ELLAGIC ACID TOXICITY AND INTERACTION WITH BENZO[A]PYRENE AND BENZO[A]PYRENE 7,8-DIHYDRODIOL IN HUMAN BRONCHIAL EPITHELIAL CELLS

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Ellagic acid, a plant phenol present in various foods consumed by humans, has been reported to have both anti-mutagenic and anti-carcinogenic potential. To evaluate the potential anti-carcinogenic property of ellagic acid, we tested its effects on the toxicity of benzo[a]pyrene and benzo[a]pyrene, 7,8-dihydrodiol and binding of benzo[a]pyrene to DNA in cultured human bronchial epithelial cells. The toxicity of ellagic acid itself for human bronchial epithelial cells was also determined. Using a colony-forming efficiency assay, it was found that a nontoxic concentration of ellagic acid (5 μg/ml) enhanced the toxicity of benzo[a]pyrene 7,8-dihydrodiol in human bronchial epithelial cells. In contrast, ellagic acid at concentrations of 1.5 and 3.0 μg/ml inhibited binding of benzo[a]pyrene metabolites to DNA in these cells. An explanation for the potentiating effect of ellagic acid on the toxicity of benzo[a]pyrene, 7,8-dihydrodiol will require further investigation into the possible mechanisms of interaction between these two compounds.

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2. Key words: benzo[a]pyrene metabolites, ellagic acids, human bronchial epithelial cells, toxicity.

3. Abbreviations: B[a]P, benzo[a]pyrene; B[a]P 7,8-DHD, (±)trans-7,8-dihydro-7,8-dihydroxybenzo[a]pyrene; B[a]PDE-1, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]PDE-2, (±) 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]PDE-1:dG, N^2-}10-{7β,8β,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]yl}:deoxyguanosine; B[a]PDE-2:dG, N^2-{10α-{7β,8α,9β-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]yl}:deoxyguanosine; CFE, colony forming efficiency; EA, ellagic acid; HBE, human bronchial epithelial.

INTRODUCTION

Several plant phenols naturally available in the human diet possess chemical structures that render them capable of acting as strong nucleophiles toward metabolites of certain polycyclic aromatic hydrocarbons (Newmark, 1984). One of these phenols, ellagic acid (EA), may act as a scavenger since it has been shown to form an adduct with benzo[a]pyrene (B[a]P) 7,8-diol-9,10-epoxide in aqueous solutions (Sayer et al., 1982). Wood et al. (1982) showed that this interaction inhibited the mutagenicity of B[a]P diol-epoxide in several strains of *Salmonella typhimurium* and in hamster V-79 cells. When painted onto the skin, EA inhibited 3-methylcholanthrene-induced skin tumors in BALB/c mice (Mukhtar et al., 1984). EA administered i.p. or in the diet inhibited B[a]P-induced pulmonary adenomas in A/J mice (Lesca, 1983); when added to the drinking water, it inhibited 3-methylcholanthrene-induced skin tumors in BALB/c mice (Mukhtar et al., 1985). EA inhibited the binding of metabolites of B[a]P to DNA in cultured explants of strain A mouse lung (Teel et al., 1985; Dixit et al., 1985) and in explants of human bronchus (Teel et al., in press).

To explore the anti-carcinogenic potential of EA, its effects on the toxicity of B[a]P 7,8-dihydrodiol (DHD) for human bronchial epithelial (HBE) cells and on the binding of B[a]P metabolites to DNA in these cells were investigated. In addition, the toxicity of EA itself for HBE cells was determined.

METHODS

Chemicals. [^{3}H] B[a]P (specific activity 30-40 Ci/mmol), [^{3}H] thymidine (specific activity 40-60 Ci/mmol), and [^{3}H] leucine (specific activity 45-70 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL. [^{3}H] B[a]P was diluted to 3 to 5 Ci/mmol with unlabeled B[a]P (Eastman Organic Chemical Co., Rochester, NY), purified through a Sep-Pak column (Waters Associates, Milford, MA), diluted further to 0.1mM in dimethyl sulfoxide (DMSO) (Burdick-Jackson, Muskegon, MI) and stored at -4°. B[a]P 7,8-DHD was obtained from the Chemical Repository of the National Cancer Institute, Bethesda, MD. EA was purchased from Aldrich Chemicals, Milwaukee, WI and hydroxylapatite (HTP-DNA grade) was obtained from Bio Rad, Richmond, CA. Unless indicated otherwise, other chemicals used were purchased from Allied-Fisher Scientific, Pittsburgh, PA.

HBE Cell Cultures. To obtain epithelial cell outgrowths from human bronchial explants, bronchial tissues obtained at autopsy within 2-6 hr of the patient's death were cut into 0.5 cm^2 pieces. A single explant was placed in the center of a 60 mm culture dish (Lux, Newberry Park, CA), containing 4 ml of REM-1 culture medium. REM-1 medium was developed for clonal growth and serial propagation of rat esophageal epithelial cells (Babcock et al., 1983). This medium was prepared from PFMR-4 medium (Lechner et al., 1980) by reducing the calcium concentration from

1.0 mM to 0.1 mM and supplementing it with 5 ng/ml epidermal growth factor (Collaborative Research Inc., Waltham, MA), 1 μM hydrocortisone (Steraloids, Wilton, NH), 1 μM each of ethanolamine and phosphoethanolamine (Calbiochem-Behring, La Jolla, CA), 5 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml cholera toxin, 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ), and 0.5 mg/ml dialyzed fetal bovine serum protein. Cultures were maintained at 36.5° in a humidified atmosphere of 3% CO_2 in air. The medium was renewed every 2-3 days.

After 11-14 days, epithelial cell outgrowths were dissociated into single cells with an enzyme solution (PET) containing 1.0% polyvinylpyrrolidone, 0.004% EGTA and 0.02% trypsin in Hepes buffered saline (HBS) (Lechner et al., 1980) and subcultured. Cells from the first to sixth passage were cryopreserved in HTBE-1 medium containing 5% DMSO. HTBE-1 medium was prepared from MCDB 151 medium (Peehl and Ham, 1980) by reducing the sodium chloride from 0.13 mM to 0.1 mM, increasing the calcium chloride from 0.03 mM to 0.1 mM, adding trace elements (Tsao et al., 1982), and supplementing with growth factors as described for REM-1 except for the addition of dialyzed fetal bovine serum and cholera toxin. Cells were coded according to the case number of the patient. For example, HBE 202 are cells obtained from outgrowths from bronchial explants from case no. 202.

Toxicity Assays. Cryopreserved HBE cells were thawed and grown to semi-confluency in 60 mm culture dishes in HTBE-1 medium. Cultures were dissociated with PET, washed with HBS, and 1×10^3 cells were plated into a series of 60 mm dishes each containing 4 ml HTBE-1 medium (3 dishes/test). After the cells attached, B[a]P (0.5, 1.0, 2.5, 5.0 μg/ml), B[a]P 7,8-DHD (0.22, 0.5, 0.75, 1.1 μg/ml) or EA (5, 10, 20 μg/ml) was added to the culture medium. Cultures were incubated at 36.5° in an atmosphere of 3% CO_2 in air. The medium was not renewed during the incubation period.

After 8-10 days of incubation, the cultures were fixed in 10% formalin and stained with 0.125% crystal violet for 4 min. For each test, the colony-forming efficiency (CFE) was determined using the following formula:

$$\text{CFE} = \frac{\text{Number of colonies per dish}}{\text{Number of viable cells inoculated per dish}} \times 100$$

The number of colonies/dish was counted manually and the mean number of colonies and the standard deviations for each test were determined.

In separate experiments, the toxicity of EA was examined by measuring the incorporation of [^{3}H] thymidine into DNA and [^{3}H] leucine into protein in cultures of HBE cells. HBE cells were grown to semi-confluency in 60 mm dishes. The medium was removed and fresh medium containing 0, 5, 10 or 20 μg/ml EA was added (six dishes/test). At this time, we added 2 μCi/ml [^{3}H] thymidine to one-half of the cultures and to the other half we added 2 μCi/ml [^{3}H] leucine. Cultures were incubated 24 hr at 36.5° in 3% CO_2 in air. The culture medium was removed and the

cultures were washed 3 × with HBS. Cells from each dish were removed by gentle scraping with a rubber policeman, incubated in 10% trichloroacetic acid at 4° for one hr and then washed repeatedly with ice-cold methanol. Cells were digested in NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL) and 100 μl aliquots were placed in scintillation vials and counted in 3a70B cocktail (Research Products International, Mt. Prospect, IL) in a Beckman LS 7800 liquid scintillation counter (Beckman Instruments, Berkeley, CA) with a counting efficiency of 40%. Mean DPM values and standard deviations were determined.

DNA-Binding Assays. Semi-confluent cultures of HBE cells were fed fresh HTBE-1 medium containing either, 0, 1.5, or 3 μg/ml EA. After 16 hr, the medium was removed and fresh medium containing the above concentrations of EA and 1 μM [^{3}H]B[a]P was added. After 24 hr, the culture medium was removed and cells were scraped from the dishes with the aid of a rubber policeman. Cells were then washed 3 times in HBS buffer and homogenized in 0.05 M sodium phosphate buffer (pH 6.5) containing 0.01M EDTA and 0.01M EDTA and 0.01M EGTA. The homogenates were made 7% (W/V) in 4-aminosalicylate-NaCl (6:1) and extracted with 1 volume of Kirby's phenol reagent (Kirby, 1965). The pooled aqueous layers were added to 2 ml 0.05 M sodium phosphate buffer (pH 6.5) containing 0.5 g hydroxylapatite. The DNA was eluted and quantitated as described by Stoner et al. (1982). After digestion in perchloric acid, the radioactivity associated with the DNA was determined by counting 100 μl aliquots in 3a70B cocktail. Binding was quantitated and expressed as pmole/mg DNA.

RESULTS

The colony forming efficiency (CFE) values shown in Table 1 express the toxicity of B[a]P, B[a]P 7,8-DHD and EA for the HBE cells. The CFE in control HBE cells was 6.7 ± 0.9. B[a]P was not toxic to HBE cells at the concentrations tested. In contrast, B[a]P 7,8-DHD exhibited toxic effects at very low concentrations and in a concentration dependent manner. There is a positive correlation between the inhibitory effects of EA on the CFE of HBE cells at concentrations of 10 and 20 μg/ml (Table 1) and the effects of EA on the incorporation of [^{3}H] thymidine and [^{3}H] leucine in HBE cells shown in Fig. 1. Although the amount of [^{3}H] thymidine and [^{3}H] leucine incorporated differed in the cells from two patients (data not shown), the inhibitory effect of EA at concentrations of 10 and 20 μg/ml occurred in cells from both patients (Fig. 1). The concentration dependent effect of EA is particularly apparent in the uptake of [^{3}H] thymidine. Data in Table 1 and Fig. 1 suggest that EA at a concentration of 5 μg/ml was nontoxic to the HBE cells.

Experiments to determine whether EA would protect against the toxicity of B[a]P 7,8-DHD for HBE cells were performed by testing toxic concentrations of B[a]P 7,8-DHD in combination with a nontoxic concentration of EA (5 μg/ml). The CFE was significantly lower in the HBE cultures treated with B[a]P 7,8-DHD and EA than

TABLE 1
Effect of Benzo[a]Pyrene, Benzo[a]Pyrene-7,8-Dihydrodiol, and Ellagic Acid on the Colony-Forming Efficiency of Human Bronchial Epithelial Cells[a]

TREATMENT[b]	COLONY-FORMING EFFICIENCY[c]
CONTROL	6.7 ± 0.9
B[a]P	
0.5	7.0 ± 1.3
1.0	5.9 ± 1.2
2.5	4.8 ± 0.7
5.0	7.9 ± 0.8
B[a]P 7,8-DHD	
0.22	5.7 ± 0.8
0.50	5.2 ± 0.3
0.75	1.7 ± 1.3
1.10	0.3 ± 0.1
EA	
5.0	6.3 ± 0.3
10.0	3.9 ± 1.5
20.0	0.9 ± 0

[a]Cells were obtained as outgrowths from bronchial explants (case No. 248, passage three) as described in Methods.
[b]Concentrations are mcg/ml culture medium.
[c]Values are the mean ± the standard deviation from three dishes. Procedures for determining the CFE are described in Methods.

in the cultures treated with B[a]P 7,8-DHD alone (Fig. 2). These data indicate that EA did not afford any protection against the toxicity of B[a]P 7,8-DHD, but rather indicate that EA has a potentiating effect on the toxicity of B[a]P 7,8-DHD.

In semi-confluent HBE cultures, EA at concentrations of 1.5 and 3.0 μg/ml inhibited the binding of B[a]P metabolites to DNA (Fig. 3). Inter-individual differences in binding to DNA were observed in the HBE cells from the four patients, but EA consistently inhibited B[a]P metabolite binding in cells from each of these patients.

DISCUSSION

Del Tito et al. (1983) reported that binding of B[a]P metabolites to calf thymus DNA *in vitro* and to rat epidermal DNA *in vivo* was inhibited by EA. The reduced cytotoxicity and mutagenicity of B[a]PDE-2 in the presence of EA was attributed to the formation of adducts between EA and the diol-epoxide (Wood et al., 1982). Dixit et al. (1985) reported the inhibition of the binding of B[a]P and B[a]P 7,8-DHD metabolites to DNA in mouse lung explants by EA. EA also inhibited B[a]P metabolite binding to DNA in human bronchial explants (Teel et al., 1985). These findings led

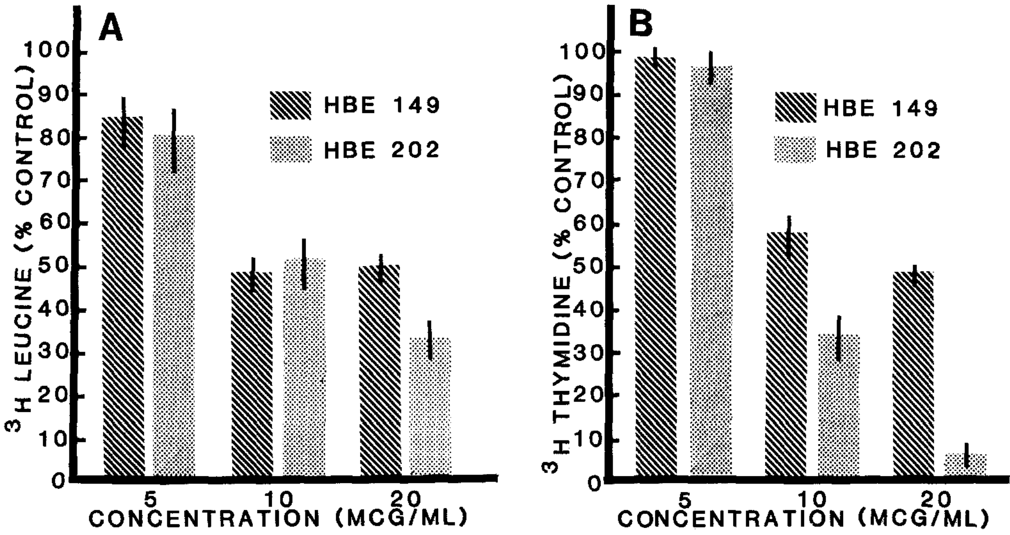

FIGURE 1. Effect of ellagic acid on the incorporation of tritiated leucine (A) into protein and tritiated thymidine (B) into DNA in human bronchial epithelial cells. Cells were obtained as outgrowths from bronchial explants, cases nos. 149 and 202, passage level 3. Percents of control values ± the standard deviation from triplicate samples are given.

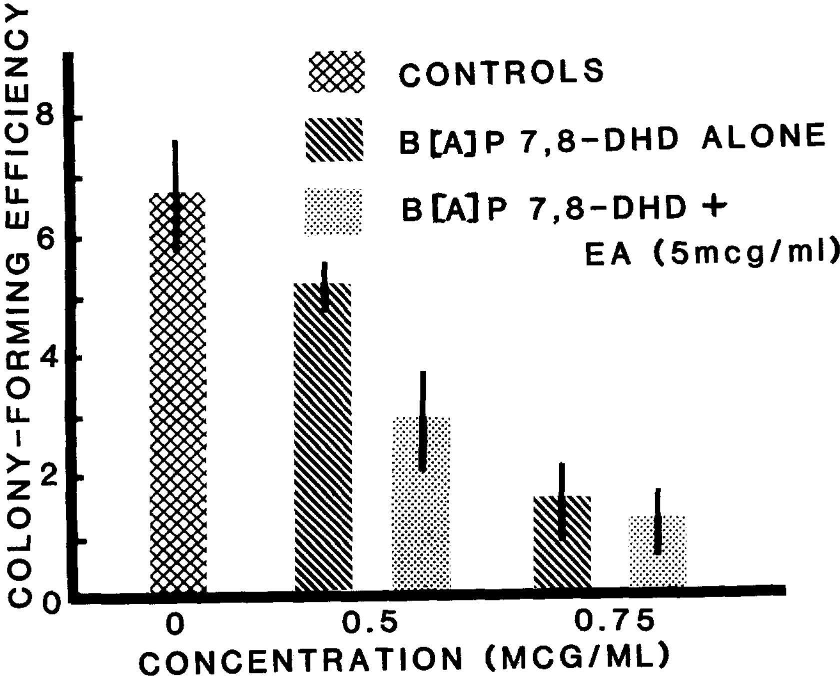

FIGURE 2. Effect of ellagic acid in combination with benzo[a]pyrene, 7,8-dihydrodiol on the colony-forming efficiency of human bronchial epithelial cells. HBE cells from an explant from case no. 248 at passage level 3 were used. CFE values are the mean ± the standard deviation from three culture dishes. Procedures for determining the CFE are described in Methods.

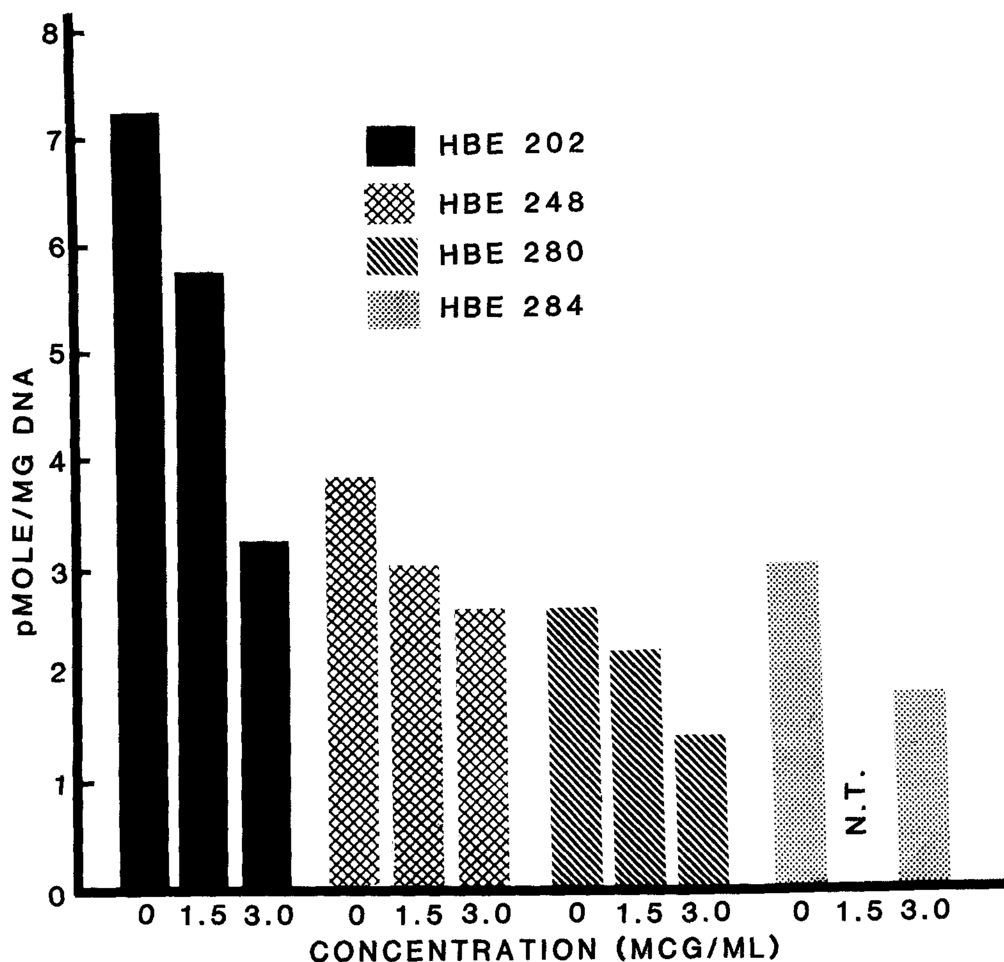

FIGURE 3. Effect of ellagic acid on the binding of benzo[a]pyrene metabolites to DNA in human bronchial epithelial cells. All cultures were at the third passage level. Binding data were obtained from five 100 mm culture dishes. Procedures for isolating DNA and quantitating bound metabolites are described in Methods. N.T. = not tested.

us to investigate the possible protective effect of EA on the toxicity of B[a]P and B[a]P 7,8-DHD in HBE cells using the CFE assay in which HBE cells were plated at low density, allowed to attach before treating, and then grown for a sufficient time to permit the development of colonies of cells. Using this assay and measuring the incorporation of [^{3}H] thymidine into DNA and [^{3}H] leucine into protein, we found that EA was nontoxic to the HBE cells at a concentration of 5 μg/ml but was toxic at 10 and 20 μg/ml (Table I, Fig. 1). B[a]P at concentrations of 5 μg/ml was nontoxic to HBE cells in the CFE assay, whereas B[a]P 7,8-DHD was toxic in a concentration dependent manner beginning at 0.5 μg/ml (Table 1). Since B[a]P 7,8-DHD is an intermediate in the metabolism of B[a]P and since EA forms adducts in aqueous

solutions with the epoxide of B[a]P 7,8-DHD, we expected that the toxicity of B[a]P 7,8-DHD would be reduced in the presence of EA. This did not occur (Fig. 2).

On the contrary, EA seemed to enhance the toxicity of B[a]P 7,8-DHD in the HBE cell cultures. Since a 5 μg/ml concentration of EA was nontoxic to HBE cells, we did not investigate the interaction of lower concentrations of EA with B[a]P 7,8-DHD. Further studies may show that lower concentrations of EA do not exhibit an enhanced toxicity of B[a]P 7,8-DHD in HBE cells. It is not surprising that B[a]P 7,8-DHD would exhibit a greater toxic effect than the parent compound, B[a]P. The data presented in Fig. 2 suggest that the adduct formation between EA and B[a]PDE in aqueous solutions reported by Wood et al. (1982) does not seem to correlate with EA's capacity to inhibit the toxicity of B[a]P 7,8-DHD in HBE cells. The mechanisms of toxicity involved are probably more complex than can be explained by the formation of B[a]PDE 1 and 2; furthermore, adduct formation between EA and B[a]PDE 1 and 2 in tissues has not been reported.

Previous reports of work done in our laboratories indicated that EA inhibited the binding of metabolites of B[a]P to DNA in explants of mouse lung (Dixit et al., 1985) and in explants of human bronchus (Teel et al., in press). In the present studies we observed that EA inhibited binding of B[a]P metabolites to DNA in cultures of human bronchial epithelial cells (Fig. 3). Studies on the mutagenicity, metabolism and DNA binding of B[a]P indicate that B[a]PDE is the major metabolite forming an adduct with DNA (Miller and Miller, 1976; Sims et al., 1974). In our hands, EA at a concentration of 100 μM inhibited the amounts of B[a]PDE-1:dG and B[a]PDE-2:dG adducts in explants of mouse lung by 70-80% (Dixit et al., 1985) and a 25 μM concentration inhibited the formation of B[a]PDE-1:dG adducts in explants of human bronchus by 50% (Teel et al., in press). The mechanism by which EA inhibits the formation of B[a]PDE:dG adducts is now under investigation in our laboratories.

The inhibition of B[a]P metabolite binding to DNA reported in this study is consistent with the findings of other investigators (Newmark, 1984; Wood et al., 1982; Mukhtar et al., 1984; Lesca, 1983; Del Tito et al., 1983), but the failure of EA to protect against the toxicity of B[a]P 7,8-DHD needs further study. We are currently investigating the interaction of EA and B[a] 7,8-DHD to find explanations for these results.

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