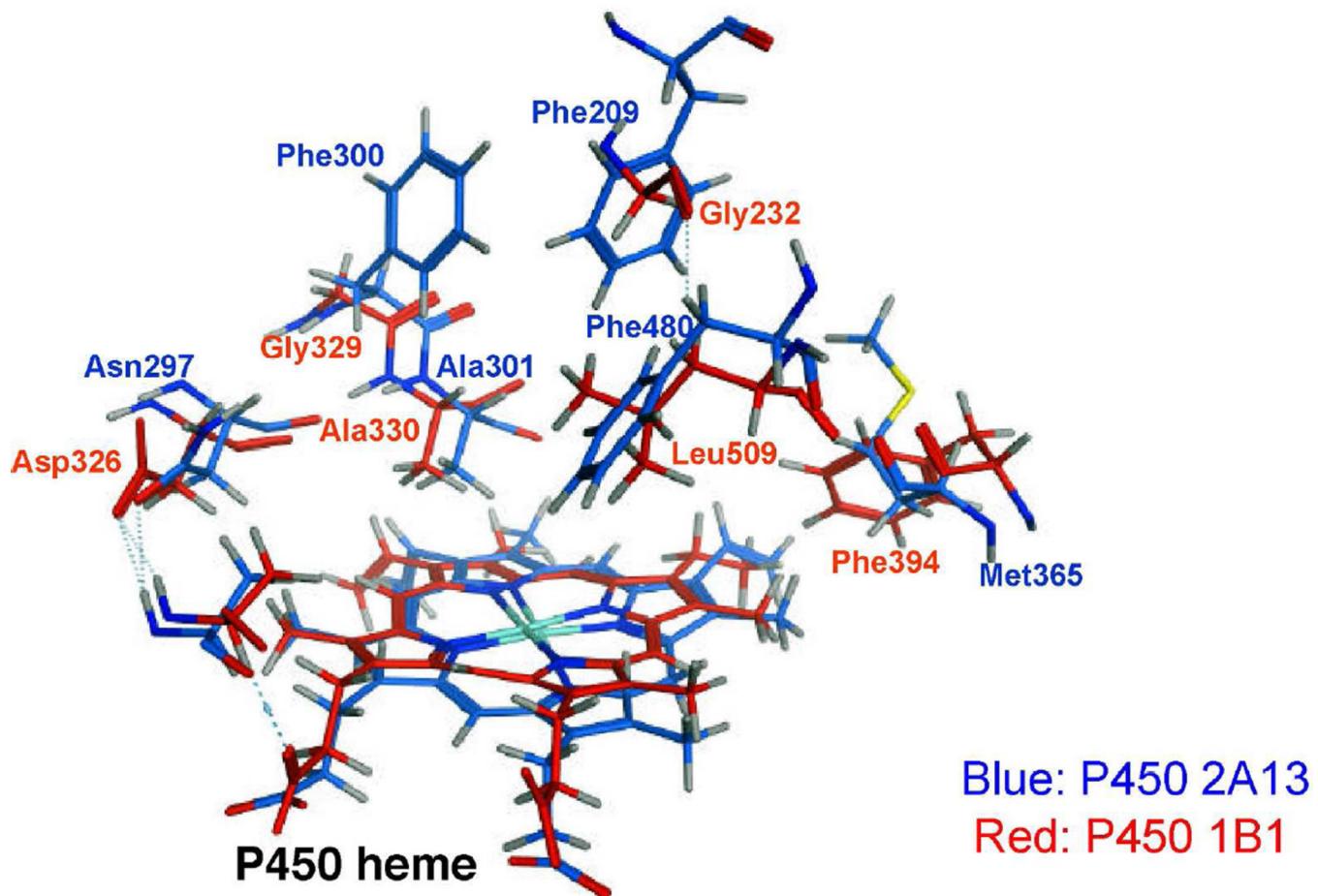


Figure 9.

Comparison of structures of active sites of P450s 2A13 and 1B1.

Table 1

Compounds that induced Type I binding spectra with P450 2A13 but not with P450 2A6

1-Acetylpyrene		7HF	
2-Acetylpyrene		1-Hydroxypyrene	
ANF		1-NP	
B[c]Phe		Pyrene	
57DHF		2,5,2',5'-TCB	
FA		357THF	
FA-2,3-diol			

Table 2

Classification of 68 chemicals into eight groups based on interaction with and inhibition of P450 2A13

Group 1 (K_s 0.4–3 μM and IC_{50} 0.4–10 μM):

acenaphthene, acenaphthylene, 1-acetylpyrene, 57DHF, 3EPh, flavanone, flavone, 7HF, 2M57DHF, 2MF, 2EN, 2-naphthalene propargyl ether (2NPE), 2-naphthalene ethyl propargyl ether (2NEPE),

Group 2 (for P450 2A13) (K_s 0.09–1.9 μM and IC_{50} 15–80 μM):

ANF, biphenyl, 2BMPE, 4Ebi, 9EPh, 3MF, naphthalene, 1NEPE, 1PP, pyrene

Group 3 (for P450 2A13) (K_s 0.06–1.6 μM and $\text{IC}_{50}>100 \mu\text{M}$):

B[c]Phe, 1EP, 2EPh, 3PPh, 1NP, 2,5,2',5'-TCB

Group 4 (P450 2A13) (K_s 2–7 μM and IC_{50} 30–90 μM):

4Bubi, 3M57DHF, 2-naphthalene methyl propargyl ether (2NMPE), phenanthrene, 357THF

Group 5 (P450 2A13) (K_s 3–7 μM and $\text{IC}_{50}>180 \mu\text{M}$):

FA, FA-2,3-diol, 1-hydroxypyrene, 4-(1-propynyl)pyrene (4PP), 1VP, 2PPh, 9PPh, resveratrol

Group 6 (P450 2A13) (K_s 10~20 μM and $\text{IC}_{50}<20 \mu\text{M}$):

2-acetylpyrene, 1-naphthalene methyl propargyl ether (1NMPE)

Group 7 (P450 2A13) ($K_s>10 \mu\text{M}$ and $\text{IC}_{50}>80 \mu\text{M}$):

2BPE, 2,2'-biphenyl dipropargyl ether (22BDPE), 4-biphenyl methyl propargyl ether (4BMPE), 2EP, 4EP, 5HF, 4Pbi

Group 8 (P450 2A13) ($K_s>100 \mu\text{M}$ and $\text{IC}_{50}>200 \mu\text{M}$):

4-ABP, anthracene, B[a]A, benzene, B[c]Phe-3,4-diol, B[b]FA, chrysene, B[a]P-4,5-diol, B[a]P-7,8-diol, 7,12-DMBA, 34DM57DHF, 3HF, 4MF, 4M57DHF, phenol, toluene, styrene