0026-895X/99/040760-08

MOLECULAR PHARMACOLOGY, **56:**760–767 (1999).

Inhibition of Aryl Hydrocarbon-Induced Cytochrome P-450 1A1 Enzyme Activity and *CYP1A1* Expression by Resveratrol

HENRY P. CIOLINO and GRACE CHAO YEH

Cellular Defense and Carcinogenesis Section, Basic Research Laboratory, Division of Basic Sciences, National Cancer Institute, Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, Maryland

Received March 31, 1999; accepted July 11, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

We investigated the effect of resveratrol, a constituent of the human diet that has been shown to inhibit aryl hydrocarbon-induced carcinogenesis in animals, on the carcinogen activation pathway regulated by the aryl hydrocarbon receptor. Resveratrol inhibited the metabolism of the environmental aryl hydrocarbon benzo[*a*]pyrene (B[a]P) catalyzed by microsomes isolated from B[a]P-treated human hepatoma HepG2 cells. Resveratrol competitively inhibited, in a concentration-dependent manner, the activity of the carcinogen activating enzymes cytochrome P-450 (CYP)1A1/CYP1A2 in microsomes and intact HepG2 cells. Resveratrol inhibited the B[a]P-induced expression of the *CYP1A1* gene, as measured at the mRNA and transcriptional levels. Resveratrol abolished the binding of B[a]P-activated nuclear aryl hydrocarbon receptor to the xenobiotic-responsive element of the *CYP1A1* promoter but did not itself bind to the receptor. Resveratrol was also effective in inhibiting *CYP1A1* transcription induced by the aryl hydrocarbon dimethylbenz[*a*]anthracene in human mammary carcinoma MCF-7 cells. These data demonstrate that resveratrol inhibits aryl hydrocarbon-induced CYP1A activity in vitro by directly inhibiting CYP1A1/1A2 enzyme activity and by inhibiting the signal transduction pathway that up-regulates the expression of carcinogen activating enzymes. These activities may be an important part of the chemopreventive activity of resveratrol in vivo.

Resveratrol (*trans*-3′,4′,5-trihydroxystilbene) is a phytochemical found in grapes and grape products such as wine, as well as other food items, and thus is a constituent of human diets (Soleas et al., 1997). Resveratrol exerts potent antioxidant and anti-inflammatory activities, which may be responsible for the beneficial effects of wine consumption in the prevention of cardiovascular disease (Constant, 1997). Recently, resveratrol was shown to inhibit the formation of preneoplastic lesions in mammary glands and block tumorigenesis in a two-stage model of skin cancer in mice exposed to the model aryl hydrocarbon (AH) dimethylbenz[*a*]anthracene (Jang et al., 1997). Potent carcinogens, AHs are activated to genotoxic metabolites by the cytochrome P-4501A (CYP1A) family of enzymes, which catalyze the oxidation of the hydrocarbon to a variety of diol epoxides, which are potent binders of DNA (Szeliga and Dipple, 1998; Guengerich and Shimada, 1998). The induction of *CYP1A1* gene transcription by AHs begins by their binding and activating the aryl hydrocarbon receptor (AHR), a cytosolic protein that, on ligand binding, translocates to the nucleus and with its partner, the aryl hydrocarbon nuclear translocator, interacts with the promoter of the *CYP1A1* gene (Rowlands and Gustafsson, 1997). This results in an up-regulation of transcription and a subsequent increase in CYP1A1 mRNA and enzyme levels. Inhibition of AHR-mediated signal transduction or CYP1A enzyme activity may be important mechanisms in the chemopreventive effect of several dietary and synthetic compounds that have been associated with a reduced risk of carcinogenesis (Wattenberg, 1996; Ciolino et al., 1998a; Singh et al., 1998).

Recently, we demonstrated that resveratrol inhibits the induction of *CYP1A1* transcription by the halogenated hydrocarbon tetrachlorodibenzo-*p*-dioxin (TCDD) (Ciolino et al., 1998b). TCDD is the most potent known ligand of the AHR, but there are several other AHR ligands that are important carcinogens. Unlike TCDD, these carcinogens, the AHs, are activated by the CYP1A enzymes to genotoxic metabolites. The goals of the current study were to determine whether resveratrol also inhibits AHR-mediated signal transduction caused by AHs, to examine the effect of resveratrol on AH metabolism, and to determine whether resveratrol directly inhibits CYP1A enzyme activity. We therefore examined the

ABBREVIATIONS: AH, aryl hydrocarbon; AHR, aryl hydrocarbon receptor; B[a]P, benzo[*a*]pyrene; CAT, chloramphenicol acetyltransferase; CYP, cytochrome P-450; DMBA, dimethylbenz[*a*]anthracene; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; EROD, ethoxyresorufin-*O*-deethylase; ETRF, ethoxyresorufin; β-Gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; poly(dI/dC), poly(deoxyinosinic/deoxycytidylic acid); RT, reverse transcription; PCR, polymerase chain reaction; TBE, Tris/borate/EDTA; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

effect of resveratrol on CYP1A enzymatic activity and *CYP1A1* expression induced by the AH benzo[*a*]pyrene (B[a]P) in HepG2 human hepatoma cells. B[a]P is a potent environmental carcinogen to which humans are exposed in cigarette smoke, industrial byproducts, and cooked meat (Lijinsky, 1991; Petruzzelli et al., 1998). We demonstrate that resveratrol inhibits B[a]P metabolism by directly inhibiting CYP1A enzymatic activity. Resveratrol also inhibits the B[a]P-induced increase in *CYP1A1* expression, thus preventing an increase in carcinogen bioactivation capacity, by blocking the binding of the activated AHR with the *CYP1A1* promoter. Resveratrol was equally effective in inhibiting *CYP1A1* expression and enzyme activity of another AH, the mammary carcinogen dimethylbenzanthracene (DMBA), in MCF-7 human mammary epithelial carcinoma cells. Thus, resveratrol may be a potent chemopreventive agent in vivo as a result of its inhibitory effects on both CYP1A enzymatic activity and AHR-mediated signal transduction.

Experimental Procedures

Materials. HepG2 and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640, glutamine, FBS, trypsin/EDTA, and PBS were purchased from BioFluids (Rockville, MD). B[a]P, DMBA, resveratrol, HEPES, EDTA, dithiothreitol (DTT), glycerol, poly(deoxyinosinic/deoxycytidylic acid) [poly(dI/dC)], sodium molybdate, ethoxyresorufin (ETRF), resorufin, Tris · HCl, and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]B[a]P (specific activity, 74 Ci/mmol) and [^{3}H]resveratrol (specific activity, 7.2 Ci/mmol) were obtained from Moravek (Brea, CA). [^{32}P]dCTP and [^{32}P]dATP were obtained from DuPont-New England Nuclear (Boston, MA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a kit from Stratagene (La Jolla, CA). Tris/borate/EDTA (TBE) gels, TBE running buffer, and high-density sample buffer were obtained from Novex (San Diego, CA). LipofectAMINE and TRIzol reagent were purchased from Life Technologies (Rockville, MD). The chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay kit was obtained from Boehringer Mannheim (Indianapolis, IN). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR and pCMV vector containing β-galactosidase (β-Gal) were obtained from Clontech (Palo Alto, CA). Aquasure was obtained from Packard (Meriden, CT). The Bradford protein assay kit was purchased from Bio-Rad (Hercules, CA). Resveratrol and B[a]P were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −80°C. Polyclonal antibody to the AHR was the kind gift of Dr. Alan Poland (Centers for Disease Control/National Institute for Occupational Safety and Health, Morgantown, WV).

Cell Culture. HepG2 and MCF-7 cells were grown in RPMI 1640 supplemented with 2 mM glutamine and 10% FBS (growth medium). Cells were subcultured weekly using 0.25% trypsin/0.05% EDTA. All experiments were carried out at 37°C and 5% CO_2 on confluent cells in growth medium except where noted.

B[a]P Metabolism. HepG2 cells were treated with 1 μM B[a]P for 24 h. Microsomes were prepared as described previously (Ciolino et al., 1998a). Microsomes (400 μg) were incubated with 5 nM [^{3}H]B[a]P and 1 mM NADPH in the presence of DMSO or resveratrol for 10 min, and the amount of [^{3}H]B[a]P was metabolized to water-soluble compounds was determined as described by Chae et al. (1991). As a negative control, microsomes isolated from untreated HepG2 cells were tested in a similar manner: there was no [^{3}H]B[a]P metabolized by these microsomes.

Microsomal CYP1A1/1A2 Activity. CYP1A1/1A2 activity was determined by ethoxyresorufin-*O*-deethylase (EROD) activity assay in the following manner: 10 μg of microsomes were brought up to 100 μl with PBS, pH 7.2. ETRF (400 nM) was added, along with DMSO or the indicated concentrations of resveratrol. The reaction was initiated by the addition of 250 μM NADPH. The reaction mixture was transferred to a 96-well plate, and EROD activity was determined in a CytoFluor II multiwell fluorescence plate reader (PerSeptive Biosystems, Framingham, MA), with an excitation wavelength of 530 nm and emission at 590 nm. For Fig. 3B, 250 μM NADPH and 100 to 1600 nM ETRF were added to 3 ml of PBS, pH 7.2. Aliquots of 410 μl were removed to which DMSO or the indicated concentration of resveratrol was added. The reaction was initiated by the addition of 45 μg of microsomal protein (final volume, 450 μl) and gently vortexed. Four 100-μl aliquots (10 μg/assay) of each were removed and placed in a 96-well plate, and EROD activity was determined. A standard curve was constructed using resorufin.

CYP1A1/1A2 Enzyme Activity in Intact Cells. Confluent cells in 24-well plates were treated with 1 ml of growth medium containing 100 nM B[a]P for HepG2 or 500 nM DMBA for MCF-7 for 9 h in the presence of DMSO or the indicated concentrations of resveratrol. At the end of the incubation, the medium was removed, and the wells were washed two times with fresh growth medium. EROD activity was determined in intact cells as described by Kennedy and Jones (1994) using 5 μM ETRF in growth medium as a substrate in the presence of 1.5 mM salicylamide to inhibit conjugating enzymes. The assay was carried out at 37°C. The fluorescence of resorufin generated from the conversion of ETRF by CYP1A1/1A2 was measured every 10 min for 60 min as described above.

RT-PCR. Confluent cells were treated with DMSO (control), 100 nM B[a]P (HepG2), or 500 nM DMBA (MCF-7) in the presence of DMSO or resveratrol for 6 h. The cells were washed twice with PBS, and total RNA was isolated using TRIzol reagent. cDNA was synthesized from 10 μg of total RNA using a Stratagene RT-PCR kit as instructed. Semiquantitative PCR for CYP1A1, CYP1A2, and GAPDH was performed in the presence of 1.5 μCi of [^{32}P]dATP. Hot start was performed by premixing AmpliTaq (Perkin–Elmer, Foster City, CA) with anti-*Taq* antibody (Clontech). Primer sequences and PCR conditions for CYP1A1 were as described by Dohr et al. (1995), and those for CYP1A2 were as described by Chung and Bresnick (1994). Primer sequences for GAPDH were from Clontech, and PCR was carried out as directed. The optimum cycle number that fell within the exponential range of response for CYP1A1 (22 cycles), CYP1A2 (25 cycles), or GAPDH (17 cycles) was used. After PCR, 5 μl of high-density sample buffer was added to the samples, and they were subjected to electrophoresis on a 10% TBE gel in 1× TBE running buffer. The gel was dried, and the results were visualized and quantified on a Bio-Rad GS-363 Molecular Imaging System (Hercules, CA). Graphs of the resulting data were generated by normalizing CYP1A1 and CYP1A2 to GAPDH.

CAT/β-Gal Assays. Cells were plated at 60,000 cells/well in 24-well plates. After 24 h, the cells were transiently transfected with 12.0 μg of a CAT reporter vector containing the full-length rat *CYP1A1* promoter (Sogawa et al., 1986) using LipofectAMINE as directed. To control for transfection efficiency, the cells were cotransfected with 1.0 μg of pCMV vector containing β-Gal. After an additional 24 h, the cells were treated with DMSO (control), 250 nM B[a]P (HepG2), or 500 nM DMBA (MCF-7) in the presence of DMSO (control) or resveratrol for 6 h. The amount of CAT transcription was determined using an enzyme-linked immunosorbent assay as directed. Transcription of β-Gal was determined by measuring enzyme activity according to the method of Rosenthal (1987). The amount of CAT transcription was normalized to β-Gal transcription.

Electrophoretic Mobility Shift Assay (EMSA) for AHR. HepG2 cells were treated with DMSO, 1 μM B[a]P, or 1 μM B[a]P in the presence of 5 or 10 μM resveratrol in growth media for 2 h. Nuclear protein was isolated, and EMSA was performed according to the method of Denison et al. (1988). Synthetic oligonucleotides containing the AHR-binding site of the xenobiotic response element (XRE) of the *CYP1A1* promoter (Chen and Tukey, 1996) were labeled with [^{32}P]dCTP. The binding reactions were carried out for 30 min and contained 5 μg of nuclear protein, 1 μg of poly(dI/dC), and

~50,000 cpm of labeled probe in a final volume of 20 μl of binding buffer (25 mM Tris, pH 7.9, 50 mM KCl, 1 mM $MgCl_2$, 1.5 mM EDTA, 0.5 mM DTT, and 5% glycerol). To determine specificity of binding to the oligonucleotide, a 200-fold excess of unlabeled XRE or 0.9 μg of a polyclonal antibody to human AHR was added to extract from B[a]P-treated cells. DNA-protein complexes were separated under nondenaturing conditions on a 4% polyacrylamide gel using 0.5× TBE (45 mM Tris, pH 7.5, 45 mM boric acid, 2 mM EDTA) as a running buffer. The gels were dried, and the DNA-protein complexes were visualized on a Bio-Rad GS-363 Molecular Imaging System.

AHR Ligand-Binding Assay. HepG2 cells were grown to confluence in 175-cm^2 flasks. The cells were washed once in PBS, harvested by trypsination, and pelleted by centrifugation at 800*g* for 10 min at 4°C. The pellet was washed once in cold PBS, repelleted as above, and resuspended in cold buffer (25 mM HEPES, 1 mM EDTA, 1 mM DTT, 20 mM sodium molybdate, and 10% glycerol, pH 7.4), with protease inhibitors as described above. The cells were homogenized by 30 strokes with a Dounce glass homogenizer on ice, and the homogenate was centrifuged at 100,000*g* for 60 min at 4°C. The supernatant (cytosol) was removed, and protein was assayed according to the method of Bradford (1976). The cytosol was aliquoted and stored at −80°C. Specific binding to the AHR was measured by sucrose density gradient centrifugation as described by Raha et al. (1990). One mg of cytosolic protein was incubated with 5 nM [^{3}H]B[a]P in the presence of DMSO (control), 5 μM B[a]P (positive control), or 0.5 or 5 μM resveratrol in a total volume of 500 μl of the above buffer for 2 h at 4°C. Samples were applied to 5 to 30% (w/v) linear sucrose density gradients in 12 ml of Beckman Quick-Seal rotor tubes (Palo Alto, CA). The gradients were centrifuged at 4°C for 2 h at 63,000 rpm (372,000*g*) in a Beckman VTI-65-1 rotor. Twenty-five fractions of 7 drops each (~500 μl) were collected from the bottom of the tubes and assayed for radioactivity using Aquasure scintillation fluid. The gradients were calibrated with catalase (11S; fraction 10) and BSA (4S; fraction 20).

Statistical Analysis. Statistical analyses were performed using StatView Statistical Analysis software (SAS Institute, San Francisco, CA). Differences between group mean values were determined by a one-factor ANOVA, followed by Fisher's protected least-significant difference post hoc analysis for pairwise comparison of mean values.

Results

Effects of Resveratrol on Metabolism of B[a]P. Incubation of [^{3}H]B[a]P with microsomes isolated from B[a]P-induced HepG2 cells and NADPH for 10 min resulted in the almost complete conversion of B[a]P to water-soluble metabolites (data not shown). This conversion results solely from CYP1A activity because the assay mixtures lacked cofactors for phase 2 (conjugating) enzymes (Chae et al., 1991). The addition of resveratrol caused a concentration-dependent decrease in the amount of B[a]P converted to water-soluble metabolites (Fig. 1).

Effect of Resveratrol on Activity of CYP1A. We analyzed the effect of resveratrol on the activity of CYP1A using the EROD assay, which is specific for the bioactivation capacity of the CYP1A enzyme family. To test whether resveratrol directly inhibits EROD activity, we examined the effect of resveratrol on EROD activity in microsomes isolated from induced cells. As shown in Fig. 2A, resveratrol inhibited microsomal EROD activity, with an IC_{50} value of approximately 1 μM. Analysis of the kinetics of inhibition by double-reciprocal (Lineweaver-Burk) plot demonstrated that the K_m value of the enzyme toward the substrate was increased in the presence of resveratrol (Fig. 2B). The K_i value was calculated as 0.42 μM.

We also examined the effect of resveratrol on EROD activity in intact cells. In untreated HepG2 cells, there was no detectable EROD activity (data not shown). B[a]P caused an increase in EROD activity to 9.14 ± 0.43 pmol/min/well. This was inhibited by resveratrol in a concentration-dependent manner, with an IC_{50} value similar to that seen in the microsomal assay (Fig. 3).

Effect of Resveratrol on Expression of *CYP1A1*. The treatment of HepG2 cells with B[a]P resulted in an approximately 6-fold increase in the mRNA levels of CYP1A1 and CYP1A2, the two major carcinogen activating enzymes in these cells (Fig. 4). The increase in both CYP1A1 and CYP1A2 mRNA was inhibited by resveratrol in a concentration-dependent manner. The transcription of an AH-sensitive CAT reporter vector that contains the *CYP1A1* promoter was examined. The treatment of cells transiently transfected with this vector with B[a]P caused a 5-fold increase in CAT transcription, which was inhibited by resveratrol in a concentration-dependent manner (Fig. 5).

Mechanism of Inhibition of *CYP1A1* Expression. We examined the effect of resveratrol on the interaction of the B[a]P-activated AHR with the XRE of the *CYP1A1* promoter. As shown in the EMSA in Fig. 6, B[a]P caused an increase in the amount of activated nuclear AHR binding to a ^{32}P-labeled oligonucleotide representing the XRE of *CYP1A1* promoter. The addition of unlabeled XRE to nuclear extracts of B[a]P-treated cells abolished XRE binding, whereas treatment with a polyclonal antibody to human AHR significantly reduced XRE binding, demonstrating the specificity of the gel shift. The increase in XRE binding caused by B[a]P was inhibited by resveratrol.

We examined the effect of resveratrol on the binding of B[a]P to the cytosolic AHR. As shown in Fig. 7, [^{3}H]B[a]P was bound to two peaks in the cytosol, located at approximately the 9S and 5S fractions at which the AHR sediments. The addition of excess unlabeled B[a]P caused a decrease in [^{3}H]B[a]P binding, demonstrating the specificity of binding. Resveratrol, at a 100- or 1000-fold excess compared with [^{3}H]B[a]P, modestly inhibited binding to the AHR. This effect was maximal at a

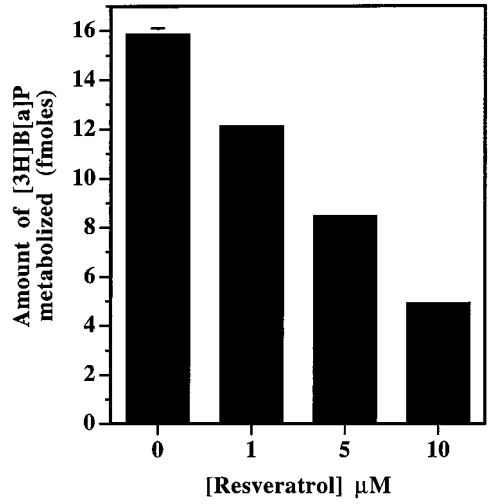

Fig. 1. Effect of resveratrol on B[a]P metabolism. Microsomes from B[a]P-induced HepG2 cells were incubated for 10 min with [^{3}H]B[a]P, and the indicated concentrations of resveratrol and the amount of water-soluble [^{3}H]B[a]P metabolites generated was determined. $n = 4 \pm$ S.E. There was a significant decrease in [^{3}H]B[a]P metabolism in the presence of all concentrations of resveratrol ($p < .05$).

1000-fold excess of resveratrol; greater amounts (up to 10,000-fold excess) did not further reduce [^{3}H]B[a]P binding (data not shown). The treatment of HepG2 cytosol with 5 nM [^{3}H]resveratrol did not result in detectable binding of [^{3}H]resveratrol to any component of the cytosol (Fig. 7).

Effect of Resveratrol on DMBA-Induced *CYP1A1* Expression in MCF-7 Cells. The expression of *CYP1A1* induced by another AH, the mammary carcinogen DMBA, was examined in MCF-7 human mammary epithelial carcinoma cells. The treatment of MCF-7 cells with DMBA resulted in an increase in EROD activity from undetectable levels to 1.20 ± 0.14 pmol/min/well. Resveratrol inhibited DMBA-induced EROD activity in intact MCF-7 cells, with an IC_{50} value of approximately 500 nM (Fig. 8A). Resveratrol also inhibited the induction of CYP1A1 mRNA by DMBA (Fig. 8B) and inhibited *CYP1A1* promoter-controlled transcription (Fig. 8C).

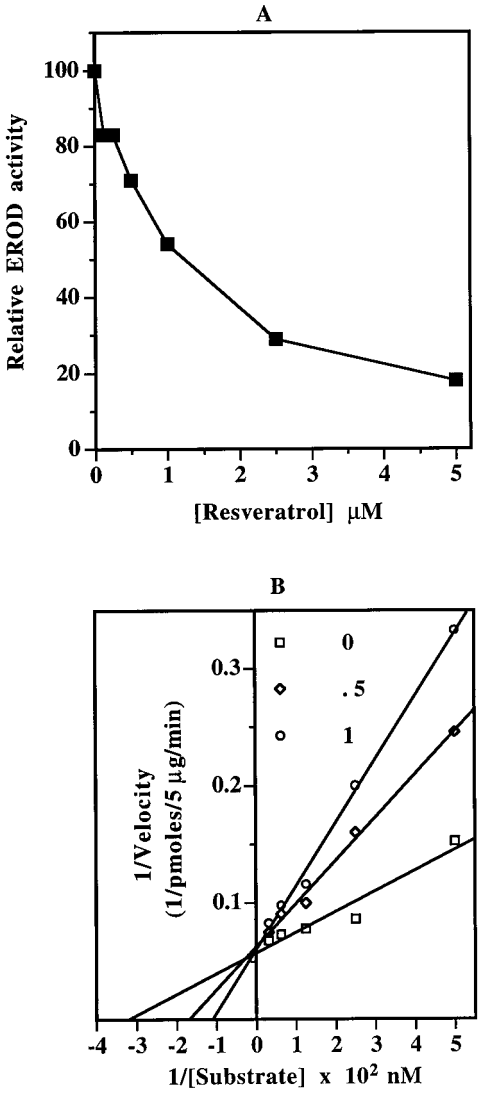

Fig. 2. Effect of resveratrol on microsomal EROD activity. A, EROD activity in 10 μg of microsomes isolated from induced HepG2 cells was determined in the presence of the indicated concentrations of resveratrol. $n = 4 \pm$ S.E. Error bars are less than symbols. There was a significant decrease EROD activity in the presence of all concentrations of resveratrol ($p < .05$). B, EROD activity in 10 μg of microsomes was determined in the presence of the indicated concentrations of ETRF and 0, 0.5, or 1 μM resveratrol. $n = 4$.

Discussion

An AH found in a variety of environmental sources, B[a]P is a potent carcinogen in animal models (Phillips, 1983). It is

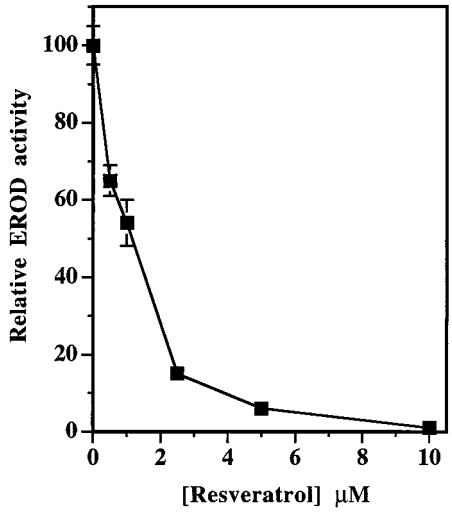

Fig. 3. Effect of resveratrol on cellular EROD activity induced by B[a]P. HepG2 cells were incubated with 100 nM B[a]P for 9 h in the presence of the indicated concentrations of resveratrol and cellular EROD activity was determined. $n = 4 \pm$ S.E. There was a significant decrease EROD activity in the presence of all concentrations of resveratrol ($p < .05$).

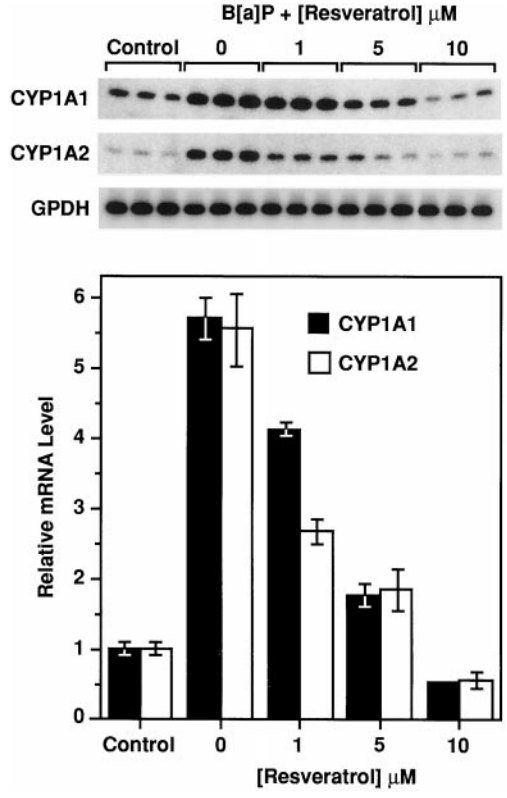

Fig. 4. Effect of resveratrol on CYP1A1 and CYP1A2 mRNA induced by B[a]P. HepG2 cells were treated for 6 h with DMSO (Control) or 100 nM B[a]P in the presence of DMSO or resveratrol at the indicated concentrations. Total RNA was isolated, cDNA was synthesized, and the amount of mRNA was determined by PCR. The results were visualized and quantified by phosphorimaging. For the graph, the levels of CYP1A1 and CYP1A2 were normalized to GAPDH level. $n = 3 \pm$ S.E. There was a significant decrease in CYP1A1 and CYP1A2 mRNA in cells treated with resveratrol ($p < .05$).

regarded as a human carcinogen because B[a]P-DNA adducts have been detected in humans (Petruzzelli et al., 1998) and B[a]P metabolites have been shown to bind to specific DNA residues in the *p53* gene, known to be "hot spots" of mutation in human carcinomas (Puisieux et al., 1991). B[a]P is metabolized to genotoxic derivatives by the action of CYP1A enzymes, which are induced by the activation of the DNA-binding capacity of the AHR for the *CYP1A1* gene promoter by B[a]P. It may be argued that the up-regulation of *CYP1A1* by the AHR is a protective mechanism, in that the AH metabolites generated by CYP1A activity are substrates of many detoxifying enzymes, such as UDP-glucuronosyltransferase (Grove et al., 1997) and glutathione-*S*-transferase (Xia et al., 1998). However, recent experiments using AHR knockout mice have indicated that the lack of the AHR, and thus an absence of *CYP1A1* induction, confers protection from the deleterious effects of B[a]P (Dertinger et al., 1998). This supports previous findings that demonstrated a positive correlation between high levels of AH hydroxylase activity and increased cancer risk (Kellermann et al., 1973; Kouri et al., 1982). Induction of *CYP1A1* by carcinogens via the AHR, therefore, appears to be detrimental to the organism or cell, and consequently inhibition of *CYP1A1* expression and CYP1A1 activity has much promise as a mechanism of chemoprevention.

Epidemiological studies have shown that diets rich in fruits and vegetables are associated with a reduced risk of cancer (Steinmetz and Potter, 1996). Much attention has accordingly been focused on discerning the mechanisms by which phytochemicals inhibit carcinogenesis. One such phytochemical, resveratrol, has been shown to inhibit AH-induced carcinogenesis in mice (Jang et al., 1997). In the present study, we therefore examined the effect of resveratrol on carcinogen activation and the carcinogen activation pathway in HepG2 cells. These cells are derived from human liver and have been extensively used in studies of B[a]P metabo-

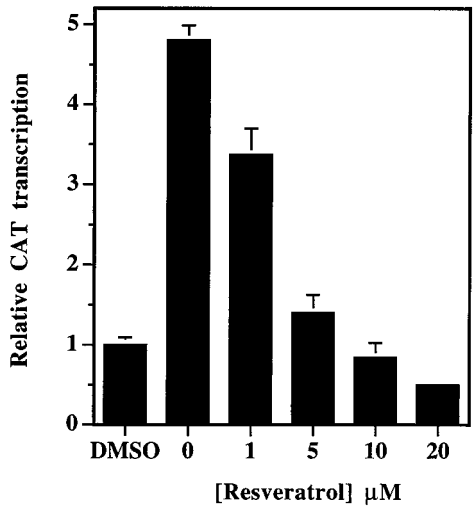

Fig. 5. Effect of resveratrol on B[a]P-induced *CYP1A1* promoter-driven transcription. HepG2 cells were transiently transfected with a CAT reporter vector containing the *CYP1A1* promoter, as well as a vector containing β-Gal, and then treated for 6 h with DMSO or 250 nM B[a]P in the presence of DMSO or resveratrol at the indicated concentrations. The levels of CAT and β-Gal were determined as described in *Experimental Procedures*, and the amount of CAT transcription was normalized to the amount of β-Gal. $n = 4 \pm$ S.E. There was a significant decrease in CAT transcription in the presence of resveratrol ($p < .05$).

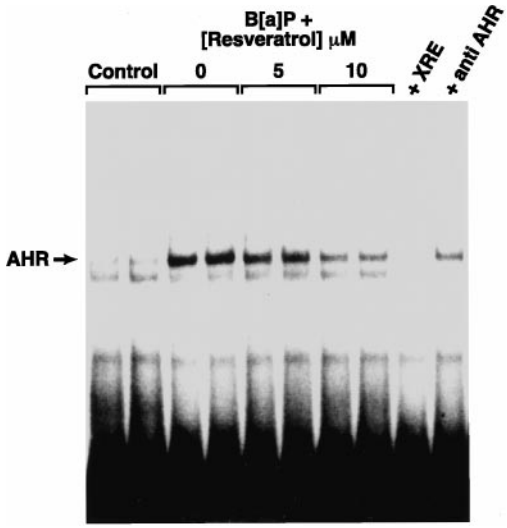

Fig. 6. Effect of resveratrol on B[a]P-induced binding of the AHR to the XRE of *CYP1A1*. HepG2 cells were treated for 2 h with DMSO (control) or 1 μM B[a]P in the presence of DMSO or the indicated concentrations of resveratrol. Nuclear protein was isolated, and the amount of activated AHR in 5 μg of protein was measured by EMSA. For competition studies, nuclear protein from B[a]P-treated cells was preincubated with a 200-fold excess of unlabeled XRE or with 0.9 μg of a polyclonal antibody to the AHR. This experiment was repeated two times with similar result.

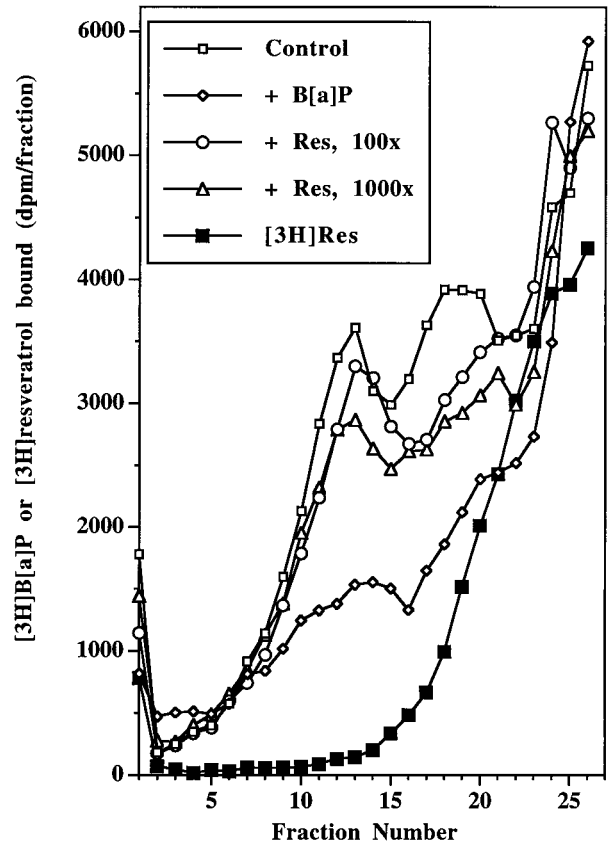

Fig. 7. Effect of resveratrol on the binding of [^{3}H]B[a]P to HepG2 cytosol. HepG2 cytosol was incubated with 5 nM [^{3}H]B[a]P in the presence of DMSO (control), 5 μM unlabeled B[a]P, 0.5 μM (100-fold) resveratrol, or 5 μM (1000-fold) resveratrol or with 5 nM [^{3}H]resveratrol. The specific binding of [^{3}H]B[a]P or [^{3}H]resveratrol was analyzed by sucrose density gradient centrifugation. This experiment was repeated three times with different cytosolic preparations with similar results. Res, resveratrol.

lism (Christou et al., 1992; Polzer et al., 1995) and of the AHR (Kress and Greenlee, 1997; Long et al., 1998).

Resveratrol inhibited the conversion of B[a]P to water-soluble metabolites by microsomes isolated from B[a]P-induced HepG2 cells (Fig. 1). This demonstrates that resveratrol must directly inhibit enzyme activity. We therefore examined the effect of resveratrol on microsomal CYP1A activity by using the EROD assay. Resveratrol inhibited microsomal EROD activity in a concentration-dependent manner (Fig. 2A). An analysis of the kinetics of enzyme inhibition by double-reciprocal (Lineweaver-Burk) plot showed that this inhibition was of a competitive type because the K_m value for the substrate was increased in the presence of resveratrol, but the V_{max} value remained unchanged (Fig. 2B). To test whether resveratrol would also inhibit cellular CYP1A activity, we measured the EROD activity in intact HepG2 cells. The treatment of cells with B[a]P caused an increase in EROD activity that was completely abolished by resveratrol (Fig. 3). This demonstrates that resveratrol is capable of entering the cells and interacting with the enzyme in situ.

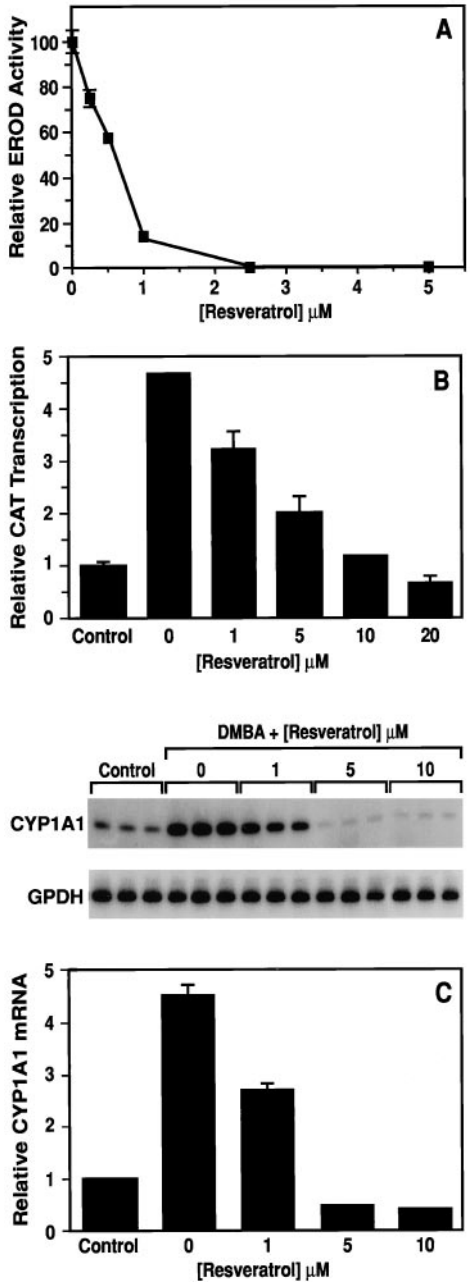

Fig. 8. Effect of resveratrol on DMBA-induced *CYP1A1* expression in MCF-7 cells. A, confluent MCF-7 cells were incubated for 9 h with 500 nM DMBA in the presence of DMSO or the indicated concentrations of resveratrol and the EROD activity was determined. $n = 4 \pm$ S.E. B, MCF-7 cells transfected with the CYP1A1 promoter containing CAT reporter vector were treated for 6 h with 500 nM DMBA and the indicated concentrations of resveratrol. C, MCF-7 cells were incubated with 500 nM DMBA and the indicated concentrations of resveratrol for 6 h, and the amount of CYP1A1 mRNA and GAPDH mRNA was determined by RT-PCR. $n = 3 \pm$ S.E. There was a significant decrease in all parameters in the presence of resveratrol ($p < .05$).

Although the decrease in B[a]P-induced CYP1A1 enzymatic activity in intact cells (Fig. 3) may be due to the direct inhibitory effect of resveratrol toward CYP1A enzymatic activity, it may also result from inhibition of AHR-mediated transcriptional activation. We recently demonstrated that resveratrol inhibits the increase in *CYP1A1* expression caused by the halogenated hydrocarbon TCDD, a ligand of the AHR (Ciolino et al., 1998b). We therefore examined the effect of resveratrol on *CYP1A1* expression induced by the AH B[a]P. The increase in CYP1A1 mRNA in HepG2 cells caused by treatment with B[a]P was inhibited by resveratrol (Fig. 4). To determine whether this inhibition occurred at the transcriptional level, we transfected cells with an AH-responsive CAT reporter vector containing the *CYP1A1* promoter. Treatment of transfected cells with B[a]P resulted in an increase in CAT transcription that was inhibited by resveratrol (Fig. 5). The decrease in CYP1A1 mRNA and *CYP1A1*-promoter driven transcription clearly demonstrates that resveratrol inhibits *CYP1A1* expression induced by B[a]P. Resveratrol also inhibited the increase in CYP1A2 mRNA caused by B[a]P (Fig. 5). Thus, the inhibition of cellular EROD activity shown in Fig. 3 may result not only from a direct inhibitory effect but also from inhibition of *CYP1A1* transcription.

To determine the mechanism of this transcriptional repression, we carried out gel shift assays to measure the amount of AHR that had been transformed to its nuclear, DNA-binding form. AHR, when activated by a ligand such as B[a]P, interacts with the XRE of the *CYP1A1* promoter, inducing transcription. As shown in Fig. 6, resveratrol inhibits the binding of B[a]P-activated nuclear AHR to ^{32}P-labeled XRE. Thus, inhibition of *CYP1A1* transcription by resveratrol is due to an inhibition of AHR-XRE interaction. This is in agreement with our previous results using TCDD to activate the AHR (Ciolino et al., 1998b). This demonstrates that resveratrol inhibits AHR-mediated signal transduction by preventing the binding of the activated receptor to the gene promoter.

Several inhibitors of *CYP1A1* transcription are known to function by binding to the ligand binding site of the AHR, blocking the binding of other ligands and thereby preventing AHR activation (Santostefano et al., 1993; Ciolino et al., 1998a). To determine whether the inhibition of AHR-XRE binding by resveratrol shown in Fig. 6 results from blocking binding of B[a]P to the AHR, HepG2 cytosol was incubated with [^{3}H]B[a]P in the presence of excess resveratrol and the specific binding was measured using sucrose density gradient centrifugation. As shown in Fig. 7, resveratrol caused a modest decrease in [^{3}H]B[a]P binding to two peaks in the gradient. This was reproducible in several different cytosolic

preparations (data not shown). However, even greater amounts of resveratrol (>1000-fold excess) did not result in a greater inhibition of binding, and [^{3}H]B[a]P binding was not reduced to the level present in an excess of unlabeled B[a]P. Furthermore, incubation of cytosol with [^{3}H]resveratrol demonstrated that there was no binding of resveratrol to any cytosolic component, indicating that resveratrol does not itself bind to the receptor (Fig. 7). The complete inhibition of B[a]P-induced *CYP1A1* transcription and AHR-XRE interaction by resveratrol demonstrated in Figs. 4, 5, and 6 occurred at concentrations of resveratrol, relative to B[a]P, much less than that required to cause partial inhibition of B[a]P binding. Thus, the inhibitory effect of resveratrol on AHR activation, and hence on *CYP1A1* expression, although it may partially result from an inhibition of B[a]P-AHR binding, would appear to be primarily through an indirect mechanism. This is consistent with our previous study (Ciolino et al., 1998b), which demonstrated that resveratrol does not inhibit the binding of TCDD to cytosolic AHR and that resveratrol inhibits the basal level of *CYP1A1* transcription in the absence of exogenous ligand. The nature of this indirect mechanism of AHR inhibition is currently under study.

Our previous study (Ciolino et al., 1998b) and current studies were carried out in HepG2 cells, a cell line derived from human liver. To test whether the inhibitory effect of resveratrol on *CYP1A1* transcription would occur in another cell line, we carried out several experiments in MCF-7 cells. The AHR pathway has also been well characterized in these cells (Moore et al., 1994; Dohr et al., 1995) and is similar to that found in normal human mammary cells in vitro (Larsen et al., 1998). Because these cells are derived from human mammary epithelial cells, we used the mammary carcinogen DMBA as a *CYP1A1* inducer, allowing us to examine not only another cell line but also another AHR ligand. As shown in Fig. 8A-C, compared with its effect on B[a]P-induced *CYP1A1* expression in HepG2 cells, resveratrol is even more effective an inhibitor of DMBA-induced CYP1A1 enzyme activity and CYP1A1 mRNA and equally effective at inhibiting DMBA-induced *CYP1A1* transcription in MCF-7 cells. Thus, resveratrol is a potent inhibitor of *CYP1A1* expression induced by different AHs in different cells types. Such an inhibitory effect may be responsible for the chemopreventive activity of resveratrol toward DMBA-induced mammary neoplastic changes as reported by Jang et al. (1997).

These experiments demonstrate that resveratrol affects the carcinogen activation pathway in vitro at two levels: it directly inhibits the activity of carcinogen activation enzymes, and it inhibits the increase in *CYP1A1* expression caused by AHs. In concert, these two activities may be responsible for the chemopreventive effect of resveratrol toward AH-induced carcinogenesis.

Acknowledgments

We thank Mr. Phillip J. Daschner for his help with EMSA and Dr. Lucy Anderson for her careful reading of the manuscript.

References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Chae YH, Marcus CB, Ho DK, Cassady JM and Baird WM (1991) Effects of synthetic and naturally occurring flavonoids on benzo[a]pyrene metabolism by hepatic microsomes prepared from rats treated with cytochrome P-450 inducers. *Cancer Lett* **60:**15–24.

Chen YH and Tukey RH (1996) Protein kinase C modulates regulation of the CYP1A1 gene by the aryl hydrocarbon receptor. *J Biol Chem* **271:**26261–26266.

Christou M, Mitchell MJ, Aoyama T, Gelboin HV, Gonzalez FJ and Jefcoate CR (1992) Selective suppression of the catalytic activity of cDNA-expressed cytochrome P4502B1 toward polycyclic hydrocarbons in the microsomal membrane: Modification of this effect by specific amino acid substitutions. *Biochemistry* **31:** 2835–41.

Chung I and Bresnick E (1994) 3-Methylcholanthrene-mediated induction of cytochrome P4501A2 in human hepatoma HepG2 cells as quantified by the reverse transcription-polymerase chain reaction. *Arch Biochem Biophys* **314:**75–81.

Ciolino HP, Daschner PJ, Wang TT and Yeh GC (1998a) Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem Pharmacol* **56:**197–206.

Ciolino HP, Daschner PJ and Yeh GC (1998b) Resveratrol inhibits transcription of *CYP1A1 in vitro* by preventing activation of the aryl hydrocarbon receptor. *Cancer Res* **58:**5707–5712.

Constant J (1997) Alcohol, ischemic heart disease, and the French paradox. *Coron Artery Dis* **8:**645–649.

Denison MS, Fisher JM and Whitlock JP Jr (1988) The DNA recognition site for the dioxin-Ah receptor complex: Nucleotide sequence and functional analysis. *J Biol Chem* **263:**17221–17224.

Dertinger SD, Silverstone AE and Gasiewicz TA (1998) Influence of aromatic hydrocarbon receptor-mediated events on the genotoxicity of cigarette smoke condensate. *Carcinogenesis* **19:**2037–2042.

Dohr O, Vogel C and Abel J (1995) Different response of 2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD]-sensitive genes in human breast cancer MCF-7 and MDA-MB 231 cells. *Arch Biochem Biophys* **321:**405–412.

Grove AD, Kessler FK, Metz RP and Ritter JK (1997) Identification of a rat oltipraz-inducible UDP-glucuronosyltransferase (UGT1A7) with activity towards benzo-(a)pyrene-7,8-dihydrodiol. *J Biol Chem* **272:**1621–1627.

Guengerich FP and Shimada T (1998) Activation of procarcinogens by human cytochrome P450 enzymes. *Mutat Res* **400:**201–213.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC and Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science (Wash DC)* **275:**218–220.

Kellermann G, Shaw CR and Luyten-Kellermann M (1973) Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. *N Engl J Med* **289:**934–937.

Kennedy S and Jones S (1994) Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal Biochem* **222:**217–223.

Kouri RE, McKinney CE, Slomiany DJ, Snodgrass DR, Wray NP and McLemore TL (1982) Positive correlation between high aryl hydrocarbon hydroxylase activity and primary lung cancer as analyzed in cryopreserved lymphocytes. *Cancer Res* **42:**5030–5037.

Kress S and Greenlee WF (1997) Cell-specific regulation of human CYP1A1 and CYP1B1 genes. *Cancer Res* **57:**1264–1269.

Larsen MC, Angus WG, Brake PB, Eltom SE, Sukow KA and Jefcoate CR (1998) Characterization of CYP1B1 and CYP1A1 expression in human mammary epithelial cells: Role of the aryl hydrocarbon receptor in polycyclic aromatic hydrocarbon metabolism. *Cancer Res* **58:**2366–2374.

Lijinsky W (1991) The formation and occurrence of polynuclear aromatic hydrocarbons associated with food. *Mutat Res* **259:**251–261.

Long WP, Pray-Grant M, Tsai JC and Perdew GH (1998) Protein kinase C activity is required for aryl hydrocarbon receptor pathway-mediated signal transduction. *Mol Pharmacol* **53:**691–700.

Moore M, Wang X, Lu YF, Wormke M, Craig A, Gerlach JH, Burghardt R, Barhoumi R and Safe S (1994) Benzo[a]pyrene-resistant MCF-7 human breast cancer cells: A unique aryl hydrocarbon-nonresponsive clone. *J Biol Chem* **269:**11751–11759.

Petruzzelli S, Celi A, Pulera N, Baliva F, Viegi G, Carrozzi L, Ciacchini G, Bottai M, Di Pede F, Paoletti P and Giuntini C (1998) Serum antibodies to benzo(a)pyrene diol epoxide-DNA adducts in the general population: Effects of air pollution, tobacco smoking, and family history of lung diseases. *Cancer Res* **58:**4122–4126.

Phillips DH (1983) Fifty years of benzo(a)pyrene. *Nature (Lond)* **303:**468–472.

Polzer RJ, Coffing SL, Marcus CB, Park SS, Gelboin HV and Baird WM (1995) Inhibition of benzo[a]pyrene metabolism by insulin, FITC-insulin and an FITC-insulin-antibody conjugate in the human hepatoma cell line HepG2. *Chem Biol Interact* **97:**307–318.

Puisieux A, Lim S, Groopman J and Ozturk M (1991) Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens. *Cancer Res* **51:**6185–6189.

Raha A, Reddy V, Houser W and Bresnick EJ (1990) Binding characteristics of 4S PAH-binding protein and Ah receptor from rats and mice. *Toxicol Environ Health* **29:**339–355.

Rosenthal N (1987) Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol* **152:**704.

Rowlands JC and Gustafsson JA (1997) Aryl hydrocarbon receptor-mediated signal transduction. *J Crit Rev Toxicol* **27:**109–134.

Santostefano M, Merchant M, Arellano L, Morrison V, Denison MS and Safe S (1993) α-Naphthoflavone-induced CYP1A1 gene expression and cytosolic aryl hydrocarbon receptor transformation. *Mol Pharmacol* **43:**200–206.

Singh SV, Benson PJ, Hu X, Pal A, Xia H, Srivastava SK, Awasthi S, Zaren HA, Orchard JL and Awasthi YC (1998) Gender-related differences in susceptibility of A/J mouse to benzo[a]pyrene-induced pulmonary and forestomach tumorigenesis. *Cancer Lett* **128:**197–204.

Sogawa K, Fujisawa-Sehara A, Yamane M and Fujii-Kuriyama Y (1986) Location of regulatory elements responsible for drug induction in the rat cytochrome P-450c gene. *Proc Natl Acad Sci USA* **83:**8044–8048.

Soleas GJ, Diamandis, EP and Goldberg DM (1997) Wine as a biological fluid: History, production, and role in disease prevention. *J Clin Lab Anal* **11:**287–313.
Steinmetz KA and Potter JD (1996) Vegetables, fruit, and cancer prevention: A review. *J Am Diet Assoc* **96:**1027–39.
Szeliga J and Dipple A (1998) DNA adduct formation by polycyclic aromatic hydrocarbon dihydrodiol epoxides. *Chem Res Toxicol* **11:**1–11.
Wattenberg LW (1996) Inhibition of tumorigenesis in animals. *IARC Sci Publ* **139:** 151–158.
Xia H, Pan SS, Hu X, Srivastava SK, Pal A and Singh SV (1998) Cloning, expression, and biochemical characterization of a functionally novel alpha class glutathione S-transferase with exceptional activity in the glutathione conjugation of (+)-anti-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene. *Arch Biochem Biophys* **353:**337–348.

Send reprint requests to: Dr. Henry P. Ciolino, Basic Research Laboratory, Building 560/Room 12-05, National Cancer Institute-Frederick Cancer Research and Development Center, National Institutes of Health, Frederick, MD 21702-1201. E-mail: hciolino@mail.ncifcrf.gov