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Reverse Type I Binding Spectra of Human Cytochrome P450 1B1 Induced by Flavonoid, Stilbene, Pyrene, Naphthalene, Phenanthrene, and Biphenyl Derivatives That Inhibit Catalytic Activity: A Structure-Function Relationship Study

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Abstract

Fifty-one chemicals including derivatives of sixteen flavonoids, three stilbenes, six pyrenes, seven naphthalenes, seven phenanthrenes, ten biphenyls, 17 β -estradiol, and estrone were examined for their abilities to induce Reverse Type I binding spectra with human cytochrome P450 (P450) 1B1 and to inhibit 7-ethoxyresorufin *O*-deethylation (EROD) activities catalyzed by P450 1B1. Forty nine chemicals showed Reverse Type I spectra with P450 1B1 and we found that 3,5,7-trihydroxyflavone, 3',4'-dimethoxy-5,7-dihydroxyflavone, 4'-methoxy-5,7-dihydroxyflavone, α - and β -naphthoflavones, 2,4,3',5'-tetramethoxystilbene, pyrene, and several acetylenic pyrenes and phenanthrenes were strong inducers of the spectra and also potent inhibitors of EROD activities catalyzed by P450 1B1. Spectral dissociation constant (K_s) and the magnitude of the binding ($\Delta A_{max}/K_s$) of 49 chemicals were correlated with the inhibition potencies of EROD activities by these chemicals (correlation coefficients (r) of 0.72 and 0.74, respectively). The K_s and $\Delta A_{max}/K_s$ values were more correlated with IC₅₀ values when compared in a group of derivatives of flavonoids, stilbenes, and estrogens ($r=0.81$ and 0.88, respectively) or a group of derivatives of pyrenes, naphthalenes, phenanthrenes, and biphenyls ($r=0.88$ and 0.91, respectively). Among 14 flavonoids examined, 3,5,7-trihydroxyflavone and 4'-methoxy- and 3',4'-dimethoxy-5,7-dihydroxyflavone were more active than flavone in interacting with P450 1B1, but the respective 7,8-dihydroxyflavones were less active. Pyrene itself was highly active in interacting with P450 1B1, but its binding was slightly decreased when substituted with acetylenic groups. In contrast, substitution of naphthalene with methyl- and ethyl propargyl ethers led to more interaction with P450 1B1 than with naphthalene itself. Similarly, substitution on phenanthrene or biphenyl with acetylenic groups and propargyl ethers increased affinities to P450 1B1. These results suggest that the Reverse Type I binding of chemicals to P450 1B1 may determine how they interact with and inhibit the catalytic activity of the enzyme. Substitutions on the compounds with various

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acetylenic groups and propargyl ethers cause an increase or decrease of their affinities to P450 1B1, depending on the parent compound used.

Introduction

P450 comprises a superfamily of enzymes that catalyze oxidative metabolism of a variety of xenobiotic chemicals including drugs, toxic chemicals, and chemical carcinogens as well as endogenous compounds such as steroids, fatty acids, prostaglandins, and lipid-soluble vitamins (1). P450 1B1 has been shown to be expressed in extrahepatic organs and has important roles in the metabolic activation of polycyclic aromatic hydrocarbons (PAHs)¹ and heterocyclic aromatic amines to active metabolites that attack DNA in the cell (2,3), as well as estrogens and possibly other endogenous compounds. Several lines of evidence have suggested that the levels of expression and catalytic roles of polymorphic P450 1B1 are related to the susceptibilities of individuals to chemical carcinogenesis in humans (4-6). Studies have also indicated that chemical inhibitors of P450 1B1 are able to decrease the incidence of cancers in experimental animals when potent carcinogens, such as PAHs, are simultaneously administered (7-10).

Chemical inhibitors of P450 1B1 have been identified in many laboratories; these include flavonoids, stilbenes, synthetic organoselenium compounds such as 1,2-, 1,3-, and 1,4-phenylenebis(methylene)selenocyanate, synthetic acetylenic PAHs such as 1-ethynylpyrene (1EP), 2-ethynylpyrene (2EP), 1-(1-propynyl)pyrene (1PP), 2-ethynylphenanthrene (2EPh), 2-(1-propynyl)phenanthrene (2PPh), and 4-(1-propynyl)biphenyl (4PB) (7,11-15). It has also been shown that many PAHs, e.g. benzo[*a*]pyrene, benz[*a*]anthracene, and 5-methylchrysene, are also potent inhibitors of human P450 1A1, 1A2, and 1B1 and that the IC₅₀ values obtained with these PAHs in inhibiting P450 activities are similar to those of known potent inhibitors, such as several synthetic acetylenic PAHs and α -naphthoflavone (ANF) (16). These results are of interest because humans are exposed to mixtures of environmental PAHs through foods and the atmosphere, and these PAHs have activities both as carcinogens (through metabolism by P450s) and as chemoprevention agents by inhibiting P450s themselves (9, 10, 17, 18). Different mechanisms have been suggested to involve in inhibition of P450 1A1, 1A2, and 1B1 by various chemicals (19). For example, 1EP and 1PP inhibit P450 1A1 in a mechanism-based manner while they directly inhibit P450 1A2 and 1B1. Similarly, several carcinogenic PAHs (e.g. benzo[*a*]pyrene, benz[*a*]anthracene, and 5-methylchrysene) are converted by P450 1A2 to active metabolites that inhibit the enzyme, but these PAHs directly inhibit P450 1A1 and 1A2 (19). Our recent studies have also suggested that there are different binding sites in the P450 1B1 molecule for a variety of PAHs, acetylenic PAHs and biphenyls as judged by spectral interactions, and the quenching of P450 1B1-derived and inhibitor-based fluorescence (20). Molecular docking studies suggest differences in the interaction of selected PAH inhibitors (e.g. benzo[*a*]pyrene, 1PP,

¹Abbreviations: ANF, α -naphthoflavone; 22BDPE, 2,2-biphenyl dipropargyl ether; 44BDPE, 4,4-biphenyl dipropargyl ether; 2BMPE, 2-biphenyl methyl propargyl ether; 4BMPE, 4-biphenyl methyl propargyl ether; BNF, β -naphthoflavone; 2BPE, 2-biphenyl propargyl ether; 4BPE, 4-biphenyl propargyl ether; 4BuB, 4-butynylbiphenyl; 34DM57DHF, 3',4'-dimethoxy-5,7-dihydroxyflavone; 34DM78DHF, 3',4'-dimethoxy-7,8-dihydroxyflavone; 34DMF, 3',4'-dimethoxyflavone; E1, estrone; E2, 17 β -estradiol; 4EB, 4-ethynylbiphenyl; 2EN, 2-ethynylphenanthrene; 1EP, 1-ethynylpyrene; 2EP, 2-ethynylpyrene; 4EP, 4-ethynylpyrene; 2EPh, 2-ethynylphenanthrene; 3EPh, 3-ethynylphenanthrene; 9EPh, 9-ethynylphenanthrene; EROD, 7-ethoxyresorufin *O*-deethylation; 2M57DHF, 2'-methoxy-5,7-dihydroxyflavone; 3M57DHF, 3'-methoxy-5,7-dihydroxyflavone; 4M57DHF, 4'-methoxy-5,7-dihydroxyflavone; 2M78DHF, 2'-methoxy-7,8-dihydroxyflavone; 3M78DHF, 3'-methoxy-7,8-dihydroxyflavone; 4M78DHF, 4'-methoxy-7,8-dihydroxyflavone; 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; 2NPE, 2-naphthalene propargyl ether; PAHs, polycyclic aromatic hydrocarbons; 4PB, 4-(1-propynyl)biphenyl; Ph, phenanthrene; 1PP, 1-(1-propynyl)pyrene; 2PPh, 2-(1-propynyl)phenanthrene; 3PPh, 3-(1-propynyl)phenanthrene; 9PPh, 9-(1-propynyl)phenanthrene; 357THF, 3,5,7-trihydroxyflavone; TMS, 2,4,3',5'-tetramethoxystilbene; 1VP, 1-vinylpyrene.

and 3-(1-propynyl)phenanthrene (3PPh) with the active sites of P450 1B1 and P450 1A2, and these results are consistent with these P450 enzymes forming different metabolites of these PAH inhibitors, thus yielding different mechanisms for inhibition of these P450 enzymes (20).

In this study, we further examined structure-function relationships of the interaction of human P450 1B1 with 51 chemicals including sixteen flavonoids, three stilbenes, six pyrenes, seven naphthalenes, seven phenanthrenes, ten biphenyls, 17 β -estradiol (E2), and estrone (E1) that have been reported to be substrates or inhibitors of P450 1B1 (7, 11). We measured the spectral interaction of chemicals with P450 1B1 and the inhibition of P450 1B1-dependent 7-ethoxyresorufin *O*-deethylation (EROD) activity by these chemical inhibitors. Of the 51 chemicals examined, 49 were found to produce Reverse Type I binding spectra with human P450 1B1 and the spectral interaction was found to correlate significantly with inhibitory potencies of P450 1B1-dependent EROD activity. We conclude that the interaction of P450 1B1 with inhibitors can be ascribed in terms of a generally common process of interaction to change the spin state of the iron in the protein, presumably by favoring the liganding of water to the distal site of the heme.

Experimental Procedures

Chemicals

Chemical structures of 51 chemicals used in this study are presented in Figures 1 and 2. Flavone, stilbenes, estrone (E1), 17 β -estradiol (E2), testosterone, pyrene, naphthalene, phenanthrene (Ph), biphenyl, 7-ethoxyresorufin, and resorufin were obtained from Sigma Chemical Co. (St. Louis, MO) or Kanto Kagaku Co. (Tokyo). Thirteen methoxylated flavonoids, with or without dihydroxy substitution at 5,7- and 7,8-positions, were synthesized and the methods for the synthesis of these chemicals have been described (22). Acetylenic pyrenes and phenanthrenes and derivatives of naphthalenes and biphenyls were also synthesized as previously described (21,23-26). Other chemicals and reagents used in this study were obtained from the sources described previously or were of the highest quality commercially available (16, 19, 20).

Enzymes

Bacterial "bicistronic" P450 1A1, 1A2, and 1B1 systems were prepared as described (16, 19). To facilitate expression and purification of P450s, six histidine residues were introduced at the position before the termination codon (C-terminal) (7). The plasmids for the expression of P450s 1A1, 1A2, or 1B1 plus human NADPH-P450 reductase were introduced into *Escherichia coli* DH5 α cells by a heat shock procedure. Bacterial membranes were prepared and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) (20).

For purification of P450 enzymes, the bacterial membranes were solubilized in 0.10 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (v/v), 0.5 M NaCl, 10 mM β -mercaptoethanol, 0.5% sodium cholate (w/v), 1% Triton N-101 (w/v), and 30 μ M ANF. The solubilized membranes were centrifuged ($10^5 \times g$, 60 min), the supernatant was applied to a nickel-nitrilotriacetic acid column (Qiagen), and the P450 proteins were purified by the method described by Chun *et al.*(7).

Enzyme Assays

EROD assays were done at 37 $^{\circ}$ C in standard reaction mixtures (1.5 mL) containing recombinant P450 1B1 (3.3 nM), P450 1A1 (5.0 nM), or P450 1A2 (100 nM), NADPH (0.83 μ M), and 7-ethoxyresorufin (4.0 μ M). Reactions were started by adding NADPH, and

product (resorufin) formation was monitored directly in a Hitachi F-4500 spectrofluorometer using an excitation wavelength of 571 nm and an emission wavelength of 585 nm (20). All of the PAH inhibitors and 7-ethoxyresorufin were dissolved in $(\text{CH}_3)_2\text{SO}$ and added directly to the incubation mixtures; the final concentration of organic solvent in the mixture was <0.4% (v/v).

The 50% inhibition concentration (IC_{50}) of EROD activities of P450 1B1, 1A1, and 1A2 were determined in a standard incubation mixture consisted of P450 enzymes, chemical inhibitors, and 7-ethoxyresorufin ($2.5 \mu\text{M}$) in a final volume of 0.5 mL of 100 mM potassium phosphate buffer (pH 7.4) and an NADPH-generating system (0.5 mM NADP^+ , 5 mM glucose 6-phosphate, and 0.5 unit of yeast glucose 6-phosphate dehydrogenase/mL) (16). Formation of resorufin from 7-ethoxyresorufin was determined fluorimetrically as described (20).

Spectral Binding Titrations

Purified P450 1B1 was diluted to $1.0 \mu\text{M}$ 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 chemical inhibitors in a Jasco V-550 spectrophotometer (Figure 3). Chemical inhibitors were also added in a buffer solution and the spectra were determined as described above. The differences in absorbance between 350 nm and 500 nm were recorded as a function of substrate concentration (Figures 3A, 3D, and 3G). The P450 spectra (with or without inhibitors) were obtained by subtracting the blank spectra (in the absence of P450) from the P450 spectra (Figures 3B, 3E, and 3H). Difference spectra for the interaction of chemicals with P450 1B1 were obtained (Figures 3C, 3F, and 3I). Spectral dissociation constants (K_s) and the magnitude of spectral binding ($\Delta A_{max}/K_s$) were estimated using nonlinear regression analysis with GraphPad Prism software (GraphPad Software, La Jolla, CA).

Other Assays

P450 and protein concentrations were estimated by the methods described previously (27, 28).

Results

Inhibition of P450 1A1-, 1A2-, and 1B1-Dependent EROD Activity by Flavonoids and other Inhibitors

We first determined the effects of flavonoids and other inhibitors on inhibition of EROD activities catalyzed by P450 1A1, 1A2, and 1B1 coexpressing NADPH-P450 reductase in membranes of *E. coli* (Figure 4). Among 10 flavonoids examined, 357THF was the most potent in inhibiting EROD activities by these three P450 enzymes and P450 1B1 was found to be more susceptible than P450 1A1 and 1A2. 3',4'-Dimethoxy-5,7-dihydroxyflavone (34DM57DHF) and 4'-methoxy-7,8-dihydroxyflavone (4M57DHF) were also potent inhibitors of P450 enzymes, particularly for P450 1B1. Pyrene and phenanthrene inhibited P450 1A2 and 1B1 more strongly than P450 1A1. Naphthalene was weak in inhibiting P450 1A1 and 1A2 but inhibited P450 1B1 with an IC_{50} of $\sim 26 \mu\text{M}$ (Figure 4). As expected, ANF and β -naphthoflavone (BNF) were potent inhibitors of these P450s, particularly for P450 1A2 and 1B1. Because these results collectively indicated that P450 1B1 was more inhibited by these selected chemicals, we focused the study to P450 1B1 for the further analysis with 51 chemicals.

Reverse Type I Spectral Changes of Human P450 1B1 Produced by Chemical Inhibitors

P450 1B1 had a wavelength maximum at ~392 nm which changed to ~414 nm upon the addition of 2NPE, 34DMF, or 4MF (Figs. 3A, 3D, and 3G). Because these chemicals had absorbance in the near-UV region, we calculated (subtracted) the absorbance due to the chemicals from the spectra of P450 1B1 interacted with the chemicals. 2-Naphthalene propargyl ether (2NPE) had weak spectral absorbance between 350 nm and 500 nm (Figure 3A) and did not cause significant interference in the absolute and difference spectra of P450 1B1 (Figure 3B and 3C, respectively). However, both 3',4'-dimethoxyflavone (34DMF) and 4'-methoxyflavone (4MF) had significant absorbance in the near-UV region (Figures 3D and 3G) and interfered in the analysis of the spectral interaction of inhibitors with P450 1B1 (Figures 3E, 3F, 3H, and 3I). Particularly with 4MF (at high concentrations), the P450 difference spectra did not show a clear trough at ~386-388 nm in the difference spectra (Figure 3I). We then compared the ratio of absorbance at 414 and 392 nm in the absolute spectra (cf. Figures 3B, 3E, and 3H) and the absorbance difference between 416 nm and 386 nm in the difference spectra (cf. Figures 3C, 3F, and 3I) for 12 chemicals (with P450 1B1) and found that there were good correlation between the data from the absolute and the difference spectra (Fig. 5). Therefore, we calculated the magnitude of spectral interaction of chemicals with P450 1B1 from either the absolute or difference spectral data.

Reverse Type I Binding Spectra and Inhibition of EROD Activity of P450 1B1 with Chemical Inhibitors

We analyzed sixteen flavonoids, three stilbenes, E1, and E2 for their abilities to induce Reverse Type I spectra and to inhibit EROD activities with P450 1B1 (Table 1 and Fig. 6). In our previous account we have obtained evidence that these chemicals directly inhibit EROD activities of P450 1B1, not in a mechanism-based manner (19,20). Among the 21 chemicals (Table 1), only 2M78DHF did not show any detectable spectral interaction with P450 1B1, even at 100 μ M. 2M78DHF was also found to be less active in inhibiting EROD (IC_{50} of >100 μ M). Flavone produced Reverse Type I spectra with P450 1B1 (K_s of 43 μ M and ΔA_{max} 0.026) and inhibited P450 1B1-dependent EROD activity (IC_{50} 0.15 μ M) (Table 1). 357THF, 4M57DHF, 34DM57DHF, and 3M57DHF were more active than flavone in interacting with and inhibiting P450 1B1, but 3M78DHF, 4M78DHF, and 34DM78DHF were not as potent as flavone. Among the three stilbenes examined, TMS was the most potent in inducing a Reverse Type I binding spectrum and inhibiting EROD activity, as compared with resveratrol and picetannol. We compared the apparent K_s values or the $\Delta A_{max}/K_s$ values with IC_{50} values for EROD inhibition with these 20 chemicals (except for 2M78DHF) and found that these correlated well, $r=0.81$ and 0.88 respectively (Fig. 6).

We also compared the spectral changes and inhibition of EROD activities with six pyrenes, seven naphthalenes, seven phenanthrenes, and ten biphenyls (Table 2 and Figure 7). It should be mentioned that these chemicals directly inhibited EROD activities of P450 1B1 except that 4PB inhibited P450 1B1 in a mechanism-based manner (results not presented). However, 4PB induced spectral changes with P450 1B1, indicating that this chemical inhibits by both a direct and in a mechanism-based manner. Among 30 chemicals examined, only naphthalene did not show any measurable spectral interaction with P450 1B1 (even at 100 μ M). Pyrene induced Reverse Type I binding spectrum, with a K_s of 1.3 μ M, and inhibited P450 1B1-EROD activity (IC_{50} 0.025 μ M); both of these values were the lowest among the pyrene derivatives examined. Naphthalene did not produce spectral changes with P450 1B1 and weakly inhibited P450 1B1-EROD activities (IC_{50} 26 μ M). The other six derivatives of naphthalene produced Reverse Type I spectra with K_s values of ~100 μ M (IC_{50} values of 1-5 μ M), except that K_s value of 2EN was 15 μ M. The K_s and IC_{50} values with phenanthrene were 14 μ M and 0.51 μ M, respectively, and other phenanthrene derivatives showed slightly lower K_s and IC_{50} values than phenanthrene. Biphenyl yielded a

K_s value of 140 μM and IC_{50} of 19 μM , and the acetylenic biphenyls were found to be more active in interacting with P450 1B1 in both spectral changes and EROD inhibition. Three propargyl ethers of biphenyl—4BMPE, 22BDPE, and 44BDPE—interacted with P450 1B1 more strongly than biphenyl itself. The other propargyl ether derivatives of biphenyl—2BPE, 4BPE, 2BMPE—were not as potent in their interactions with P450 1B1.

Correlation between K_s or $\Delta A_{\text{max}}/K_s$ values (for Reverse Type I spectra) and IC_{50} values for P450 1B1-dependent EROD inhibition were compared for a combined set of 6 pyrenes, 6 naphthalenes (except naphthalene), 7 phenanthrenes, and 10 biphenyls and the correlation coefficients (r) were 0.88 and 0.91, respectively (Figure 7).

We also calculated the correlation coefficients between spectral data and IC_{50} values in several groups of chemical inhibitors determined in this study (Table 3). When the IC_{50} values for EROD activity were compared with K_s or $\Delta A_{\text{max}}/K_s$ values for Reverse Type I binding spectra with a total of 49 chemicals, the correlation coefficients (r) were 0.72 and 0.74, respectively. However, these correlations became higher when these values were calculated in a set of flavonoids and stilbenes ($r=0.81$ and 0.86 , respectively in $n=16$), a set of biphenyls ($r=0.75$ and 0.89 , respectively in $n=10$), or a group of phenanthrenes ($r=0.80$ and 0.77 , respectively, $n=7$). The relationships in sets of pyrenes and naphthalenes were not so high, except that IC_{50} and K_s values were well correlated in a group of pyrenes ($r=0.78$). In all cases, the correlation coefficients between ΔA_{max} and IC_{50} values were not so high.

Discussion

Chemical inhibitors (as well as substrates) for P450 enzymes have been shown to induce spectral changes with oxidized state of the enzymes and at least three types of spectral changes— Type I, Type II, and Reverse Type I—are found upon binding of different chemicals with various forms of P450s (29,30). Spectral dissociation constants (K_s) and magnitudes of spectral binding ($\Delta A_{\text{max}}/K_s$) have been used as parameters for the affinity and potencies of chemicals to interact with P450 enzymes (31). Of 51 chemicals examined in this study, 49 were found to induce Reverse Type I binding spectra in interacting with human P450 1B1; only naphthalene and 2M78DHF did not show any spectral interaction, even at 100 μM . Our studies showed that all of the 49 chemicals caused almost the same spectral changes with P450 1B1, so-called Reverse Type I spectra or “Modified Type II” spectra (30). It should be noted that some of these compounds themselves had absorbance in the near-UV region, and these spectra sometimes caused interference in calculating the magnitude of the absorbance change in the difference spectra (Figure 3). Since good correlation was noted between the ratio of 392 nm versus 414 nm in the absolute spectra and the absorbance difference in 416 nm and 386 nm in the difference spectra, we determined the magnitude of interaction using either the results from absolute spectra or from difference spectra (Figure 4).

Previously, the chemicals that produce Reverse Type I spectra with P450 enzymes in several animal species have been suggested to have hydroxyl groups in the molecule and to ligand to the heme iron of the cytochrome (29-32). Our present results showed that several of the chemicals, such as hydroxylated flavones, stilbenes, and estrogens, contain hydroxyl groups in the molecule, however, there are many exceptions in that flavone and several flavonoids, pyrene, phenanthrene, biphenyl, and many other compounds do not have hydroxyl groups but have the ability to induce Reverse Type I binding spectra with P450 1B1. A more likely explanation for the ligand-induced conversion of high- to low-spin iron is that the ligands bind and alter the active site pocket of the P450 (1B1) to admit water as the distal ligand, in a similar way that the introduction of ligands into P450 3A4 active site causes the protein to release bound distal ligand waters (32).

Methoxylated flavones have been reported to be more resistant to metabolism by xenobiotic-metabolizing enzymes (e.g. UDP-glucuronyltransferases and sulfotransferases) than the unmethoxylated ones and thus accumulate in tissues and increase their biological potencies, including chemoprevention activities (15,34-37). Our results showed that (di)hydroxylation of flavones at the 5- and 7-positions of 3'-methoxy-, 4'-methoxy-, and 3',4'-dimethoxy-flavones was found to stimulate both the magnitude of spectral binding and the potencies of EROD inhibition, but (di)hydroxylation at the 7- and 8-positions of the respective flavones decreased their affinities to P450 1B1 as compared with those of the parent flavone. Potent inhibition of human P450 1B1 by 5,7-dihydroxylated flavonoids has recently been reported by Kim *et al.* (38) using 5,7-dihydroxyflavones, 5,7-dihydroxyflavones, 4'-hydroxy-5,7-dihydroxyflavone, and 3',4'-dihydroxy-5,7-dihydroxyflavone. These results suggest that hydroxylation position affects the interaction with P450 1B1, but that hydroxylation is not critical in determining the magnitude of the Reverse Type I binding spectra of chemicals with P450s.

Addition of any of several acetylenic groups to the pyrene molecule slightly increased both K_s (2.7~4.4 μM) and IC_{50} values (0.036-0.17 μM). It is not known at present whether or not acetylene moieties attached to pyrene affect affinities toward P450 1B1; however, our previous studies of molecular docking of human P450 1B1 with acetylenic PAH inhibitors have suggested that the aromatic positions of 1PP and 3PPh dock with the P450 1B1 heme, resulting in the formation of oxidative products at aromatic position that lose ability to inhibit P450 1B1 (20). In this regard, Sohl *et al.* (39) showed that pyrene binds to rabbit P450 1A2 and induces Type I binding spectra with a K_s value of 0.036 μM and that this value is increased to 0.2 μM for 1-hydroxypyrene.

2EN, 1NMPE, 1NEPE, 2NPE, 2NMPE, and 2NEPE were all found to induce Reverse Type I spectra with K_s values of 15-110 μM and inhibited EROD activities with IC_{50} values of 0.92-5.2 μM , although naphthalene itself did not show any spectra with P450 1B1, even at a 100 μM concentration, and weakly inhibited EROD activity catalyzed by P450 1B1. Similarly, substitution of phenanthrene with acetylenic groups increased the affinity towards P450 1B1, in terms of Reverse Type I binding spectra and inhibition of P450 1B1-dependent EROD activity. Biphenyl was also not very potent in producing Reverse Type I binding spectra or inhibiting catalytic activity, but substitution with either a 4-ethynyl-, 4-(1-propynyl)-, or 4-butynyl group caused an increase in affinity towards P450 1B1 (K_s values of 7.6, 12, and 54 μM , respectively). Substitution on biphenyl with mono- and di-propargyl ether also increased affinity toward P450 1B1. These results suggest that the substituted naphthalenes, phenanthrenes, and biphenyls—but not pyrenes—fit better into the pocket of P450 1B1 and thus significantly inhibit P450 1B1 catalytic activity.

Chun *et al.* (7) reported that TMS induces Reverse Type I binding spectra with P450 1B1 and inhibits P450 1B1-dependent EROD activity. In this work we found that resveratrol and piceatannol, as well as TMS, induced Reverse Type I binding spectra and inhibited EROD activity, although the former two polyphenols were less active than TMS in interacting with P450 1B1. These three polyphenols have been reported to have chemopreventive activity and several mechanisms for inhibition of cell growth have been presented (40,41). Interestingly, Potter *et al.* (42) have shown that resveratrol is converted by human P450 1B1 to piceatannol, which shows anti-leukemic activity and inhibits tyrosine kinase activity (43).

In conclusion, our current studies showed that 49 of 51 chemicals induced Reverse Type I binding spectra with human P450 1B1 and the magnitude of spectral binding (K_s and $\Delta A_{max}/K_s$) were apparently related to the inhibition potencies (IC_{50} values) of these chemicals against P450 1B1. It was found that substitution of pyrene with acetylenic groups decreased the affinities to P450 1B1 while substitution of naphthalene and phenanthrene with

acetylenic groups or propargyl ethers increased the affinity for P450 1B1. Substitution of biphenyl with these groups also increased affinity towards P450 1B1. In cases of flavonoids, 5,7-dihydroxy substituted flavonoids showed more interaction with P450 1B1 while 7,8-dihydroxylation decreased the affinity to P450 1B1. Collectively our current studies showed, for the first time, that interactions of chemicals with P450 1B1 (as determined by Reverse Type I binding spectra) may determine how these chemicals inhibit catalytic activity of P450 1B1 and that substitution on these particular chemical scaffolds sometimes affect their affinities to P450 1B1. The interaction of P450 1B1 with inhibitors can be ascribed in terms of a generally common process of interaction to change the spin state of the iron in the protein, presumably by favoring the liganding of water to the distal site of the heme. Our present results on structure–function relationships of these chemicals with P450 1B1 may be of use for further studies on molecular mechanisms of substrate and inhibitor binding at P450 1B1 active sites.

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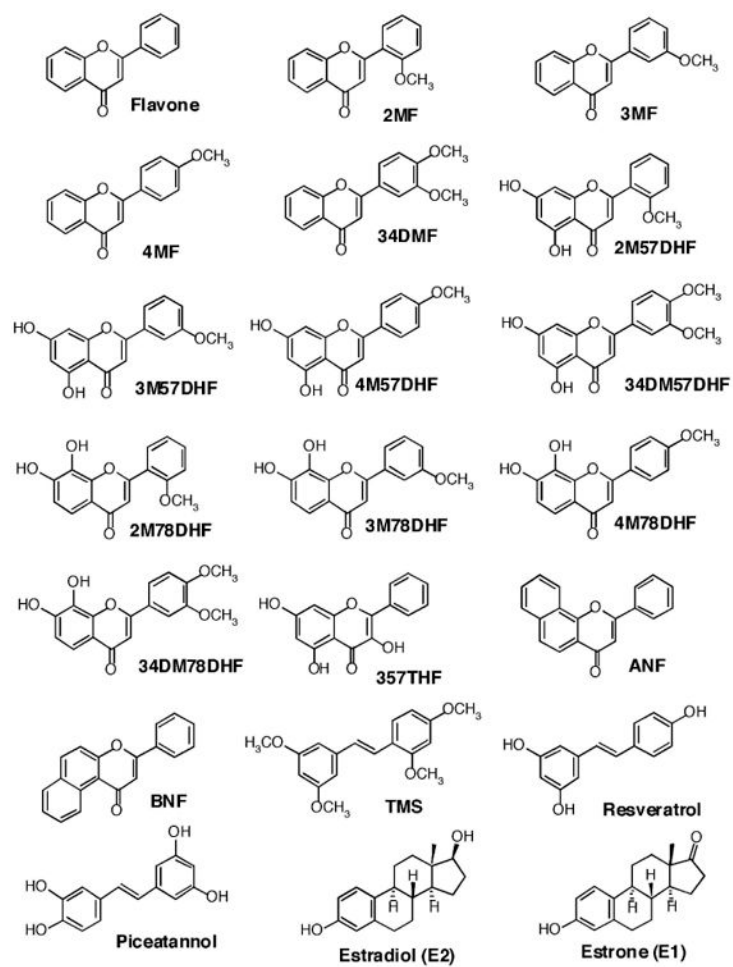


Figure 1.
Structures of flavonoids, isoflavones, benzoflavones, and estrogens.

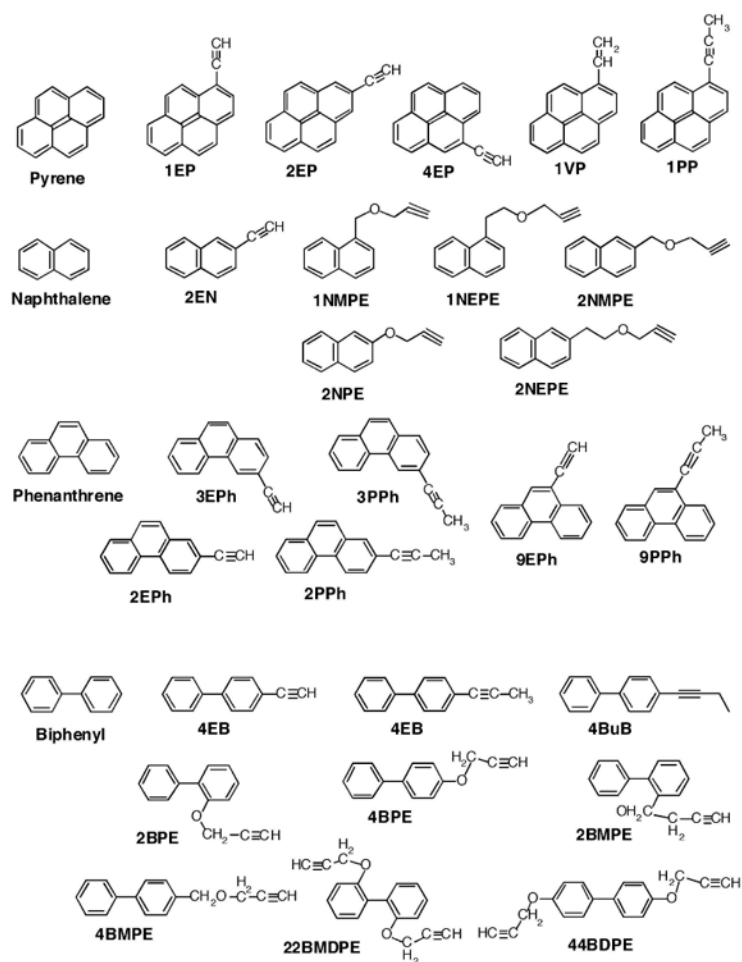


Figure 2.
Structures of derivatives of pyrenes, naphthalenes, phenanthrenes, and biphenyls.

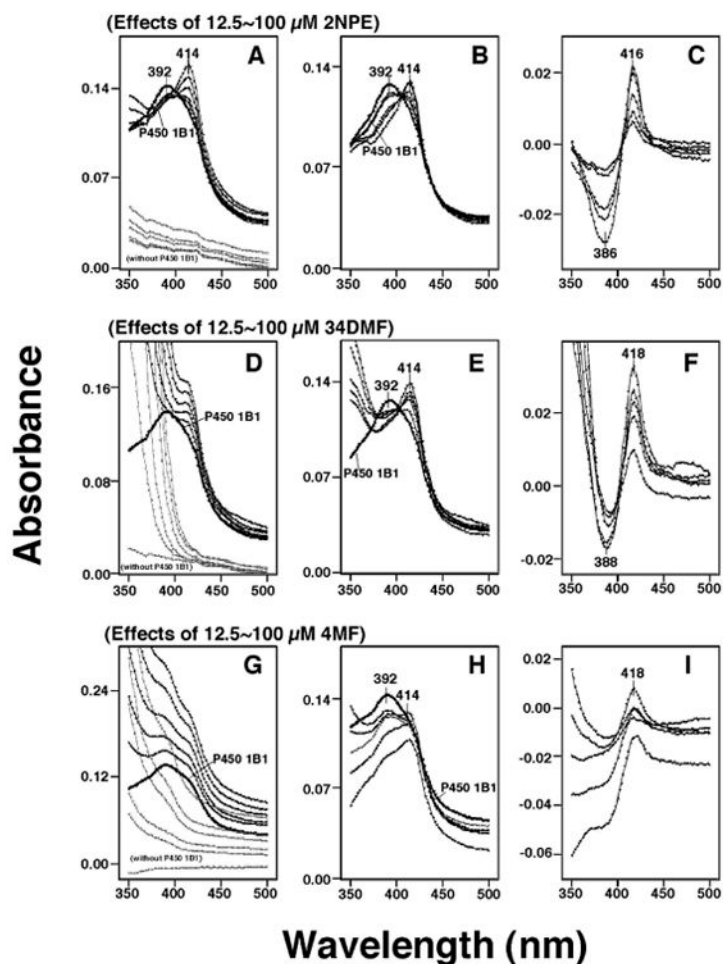


Figure 3. Spectral interactions of 2NPE (A, B, and C), 34DMF (D, E, and F), and 4MF (G, H, and I). Chemicals (at concentrations of 12.5, 25, 50, 75, and 100 μM) were added to the buffer, with or without 1 μM P450 1B1, and the spectra were recorded between 350 nm and 500 nm (A, D, and G). P450 spectra were obtained by subtracting the blank spectra (in the presence of chemicals and in the absence of P450 1B1) from the P450 spectra (containing 0-100 μM chemicals and P450 1B1) (B, E, and H). The difference spectra, showing the interaction of chemicals with P450 1B1 were also recorded (C, F, and I).

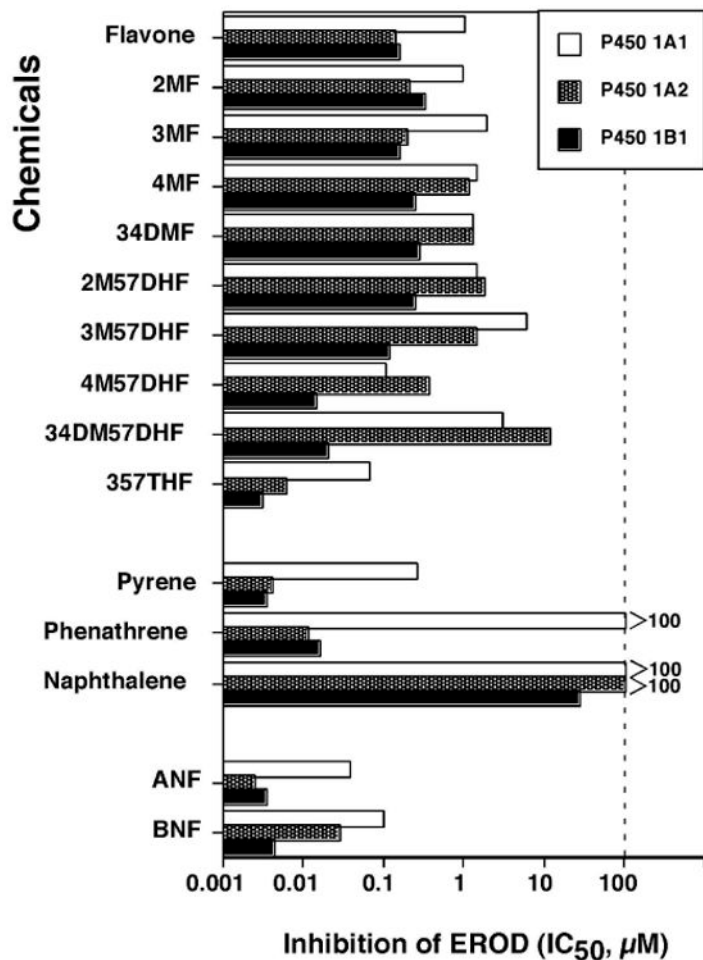


Figure 4.

Effects of flavone and nine flavonoids, pyrene, phenanthrene, naphthalene, ANF and BNF on EROD activities catalyzed by P450 1A1 (□), P450 1A2 (shaded column, ▨), and P450 1B1 (■). The data presented are mean IC_{50} values ($n=3$) and the standard deviations in these cases were less than 15% of the values. Control activities for EROD by P450 1A1, 1A2, and 1B1 were 45 ± 5 , 6.5 ± 0.8 , and 26 ± 4 nmol/min/nmol P450, respectively.

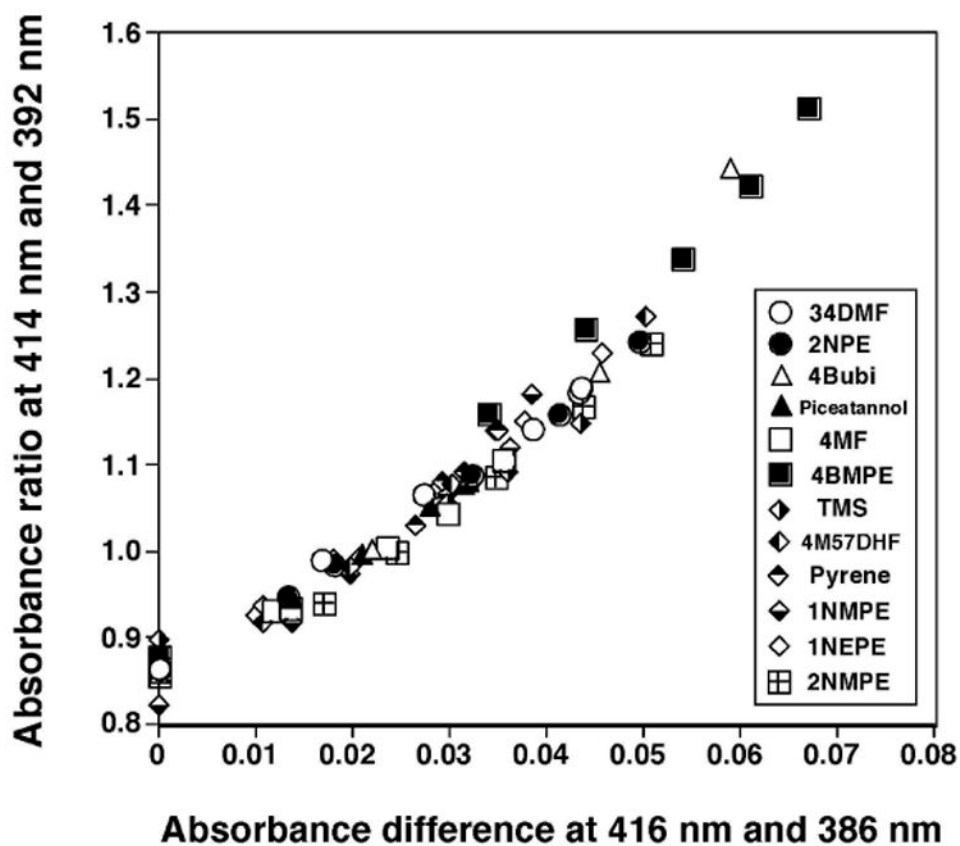


Figure 5. Correlation between the peak ratio of 414 nm vs 392 nm in the absolute spectra of P450 and the absorbance difference in 416 nm and 386 nm in the difference spectra for 12 chemical inhibitors. Methods for determination of the spectra are presented in Experimental Procedures. Concentrations of chemicals used were 12.5-100 μ M except for TMS, 4M57DHF, and pyrene, for which concentrations of 1.25-10 μ M were used.

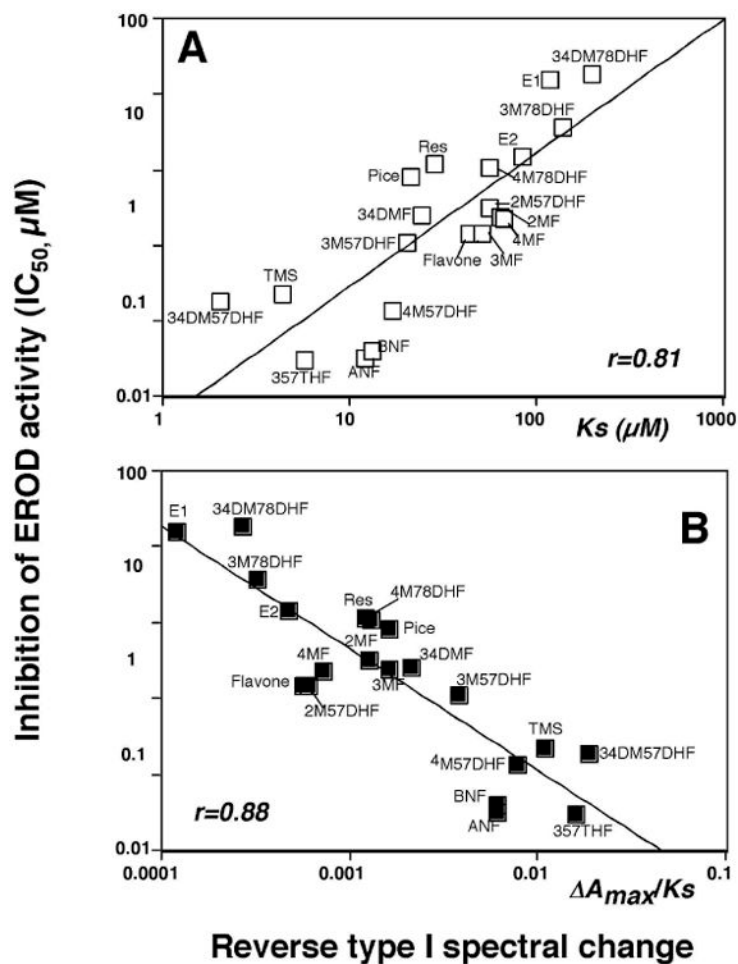


Figure 6. Relationship between IC_{50} values and K_s values (A) or $\Delta A_{max}/K_s$ (B) in the interactions of flavone and flavonoids, isoflavones, E1, and E2 with P450 1B1.

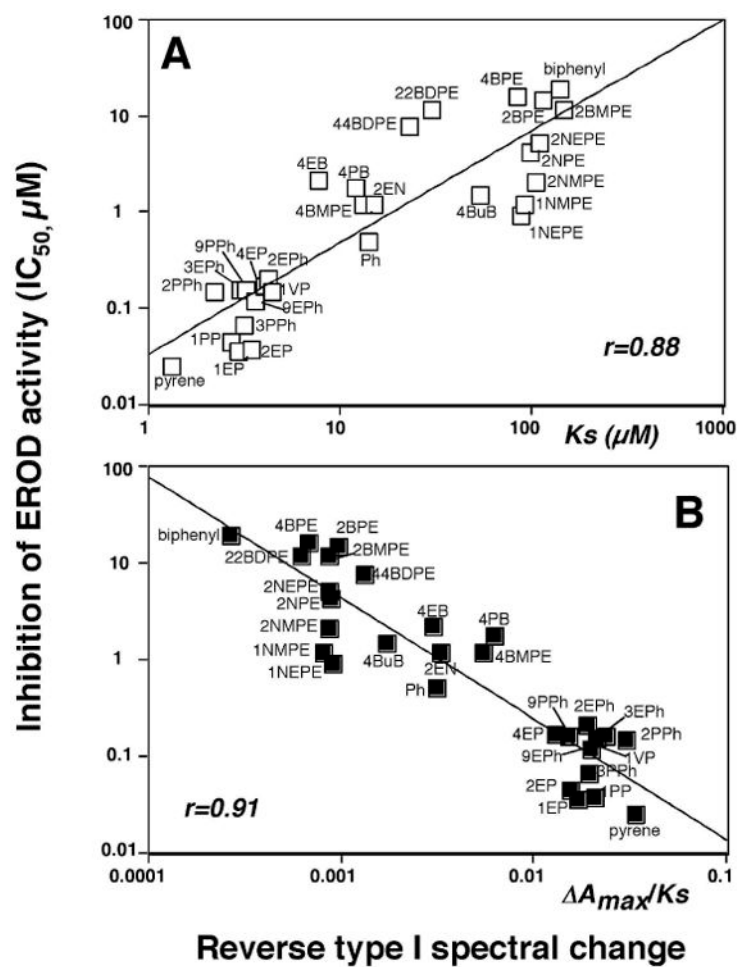


Figure 7. Relationship between IC_{50} values and K_s values (A) or $\Delta A_{max}/K_s$ (B) in the interaction of derivatives of pyrenes, naphthalenes, phenanthrenes, and biphenyls with P450 1B1.

Table 1
Reverse Type I spectral changes and inhibition of EROD activities of human P450 1B1 with derivatives of flavonoids, stilbenes, and estrogens

chemicals	Reverse Type I spectra			inhibition of EROD
	K_s (μM)	ΔA_{max}	$\Delta A_{\text{max}}/K_s$	IC_{50} (μM)
flavone	43 \pm 4.7	0.026 \pm 0.002	0.0006	0.15 \pm 0.02
2MF	56 \pm 8.0	0.070 \pm 0.005	0.0013	0.32 \pm 0.06
3MF	51 \pm 4.1	0.029 \pm 0.001	0.0006	0.15 \pm 0.02
4MF	66 \pm 11	0.048 \pm 0.005	0.0007	0.23 \pm 0.03
34DMF	24 \pm 3.2	0.051 \pm 0.003	0.0021	0.26 \pm 0.03
2M57DHF	64 \pm 10	0.100 \pm 0.011	0.0016	0.24 \pm 0.02
3M57DHF	20 \pm 3.0	0.075 \pm 0.008	0.0037	0.11 \pm 0.02
4M57DHF	17 \pm 0.7	0.130 \pm 0.004	0.0078	0.014 \pm 0.002
34DM57DHF	2.0 \pm 0.3	0.037 \pm 0.002	0.0180	0.019 \pm 0.003
2M78DHF	ND	ND	ND	>100
3M78DHF	140 \pm 15	0.044 \pm 0.005	0.0003	3.7 \pm 0.5
4M78DHF	56 \pm 15	0.073 \pm 0.009	0.0013	1.1 \pm 0.2
34DM78DHF	190 \pm 17	0.053 \pm 0.003	0.0003	19 \pm 2.3
357THF	5.7 \pm 0.9	0.090 \pm 0.007	0.0160	0.0031 \pm 0.0002
ANF	12 \pm 1.6	0.073 \pm 0.007	0.0061	0.0033 \pm 0.0003
BNF	13 \pm 1.2	0.079 \pm 0.009	0.0061	0.0041 \pm 0.0004
TMS	4.3 \pm 0.7	0.047 \pm 0.003	0.0110	0.023 \pm 0.003
resveratrol (Res)	28 \pm 3.3	0.034 \pm 0.004	0.0012	1.2 \pm 0.2
Piceatannol (Pice)	21 \pm 1.1	0.034 \pm 0.001	0.0016	0.83 \pm 0.14
E2	83 \pm 18	0.039 \pm 0.007	0.0005	1.5 \pm 0.2
E1	120 \pm 13	0.014 \pm 0.002	0.0001	16 \pm 1.8

ND, not detected at the highest concentration of 100 μM . K_s , ΔA_{max} , and IC_{50} values presented are the means \pm SEs obtained from the experiments with 5-8 different concentrations of the chemicals.

Table 2
Reverse Type I spectral changes and inhibition of EROD activities of human P450 1B1 with pyrenes, naphthalenes, phenanthrenes, and biphenyls

chemicals	Reverse Type I spectra			inhibition of EROD
	K_s (μM)	ΔA_{max}	$\Delta A_{\text{max}}/K_s$	IC_{50} (μM)
pyrene	1.3 \pm 0.1	0.044 \pm 0.001	0.0338	0.025 \pm 0.003
1EP	2.9 \pm 0.3	0.049 \pm 0.005	0.0169	0.036 \pm 0.005
2EP	3.4 \pm 0.2	0.071 \pm 0.001	0.0209	0.038 \pm 0.004
4EP	4.0 \pm 0.5	0.052 \pm 0.004	0.0130	0.17 \pm 0.03
1VP	4.4 \pm 0.3	0.092 \pm 0.003	0.0209	0.15 \pm 0.02
1PP	2.7 \pm 0.3	0.042 \pm 0.002	0.0156	0.045 \pm 0.005
naphthalene	ND	ND	ND	26 \pm 3.2
2EN	15 \pm 0.3	0.049 \pm 0.002	0.0033	1.2 \pm 0.2
1NMPE	92 \pm 10	0.074 \pm 0.005	0.0008	1.2 \pm 0.3
1NEPE	88 \pm 18	0.079 \pm 0.009	0.0009	0.92 \pm 0.15
2NPE	97 \pm 19	0.086 \pm 0.008	0.0009	4.3 \pm 0.6
2NMPE	100 \pm 17	0.090 \pm 0.009	0.0009	2.1 \pm 0.3
2NEPE	110 \pm 3.1	0.094 \pm 0.016	0.0009	5.2 \pm 0.7
Phenanthrene	14 \pm 1.7	0.044 \pm 0.002	0.0031	0.51 \pm 0.10
2EPh	4.2 \pm 0.5	0.080 \pm 0.004	0.0190	0.21 \pm 0.03
3EPh	3.0 \pm 0.3	0.071 \pm 0.001	0.0237	0.16 \pm 0.02
9EPh	3.6 \pm 0.4	0.071 \pm 0.004	0.0197	0.12 \pm 0.01
2PPh	2.2 \pm 0.2	0.067 \pm 0.005	0.0305	0.15 \pm 0.02
3PPh	3.1 \pm 0.4	0.060 \pm 0.006	0.0194	0.067 \pm 0.008
9PPh	3.2 \pm 0.4	0.049 \pm 0.002	0.0153	0.16 \pm 0.03
biphenyl	140 \pm 19	0.037 \pm 0.008	0.0003	19 \pm 2.3
4EB	7.6 \pm 1.2	0.023 \pm 0.001	0.0030	2.2 \pm 0.3
4PB	12 \pm 1.8	0.074 \pm 0.008	0.0062	1.8 \pm 0.2
4BuB	54 \pm 6.2	0.092 \pm 0.007	0.0017	1.5 \pm 0.3
2BPE	114 \pm 16	0.110 \pm 0.009	0.0010	15 \pm 0.2
4BPE	83 \pm 26	0.056 \pm 0.010	0.0007	16 \pm 0.3
2BMPE	146 \pm 15	0.130 \pm 0.009	0.0009	12 \pm 0.2
4BMPE	13 \pm 1.5	0.071 \pm 0.002	0.0055	1.2 \pm 0.1
22BDPE	30 \pm 4.5	0.018 \pm 0.001	0.0006	12 \pm 1.7
44BDPE	23 \pm 4.3	0.031 \pm 0.002	0.0013	7.8 \pm 1.0

ND, not detected at the highest concentration of 100 μM . K_s , ΔA_{max} , and IC_{50} values presented are the means \pm SEs obtained from the experiments with 5-8 different concentrations of the chemicals.

Table 3
Correlation between IC₅₀ values for EROD inhibition and spectral parameters (K_s, ΔA_{max}, and ΔA_{max}/K_s) with various groups of chemical inhibitors in human P450 1B1 enzyme system

Chemicals	Comparison	correlation coefficient (<i>r</i>)
Total (n=49)	IC ₅₀ vs K _s	0.72
	IC ₅₀ vs ΔA _{max}	0.21
	IC ₅₀ vs ΔA _{max} /K _s	0.74
flavonoids, stilbenes, E1, and E2 (n=20)	IC ₅₀ vs K _s	0.81
	IC ₅₀ vs ΔA _{max}	0.29
	IC ₅₀ vs ΔA _{max} /K _s	0.86
biphenyls (n=10)	IC ₅₀ vs K _s	0.75
	IC ₅₀ vs ΔA _{max}	0.12
	IC ₅₀ vs ΔA _{max} /K _s	0.89
pyrenes (n=6)	IC ₅₀ vs K _s	0.78
	IC ₅₀ vs ΔA _{max}	0.52
	IC ₅₀ vs ΔA _{max} /K _s	0.55
naphthalene (n=6)	IC ₅₀ vs K _s	0.42
	IC ₅₀ vs ΔA _{max}	0.60
	IC ₅₀ vs ΔA _{max} /K _s	0.33
phenanthrenes (n=7)	IC ₅₀ vs K _s	0.80
	IC ₅₀ vs ΔA _{max}	0.43
	IC ₅₀ vs ΔA _{max} /K _s	0.77