Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: Exceptional activity of ellagic acid

(ultimate carcinogen Ames' Salmonella typhimurium/Chinese hamster V79 cells/kinetics of epoxide disappearance)

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ABSTRACT Ferulic, caffeic, chlorogenic, and ellagic acids, four naturally occurring plant phenols, inhibit the mutagenicity and cytotoxicity of 7,8-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P 7,8-diol-9,10-epoxide-2), the only known ultimate carcinogenic metabolite of benzo[a]pyrene. The mutagenicity of 0.05 nmol of B[a]P 7,8-diol-9,10-epoxide-2 in strain TA100 of Salmonella typhimurium is inhibited 50% by incubation of the bacteria and the diol epoxide with 150 nmol of ferulic acid, 75 nmol of caffeic acid, 50 nmol of chlorogenic acid or, most strikingly, 1 nmol of ellagic acid in the 0.5-M1 incubation mixture. A 5-nmol dose of ellagic acid inhibits mutation induction by 90%. Ellagic acid is a potent antagonist of B[a]P 7,8-diol-9,10-epoxide-2 in Chinese hamster V79 cells. Mutations to 8-aza-guanine resistance induced by 0.2 M1 diol epoxide are reduced by 50% when tissue culture media also contains 2 μM ellagic acid. Similar to results obtained with the bacteria, ferulic, caffeic, and chlorogenic acids are approximately two orders of magnitude less active than ellagic acid in the mammalian cell assay. The anti-mutagenic effects of the plant phenols result from their direct interaction with B[a]P 7,8-diol-9,10-epoxide-2, because a concentration-dependent increase in the rate of diol epoxide disappearance in cell-free solutions of 1:9 diene/water, pH 7.0, is observed with all four phenols. In parallel with the mutagenicity studies, ellagic acid is 80–300 times more effective than the other phenols in accelerating the disappearance of B[a]P 7,8-diol-9,10-epoxide-2. Ellagic acid at 10 μM increases the disappearance of B[a]P 7,8-diol-9,10-epoxide-2 by approximately 20-fold relative to the spontaneous and hydronium ion-catalyzed hydrolysis of the diol epoxide at pH 7.0. Ellagic acid is a highly potent inhibitor of the mutagenic activity of bay-region diol epoxides of benzo[a]pyrene, dibenzo[a,]pyrene, and dibenzo[a,]pyrene, but higher concentrations of ellagic acid are needed to inhibit the mutagenic activity of the chemically less reactive bay-region diol epoxides of benzo[a]anthracene, chrysene, and benzo[c]phenanthrene. These studies demonstrate that ellagic acid is a potent antagonist of the adverse biological effects of the ultimate carcinogenic metabolites of several polycyclic aromatic hydrocarbons and suggest that this naturally occurring plant phenol, normally ingested by humans, may inhibit the carcinogenicity of polycyclic aromatic hydrocarbons.

Polycyclic aromatic hydrocarbons are relatively inert environmental precarcinogens that must be metabolized by mammalian enzymes to their biologically active products (ultimate carcinogens). Mutagenicity, metabolism, DNA-binding, and tumorigenicity studies during the past 8 years have shown that benzo-

ring diol epoxides, in which the epoxide moiety forms part of the bay region (1, 2) of the hydrocarbons, are ultimate carcinogens of polycyclic hydrocarbons (3–6).

Chemical and kinetic studies have demonstrated that bay-region diol epoxides are subject to general acid catalysis to form chemically unreactive and biologically inactive tetaols (7–12) as well as to form covalent adducts with low molecular weight nucleophiles such as p-nitrosothiophenol and 2-mercaptoethanol (7, 8, 12). Thus a chemical basis exists for a rational approach to the identification of nontoxic compounds that can block the adverse biological effects of the ultimate carcinogens of polycyclic aromatic hydrocarbons. We have recently shown (13) that riboflavin 5'-phosphate (FMN) can markedly diminish the mutagenicity of the trans diastereomer of the bay-region diol epoxide of benzo[a]pyrene (B[a]P 7,8-diol-9,10-epoxide-2) by a mechanism involving complex formation followed by general acid catalyzed hydrolysis of the diol epoxide to tetaols. These results with FMN have demonstrated the feasibility of blocking the adverse biological effects of ultimate carcinogens and have served as an impetus to identify additional compounds with even better capacity to interact with and detoxify bay-region diol epoxides. In the present study, we find that ferulic, caffeic, chlorogenic, and ellagic acids, four naturally occurring plant phenols that share the common structural elements of a meta- or para-hydroxylated benzoic or cinnamic acid (Fig. 1) can inhibit the mutagenicity of the ultimate carcinogenic metabolite of B[a]P. One of these phenols, ellagic acid, is an ex-

Abbreviations: B[a]P, benzo[a]pyrene; B[a]P 7,8-diol-9,10-epoxide-1, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]P 7,8-diol-9,10-epoxide-2, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]P H2,9,10-epoxide, 9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; DB[a], H, 1,2-diol-3,4-epoxide-2, (±)-1β,2α-dihydroxy-3a,4α-epoxy-1,2,3,4-tetrahydrodibenzo[a,]pyrene; DB[a], H, 1,4-diol-1,2-epoxide-2, (±)-1β,3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrodibenzo[a,]pyrene; DB[a], H, 3,4-diol-1,2-epoxide-2, (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrodibenzo[a,]pyrene; DB[a], H, 1,2,3,4-diol-3,4-epoxide-2, (±)-1β,2α-dihydroxy-3a,4α-epoxy-1,2,3,4-tetrahydrochrysene; DB[a], H, 1,2,3,4-diol-1,2-epoxide-2, (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene; Me2SO, dimethyl sulfoxide. All compounds are racemic mixtures where optical enantiomers are possible.

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‡ As indicated in the abbreviations, benzo-ring diol epoxides of trans dihydrodiols exist as diastereomeric pairs in which the benzylic hydroxyl group and the epoxide oxygen are either cis (isomer-1 series) or trans (isomer-2 series) to each other. Fig. 1 depicts the bay-region diol epoxide-2 isomer of benzo[a]pyrene.
ceptional antagonist of the mutagenicity of bay-region diol epoxides of several polycyclic aromatic hydrocarbons.

MATERIALS AND METHODS

Materials. Ferulic, caffeic, chlorogenic, and ellagic acids were obtained from Aldrich (Milwaukee, WI), and all four compounds were essentially pure as determined by reverse-phase high-pressure liquid chromatography. For the kinetic studies, caffeic, ferulic, and ellagic acids were recrystallized from ethanol/water, me thanol/water, and isopropyl alcohol/water, respectively. The bay-region diol epoxides of B[a]P (7, 8), dibenz[a,h]pyrene (DB[a,h]P) (14), dibenz[a,i]pyrene (DB[a,i]P) (15), benz[a]anthracene B[a]A (15), chrysene (16, 17), and benzo[c]phenanthrene B[c]Phen (12) were synthesized as previously described, as were B[a]P H4,9,10-epoxide (18) and B[a]P 4,5-oxide (19). All compounds were free of detectable impurities, and the diol epoxides were free of diastereomeric contamination, as determined by mass spectral and nuclear magnetic resonance analyses. Media for culturing bacterial and mammalian cells were obtained from Becton Dickinson (Cockeysville, MD) and GIBCO, respectively. Fetal calf serum was obtained from Reheis (Kankakee, IL). Dimethyl sulfoxide (Me2SO) was distilled from calcium hydride under reduced pressure, and dioxane was distilled from sodium. 

Mutagenesis Assays with Bacteria. Strain TA100 of histidine-independent Salmonella typhimurium was obtained from B. Ames (University of California, Berkeley, CA) and was cultured as described (20). Test compounds were dissolved in anhydrous Me2SO. The bacteria (2 × 108) were suspended in 0.5 ml of anisotonic phosphate-buffered saline (5 mM potassium phosphate/150 mM sodium chloride, pH 7.0), were preincubated for 1 min at 37°C with 10 μl of Me2SO or various amounts of the plant phenols added in 10 μl of Me2SO. After an additional 5-min incubation at 37°C, 2 ml of top agar was added, and the entire mixture was poured onto a histidine-deficient Vogel-Bonner agar medium in a 15 × 100 mm Petri dish. Mutations to histidine-independent growth were assessed by counting the macroscopic colonies of bacteria after a 2-day incubation of the plates at 37°C. All experiments were performed in triplicate.

Mutagenesis Assays with Mammalian Cells. Line V79-6 of Chinese hamster cells was a generous gift of E. H. Y. Chu (University of Michigan, Ann Arbor, MI). Assays assessing cytotoxicity and intrinsic mutagenicity were adapted from the procedure of Chu (21) and were performed as described (20). The V79 cells were seeded in 60-mm culture dishes at concentrations of 104 cells per dish (4 dishes) and 105 cells per dish (16 dishes) for experiments assessing cell survival and mutagenicity, respectively. After the cells were cultured for 18 hr in 5 ml of medium, the plant phenol was added in 10 μl of anhydrous Me2SO, and B[a]P 7,8-diol-9,10-epoxide-2 was added 20 min later in 15 μl of Me2SO. Control samples were treated with an equal amount of Me2SO. After a 1-hr incubation at 37°C, the medium and compounds were removed from the culture dish, and the cells were washed once with 5 ml of phosphatebuffered saline (9.5 mM sodium phosphate/140 mM sodium chloride/3 mM potassium chloride, final pH 7.2) and then cultured in 5 ml of fresh medium. To assess cell survival, culture dishes that contained 105 cells were incubated at 37°C for 7 days, at which time the macroscopic colonies of cells were fixed with methanol, stained with Giemsa stain, and counted. To assess mutagenic activity, the culture dishes containing 104 cells were incubated at 37°C for 3 days. At this time, mutant cells resistant to 8-azaguanine were selected by addition of 10 μg of 8-azaguanine per ml of culture medium. On day 7, the medium was replaced with 5 ml of fresh medium containing the same amount of 8-azaguanine. One week later, colonies resistant to 8-azaguanine were fixed with methanol, stained with Giemsa stain, and counted.

Kinetics of Diol Epoxide Disappearance. Rates for the disappearance of B[a]P 7,8-diol-9,10-epoxide-2 in the presence of the phenolic compounds were measured at 25°C in 1:9 (vol/vol) dioxane/water solutions, 0.1 M in NaClO4. The pH was maintained at 7.0 with 2 mM Tris-HCl buffer. Reactions were followed by measuring absorbance at 346 nm with a Cary 219 spectrophotometer. Reactions were initiated by addition of 5–20 μl of a stock solution of the diol epoxide in dioxane to 3 ml of each reaction mixture containing a buffered solution of the appropriate phenol to give a final diol epoxide concentration of 7–9 μM (with caffeic, ferulic, and chlorogenic acids) or 1.6–3.3 μM (with ellagic acid). In all experiments, the ratio of phenol to diol epoxide was equal to or greater than 5:3:1, and at the lowest diol epoxide-to-phenol ratios no significant deviations from pseudo-first-order kinetics were observed. Pseudo-first-order rate constants for reactions of the diol epoxide were determined as described (13). Apparent second-order rate constants, kapp, at pH 7.0, were determined from the slopes of linear plots of the observed pseudo-first-order rate constants vs. the concentrations of the phenols.

RESULTS

The dose-dependent inhibitory effects of ferulic, caffeic, chlorogenic, and ellagic acids on the mutagenicity of 0.05 nmol of B[a]P 7,8-diol-9,10-epoxide-2 in strain TA100 of Salmonella typhimurium are shown in Fig. 2. Mutations induced by the diol epoxide were inhibited 50% by doses of 150, 75, and 50 nmol, respectively, of ferulic, caffeic, and chlorogenic acids. More strikingly, only 1 nmol of ellagic acid was needed to reduce the mutagenicity of B[a]P 7,8-diol-9,10-epoxide-2 by 50%, and 3 nmol of ellagic acid reduced mutations by 90%. Comparison of these results with those previously reported (13) for riboflavin 5'-phosphate (FMN) (dotted line Fig. 2) indicated that FMN was 6–20 times more potent than ferulic, caffeic, and chlorogenic acids but was only about 1/10th as active as ellagic acid. Thus 2 μM ellagic acid was sufficient to inhibit the mutagenicity of B[a]P 7,8-diol-9,10-epoxide-2 by 50% in the bacterial test system.
Ellagic acid was also a potent inhibitor of the cytotoxic (Fig. 3) and mutagenic (Fig. 4) effects of B[a]P 7,8-diol-9,10-epoxide-2 in Chinese hamster V79 cells. Although only 1% of the mammalian cells survived exposure to 0.6 nmol/ml of the diol epoxide in the absence of ellagic acid, cell survival was increased to 50% by an 8-fold molar excess (5 nmol/ml) of ellagic acid (Fig. 3). 8-Azaguanine-resistant mutants induced in the V79 cells by a 1-hr incubation with B[a]P 7,8-diol-9,10-epoxide-2 at 0.2 nmol/ml were reduced by 50% when the cells were pretreated with a 10-fold molar excess (2 nmol/ml) of ellagic acid. As was found in the bacterial test system, considerably higher concentrations of chlorogenic, caffeic, and ferulic acids were required to block the mutagenicity (Fig. 4) and toxicity (data not shown) of B[a]P 7,8-diol-9,10-epoxide-2, but the relative protective effect of these three compounds was essentially the same in the mammalian and bacterial cells. No cytotoxicity (>80% cell survival) or mutagenicity was observed in the Chinese hamster V79 cells when relatively high concentrations of ellagic acid (20 nmol/ml), chlorogenic acid (500 nmol/ml), caffeic acid (500 nmol/ml), or ferulic acid (500 nmol/ml) were incubated with the cells in the absence of B[a]P 7,8-diol-9,10-epoxide-2.

The effect of ellagic acid on the mutagenicity of several bay-region diol epoxides was determined in strain TA100 of S. typhimurium (Table 1). Ellagic acid had equivalent antimutagenic activity toward B[a]P 7,8-diol-9,10-epoxide-1, B[a]P 7,8-diol-9,10-epoxide-2, and B[a]P H2-9,10-epoxide, a highly mutagenic epoxide in which the hydroxyl groups of the angular benzo-ring have been replaced by hydrogen. Ellagic acid was much less effective in blocking the mutagenicity of the B[a]P 4,5-oxide (K-region oxide), a primary oxidative metabolite of B[a]P that has little or no carcinogenic activity (22). Ellagic acid was also a potent inhibitor of the mutagenic activity of the bay-region diol epoxides of DB[a,h]P and DB[a,i]P but was less effective in inhibiting mutations induced by the bay-region diol epoxides of B[a]A, chrysene, and B[c]phen.

Ferulic, caffeic, chlorogenic, and ellagic acids accelerate the
disappearance of B[a]P 7,8-diol-9,10-epoxide-2 from aqueous solution (1:9 dioxane/water, pH 7/0.1 M NaClO₄) as shown by the second-order rate constants for diol epoxide disappearance in Table 2. This observation indicates that the antimutagenic activity of the phenolic compounds results from their interaction with the diol epoxide, rather than with the target cells. If the most active compound, ellagic acid, is assigned an arbitrary rate of 100 for its reaction with the diol epoxide, the relative rates for chlorogenic, caffeic, and ferulic acids are 1.1, 0.6, and 0.3, respectively. This order of reactivity, as well as the magnitude of the differences in rate among the phenolic compounds, parallels closely their effectiveness in inhibiting mutagenesis. The effect of ellagic acid on diol epoxide disappearance is particularly striking. Under the experimental conditions described above, the half-life of the diol epoxide was decreased by 40 min in the absence of ellagic acid to 2 min in the presence of 10 μM ellagic acid. The data in Table 2 also show that the rate constant for the disappearance of the diol epoxide in the presence of ellagic acid is about 12 times greater than the analogous constant calculated for the disappearance of the diol epoxide in the presence of FMN, a finding that is consistent with the approximate 10-fold difference in antimutagenic potency between FMN and ellagic acid (Fig. 2).

**DISCUSSION**

The results of the present study indicate that the naturally occurring plant phenols ferulic, caffeic, chlorogenic, and ellagic acids can block the mutagenic activity of B[a]P 7,8-diol-9,10-epoxide-2, the only known ultimate carcinogen of B[a]P. Of the four compounds tested, ellagic acid (a phenolic lactone) was shown to be an exceptional antimutagen and was effective at concentrations 1/10th of those described (13) for FMN. Ferulic, caffeic, and chlorogenic acids all induced concentration-dependent decreases in the mutagenicity of B[a]P 7,8-diol-9,10-epoxide-2, but these three plant phenols were considerably less active than ellagic acid and FMN.

The kinetic data (Table 2) for the disappearance of B[a]P 7,8-diol-9,10-epoxide-2 from aqueous solution indicate that the antimutagenic activity of ellagic acid and the other three compounds results from a direct interaction between the phenol and the diol epoxide as opposed to an effect on the cells. Previous studies have shown that phenol itself is an acid catalyst of B[a]P 7,8-diol-9,10-epoxide-2 hydrolysis and that significant amounts of hydrocarbon-phenol adduct(s) can be formed (10). Similarly, recent studies (unpublished observations) have shown that ellagic acid forms an adduct with B[a]P 7,8-diol-9,10-epoxide-2 via a phenolic hydroxyl group, but the polyphenol is at least 3,000 times more reactive than phenol. With FMN, general acid catalysis by the phosphate monoanion is observed in the absence of any adduct formation, and all of the B[a]P 7,8-diol-9,10-epoxide-2 is hydrolyzed to tetaols (13). Studies on the mechanism of the antimutagenic effect of ellagic acid may provide a basis for the observation that ellagic acid inhibits the mutagenicity of bay-region diol epoxides of B[a]P, DB[a,h]P, and DB[a,i]P more readily than the less reactive bay-region diol epoxides of B[a]A, chrysele, and B[c]Phen (Table 1). Both theoretical quantum mechanical calculations (2) based on the π-electron energy changes and kinetic data for solvolysis of diol epoxides (9, 10, 12, 16, 19) have indicated that the diol epoxides of B[a]P, DB[a,h]P, and DB[a,i]P are the most reactive of the bay-region diol epoxides that have been studied to date.

None of the four plant phenols had detectable mutagenic activity in strain TA100 of *S. typhimurium* or in Chinese hamster V79 cells. The bacterial studies were done in both the absence and presence of a hepatic 9,000 × g supernatant fraction from Aroclor 1254-induced rats, which serves as a metabolic activation system for premutagens that must be metabolized to their active products. Recent studies have suggested that caffeic and chlorogenic acids are mutagenic and clastogenic when tested in the presence of a transition metal (24, 25). However, previous reports (26–28) have indicated that transition metals catalyze the oxidation of catechols with the generation of hydrogen peroxide. It is likely that the mutagenicity of caffeic and chlorogenic acids in the *in vitro* test systems results from the formation of unphysiologically high concentrations of hydrogen peroxide or related active oxygen species such as superoxide (26). No toxicity was observed when rats were fed a diet containing 0.5% caffeic acid for 6 months. Indeed, these rats had a lower incidence of spontaneous tumors than rats fed a control diet (30).

All four of the phenolic compounds described in this report

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**Table 1. Effect of ellagic acid on the mutagenic activity of bay-region diol epoxides and other oxides of several polycyclic aromatic hydrocarbons**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount, nmol*</th>
<th>Histidine revertants per plate</th>
<th>Inhibition by ellagic acid, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.625</td>
</tr>
<tr>
<td>B[a]P 7,8-diol-9,10-epoxide-2</td>
<td>0.05</td>
<td>1,190</td>
<td>50</td>
</tr>
<tr>
<td>B[a]P 7,8-diol-9,10-epoxide-1</td>
<td>0.05</td>
<td>1,060</td>
<td>23</td>
</tr>
<tr>
<td>B[a]P H2,9,10-epoxide</td>
<td>0.05</td>
<td>1,240</td>
<td>37</td>
</tr>
<tr>
<td>B[a]P 4,5-oxide</td>
<td>0.50</td>
<td>890</td>
<td>—</td>
</tr>
<tr>
<td>DB[a,h]P 4,5-oxide</td>
<td>0.15</td>
<td>360</td>
<td>35</td>
</tr>
<tr>
<td>DB[a,i]P 4,5-oxide</td>
<td>0.15</td>
<td>360</td>
<td>36</td>
</tr>
<tr>
<td>B[c]Phen 4,5-oxide</td>
<td>0.15</td>
<td>440</td>
<td>—</td>
</tr>
<tr>
<td>Chrysele 4,5-oxide</td>
<td>0.50</td>
<td>360</td>
<td>—</td>
</tr>
</tbody>
</table>

* Amounts of the compounds were selected from linear portions of their dose–response curves with strain TA100 of *S. typhimurium.*

† Average number (n = 3) of histidine-independent revertants induced per plate in strain TA100 of *S. typhimurium* by the indicated amounts of each epoxide, after subtraction of the spontaneous mutation frequency of 140 revertants per plate.

**Table 2. Apparent second-order rate constants for the disappearance of B[a]P 7,8-diol-9,10-epoxide-2 at 25°C in 1:9 dioxane/water, ionic strength 0.1 M, pH 7.0**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. range, μM</th>
<th>k app M⁻¹ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>250–980</td>
<td>1.78</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>250–980</td>
<td>3.13</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>63–300</td>
<td>6.3</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>8.8–35</td>
<td>560</td>
</tr>
<tr>
<td>FMN</td>
<td>45</td>
<td>140</td>
</tr>
</tbody>
</table>

* Calculated from k₅k₆ for this compound at pH 7.0 (13).
are widely distributed among fruits and vegetables that are regularly consumed by humans (31–33). Caffeic and ferulic acids are ubiquitous; high concentrations of ellagic acid are found in grapes, certain nuts, and strawberry preserves; and chlorogenic acid is found in relatively high concentrations in coffee, apples, and potatoes.

Ellagic acid is pharmacologically active and has been found to control hemorrhage in animals (34, 35) and humans (36, 37), presumably by activating the intrinsic blood coagulation system (38). Hypotensive and sedative effects of ellagic acid have also been reported in rodents (39). Rats fed ellagic acid at a dose of 50 mg/kg per day for up to 45 days did not show any evidence of toxicity (40), and doses of ellagic acid of 0.2 mg/kg, intravenously, were well tolerated in humans (37).

Caffeic and ferulic acids have been shown to diminish the incidence of forestomach tumors in mice treated with Bl[a]P (41). The ability of both caffeic and ferulic acids to block the mutagenicity of Bl[a]P 7,8-diol-9,10-epoxide-2 may be related to their anticarcinogenic activity. To our knowledge, ellagic and chlorogenic acids have not been tested for anticarcinogenic activity, although chlorogenic acid has recently been shown to block the formation of mutagenic compounds resulting from the pyrolysis of protein (42). The results of the present study indicate that ellagic acid is an exceptional antagonist of the mutagenicity of bay-region diol-epoxides derived from several polycyclic aromatic hydrocarbons. Additional research is needed to determine whether or not ellagic acid can block the carcinogenicity of polycyclic hydrocarbons in animals.

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