

Inhibitory effects of naturally occurring coumarins on the metabolic activation of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene in cultured mouse keratinocytes

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Several naturally occurring coumarins to which humans are routinely exposed have been previously found to be potent inhibitors and inactivators of cytochrome P450 (P450) 1A1-mediated monooxygenase in both murine hepatic microsomes and in a reconstituted system using purified human P450 1A1 [Cai *et al.* (1993) *Chem. Res. Toxicol.*, **6**, 872–879 and Cai *et al.* (1996) *Chem. Res. Toxicol.*, **9**, 729–736]. In the present study, several of these coumarins were investigated for their inhibitory effects on the metabolism and metabolic activation of benzo[*a*]pyrene (B[*a*]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA) in cultured mouse keratinocytes. Initial analysis of B[*a*]P metabolism in cultured keratinocytes showed that imperatorin, isoimperatorin, coriandrin, and bergamottin, at concentrations of 2 nM equal with B[*a*]P, reduced the formation of water-soluble metabolites of B[*a*]P by 33% to 57%. Bergamottin and coriandrin were the most potent inhibitors of the compounds examined. HPLC analysis of organic solvent-soluble metabolites of B[*a*]P indicated that all the coumarins tested significantly reduced the formation of individual B[*a*]P metabolites (including phenols, diols and tetraols). However, the greatest effect was on the formation of B[*a*]P tetraols. Additional experiments determined the ability of selected coumarins to block covalent binding of B[*a*]P and DMBA to DNA in keratinocytes. Bergamottin preferentially inhibited the binding of B[*a*]P to DNA by 56%, while coriandrin preferentially inhibited the binding of DMBA to DNA by 48%. Notably, analysis of individual DNA adducts formed from B[*a*]P and DMBA indicated that both bergamottin and coriandrin specifically inhibited the formation of anti diol-epoxide DNA adducts derived from both hydrocarbons. The preferential inhibitory effect of bergamottin and coriandrin on the formation of anti diol-epoxide adducts derived from DMBA was further confirmed by separation of anti- and syn-diol-

epoxide–DNA adducts using immobilized boronate chromatography. The current study demonstrates that certain naturally occurring coumarins inhibited metabolic activation of B[*a*]P and DMBA in cultured mouse keratinocytes and specifically inhibited the formation of DNA adducts derived from the anti diol-epoxide diastereomers from either hydrocarbon. The current data also suggest that certain naturally occurring coumarins may possess anticarcinogenic activity toward polycyclic aromatic hydrocarbons.

Introduction

Polycyclic aromatic hydrocarbons (PAH*) are widely distributed in the environment. They have been identified as carcinogens in experimental animals and in humans and have been found in tissues such as skin, esophagus, pancreas, prostate, and lung (1–4). In order to exert their cytotoxic, mutagenic, and carcinogenic properties, PAHs must be metabolically activated to reactive intermediates (5,6). Benzo[*a*]pyrene (B[*a*]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA), both effective tumor initiators and complete carcinogens in mouse skin (7,8), require metabolic activation through a two-step oxidation process by cytochrome(s) P450 to diol-epoxide intermediates (9). In many target tissues, diol-epoxides are the major metabolites that form adducts with DNA (9). The major stable DNA adducts derived from B[*a*]P or DMBA are formed either by reaction of anti-B[*a*]P diol-epoxide (anti-BPDE) with deoxyguanosine or reaction of anti- and syn-DMBA diol-epoxide (anti- and syn-DMBADE) with deoxy-guanosine and/or deoxyadenosine (9–11). It has been shown that the levels of diol-epoxide DNA adducts formed from B[*a*]P and DMBA are quantitatively correlated with the tumor initiating activity of these PAHs in mouse skin (7,12,13).

B[*a*]P and DMBA are metabolized by multiple forms of cytochrome(s) P450 and it is known that the different cytochrome(s) P450 have different regio-selectivity in metabolism of these PAHs. For example, P450 1A1 is the major isozyme that catalyzes the two-step oxidation of B[*a*]P to its bay region diol-epoxide intermediates in both animal and human tissues (6,14,15). In addition, both rodent and human P450 1A1 efficiently convert B[*a*]P to a variety of other metabolites (16). Other P450s can metabolize B[*a*]P, including P450 1A2 and human P450 2C9 but may be quantitatively less important than P450 1A1 depending on the tissue (16). It has been shown that both rodent and human P450 1A1 is highly stereoselective in converting B[*a*]P-7,8-diol to the (+) enantiomer of anti-BPDE (16). Recent evidence demonstrates that the cytochrome(s) P450 involved in the metabolic activation of DMBA are different from those involved in the metabolic activation of B[*a*]P. In this regard, P450 1B1 in benz[*a*]anthracene (BA)-treated C3H/10T1/2 cells and 2C6 in rat hepatic microsomes efficiently metabolized DMBA to DMBA 3,4-diol, an approximate carcinogen, compared with

*Abbreviations: PAH, polycyclic aromatic hydrocarbon; B[*a*]P, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; BA, benz[*a*]anthracene; P450, cytochrome P450; DMH, dimethylhydrazine; DAS, diallyl sulfide; PEITC, phenethyl isothiocyanate; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 7,8-BF, 7,8-benzoflavone; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; 1-EP, 1-ethynylpyrene; MC, 3-methylcholanthrene; PB, phenobarbital; 3-OH-B[*a*]P, 3-hydroxy-B[*a*]P; (+) anti-BPDE, (+)-7 β , 8 α -dihydroxy-9 α , 10 α -epoxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene; (–) syn-BPDE, (–)-7 β , 8 α -dihydroxy-9 β ,10 β -epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; anti-DMBADE, (±) 1 β , 2 β -epoxy-3 β , 4 α -dihydroxyl-1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene; syn-DMBADE, (±) 1 α , 2 α -epoxy-3 β , 4 α -dihydroxyl-1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene; dGuo, deoxyguanosine; dAdo, deoxyadenosine.

other cytochrome(s) P450, while P450 1A1 in BA-treated mouse hepa-1 cells produced very low amounts of DMBA-3,4-diol (17,18). Furthermore, human P450 1B1 was recently shown to be ineffective at converting DMBA to a mutagen but very effective at converting DMBA-3,4-diol to a mutagenic metabolite (19). In contrast, human P450 1A1 possessed very little ability to convert either DMBA or DMBA-3,4-diol to mutagenic metabolites in this latter study (19).

Several agents, both naturally occurring and synthetic, which selectively inhibit specific cytochrome(s) P450, have been reported to effectively inhibit tumorigenesis caused by specific carcinogens. For example, 1-ethynylpyrene (1-EP), an inhibitor of both rat P450 1A1 and 1B1 (20,21), has been reported to inhibit both B[a]P- and DMBA-initiated mouse skin tumors (22). In addition, diallyl sulfide (DAS) and phenethyl isothiocyanate (PEITC), compounds found naturally in garlic, selectively inhibit the activity of P450 2E1 (23,24) and modulate carcinogenesis induced by carcinogens that are activated by this P450. In particular, DAS was shown to inhibit 1,2-dimethylhydrazine (DMH)-induced hepatotoxicity and colon carcinogenesis in rats (25,26), whereas PEITC produced a large decrease in the rate of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) oxidation in microsomes and was inhibitory against NNK-induced lung tumorigenesis in mice (27).

Recently, we reported that certain naturally occurring coumarins found in the human diet, including the linear furanocoumarins imperatorin, bergamottin, and isopimpinellin, the linear furoisocoumarin coriandrin, and the simple coumarin ostruthin were potent inhibitors or inactivators of either P450 1A1-mediated ethoxyresorufin-*O*-dealkylase activity in hepatic microsomes from 3-methylcholanthrene (MC)-pretreated mice or P450 2B1-mediated pentoxyresorufin-*O*-dealkylase activity in hepatic microsomes from phenobarbital (PB)-pretreated mice (28). Further studies also demonstrated that coriandrin and bergamottin effectively inhibited purified human P450 1A1 and had modest inhibitory activity toward purified human P450 1A2 (29). In these studies, coriandrin was found to be a mechanism-based inactivator of both mouse and human P450 1A1 (29). All of these data suggested that naturally occurring coumarins ingested by man may have marked effects on the metabolic activation of PAHs and other carcinogens. Earlier studies, in which imperatorin was reported to be an effective antimutagen for 2-aminoanthracene and B[a]P in *Salmonella typhimurium* in the presence of a hepatic S_9 activating system, also support this suggestion (30).

The present study has investigated the ability of several natural coumarins for their effects on the metabolism and metabolic activation of B[a]P and DMBA in cultured mouse keratinocytes. To achieve this goal, we have analysed the effects of bergamottin, coriandrin, imperatorin, isoimperatorin, and ostruthin on the overall metabolism of B[a]P. In addition, we analysed the effect of these coumarins on the covalent binding of both B[a]P and DMBA to the DNA of cultured keratinocytes maintained in high Ca^{2+} medium. The results demonstrate that these naturally occurring coumarins are potent inhibitors of the metabolic activation of B[a]P and DMBA in this cell culture model system of mouse epidermis. Furthermore, the data suggest that naturally occurring coumarins may possess anticarcinogenic activity *in vivo*.

Materials and methods

Materials

B[a]P and 7,8-benzoflavone (7,8-BF) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). DMBA was obtained from Eastman Kodak

Co. (Rochester, NY). [^3H]B[a]P (specific activity, 66–70 Ci/mmol) and [^3H]DMBA (specific activity, 50 Ci/mmol) were obtained from Amersham Co. (Arlington Heights, IL) and diluted with unlabelled B[a]P to specific activities of 1 Ci/mmol or 3 Ci/mmol as indicated. Imperatorin was from Indofine Chemical Co. (Belle Mead, NJ). Coriandrin was obtained as previously described (31). Ostruthin, bergamottin and isoimperatorin were obtained from Dr Warren Steck, National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Fetal bovine serum (FBS) was purchased from Irvine Scientific Co. (Santa Ana, CA). Trypsin and guanidine isothiocyanate were purchased from Gibco BRL (Grand Island, NY). Eagle's minimal essential medium (EMEM) was obtained from Biowhittaker (Walkersville, MD). Percoll was purchased from Pharmacia (Uppsala, Sweden). DNase I (bovine pancreas, EC 3.1.4.1), alkaline phosphatase (*E. coli*, type III, EC 3.1.3.1.), and snake venom phosphodiesterase (Crotalus atrox, EC 3.1.4.1) were purchased from Sigma Chemical Co. (St Louis, MO). Sephadex LH-20 was supplied by Pharmacia Inc. (Piscataway, NJ). Servacel dihydroxyboryl-cellulose was purchased from Accurate Chem. (Westbury, NY). Other chemicals and reagents were obtained commercially and were the highest purity deemed necessary. All chemicals used in the current study were >96% pure as judged by HPLC.

Cell cultures

Primary cultures of keratinocytes from dorsal skins of adult female SENCAR mice (NCI, Frederick, MD) were prepared according to established procedures (32,33). Cells were seeded at a density of $\sim 2.5 \times 10^6$ per 35 mm or 8×10^6 per 100 mm dish for analysis of B[a]P metabolism or DNA adducts, respectively, in low Ca^{2+} modified EMEM with growth factor supplements and 1% FBS. 40 h later, the cultures were switched to high Ca^{2+} (1.4 mM) modified EMEM without growth factor supplements. 24 h later, cells were treated with [^3H]B[a]P (1 Ci/mmol for metabolism studies, 3 Ci/mmol for B[a]P DNA adduct studies) or [^3H]DMBA (1 Ci/mmol for DNA adducts studies) at a final media concentration of 2 nM and various coumarins at a concentration equimolar with the hydrocarbon.

Extraction of extracellular metabolites of B[a]P

The medium from duplicate dishes was collected and pooled 24 h after initial treatment with [^3H]B[a]P and coumarin. One ml media sample was extracted twice with 2 volumes of ethylacetate:acetone (2:1). The organic extracts were pooled and evaporated to dryness and the residues were dissolved in methanol for analysis of organic solvent-soluble metabolites. Another 1 ml media sample was analysed for the presence of glucuronide conjugates by treatment with β -glucuronidase prior to extraction according to our previously published procedure (32,34).

Analysis of DNA binding and hydrocarbon-DNA adduct formation

After the medium was removed, the cells were washed twice with PBS. The cells were lysed with 0.75 M guanidine isothiocyanate and the DNA was subsequently isolated as described (35). The extracted DNA was dissolved in 0.01 M Tris-MgCl₂ buffer (pH 7.0) and quantitated spectrophotometrically at 260 nm. The purity of DNA was determined as previously described (35). The radioactivity associated with purified DNA was measured using a Beckman LS 1800 liquid scintillation counter after digestion with DNase I. DNase-digested samples were further hydrolyzed using snake venom phosphodiesterase and alkaline phosphatase, sequentially as described previously (36). DNA hydrolysates were also processed through a short Sephadex LH-20 column as previously described (36). Some DNA hydrolysates were subjected to a dihydrobromate column of Servacel DHB[®] (0.9 × 3 cm) using the procedure essentially as described by Sawicki *et al.* (37) to separate anti and syn diol-epoxide-nucleoside adducts. One ml fractions were collected from Servacel columns and radioactivity in each fraction was determined. Fractions for either anti or syn diol-epoxide adducts were combined, respectively, for HPLC analysis.

HPLC analysis

HPLC analyses were performed using a Dupont Series 8800 HPLC equipped with an Altex Ultrasphere ODS column (46 mm × 25 cm). B[a]P metabolites were separated by sequential elution using the following gradient: 50–70% methanol in water (linear, over 20 min); 70% methanol in water (10 min); 70–85% methanol in water (linear, over 20 min); 85–100% methanol in water (linear, over 10 min). The gradient system for B[a]P-DNA adducts was as follows: 45% methanol in water (50 min); 45–60% methanol in water (linear, over 40 min); 60–100% methanol in water (linear, over 15 min). For analysis of DMBA-DNA adducts, a multistep gradient program was also used: 40–50% methanol in water (linear, over 50 min); 10 min hold at 50% methanol in water; 50–60% methanol in water (linear, over 40 min); and 60–100% methanol in water (linear, over 15 min). The column flow rate was 1 ml/min for all analyses. Individual 0.5 ml fractions were collected in scintillation vials. Radioactivity in each fraction was determined using a Beckman LS 1800 liquid scintillation counter.

Table I. Inhibitory effect of coumarins on the formation of water-soluble metabolites of B[a]P^a

Treatment	Water-soluble metabolites ^b % of control ^d	Glucuronide ^c % of control ^e
Control	100	100
7,8-BF	52	—
Bergamottin	44	32
Coriandrin	56	61
Isoimperatorin	59	64
Imperatorin	64	71
Ostruthin	83	86

^aThe data in the table represent an average of the results of two separate experiments with a variation from 4–6% for water soluble metabolites and 4–20% for glucuronides.

^bRadioactivity in the aqueous phase following extraction of the medium with ethylacetate:acetone (2:1) (expressed as a percentage of the total radioactivity recovered in medium).

^cAdditional radioactivity released into the organic extract by β -glucuronidase treatment of the medium (expressed as a percentage of the total radioactivity recovered in the medium).

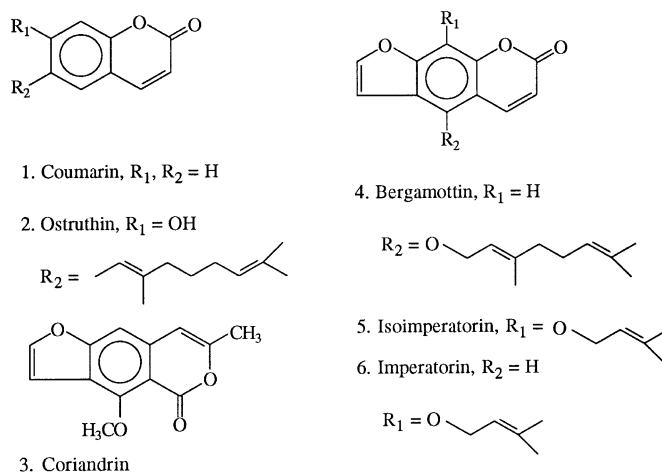
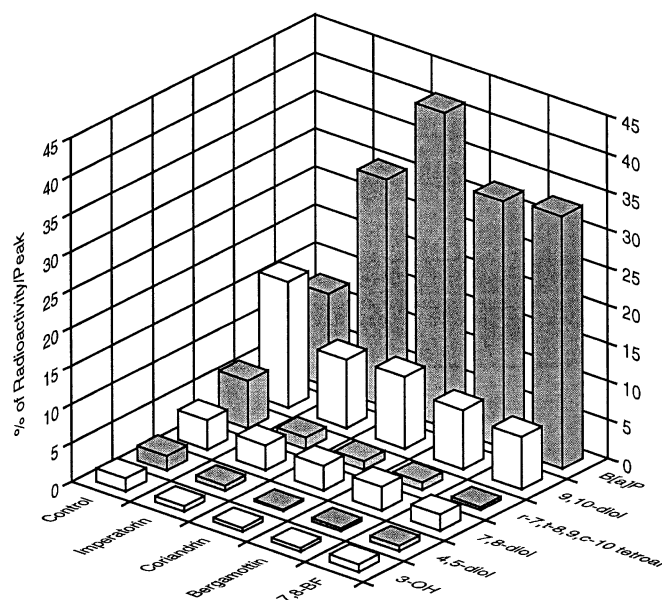
^dThe value in control group for % of water-soluble metabolites is 49%.

^eThe value in control group for % of glucuronide is 28%.

Results

Inhibitory effect of coumarins on B[a]P metabolism in mouse keratinocytes

Keratinocytes in high Ca^{2+} medium were treated with equimolar concentrations (2 nM) of coumarins and ^3H -B[a]P for 24 h. Media from duplicate plates was collected and extracted with organic solvent. The percentage of water soluble metabolites and glucuronides in the medium was determined. As shown in Table I, imperatorin, isoimperatorin, coriandrin, and bergamottin decreased the formation of water-soluble metabolites of B[a]P by 36–64% compared to the control cultures treated with the acetone vehicle instead of the coumarins. Glucuronide metabolites of B[a]P were also reduced in the cultures exposed to these coumarins. Analysis of the B[a]P metabolites released after digesting with β -glucuronidase revealed that the major glucuronide metabolite, 3-hydroxy-B[a]P (3-OH-B[a]P), was also reduced in the presence of the same coumarins (data not shown). The inhibition of the formation of water soluble metabolites of B[a]P by bergamottin, coriandrin, and isoimperatorin was very similar to that observed with 7,8-BF, a potent inhibitor of cytochrome P450 1A1 (38,39) (Table I). Bergamottin, which inhibited formation of water soluble metabolites from B[a]P by 56%, was the most effective inhibitor of B[a]P metabolism, while ostruthin, a simple coumarin, had little or no effect on overall metabolism of B[a]P (Table I). The analysis of organic solvent soluble metabolites of B[a]P in the medium indicated that the major metabolites formed by keratinocytes were 3-OH-B[a]P, B[a]P-9,10-diol, B[a]P-7,8-diol, and the (+)anti-BPDE tetraol. Figure 2 shows that bergamottin, coriandrin, and imperatorin dramatically decreased B[a]P tetraol formation by 70–84% and other metabolites of B[a]P such as the 3-OH, 9,10-diol, and 4,5-diol metabolites to a significant but lesser extent relative to acetone-treated keratinocytes. However, levels of the 7,8-diol were not significantly inhibited by the coumarins. Bergamottin was the most effective inhibitor of B[a]P metabolism in cultured mouse keratinocytes and gave an overall inhibition of B[a]P metabolism similar to that of 7,8-BF (Figure 2).

**Fig. 1.** Chemical structures of naturally occurring coumarins used in the present study.**Fig. 2.** Effect of coumarins on the formation of organic solvent-soluble metabolites of B[a]P. Each bar represents the radioactivity for each individual metabolite expressed as a percentage of the total organic-solvent soluble radioactivity and represents an average of three separate experiments (25% maximal variation). There were significant differences between control and all treated groups ($P < 0.05$) except 7,8-diol for coumarin treated groups and 3-OH-B[a]P for the 7,8-BF treated group according to the Mann-Whitney U -test.

Effects of selected coumarins on covalent binding of B[a]P and DMBA to DNA in cultured mouse keratinocytes.

The effect of equimolar concentrations of bergamottin or coriandrin on B[a]P and DMBA-DNA adduct formation in cultured keratinocytes is shown in Table II. The covalent binding of both PAH to keratinocyte DNA was significantly reduced in cells treated with either coumarin compared to cells exposed only to B[a]P or DMBA. However, a differential inhibitory effect of the coumarins was observed depending on whether B[a]P or DMBA was used. Bergamottin effectively inhibited covalent binding of B[a]P to DNA by 56% but had lesser inhibitory effect on the binding of DMBA to DNA (25% inhibition). In contrast, coriandrin produced greater inhibition of covalent binding of DMBA to DNA (48%) and was less effective at inhibiting covalent binding of B[a]P to

Table II. Inhibitory effects of coumarins on covalent binding of [³H]B[a]P and [³H]DMBA metabolites to DNA in mouse keratinocytes^a

Modifier treatment	Covalent binding (pmol/mg of DNA) B[a]P	DMBA
Control	27±3	108±5
Bergamottin	12±2 ^b (56)	81±7 ^{b,c} (25)
Coriandrin	22±1 ^{b,c} (19)	56±6 ^{b,c} (48)

^aValues in the table represent an average of at least three separate experiments ±SE. Numbers in parentheses represent the % inhibition relative to control values.

^bSignificantly less than the control ($P < 0.05$) by Student's *t*-test.

^cSignificantly less (for B[a]P) or greater (for DMBA) than the group treated with bergamottin ($P < 0.05$) by Student's *t*-test.

DNA (19% inhibition). Statistical analyses indicated that there was a significant difference between control and all coumarin groups ($P < 0.05$). In addition, the differences between each coumarin for a given hydrocarbon were also statistically significant ($P < 0.05$).

Effects of selected coumarins on the formation of specific DNA adducts derived from B[a]P and DMBA

The effects of bergamottin and coriandrin on the formation of individual B[a]P and DMBA DNA adducts were analysed by HPLC after hydrolyzing DNA samples to deoxyribonucleosides. Figure 3 shows the effects of bergamottin and coriandrin on the formation of individual B[a]P-DNA adducts in keratinocytes. As shown, four B[a]P-DNA adduct peaks were routinely detected in cultured keratinocytes, including (+) anti-BPDE-dGuo (peak III in Figure 3A), 9-hydroxyl-4,5-oxide-B[a]P-dGuo (peak I in Figure 3A), syn-BPDE-dGuo (peak IV in Figure 3A), and an unknown adduct (peak II in Figure 3A). The (+) anti-BPDE-dGuo adduct was the major DNA adduct detected in cultured keratinocytes exposed to B[a]P, a finding consistent with earlier studies from our laboratory (6). Bergamottin and coriandrin dramatically inhibited formation of both the (+) anti-BPDE-dGuo and 9-hydroxyl-4,5-oxide B[a]P-dGuo adducts compared to the control but did not decrease the formation of peaks II and IV (an unidentified adduct and a syn-BPDE-dGuo adduct, respectively). The results from these experiments are summarized in Table III and show that the relative distribution of B[a]P-DNA adducts was noticeably changed in the cells treated with bergamottin and coriandrin. In this regard, there was an increase in the level of the syn-BPDE adduct and a decrease in the level of the (+) anti-BPDE adducts in cells exposed to both coumarins. Thus, the ratio of the (+) anti-BPDE-dGuo:syn-BPDE-dGuo adducts changed from 11:1 in control cultures to 3.9:1 (bergamottin) and 3.2:1 (coriandrin) in coumarin treated cultures. The results presented in Figure 3 and Table III also demonstrate that bergamottin was a slightly more effective inhibitor of (+) anti-BPDE-dGuo adduct formation than coriandrin, consistent with its effects on total covalent binding of B[a]P to DNA.

Cultured keratinocytes exposed to DMBA for 24 h generated three major DNA adduct peaks which were identified as anti-DMBADE-dGuo (peak I in Figures 4A and C), syn-DMBADE-dAdo (peak II in Figures 4A and C), and anti-DMBADE-dAdo (peak III in Figures 4A and C). In addition, seven minor DNA adducts (peaks a, b, c, d, e, f, and g in Figures 4A and C) were also detectably present. As shown in Figures 4B and D, a marked reduction of anti-DMBA-diol

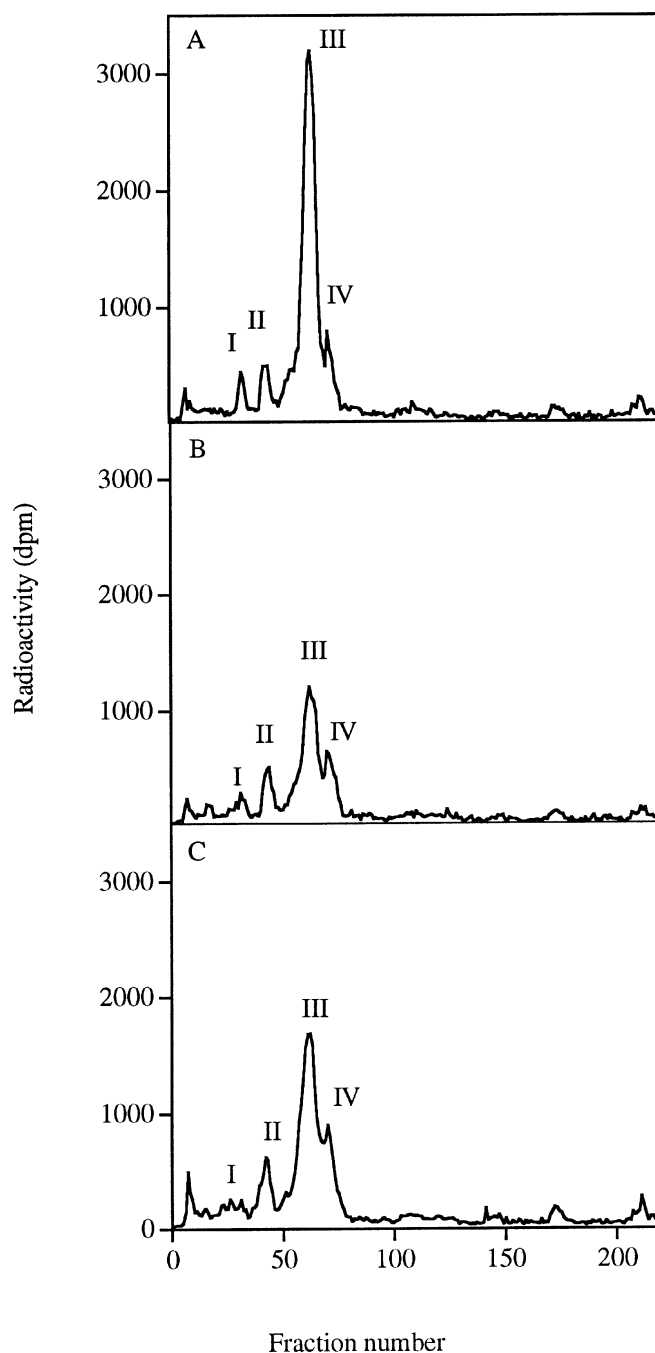


Fig. 3. HPLC profile of DNA adducts formed in cultured mouse keratinocytes 24 h after exposure to B[a]P. The chromatograms were normalized by the inclusion of two UV-absorbing markers (B[a]P-9,10-diol and B[a]P-4,5-diol) in the samples. (Peak I) 9-hydroxyl-4,5-oxide B[a]P-dGuo; (Peak II) unknown adduct; (Peak III) (+)-anti-BPDE-dGuo; (Peak IV) syn-BPDE-dGuo. (A) B[a]P-DNA adducts from acetone; (B) bergamottin-treated keratinocytes; (C) B[a]P-DNA adducts from coriandrin-treated keratinocytes. The Y-axis represents the radioactivity in each fraction which has been based on sample recovery from the column and normalized for equivalent amount of DNA in each sample.

epoxide-dGuo and dAdo adducts (peaks I and III, respectively) was observed in the keratinocytes treated with either bergamottin or coriandrin. In contrast, the major syn-DMBA-diol epoxide-dAdo adduct (peak II in Figures 4B and D) was only slightly decreased as was the case for the other DNA adduct peaks (peaks a–g). The specific inhibition of anti-DMBADE adduct formation by the two coumarins can be readily seen in

Table III. Distribution of individual B[a]P- and DMBA-DNA adducts in coumarin-treated keratinocytes^a

Treatment	B[a]P-DNA adducts ^b (pmol/mg of DNA)				DMBA-DNA adducts ^c (pmol/mg of DNA)		
	I	II	III	IV	I	II	III
Control	1.1	1.4	16.4	1.5	25.0	18.3	13.4
Bergamottin	0.7	2.1	7.7	2.0	7.9	17.0	4.4
Coriandrin	0.3	2.1	9.6	3.0	5.7	11.7	2.6

^aThe results represent an average from two separate experiments with the variation from 10–30% for B[a]P and 7–25% for DMBA.

^bB[a]P-DNA adduct peaks: peak I, 9-hydroxy-4,5-oxide-B[a]P-dGuo; peak II, unknown; peak III, (+) anti-B-PDE-dGuo; peak IV, syn-B-PDE-dGuo.

^cDMBA-DNA adducts: peak I, anti-diol-epoxide-dGuo; peak II, syn-diol-epoxide-dAdo; peak III, anti-diol-epoxide-dAdo.

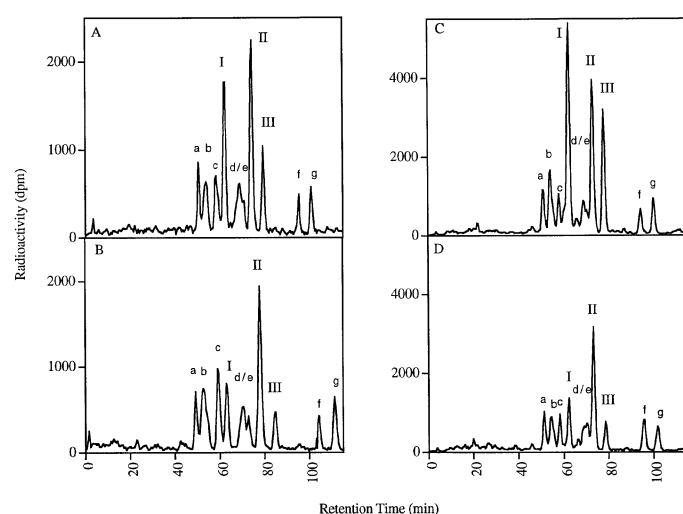


Fig. 4. HPLC profiles of the DNA adducts isolated from mouse keratinocytes 24 h after exposure to DMBA. The chromatograms were normalized by the inclusion of two UV-absorbing markers (B[a]P-9,10-diol and B[a]P-4,5-diol) in the samples. (A and B) DMBA-DNA adducts from acetone and bergamottin-treated keratinocytes; (C and D) DMBA-DNA adducts from acetone and coriandrin-treated keratinocytes. (Peaks I, II, and III) are anti-diol-epoxide-dGuo, syn-diol-epoxide-dAdo, and anti-diol-epoxide-dAdo, respectively. The Y-axis represents the radioactivity in each fraction which has been based on sample recovery from the column and normalized for equivalent amount of DNA in each sample.

Table III where the level of anti-DMBADE adducts formed from DMBA was markedly lower in cells treated with the coumarins. In contrast, the level of syn-DMBADE adducts remained the same (bergamottin) or slightly decreased (coriandrin). The inhibitory effect of coriandrin on the formation of specific DMBA DNA adducts was greater than that of bergamottin, a finding consistent with the data in Table II.

Selective inhibition of anti-diol epoxide-DNA adducts derived from DMBA by bergamottin and coriandrin

The relative proportion of syn- and anti-DMBADE-DNA adducts was further analysed in cultured keratinocytes treated with coumarins to confirm their selective inhibitory effect on anti-DMBADE-DNA adduct formation. For these experiments, Servacel DHB[®] columns were used to separate the anti- and syn-DMBADE-DNA adducts prior to HPLC. As shown in Figure 5, coriandrin dramatically inhibited the formation of total anti-DMBADE-DNA adducts (peak 2 in Figure 5) but had much less effect on total syn-DMBADE-DNA adducts

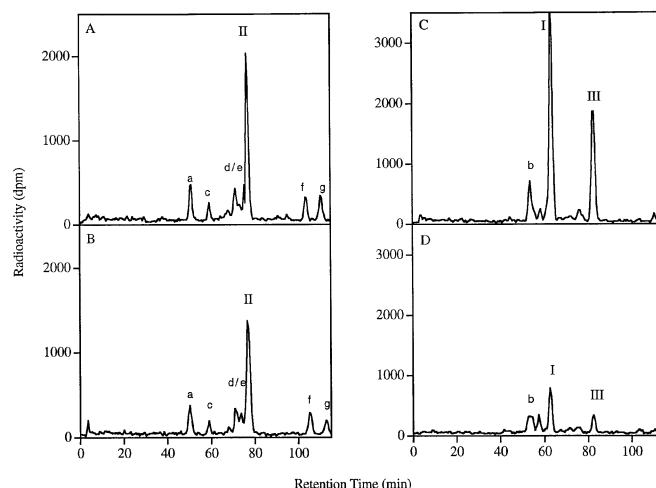


Fig. 5. Servacel DHB column chromatography profiles of DMBA-DNA adducts isolated from keratinocytes. Solid line, DMBA-DNA adducts from acetone-treated cells; Dashed line, DMBA-DNA adducts from coriandrin-treated cells. (Peak I) syn-diol-epoxide-DNA adducts; (Peak II), anti-diol-epoxide-DNA adducts. The chromatograms shown are representative with repeat experiments giving essentially identical results. The Y-axis represents the radioactivity in each fraction which has been based on sample recovery from the column and normalized for equivalent amount of DNA in each sample.

(peak 1 in Figure 5) relative to the control. Table IV summarizes the results from two separate experiments using both coriandrin and bergamottin, which clearly shows that both coumarins effectively decreased the total anti-DMBADE-DNA adducts by 41% and 65%, respectively. In contrast, only a slight reduction of syn-DMBADE-DNA adducts was observed (6% and 11%, respectively). Thus, the ratios of anti- to syn-DMBADE adducts in coumarin-treated cells was 1.44:1 and 0.57:1 for control and coriandrin-treated cultures, respectively, and 1.7:1 and 1:1 for control and bergamottin-treated cultures, respectively. Further analysis of individual DNA adducts derived from the anti-DMBADE and syn-DMBADE by HPLC again showed that coriandrin (and bergamottin to a lesser extent, data not shown) greatly decreased all individual anti-DMBADE-DNA adducts (peaks b, I, and III in Figure 5D) and only slightly decreased individual syn-DMBADE-DNA adducts (peaks a, c, d, II, e, and f), further confirming the apparent selectivity for inhibiting formation of anti-DMBADE-DNA adducts.

Discussion

The present study demonstrates that several naturally occurring coumarins including bergamottin, coriandrin, isomerperatorin, and imperatorin effectively inhibited the metabolic activation of B[a]P and/or DMBA in cultured mouse keratinocytes. The major findings of this study are as follows: (a) natural coumarins inhibited the formation of water-soluble and organic-solvent soluble metabolites of B[a]P in cultured mouse keratinocytes with the following order of effectiveness: bergamottin > coriandrin > isomerperatorin > imperatorin; (b) both bergamottin and coriandrin were found to inhibit DNA adduct formation from both B[a]P and DMBA in mouse keratinocytes; (c) bergamottin was more effective at inhibiting covalent binding of B[a]P to keratinocyte DNA whereas coriandrin was more effective at inhibiting covalent binding of DMBA to keratinocyte DNA; and (d) both bergamottin and coriandrin selectively inhibited the formation of anti-diol-epoxide-DNA

Table IV. Stereoselective inhibition of DMBA–DNA adducts in keratinocytes by coumarins^a

Adducts	Experiment I				Experiment II			
	Control		Bergamottin		Control		Coriandrin	
	syn-	anti-	syn-	anti-	syn-	anti-	syn-	anti-
pmol/mg of DNA	33	56	31	33	39	56	35	20
% of control	100	100	94	59	100	100	89	35
Ratio of anti to syn	1.7 : 1		1.06 : 1		1.44 : 1		0.57 : 1	

^aResults represent an average from two separate experiments with similar results.

adducts from both hydrocarbons. Overall, the current data suggest that certain natural coumarins have the ability to block the metabolic activation of PAH in a cell culture model system of mouse epidermis.

Based on the current literature, both rodent and human P450 1A1 appears to be a predominant P450 involved in converting B[a]P to various metabolites, including 3-OH-B[a]P, B[a]-9,10-diol, B[a]P-7,8-diol, and (+) anti-BPDE (16). The pattern of organic solvent soluble metabolites of B[a]P formed in cultured mouse keratinocytes in our studies was very similar to that catalyzed by cDNA-expressed mouse P450 1A1 (16). P450 1A1 is expressed in mouse keratinocytes (40) and this isozyme is highly inducible by PAH (41). In our previous studies, bergamottin, coriandrin, isoimperatorin, imperatorin, and ostruthin were found to be effective inhibitors of P450 1A1 in hepatic microsomes from mice pretreated with MC (28). It is interesting to note that the ability of this series of naturally occurring coumarins to inhibit P450 1A1 correlated with their ability to inhibit B[a]P metabolism in cultured keratinocytes. Collectively, these data suggest that P450 1A1 may be the predominant P450 involved in metabolism of B[a]P in cultured mouse keratinocytes maintained in high-Ca²⁺ media (32,40). It is also interesting to note that none of the coumarins appeared to significantly inhibit formation of B[a]P-7,8-diol, the proximate carcinogenic metabolite, and yet the formation of the (+) anti-BPDE was dramatically inhibited as measured by the presence of the (+) anti-BPDE tetraol (Figure 2). This result may be explained by the fact that P450 1A1 more efficiently catalyzes the second oxidation step in the metabolic activation of B[a]P. In fact, a recent study from Shou *et al.* (16) reported a higher turnover rate of cDNA expressed rodent P450 1A1 for conversion of B[a]P-7,8-diol to BPDE than for B[a]P to B[a]P-7,8-diol. Thus, the coumarins may preferentially inhibit formation of the anti-BPDE leading to an accumulation of B[a]P-7,8-diol due to its decreased disposition.

Analysis of the effects of both bergamottin and coriandrin on B[a]P–DNA adduct formation provides further support for a predominant role of P450 1A1 in the metabolism of B[a]P in this system and in the mechanism for their inhibitory effects. In this regard, analysis of the B[a]P–DNA adduct profiles in Figure 3 revealed the presence of several B[a]P–DNA adducts, including the 9-OH-B[a]P-4,5-oxide–dGuo adduct and the (+)anti-BPDE–dGuo adduct. Current evidence indicates that the (+)anti-BPDE is formed through the stereoselective metabolism of B[a]P-7,8-diol by rodent and human P450 1A1 as well as other human P450 isozymes such as P450 1A2, 2C9 and P450 3A (16). Our results demonstrate that both bergamottin and coriandrin selectively decreased (+) anti-BPDE and 9-OH-B[a]P-4,5-oxide but did not dramatically

reduce formation of other DNA adducts formation [including a syn-BPDE–dGuo adduct]. These results suggest that bergamottin and coriandrin, through inhibition of P450 1A1, selectively inhibited formation of the (+) anti-BPDE–dGuo adduct. The fact that bergamottin and coriandrin also selectively decreased formation of the DNA adduct tentatively identified as the 9-OH-B[a]P-4,5-oxide–dGuo adduct further supports this hypothesis since the metabolism of B[a]P to 9-OH-B[a]P or B[a]P-4,5-oxide has been shown to be catalyzed by both rodent and human P450 1A1 (16) and the formation of DNA adducts derived from this metabolite can be blocked by antibody against MC-induced P450s (6).

In the present study we also examined the effects of bergamottin and coriandrin on the formation of DNA adducts from DMBA in cultured mouse keratinocytes. The metabolism and metabolic activation of DMBA is less well understood than B[a]P. However, recent studies have indicated the involvement of specific P450s in the metabolism and metabolic activation of this PAH. In this regard, P450 2C6 in rat hepatic microsomes has been shown to convert DMBA to its proximate carcinogenic 3,4-diol (18). Recently, Pottenger *et al.* (17) further demonstrated that P450 1B1 in BA-treated C3H/10T1/2 cells was very active in converting DMBA to DMBA-3,4-diol, while P450 1A1 in BA-treated mouse hepatoma cells was much less active in this regard (17). In addition, Shimada *et al.* recently reported that human P450 1B1 possessed high catalytic activity in converting DMBA-3,4-diol but very low catalytic activity in converting DMBA to mutagenic metabolites (19). The pattern of metabolites produced by both mouse and human P450 1B1 was very different from the pattern generated by both mouse and human P450 1A1 in these studies (17,19). Furthermore, it has been demonstrated that P450 1A1 in rat hepatic microsomes is quantitatively important in the formation of the DMBA-5,6-diol and DMBA-8,9-diol metabolites (42). However, these metabolites contribute little, if any, to the covalent binding of DMBA to DNA (43). That P450 1A1 is quantitatively more important in the metabolic activation of B[a]P than DMBA in mouse keratinocytes is also supported by the differential effect of bergamottin and coriandrin. In this regard, the potency for the inhibition of covalent binding of B[a]P to DNA [bergamottin > coriandrin (see Table II)] correlated with their ability to inhibit P450 1A1, while the reverse relationship was observed with DMBA. Our previous study showed that coriandrin is a suicide inhibitor of both mouse and human P450 1A1 (29). It is interesting to note that 1-ethynylpyrene, previously reported to be a selective suicide inhibitor of rat P450 1A1 (20), was more effective at inhibiting covalent binding of DMBA than B[a]P to DNA in mouse epidermis (20). This compound was recently found to be a potent inhibitor of rat P450 1B1 in cultured adrenocortical

cells (21). This finding raises the interesting possibility that coriandrin could have a similar inhibitory effect on P450 1B1 and that this P450 is quantitatively important in the metabolic activation of DMBA in cultured mouse keratinocytes. P450 1B1 is expressed in both cultured mouse and human keratinocytes (44, 45) and thus may be important in the metabolic activation of DMBA in this tissue. In addition, the importance of other P450s that may be expressed in cultured keratinocytes (and mouse epidermis *in vivo*) (46,47) remains to be determined. Further studies in progress are investigating these possibilities.

Another interesting observation in our current study was the finding that both bergamottin and coriandrin at concentrations equimolar with DMBA reduced the formation of anti-DMBADE–DNA adducts while having little or no effect on the formation of DNA adducts from the syn-DMBADE. Recent studies in cultured mouse embryo cells and mouse epidermis *in vivo* have indicated that the major anti-DMBADE–DNA adducts were derived from the metabolism of DMBA-(3R,4R)-dihydrodiol to the DMBA-(4R,3S)-diol-(2S,1R)-epoxide, whereas the major syn-DMBADE–DNA adducts resulted from the binding of DMBA-(4S,3R)-diol-(2S,1R)-epoxide that is produced by activation of DMBA-(3S,4S)-dihydrodiol (48,49). In studies with MCF-7 cells, Lau *et al.* (50) found that the predominant DNA adducts formed from DMBA were anti-diol-epoxide adducts and suggested that this was due to constitutive expression of P450 1B1 in human MCF-7 cells. In fact, MCF-7 cells were shown to contain metabolic activity toward DMBA that could be inhibited by antibodies against P450 1B1 (51). These data further support the hypothesis that both bergamottin and coriandrin may inhibit a different P450 in mouse keratinocytes in bringing about a specific reduction in anti diol-epoxide–DNA adducts derived from DMBA. In addition, the potency for inhibition of this ‘other P450’ appears to be reversed for bergamottin and coriandrin compared with their effects on P450 1A1.

In conclusion, our studies demonstrate that several naturally occurring coumarins, to which humans are routinely exposed, are potent inhibitors of the metabolism and metabolic activation of B[a]P and DMBA in cultured mouse keratinocytes. Several of these compounds (bergamottin and coriandrin), upon further examination, demonstrated some selectivity toward the formation of specific metabolites of both hydrocarbons. These results support the involvement of distinct P450s in the metabolism and metabolic activation of B[a]P vs DMBA in mouse keratinocytes. As a class of compounds the coumarins may prove useful in evaluating specific P450s involved in the metabolic activation of B[a]P and/or DMBA in a specific tissue. In addition, these compounds may have the ability to block tumor initiation in mouse skin and other tissues *in vivo*. Experiments dealing with both of these areas are currently underway.

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