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SUICIDE INHIBITORS OF CYTOCHROME P450 1A1 AND P450 2B1*

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Abstract—The inhibition of the P450 1A1 dependent de-ethylation of 7-ethoxyphenoxazone (7EPO) and the P450 2B1 dependent de-pentylation of 7-pentoxyphenoxazone (7PPO) by 1-ethynylnaphthalene (1EN), 2-ethynylnaphthalene (2EN), 1-ethynylanthracene (1EA), 2-ethynylanthracene (2EA), 9-ethynylanthracene (9EA), 2-ethynylphenathrene (2EPh), 3-ethynylphenanthrene (3EPh), 9-ethynylphenanthrene (9EPh), 1-ethynylpyrene (1EP) and 2-ethynylpyrene (2EP) was studied in hepatic microsomal preparations from rats. Although all of the polycyclic aromatic acetylenes studied inhibited the dealkylation of 7EPO or 7PPO, only some of the acetylenes produced a mechanism-based irreversible inactivation (suicide inhibition) of the P450 dependent dealkylation of 7EPO or 7PPO. Of the molecules tested, only 1EP, 1EN, 2EN, 2EPh and 3EPh were effective suicide inhibitors of the P450 1A1 dependent de-ethylation of 7EPO and only 1EN, 2EN, 1EA and 9EPh were effective suicide inhibitors of the P450 2B1 dependent de-pentylation of 7PPO. In addition to the size and shape of the polycyclic aromatic ring system, placement of the carbon—carbon triple bond on the ring system was critical for suicide inhibition. In contrast to 1EP, 2EP was not a mechanism-based inhibitor of P450 1A1; 9EPh, but not 2EPh or 3EPh, was a suicide inhibitor of P450 2B1. None of the aryl acetylenes tested produced heme destruction under assay conditions that produced the suicide inhibition of the P450 dependent 7EPO or 7PPO dealkylation activities. Because a precise orientation of the terminal acetylene is required to produce suicide inhibition without heme destruction, acetylenic suicide inhibitors can potentially be used to differentiate between P450 isozymes and to establish some distinguishing geometric features of the active site of these isozymes.

The cytochrome P450 dependent monooxygenases are a group of hemeproteins involved in the oxidation of both endogenous substrates such as steroids, fatty acids, and lipophilic vitamins [1–4], and of xenobiotic substrates such as drugs and procarcinogens [5–8]. A "superfamily" of genes in yeast, bacteria, and mammals directs the synthesis of a large number of different P450 dependent monooxygenase isozymes [9–12]. A relatively few P450 isozymes in humans appear to be responsible for the metabolic activation and the detoxification of the known chemical procarcinogens [13].

Based upon the observation of Ortiz de Montellano and Kunze [14] that a range of acetylene structures can produce a time-dependent destruction of the heme chromophore in P450 dependent monooxygenases, we initiated a study of the mechanism-based inactivation (suicide inhibition) of cytochrome P450 dependent enzymatic activities by compounds containing carbon–carbon triple bonds (acetylenes). Our initial efforts focused on P450 1A1 and P450 1A2, P450 isozymes that are known to metabolically activate important classes of chemical procarcinogens. We have reported that different aryl acetylenes can function *in vitro* as effective suicide inhibitors of the P450 1A1 dependent metabolism of the polycyclic aromatic hydrocarbon (PAH)‡ procarcinogen benzo[*a*]pyrene [15–17] and the P450 1A2 dependent metabolism of the aromatic amine procarcinogen 2-naphthylamine [18]. Aryl acetylenes have also been demonstrated to inhibit the metabolic activation of procarcinogens *in vivo* [19, 20].

This paper describes structure–activity relationships for the inhibitors of P450 1A1 and P450 2B1 dependent reactions in rat liver microsomes by a series of aryl acetylenes. The results establish that in addition to the size and shape of a polycyclic aromatic ring system, the position of attachment of the carbon–carbon triple bond on the aromatic system is critically important in producing a selective suicide inhibition of P450 dependent enzymatic activities. We also found that the time-dependent loss of P450 dependent activities produced by incubation of liver microsomes with aryl acetylenes occurred without a concomitant loss of the P450 heme chromophore.

MATERIALS AND METHODS

Chemicals

Prior to use, tetrahydrofuran was distilled from

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‡ Abbreviations: PAH, polycyclic aromatic hydrocarbons; 7PPO, 7-pentoxyphenoxazone; 7EPO, 7-ethoxyphenoxazone; 1AA, 1-acetylanthracene; 2AA, 2-acetylanthracene; VP, 1-vinylpyrene; 2BrP, 2-bromopyrene; 2BTHP, 2-bromo-4,5,9,10-tetrahydropyrene; 2EP, 2-ethynylpyrene; 2TMSEP, 2-(trimethylsilyl)ethynylpyrene; 1EP, 1-ethynylpyrene; 1EN, 1-ethynylnaphthalene; 2EN, 2-ethynylnaphthalene; 1EA, ethynylanthracene; 2EA, 2-ethynylanthracene; 9EA, 9,ethynylanthracene; 2EPh, 2-ethynylphenanthrene; 3EPh, 3-ethynylphenanthrene; 9EPh, 9-ethynylphenanthrene; BNF, β-naphthoflavone; PB, phenobarbital; G6P, glucose-6-phosphate; and PAA, polycyclic aromatic acetylene.

sodium benzophenone dianion under nitrogen atmosphere and benzene, toluene, and petroleum ether were distilled under nitrogen atmosphere. Other solvents were reagent grade and used without further purification.

1-Acetylpyrene was obtained from Chemsyn Science Laboratories (Lenexa, KS). 2'-Acetonaphthone and anthracene were purchased from Eastman Kodak (Rochester, NY) and 9-acetylanthracene was purchased from Lancaster Synthesis, Ltd. (Windham, NH). Carbon monoxide, research grade, was obtained from Matheson Gas Products, Inc. (Secaucus, NJ), 10% Pd/C from Engelhard Industries (Newark, NJ), copper(II) acetate from Alfa Products (Danvers, MA), and dichlorobis(benzonitrile)palladium(II) from Johnson Matthey (Danvers, MA). 1'-Acetonaphthone, 2-, 3- and 9-acetylphenanthrene, *n*-butyllithium, diisopropylamine, sodium phenoxazone, pyrene, diethylchlorophosphate, *o*-chloranil, triphenyl phosphine, *β*-naphthoflavone, trimethylsilylacetylene and methyltriphenylphosphonium bromide were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase, Type XXIII (EC 1.1.1.49), and phenobarbital were obtained from the Sigma Chemical Co. (St. Louis, MO). 7-Pentoxyphenoxazone (7PPO) and 7-ethoxyphenoxazone (7EPO) were prepared by Rajeev Khatkhate, Tulane University, New Orleans, LA, by the method of Prough *et al.* [21].

Instrumentation

Proton NMR spectra were collected on a Bruker AC200 NR/200FT NMR spectrometer. Mass spectral data were obtained with a Hewlett–Packard Mode 5995 gas chromatograph/quadropole mass spectrometer equipped with a capillary column operating in the electron impact mode at 70 eV. All UV-visible spectra were obtained with a 8451A Hewlett–Packard diode array spectrophotometer. All spectrofluorometric data were obtained with a SPEX Fluorolog 2 Spectrofluorometer equipped with a 450 W Xenon lamp. Elemental analyses were performed by Spang Microanalytical Laboratory (Eagle Harbor, MI).

Synthesis

Synthesis of 1-acetylanthracene (1AA). 1AA (m.p. 108–109°) was synthesized by a modification of the method of Bassilios *et al.* [22]. In a 500-mL reaction vessel equipped with a mechanical stirrer, anthracene (10 g, 56 mmol) and aluminium chloride (9 g, 67 mmol) were dissolved in 200 mL of dry 1,1-dichloroethane and cooled to 0°. Fifty milliliters of 10% acetyl chloride in 1,1-dichloroethane (70 mmol of acetyl chloride) was slowly added over 15 min while maintaining the reaction of 0°. The reaction mixture was stirred for an additional 2 hr and filtered. The red precipitate was washed twice with cold 1,1-dichloroethane and twice with cold water. The precipitate was decomposed with ice-cold 1 N HCl and allowed to dry. The green crystals were recrystallized from absolute ethanol yielding 4.7 g (21 mmol) of orange crystals (35% yield). The 1AA was >99% pure by GC–MS. Mass spectral fragmentation pattern: M^+ at 220 (89%); M^+-CH_3 at 205 (91%); M^+-$COCH_3$ at 177 (100%); M^+-$C_7H_4COCH_3$ at 88 (40%).

Synthesis of 2-acetylanthracene (*2AA*). 2AA (m.p. 190–192°) was synthesized by the method of Bassilios *et al.* [23]. Initial purification was accomplished by flash chromatography by the method of Still *et al.* [24] with benzene as the eluting solvent. The enriched 2AA fraction was washed with hot absolute ethanol to remove any remaining 1AA and recrystallized from glacial acetic acid. The bright yellow crystals were >99% pure by GC–MS. Mass spectral fragmentation pattern: M^+ at 220 (100%); M^+-CH_3 at 205 (96%); M^+-$COCH_3$ at 177 (99%); M^+-$C_7H_4COCH_3$ at 88 (34%).

Synthesis of 1-vinylpyrene (VP). VP (m.p. 82–84°) was synthesized from 1-formylpyrene and purified as previously described [17]. The identity and purity were checked by GC–MS and proton NMR. Purity was >99% and NMR gave C=CH_2, d, 5.58 ppm, and d, 5.95 ppm, *J* 1.8 Hz. Mass spectral fragmentation pattern: M^+ at 228 (97%); M^+-H at 227 (100%); M^+-H_2 at 226 (72%); M^+-C_9H_7 at 113 (43%).

Synthesis of 4,5,9,10-tetrahydropyrene. 4,5,9,10-Tetrahydropyrene (m.p. 84°) was synthesized by a modification of the method of Fu *et al.* [25]. Ten grams (49.5 mmol) of pyrene, dissolved in 300 mL of ethyl acetate, was hydrogenated for 8 days at room temperature in a Parr shaker-type hydrogenation apparatus with 10 g of 10% Pd/C and 60 psi of H_2. The crude product was recrystallized from ethanol and water yielding 6.20 g of white crystals (30.0 mmol, 61% yield), >97% pure by GC–MS. Mass spectral fragmentation pattern: M^+ at 206 (100%); M^+-H_4 at 202 (33%); M^+-CH_5 at 189 (14%); M^+-C_8H_9 at 101 (18%).

Synthesis of 2-bromopyrene (2BrP). 2-Bromo-4,5,9,10-tetrahydropyrene (2BTHP, m.p. 98.5°) was prepared from 4,5,9,10-tetrahydropyrene by the bromination procedure described by Harvey *et al.* [26] and purified by flash chromatography on a silica gel column with petroleum ether as the eluting solvent. The 2BTHP was recrystallized from aqueous ethanol and was pure by GC–MS. Mass spectral fragmentation pattern: M^+ at 284 (100%) and 286 (96%); M^+-H_2Br at 203 (68%); M^+-C_8H_8Br at 101 (51%). 2BTHP (0.81 g, 2.8 mmol) and *o*-chloranil were refluxed in dry benzene for 48 hr and the resulting 2BrP (m.p. 97°) was purified by flash chromatography on a florasil column with cyclohexane as the solvent (0.38 g, 1.3 mmol, 47% yield). Purity was >98% by GC–MS. Mass spectral fragmentation pattern: M^+ at 282 (97%) and 280 (100%); M^+-Br at 201 (55%); M^+-C_8H_8Br at 100 (42%).

Synthesis of 2-ethynylpyrene (2EP). 2EP (m.p. 104°) was synthesized from 2BrP (0.5 g, 1.8 mmol) by coupling with trimethylsilylacetylene according to the procedure described by Neenan and Whitesides [27]. The 2-(trimethylsilyl)ethynylpyrene (2TMSEP) was dissolved in dichloromethane and washed with 15% HCl, followed by three water washes. The 2TMSEP was purified on a florasil flash chromatography column with petroleum ether as the solvent, yielding crystals (0.3726 g, 1.06 mmol, 70% yield) that were >99% pure by GC–MS. Mass

spectral fragmentation pattern: M^+ at 298 (70%); M^+-CH_3 at 283 (100%). The protecting group was removed by stirring the 2TMSEP in 30 mL of a methanol solution of 0.07 g of KOH for 1 hr at room temperature. The product was added to water and extracted into petroleum ether. The solvent was removed *in vacuo* and the crude 2EP was treated with charcoal and recrystallized from methanol. The 2EP (0.2296 g, 1.02 mmol, 81% yield) was >98% pure by GS–MS. NMR gave a —C≡H = s, 3.25 ppm, singlet; ArH (9H) = 8.00–8.30 ppm, multiplet. Mass spectral fragmentation: M^+ at 226 (100%); M^+-C_9H_6 at 112 (19%). Analysis: Calculated: C, 95.54; H, 4.46. Found: C, 95.44; H, 4.42. The analytically pure crystalline product was off-white.

Synthesis of other acetylenic polyaromatic hydrocarbons. 1-Ethynylpyrene (1EP, m.p. 105–106°), 1-ethynylnaphthalene (1EN) and 2-ethynylnaphthalene (2EN, m.p. 40–42°), 1-ethynylanthracene (1EA, m.p. 88°), 2-ethynylanthracene (2EA, m.p. 174–175°), 9-ethynylanthracene (9EA, m.p. 60–61°), 2-ethynylphenanthrene (2EPh, m.p. 77–78°), 3-ethynylphenanthrene (3EPh, m.p. 109°), and 9-ethynylphenanthrene (9EPh, m.p. 62°) were synthesized from the corresponding methyl ketones by previously published methods [17]. Initial purification was by flash chromatography with either petroleum ether:toluene (95:5) [1EN, 2-EN and 1EP] or benzene [1EA, 2EA, 9EA, 2EPh, 3EPh and 9EPh] as the eluting solvent. 1EA, 2EA, 9EA, 2EPh, 3EPh and 9EPh were recrystallized from methanol. Identity and purity were checked by GS–MS and proton NMR. Purity was at least 98%. Mass and proton NMR ($CDCl_3$/TMS) spectral correlations and elemental analyses for new compounds are as follows:

1EA–NMR-≡C—H = 3.58 ppm; ArH (3H) = 7.36–7.51 ppm, multiplet; ArH (1H) = 7.73–7.76 ppm, doublet; ArH (3H) = 7.99–8.10 ppm, multiplet; ArH (1H) = 8.43 ppm, singlet; ArH (1H) = 8.92 ppm, singlet. Mass spectral fragmentation pattern: M^+ at 202 (100%); m^+-C_8H_5 at 101 (11%); M^+-C_9H_6 at 88 (9%). Analysis: Calculated for $C_{16}H_{10}$: C, 95.02; H, 4.98. Found: C, 95.08; H, 5.08. The product was dark yellow crystals.

2EA—NMR-≡C—H = 3.20 ppm, singlet; ArH (3H) = 7.36–7.51 ppm, multiplet; ArH (1H) = 7.91 ppm, singlet; ArH (2H) = 7.96–8.02 ppm, multiplet; ArH (1H = 8.19 ppm, singlet; ArH (2H) = 8.37 ppm, singlet. Mass spectral fragmentation pattern: M^+ at 202 (100%); M^+-C_8H_5 at 101 (14%); M^+-C_9H_6 at 88 (11%). Analysis: Calculated for $C_{16}H_{10}$: C, 95.02; H, 4.98. Found: C, 95.06; H, 5.00. The product was yellow-gold crystals.

9EA—NMR-≡C—H = 3.99 ppm, singlet; ArH (4H) = 7.46–7.62 ppm, multiplet; ArH (2H = 7.98–8.02 ppm, triplet; ArH (1H) = 8.45 ppm, singlet; ArH (2H) = 8.55–8.60 ppm, doublet. Mass spectral fragmentation pattern: M^+ at 202 (100%); M^+-C_8H_6 at 100 (11%); M^+-C_9H_6 at 88 (7%). Analysis: Calculated for $C_{16}H_{10}$: C, 95.02; H, 4.98. Found: C, 94.98; H, 4.91. The product was dark orange-red crystals.

2EPh—NMR-≡C—H = 3.19 ppm, singlet; ArH (5H) = 7.61–7.70 ppm, multiplet; ArH (1H) = 7.74–7.91 ppm, multiplet; ArH (1H) = 8.05–8.06 ppm, doublet; ArH (2H) = 8.60–8.65 ppm, multiplet. Mass spectral fragmentation pattern: M^+ at 202 (100%); M^+-C_8H_5 at 101 (13%). Analysis: Calculated for $C_{16}H_{10}$: C, 95.02; H, 4.98. Found: C, 95.11; H, 5.03. The product was light yellow crystals.

3EPh—NMR-≡C—H = 3.20 ppm, singlet; ArH (6H) = 7.61–8.05 ppm, multiplet; ArH (2H) = 8.59–8.66 ppm, multiplet; ArH (1H) = 8.85 ppm, singlet. Mass spectral fragmentation pattern: M^+ at 202 (100%); M^+-C_8H_5 at 100 (11%). Analysis: Calculated for $C_{16}H_{10}$: C, 95.02; H, 4.98. Found: C, 95.07; H, 4.87. The product was beige crystals.

9EPh—NMR-≡C—H = 3.47 ppm, singlet; ArH (4H) = 7.59–7.71 ppm, multiplet; ArH (1H) = 7.82–7.86 ppm, triplet; ArH (1H) = 8.06 ppm, singlet; ArH (1H) = 8.44–8.49 ppm, multiplet; ArH (2H) = 8.63–8.70 ppm, triplet. Mass spectral fragmentation pattern: M^+ at 202 (100%); M^+-C_8H_5 at 100 (11%). Analysis: Calculated for $C_{16}H_{10}$: C, 95.02; H, 4.98. Found: C, 95.14; H, 5.04. The product was yellow crystals.

Mass spectral fragmentation patterns for other compounds. 1EP—M^+ at 226 (100%); M^+-C_9H_5 at 133 (26%). Proton NMR-≡C—H 3.56 ppm, singlet. Other data were published previously in Ref. 15.

1EN—M^+ at 152 (100%); M^+-C_2H_2 at 126 (7%); M^+-C_6H_4 at 76 (17%); M^+-C_7H_6 at 63 (17%). Proton NMR≡C-H 3.46 ppm, singlet. Other data were published previously in Ref. 18.

2EN—M^+ at 152 (100%); M^+-C_6H_4 at 76 (13%); M^+-C_7H_5 at 63 (12%). Proton NMR-≡C—H 3.14 ppm, singlet. Other data were published previously in Ref. 18.

Microsomal preparation

Liver microsomes were prepared from male Sprague–Dawley rats (Charles River Laboratories) (150–200 g) by a modification of the method of Guengerich [28]. P450 1A1 was induced by i.p. injection of the rats with 50 mg/kg β-naphthoflavone (BNF) in 1 mL corn oil for 3 consecutive days and the rats were killed by CO_2 asphyxiation on day 4. P450 2B1 was induced by addition of 0.1% phenobarbitol (PB) to the drinking water for 1 week prior to killing the animals. The microsomes were prepared in 0.25 M sucrose in 10 mM Tris acetate buffer, pH 7.4, and the microsomal pellet (105,000 *g* for 90 min) was resuspended in 0.15 M KCl in 10 mM Tris acetate buffer, pH 7.4, and recentrifuged. The microsomes were resuspended in sufficient 10 mM Tris acetate buffer, pH 7.4, containing 1 mM EDTA and 20% (w/v) glycerol (microsomal storage buffer) so that 1 mL of microsomal suspension contained the material from 0.5 g liver (wet weight). The microsomal suspension was stored in 1-mL aliquots in plastic tubes at −70°; a fresh vial was used for each day's experiments. Protein concentration was determined by the method of Bradford [29] with bovine γ-globulin as the standard.

Assay of 7-alkoxyphenoxazone dealkylation

7-Ethoxyphenoxazone (7EPO) de-ethylation and 7-pentoxyphenoxazone (7PPO) de-pentylation were determined by a modification of the method of Burke *et al.* [30].

7EPO de-ethylation. The reaction was carried out in a 1-cm quartz cuvette in 0.1 M Na^+/K^+ phosphate buffer, pH 7.6, containing 5 mM $MgCl_2$ and 2 μM 7EPO [5 μL of 0.4 mM 7EPO in dimethyl sulfoxide (DMSO)]. Five microliters of microsomal protein (~12 μg) in microsomal storage buffer was added. Inhibitors were dissolved in DMSO to give the desired concentration when 5 μL/mL reaction mixture was added. Control assays contained 5 μL/mL of DMSO. The reaction was initiated by the addition of 100 μL of an NADPH-regenerating solution [1 mM NADP, 4.5 mM glucose-6-phosphate (G6P), 3 mM $MgCl_2$, and 3 U/mL G6P dehydrogenase, Type XXIII, in 0.1 M Na^+/K^+ phosphate buffer, pH 7.8]. Total volume of the reaction mixture was 1.0 mL. The production of 7-hydroxyphenoxazone anion was monitored at 530 nm excitation and 585 nm emission, slit width 2 nm. The reaction was carried out at 37° and the reaction was linear for at least 10 min when no inhibitor was added.

7PPO de-pentylation. 7PPO de-pentylation was assayed as above except that the reaction was carried out in 0.05 M Tris–HCl buffer, pH 7.5 and 5 μM 7PPO was used as a substrate (5 μL of 1 mM 7PPO dissolved in DMSO). Microsomal protein from PB-pretreated rats (~26 μg) was added in 5 μL microsomal storage buffer. The NADPH-regenerating solution was dissolved in 0.05 M Tris–HCl buffer, pH 7.8. The reaction rate was linear for at least 10 min, in the absence of inhibitors.

Assay of VP metabolism

The metabolism of VP was assayed measuring the formation of a fluorescent metabolite (emission 376 nm, excitation 330 nm, slit width 2 nm) at 37°. VP metabolism by BNF microsomes was assayed in 0.1 M phosphate buffer, pH 7.6, with approximately 62 μg of microsomal protein and 5 μL of the appropriate concentrations of VP dissolved in DMSO. The reaction was started by the addition of 100 μL of NADPH-regenerating solution as used in the 7EPO assay. Total volume of the assay mixture was 1 mL (0.5% DMSO) in a 1-cm quartz cuvette.

VP metabolism by PB microsomes was assayed in 0.05 M Tris–HCl buffer, pH 7.5, at 37°, with approximately 78 μg of microsomal protein, 5 μL of the appropriate VP solution in DMSO, and the reaction was initiated by addition of 100 μL of the NADPH-regenerating solution as used in the 7PPO reaction.

Cytochrome P450 destruction assay

Cytochrome P450 content was determined by the method of Fowler *et al.* [31]. An assay mixture was prepared to contain 1.5 mM KCl, 0.1 mM EDTA, 10 μL/mL of DMSO with the appropriate inhibitor concentration and 1 mg/mL microsomal protein final concentration and incubated for 3 min at 37°. NADPH-regenerating solution (1 mM NADP, 4.5 mM G6P, 3 mM $MgCl_2$, and 3 U/mL G6P dehydrogenase, Type XXIII, final concentration) was added to initiate the reaction. Reaction mixture (1.0 mL) was immediately removed and placed in an iced cuvette to stop the reaction (zero time). The remaining solution was incubated for 10 min at 37° and 1.0 mL was removed to an iced cuvette to stop the reaction. A control sample with 10 μL of DMSO in place of inhibitor was assayed in the same manner for each set of content assays. For each content determination, solid sodium dithionate (~1 mg) was added to the sample cuvette and a reference spectrum from 400 to 500 nm was obtained. The solution in the cuvette was gently gassed with CO for about 1 min, the cuvette was then capped and the spectrum was obtained from 400 to 500 nm. The change in absorbance at 450 nm relative to 490 nm was converted to P450 content using the millimolar extinction coefficient of 91.

RESULTS

Investigations have shown that for rat liver microsomes 7EPO de-ethylase activity is a measure of P450 1A1 activity and 7PPO de-pentylase activity is a measure of P450 2B1 activity [30].

The ethynyl-substituted compounds 1EP, 2EP, 1EN, 2EN, 1EA, 2EA, 9EA, 2EPh, 3EPh and 9EPh and the olefin-substituted compound VP (Fig. 1) were each examined for inhibitory activity towards P450 1A1 and P450 2B1 in microsomal systems at concentrations of 10 μM and 1 μM. All of the acetylenic compounds were found to inhibit alkoxyphenoxazone dealkylation activity at both concentrations. The results at a 1 μM concentration are shown in Table 1. Although 2EA only slightly inhibited 7PPO de-pentylase activity of PB microsomes at 1 μM, 10 μM 2EA gave 43% inhibition of activity after 10 min. A 10 μM concentration of 1EN inhibited 51% 7EPO de-ethylase activity by BNF microsomes at 10 min. VP strongly inhibited dealkylation of both EPO and PPO initially, but we observed a time-dependent increase in dealkylation as the incubation with VP proceeded.

VP was found to be readily metabolized by both PB and BNF microsomes. At least one fluorescent metabolite was formed. This metabolite was found to have an emission at 376 nm when excited at 330 nm. The K_m for the formation of this metabolite from VP was found to be 0.9 μM with BNF microsomes and 2.1 μM with PB microsomes. This compares with the K_m of 7-EPO with BNF microsomes of 0.4 μM and the K_m of 7-PPO with PB microsomes of 1.7 μM.

All ethynyl-substituted PAHs were further tested to determine if the loss of activity was time dependent. Each inhibitor was assayed in each system at four or more concentrations that gave 25–90% inhibition after 10 min. An appropriate control was assayed for each inhibitor tested. Only 1EN and 2EN caused time-dependent loss of activity in both systems. The loss of activity by PB microsomes was far greater than that by BNF microsomes at a given concentration of either naphthylacetylene. 1EP, 2EPh and 3EPh caused considerable time-dependent 7EPO de-ethylase activity loss with BNF microsomes, but the 7PPO de-pentylase activity by PB microsomes was linear over 10 min when these compounds were added as inhibitors. 1EA and 9EPh caused large decreases of 7PPO de-pentylation by PB microsomes over time, but the 7EPO de-ethylase activity by BNF microsomes was linear for the 10-min incubation

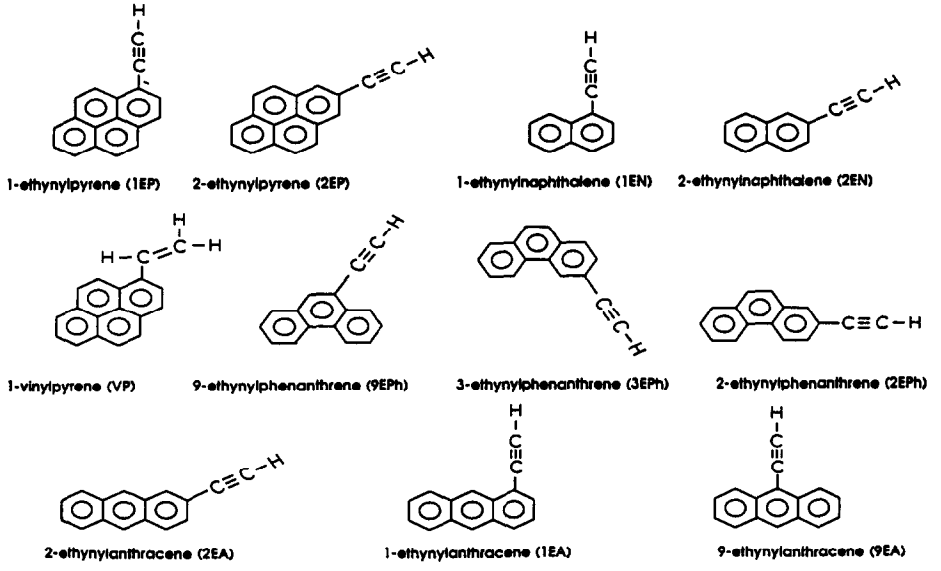

Fig. 1. Structures of the polycyclic aromatic acetylenes (PAAs) and VP studied as inhibitors of P450 1A1 catalyzed 7EPO de-ethylase activity and as inhibitors of P450 2B1 catalyzed 7PPO de-pentylase activity.

Table 1. Observed 7EPO or 7PPO dealkylase activity after 10-min incubations with polycyclic aromatic acetylenes (PAAs)

PAA added (1 μM)	7PPO activity in PB microsomes* (nmol/10 min/mg)	7EPO activity in BNF microsomes* (nmol/10 min/mg)
1EP	0.3 (3)†	0.1 (<1)†
2EP	0.3 (2)	0.3 (4)
1EN	1.5 (10)	18.4 (97)
2EN	1.9 (15)	18.4 (83)
1EA	4.2 (36)	7.2 (45)
2EA	10.2 (92)	5.9 (39)
9EA	1.2 (11)	5.6 (29)
2EPh	3.4 (46)	4.0 (23)
3EPh	1.0 (14)	2.7 (17)
9EPh	0.4 (3)	5.7 (36)

* Activity is represented as the nmol 7-hydroxyphenoxazone anion produced after 10 min. Assays were carried out as described in Materials and Methods.

† The values in parenthesis indicate the percentage of product formed in a 10-min incubation in the presence of 1 μM added inhibitor relative to that formed in a control incubation under identical conditions in the absence of added inhibitor carried out with the same microsomal preparation on the same day.

period with these compounds. 9EA showed a slight time-dependent loss of 7EPO de-ethylation but the loss was minor when compared to the loss in initial velocity of the reaction. 2EA and 2EP caused no time-dependent loss in either system.

A polynomial expression that produced the best fit of the experimental data was determined using the Enzfitter™ graphics package. The log values of the activity were calculated from this polynomial expression, and a linear regression fit of these log values versus incubation time was calculated (Fig. 2). The time required for the loss of 50% of the initially observed activity ($T_{1/2}$) was established for each concentration of inhibitor that showed time-dependent activity loss. In some cases, the loss of enzyme activity displayed non-pseudo first-order rates. For these cases the initial rates of loss were used to estimate $T_{1/2}$ values as described by Silverman [32]. Although this introduced some error into the calculation of $T_{1/2}$ and thus K_I, comparisons of inactivators can be directly made under identical conditions so long as K_I values are viewed as estimates or lower limits rather than exact values. The $T_{1/2}$ values determined were plotted versus the reciprocals of the concentrations as Kitz and Wilson plots [33] (Fig. 3). The limiting $T_{1/2}$ (time to cause the loss of one-half the initial activity at infinite inhibitor concentration) was determined from the y-intercept. The x-intercept obtained by extrapolation ($-1/K_I$) yielded K_I (the dissociation constant of the enzyme–inhibitor complexes that lead to enzyme inactivation).

Dixon plots were used to calculate K_i values for reversible type inhibition for the initial inhibition of activity seen with all the inhibitors tested (data not shown). The kinetic parameters for inhibition by the acetylenic PAH studies are summarized in Tables 2 and 3.

Most of the values we obtained for K_i and K_I can be considered to be nearly equal, since the values of K_I were obtained using the method of Kitz and Wilson [33] rather than more rigorous methods such as those of Waley [34] or Tatsunami *et al.* [35]. We feel that this treatment is adequate when microsomes are used as the source of enzymatic activity.

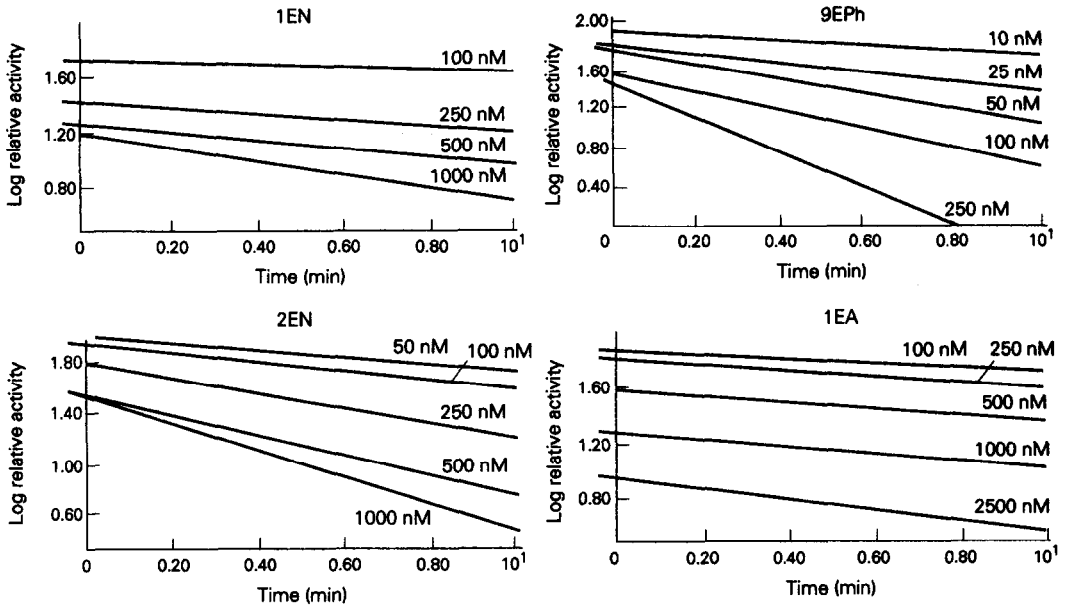

Fig. 2. Log values of 7PPO de-pentylase activity in PB microsomes versus time relative to those of suitable controls for 2EN, 9EPh, 1EN and 1EA. The other PAAs investigated did not show time-dependent increases in inhibition in the system. The $T_{1/2}$ for each inhibitor concentration was determined from the slopes.

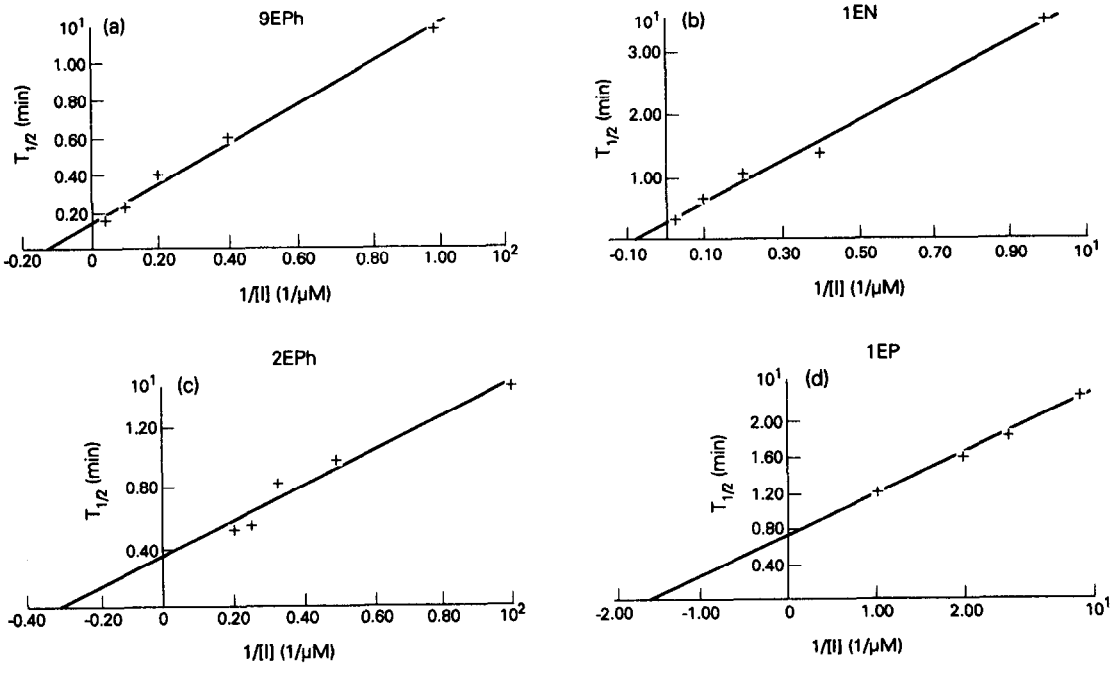

Fig. 3. Kitz–Wilson plots for the inhibition of 2B1 by 9EPh (a) and by 1EN (b) and for the inhibition of 1A1 by 2EPh (c) and by 1EP (d). The $T_{1/2}$ values determined from plots of the log of the dealkylase activities versus time as illustrated in Fig. 2 are plotted versus the reciprocals of the inhibitor concentrations. The y-intercept indicates the limiting $T_{1/2}$ for 7PPO de-pentylase activity (a, b) or for 7EPO de-ethylase activity (c, d) at infinite inhibitor concentrations. K_I for each suicide inhibitor in each assay system is obtained by extrapolation to the x-intercept $(-1/K_I)$.

Table 2. Effects of polycyclic aromatic acetylenes on P450 2B1 dependent 7PPO de-pentylase activity in PB microsomal preparations

PAA added	K_i* (nM)	K_I† (nM)	$T_{1/2}$‡ (min)
1EP	80	ND§	ND
2EP	20	ND	ND
1EN	300	1,300	2.7
2EN	460	140	3.0
1EA	560	130	9.5
2EA	16,000	ND	ND
9EA	160	ND	ND
2EPh	420	ND	ND
3EPh	160	ND	ND
9EPh	100	70	1.5

* Apparent dissociation constants for reversible type inhibition as determined from Dixon plots.

† Apparent dissociation constants of enzyme–inhibitor complexes leading to loss of 7PPO de-pentylase activity.

‡ Calculated half-lives of the 7PPO de-pentylase activity at infinite inhibitor concentration.

§ ND: no time-dependent loss of activity was observed under the assay conditions.

Table 3. Effects of polycyclic aromatic acetylenes on P450 1A1 dependent 7EPO de-ethylase activity in BNF microsomal preparations

PAA added	K_i* (nM)	K_I† (nM)	$T_{1/2}$‡ (min)
1EP	20	70	6.8
2EP	40	ND§	ND
1EN	10,000	5,800	10.2
2EN	6,000	13,000	6.8
1EA	670	ND	ND
2EA	1,600	ND	ND
9EA	340	NM‖	>30
2EPh	440	350	3.4
3EPh	220	260	4.3
9EPh	450	ND	ND

* Apparent dissociation constants for reversible type inhibition as determined from Dixon plots.

† Apparent dissociation constants of enzyme–inhibitor complexes leading to loss of 7EPO de-ethylase activity.

‡ Calculated half-lives of the 7EPO de-ethylase activity at infinite inhibitor concentration.

§ ND: no time-dependent loss of 7EPO de-ethylase activity was observed under the assay conditions.

‖ NM: K_I value could not be obtained under the assay conditions.

However, the values of K_i and K_I for the inhibitors 1EN and 2EN with both systems and 1EA with PB microsomes are clearly different. The values of K_i and K_I are often the same for mechanism-based inhibitors. However, as reviewed and evaluated by Silverman [32], this is true only when the rate of formation of the activated inhibitor–enzyme complex is the rate-determining step. If the rate of formation of covalently bound inhibitor–enzyme from the activated inhibitor–enzyme complex is rate determining, then K_I may be greater than K_i. Also, interaction of the inhibitor and substrate with the enzyme may be such that two distinctly different complexes are formed. Such complexes of P450 1A1 have been described by Turner *et al.* [36]. The observations that 1EP manifests noncompetitive inhibition patterns of 7PPO de-pentylase and 2EA mixed-type inhibition patterns of 7PPO and 7EPO dealkylase activities (see below) indicate that ternary complexes of P450 isozyme, alkoxyphenoxazone substrate, and aryl acetylene can be generated in these systems. If one complex leads to enzyme inactivation (K_I) and the other leads to product formation (K_i), they the two constants would be different or only coincidently equal.

For those inhibitors causing time-dependent loss of activity the P450 destruction was assayed by comparing the P450 content of microsomes that were incubated without inhibitor added to that of microsomes incubated in the presence of inhibitor concentrations that gave at least 90% loss of activity. P450 content assays made immediately after adding the NADPH-regenerating solution (zero time) and after 10 min. There was no significant difference between the P450 content after the 10-min incubation with or without inhibitor for any aryl acetylene tested. The measured P450 content at zero time and after a 10-min incubation with or without the acetylene were the same within +/−5%.

The types of reversible inhibition by 1EP with PB microsomes and by 2EA with both microsomes were studied. 1EP and 2EA were assayed at various concentrations with PB microsomes as outlined in Materials and Methods except that various concentrations of 7PPO were used for each inhibitor concentration. 2EA was similarly assayed with BNF microsomes with various concentrations of 7EPO. The initial velocity of each assay was determined and the double-reciprocal plots for each inhibitor concentration were plotted. 1EP showed noncompetitive type inhibition in the 7PPO de-pentylase system, while 2EA showed mixed inhibition (competitive–noncompetitive) for both systems.

DISCUSSION

The results of these studies establish that the size of an aromatic ring system and the placement of the carbon—carbon triple bond (ethynyl group) on a polycyclic aromatic acetylene both influence the effectiveness of the inhibition of 7EPO de-ethylation catalyzed by P450 1A1 and of 7PPO de-pentylation catalyzed by P450 2B1. Although all of the aryl acetylenes tested caused a decrease in velocity of the dealkylation of phenoxazone substrates in both systems studied, selective suicide inhibition resulting in a time-dependent loss of the dealkylase activity was only observed with certain of these potential suicide inhibitors. The data emphasize that this selective suicide inhibition is dependent upon the position of the ethynyl group.

Olefins have been shown to be mechanism-based inhibitors of P450 in many studies, especially at high concentrations (mM) [37]. Although VP caused a decrease in initial velocity in both systems studied,

VP was metabolized by P450 1A1 and P450 2B1 to less inhibitory products. In the presence of VP the velocity of the dealkylation reactions *increased* with time; no evidence of a time-dependent loss of the dealkylase activity due to VP was obtained. The ethynyl group appears to be necessary for mechanism-based inhibition by this series of compounds; it cannot be replaced by a vinyl group.

Ortiz de Montellano and Komives [38] have proposed that the P450 dependent oxidation of the carbon—carbon triple bond in a terminal acetylene can occur with different regiospecificity. The catalyzed transfer of oxygen from the P450 perferryl oxygen to the *internal carbon* generates a reactive intermediate that results in heme alkylation and destruction of the P450 chromophore. In contrast, the transfer of oxygen from the P450 perferryl oxygen to the *terminal carbon* of the acetylene results in an intermediate that rearranges, via a 1,2-shift of hydrogen, to generate a ketene. Although this ketene intermediate can be hydrolyzed to produce carboxylic acid products, it also may react with nucleophilic amino acid residues within the P450 active site. CaJacob *et al.* [39] have proposed that the covalent modification of protein by a ketene generated by a $P450_{LA\omega}$ hydroxylase (P450 4A1 [11]) dependent oxidation of the terminal acetylenic carbon is responsible for suicide inhibition of this enzyme by 10-undecynoic acid.

In these studies with aryl acetylenes we observed no measurable destruction of heme even with concentrations of mechanism-based inhibitors that produced 95% loss of the P450 dependent catalytic activity. We conclude that the mechanism-based inhibition of P450 1A1 and P450 2B1 isozymes by aryl acetylenes does not proceed by destruction of the heme. The generation of a ketene intermediate within the active site of the enzyme by a regiospecific oxidation of the terminal ethynyl carbon atom as proposed by Ortiz de Montellano and Komives [38] and by CaJacob *et al.* [39] could result in covalent modification of the P450 protein and the observed loss of catalytic activity concomitant heme destruction. Since the regiospecific oxidation of the acetylene on the terminal carbon is required for formation of the ketene intermediate, this proposed mechanism is fully consistent with the results of these experiments which emphasize the critical importance of the proper orientation of an ethynyl group within the active site of a P450 isozyme to produce suicide inhibition. Observations that the inactivation of P450 isozymes by aryl acetylenes is accomplished by a covalent labeling of the P450 protein by the acetylene are also consistent with this proposed mechanism of action [15, 16, 18, 39].

All of the aryl acetylenes studied were reversible inhibitors of P450 2B1 and 1A1. 1EP and 2EP were somewhat better reversible inhibitors of 2B1 than 1EN, 2EN, 1EA and 3EPh, while 2EA was a relatively poor inhibitor of 2B1 with an apparent K_i that was nearly two orders of magnitude greater than that of any of the other compounds studied. 1EP, 2EP, 2EPh, 3EPh, 9EPh and 9EA were all very effective reversible inhibitors of 1A1. Although the range of K_i values varied over three orders of magnitude, even 1EN ($K_i = 10\ \mu M$) in the P450 2B1 system and 2EA ($K_i = 16\ \mu M$) in the P450 1A1 system would normally be considered good inhibitors.

Only 1EN, 2EN, 1EA and 9EPh caused the time-dependent loss of the P450 2B1 dependent 7PPO de-pentylation activity. It appears that aryl acetylenes must be relatively small and compact for the proper orientation of the ethynyl group in the active site of P450 2B1 to produce a mechanism-based inactivation of this isozyme.

1EN and 2EN were both effective suicide inhibitors with limiting $T_{1/2}$ values for the inactivation of P450 2B1 dependent activity of ≤ 3 min. 2EN apparently binds in at least one preferred orientation with the ethynyl group properly positioned relative to the perferryl oxygen function for regiospecific oxidation of the terminal carbon, and the K_I for suicide inhibition by 2EN was only one-tenth that of 1EN. 1EA was also an effective mechanism-based inhibitor of P450 2B1. It had a K_I similar to that of 2EN, but limiting $T_{1/2}$ was about three times that of 2EN, indicating a slower rate of the catalyzed oxidation that leads to enzyme destruction.

9EPh was a very effective suicide inhibitor of the P450 2B1 dependent activity. The selective suicide inhibition of P450 2B1 by this specific ethynylphenanthrene isomer demonstrated clearly the critical importance of the position of the acetylenic substituent for the suicide inhibition of P450 dependent isozymes. Even though 9EPh possesses a third fused benzo ring and is therefore larger than 2EN, the K_I and the limiting $T_{1/2}$ for suicide inhibition by 9EPh were about one-half those found for 2EN. The third aromatic ring apparently enhanced preferential binding of the 9EPh to the active site of P450 2B1 with the ethynyl group correctly positioned in juxtaposition to the perferryl oxygen to produce suicide inhibition. The position of a third aromatic ring in relationship with the ethynyl moiety was of critical importance in this series of inhibitors. No suicide inhibition of P450 2B1 was produced by 2EPh, 2EA, or by 3EPh even though each of these compounds, like 9EPh, can be viewed as a 2EN molecule possessing an additional fused benzo ring (Fig. 4).

1EP and 2EP appeared to be too large to bind within critical regions of the active site of P450 2B1 in an orientation that will lead to suicide inhibition under the assay conditions. Kinetic studies showed that 1EP functions as a non-competitive inhibitor that binds to the enzyme without displacing the phenoxazone substrate. 1EP could be binding to lipophilic areas of P450 and changing the conformation of the active site to prevent substrate binding and/or oxidation or the binding of 1EP might alter the P450 conformation and disrupt the interaction of the P450 and the reductase.

1EP, 1EN, 2EN, 3EPh and 2EPh were also effective suicide inhibitors of the P450 1A1 dependent EPO de-ethylase activity. Although 1EP had a lower K_I for suicide inhibition than either 2EPh or 3EPh, the limiting $T_{1/2}$ was slightly longer for 1EP. 2EN had a limiting $T_{1/2}$ nearly identical to that of 1EP but the K_I for 2EN was nearly 200 times greater than that of 1EP. 1EN had both a longer limiting $T_{1/2}$ and a higher K_I, while 9EA produced only slight suicide inhibition of the P450 1A1 isozyme.

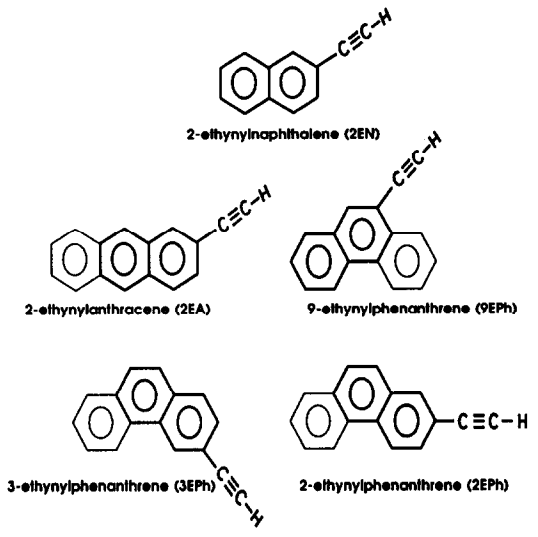

Fig. 4. Illustration to emphasize that 2EA, 9EPh, 3EPh and 2EPh can all be derived from 2EN by the addition of another benzo ring. The added ring in 9EPh enhanced the suicide inhibition of 7PPO de-pentylase activity but 2EA, 3EPh and 2EPh were not suicide inhibitors of this system.

1EP was most likely a better inhibitor of P450 1A1 because the larger polycyclic aromatic system produces a more favourable binding to the large active site region proposed for this isozyme. In at least one accessible orientation, the ethynyl group of 1EP was properly positioned to cause suicide inhibition. In contrast, 2EP did not produce a suicide inhibition of P450 1A1. This experimental result again emphasizes the importance of the proper placement of the acetylenic group on the polycyclic aromatic system to produce the selective suicide inhibition of P450 dependent isozymes.

3EPh and 2EPh could also be bound within the active site of P450 1A1 with the ethynyl group properly positioned to cause suicide inhibition, but, on the other hand, 9EPh, with the ethynyl group attached to a different ring of the phenanthrene ring system could not. The contrast with the effect of 9EPh on P450 2B1 was especially striking.

P450 isozymes show extensive overlap in their ability to metabolize different substrates. Nevertheless a recent review [13] emphasizes the possibility of identifying substrates that are preferentially metabolized by individual P450 isozymes. Because of the precise orientation required for a terminal acetylene to produce suicide inhibition of a P450 isozyme without concomitant heme destruction, potential acetylenic suicide inhibitors can also be used to differentiate between P450 isozymes and to establish some distinguishing geometric features of the active site regions of these isozymes. Covey *et al.* [40] established that a precise complementary fit is required for a terminal acetylene to produce suicide inhibition of a P450 dependent enzyme when they discovered that only the 10-[(1*S*)-1-hydroxy-2-propynyl]estr-4-ene-3,17-dione enantiomer produces suicide of human placental atomatase, even though both the 1*S* and 1*R* enantiomers are potent inhibitors of this enzymatic activity. We have extended these observations and have demonstrated that changes in the placement of an ethynyl substituent on a planar polycyclic aromatic ring system can produce selective suicide inhibition of particular P450 isozymes. Further development of structure–activity relationships in this area should make it possible to design relatively simple molecules that will be more specific suicide inhibitors of the P450 isozymes. Such reagents would be useful in mapping, indirectly, the active site regions of different P450 dependent enzymes and should also function as effective inhibitors of the metabolic activation of procarcinogens and promutagens catalyzed by some P450 isozymes.

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