

Effect of naturally occurring coumarins on the formation of epidermal DNA adducts and skin tumors induced by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene in SENCAR mice

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Several naturally occurring coumarins previously found to be potent inhibitors of mouse hepatic ethoxyresorufin-O-deethylase (EROD) and/or pentoxyresorufin-O-dealkylase (PROD) were examined for their effects on formation of benzo[*a*]pyrene (B[*a*]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA) DNA adducts in mouse epidermis, as well as, their effects on skin tumor initiation by these polycyclic aromatic hydrocarbons (PAH). Bergamottin, a potent inhibitor of hepatic EROD, given topically 5 min prior to an initiating dose of B[*a*]P, significantly decreased total covalent binding of B[*a*]P to DNA in a dose-dependent manner 24 h after treatment. A dose of 400 nmol bergamottin reduced covalent binding of B[*a*]P by 72%. Coriandrin, at a dose of 400 nmol also significantly reduced total covalent binding of B[*a*]P by 59%. In addition, formation of the major (+)anti-B[*a*]P-diol epoxide-N2-dGuo adduct was selectively reduced by both of these coumarins. In contrast, bergamottin and coriandrin did not significantly decrease covalent binding of DMBA to epidermal DNA at doses of either 400 nmol or 800 nmol. Imperatorin and isopimpinellin, which are more potent inhibitors of hepatic PROD activity, significantly reduced overall binding of DMBA to epidermal DNA by 67% and 52%, respectively, when applied at doses of 400 nmol. These two coumarins also inhibited B[*a*]P-DNA adduct formation at similar doses but to a lesser extent. Imperatorin at a dose of 400 nmol dramatically decreased formation of covalent DNA adducts derived from both the anti and syn diol epoxides of DMBA. Bergamottin was a potent inhibitor of tumor initiation by B[*a*]P while coriandrin was less effective in this regard. Imperatorin was an effective inhibitor of skin tumor initiation by DMBA and also inhibited complete carcinogenesis by this PAH. At dose levels higher than those effective against DMBA, imperatorin also inhibited tumor initiation by B[*a*]P. The results demonstrate that several naturally occurring coumarins possess the ability to block DNA adduct formation and tumor initiation by PAHs such as

B[*a*]P and DMBA. The mechanism for reduced DNA adduct formation and tumor initiation appears to involve inhibition of the P450s involved in the metabolic activation of these hydrocarbons. Finally, the differential effects of certain coumarins on B[*a*]P vs DMBA DNA adduct formation and tumor initiation may be useful for dissecting the role of specific cytochromes P450 in their metabolic activation.

Introduction

The process of skin carcinogenesis in mice can be effected by using either a complete carcinogenesis protocol (one large dose or multiple low doses of a carcinogen) or a multistage protocol involving the operational and mechanistically distinct stages of initiation and promotion. It is generally accepted that the stages of initiation and promotion are present during complete carcinogenesis but that they may occur by distinct mechanisms (1). It is highly likely that both types of carcinogenic processes occur in humans as there are clear examples of complete carcinogenesis in humans (e.g. UV-induced skin cancer; cigarette-induced lung cancer). During the initiation stage, carcinogens are metabolically activated to form ultimate carcinogens, which react with DNA at critical target genes such as the *c-Ha-ras* protooncogene resulting in mutations (1). Since the initiation stage is irreversible, inhibition of the formation of carcinogen-DNA adducts, in theory, should reduce the formation of tumors. Many chemicals have been shown capable of inhibiting the tumor initiation stage of chemical carcinogenesis in mouse skin as well as other model systems (reviewed in 1–3) supporting this hypothesis.

Benzo[*a*]pyrene (B[*a*]P*) and 7,12-dimethylbenz[*a*]anthracene (DMBA) have been identified as effective tumor initiators and complete carcinogens in the mouse skin model, with the latter compound being particularly potent in this regard (1–3). These polycyclic aromatic hydrocarbons (PAH) are metabolically activated by cytochrome(s) P450 to diol-epoxide intermediates (4). The major DNA adducts derived from B[*a*]P are formed by reaction of (+)anti-B[*a*]P-diol epoxide [(+)anti-BPDE] with deoxyguanosine (dGuo) or from DMBA by reaction of both the anti- or syn-DMBA-diol epoxides (anti-DMBADE or syn-DMBADE) with dGuo and/or deoxyadenosine (dAdo) (5,6). It has been shown that the formation of some of these DNA adducts are quantitatively correlated with the tumor initiating activity of these PAH (7,8). Furthermore, agents that decrease the formation of DNA adducts derived from B[*a*]P and DMBA have been shown to inhibit tumor initiation by these hydrocarbons. These agents include: 7,8-benzoflavone (7,8-BF), 1-ethynylpyrene (1-EP), and polyphenols from green tea (2,3,9,10).

Previous studies from our laboratory have shown that several naturally occurring coumarins, including the furanocoumarins bergamottin, imperatorin, and isopimpinellin (abundant in citrus oil) and a furoisocoumarin, coriandrin (present in coriander leaves), inhibited mouse hepatic EROD or PROD activity

*Abbreviations: AHH, aryl hydrocarbon hydroxylase; AP, aminopyrene; (+) anti-BPDE, (+)-7b, 8a-dihydroxy-9a, 10a-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; anti-DMBADE, (±) 1b, 2b-epoxy-3b, 4a-dihydroxyl-1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene; BA, benz[*a*]anthracene; B[*a*]P, benzo[*a*]pyrene; 7,8-BF, 7,8-benzoflavone; dAdo, deoxyadenosine; DMBA, 7,12-dimethylbenz[*a*]anthracene; 1-EP, 1-ethynylpyrene; P450, cytochrome P450; PAH, polycyclic aromatic hydrocarbon; SCCs, squamous cell carcinomas; syn-DMBADE, (±) 1a, 2a-epoxy-3b, 4a-dihydroxyl-1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

in vitro (11). Coriandrin and bergamottin also were shown to effectively inhibit purified human P450 1A1 in a reconstituted system (12). Recent studies using cultured mouse keratinocytes have further demonstrated that these compounds inhibited metabolic activation of both B[a]P and DMBA by reducing the formation of DNA adducts derived from these hydrocarbons (13). All of these data have suggested that one or more of these naturally occurring coumarins ingested by humans may have marked effects on tumorigenesis induced by PAH and possibly other carcinogens.

The present study was designed to investigate the ability of several naturally occurring coumarins to inhibit PAH-induced skin carcinogenesis in SENCAR mice. To this end, we have determined the effect of coumarins on the formation of DNA adducts derived from B[a]P and DMBA in mouse epidermis *in vivo*. In addition, we examined the effects of selected coumarins on tumor initiation induced by B[a]P and DMBA in mouse skin using a standard two-stage initiation-promotion protocol, as well as, the effect of one coumarin on complete carcinogenesis by DMBA. The results demonstrate that at least one naturally occurring coumarin (imperatorin) effectively inhibited the formation of DNA adducts and also inhibited tumorigenesis induced by both B[a]P and DMBA while another naturally occurring coumarin (bergamottin) was a potent and selective inhibitor of B[a]P DNA adduct formation and tumor initiation. The results have led to an hypothesis regarding the most effective type of inhibitory agent that will be active against tumor initiation by a broad number of PAH, including both B[a]P and DMBA.

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 (sp. act. 66–70 Ci/mmol) and [3H]DMBA (sp. act. 50 Ci/mmol) were obtained from Amersham Co. (Arlington Heights, IL) and diluted with unlabeled B[a]P or DMBA to specific activities of 1 Ci/mmol or 10 Ci/mmol as indicated. Bergamottin and isopimpinellin were purchased from Indofine Chemical Co. (Belle Mead, NJ). Coriandrin (14) was provided by Dr Mike Ashwood-Smith (University of Victoria, Victoria, B.C.). Ostruthin was obtained from Dr Warren Steck (National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan). Imperatorin was synthesized as previously described (15). DNase I (bovine pancreas, EC 3.1.4.1), alkaline phosphatase (*Escherichia 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). 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.

Analysis of hydrocarbon-DNA adduct formation

The backs of SENCAR mice (7–9 weeks of age) were shaved two days before treatment and only those mice in the resting phase of the hair cycle were used. Several dose levels of individual coumarins were applied to the dorsal skin of mice (3 mice per group) 5 min prior to treatment with either [3H]B[a]P (200 nmol with specific radioactivity 1 Ci/mmol) or [3H]DMBA (10 nmol with specific radioactivity 10 Ci/mmol). The control group of mice was treated with the acetone vehicle (0.2 ml) 5 min prior to the hydrocarbon treatment. The mice were killed 24 h after treatment and the epidermis from mice in each group was scraped and pooled for DNA isolation and DNA-adduct analysis.

The epidermis from groups of mice was lysed with 0.75 M guanidine isothiocyanate and homogenized using a syringe with a 16 G needle. The DNA was subsequently isolated as previously described (16). The extracted DNA was dissolved in 0.01 M Tris–MgCl₂ buffer (pH 7.0) and quantitated spectrophotometrically at 260 nm. The radioactivity associated with purified DNA was measured using a Beckman LS 1800 liquid scintillation counter after digestion with DNase I. DNase-digested DNA samples were further hydrolyzed by sequentially adding snake venom phosphodiesterase and alkaline

phosphatase as described previously (17). DNA hydrolysates were processed through a short Sephadex LH-20 column as described by DiGiovanni *et al.* (17) to separate nucleoside adducts from free nucleosides. The adducts derived from B[a]P and DMBA were analyzed by HPLC (17).

HPLC analysis

HPLC analyses were performed using a Shimadzu SCL-6A HPLC system equipped with an Altex Ultrasphere ODS column (46mm×25cm). The gradient system for B[a]P–DNA adducts was as follows: 45% methanol in water (over 50 min); 45–60% methanol in water (linear, 40 min); 60–100% methanol in water (linear, 15 min). For analysis of DMBA–DNA adducts, a multistep gradient program was also used: 40–50% methanol in water (linear, 50 min); 10 min hold at 50% methanol in water; 50–60% methanol in water (linear, 40 min); and 60–100% methanol in water (linear, 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.

Tumor experiments

Groups of 25 or 30 mice 7–9 weeks of age were used for the initiation-promotion and the complete carcinogenesis studies, respectively. For the former studies, groups of 25 mice each were treated with various doses of coumarins 5 min prior to initiation with B[a]P (200 nmol per mouse). Control mice received the vehicle, acetone (0.2 ml), 5 min prior to B[a]P treatment. Two weeks after initiation, mice received topical applications of TPA (1.7 nmol per mouse) given twice-weekly. The promotion stage was continued in each experimental group until a maximal papilloma response was achieved. As a control for any coumarin related effects, groups of 25 or 30 mice were treated with the coumarins at the initiation stage followed by twice-weekly treatment with TPA. The incidence and multiplicity of skin papillomas were recorded weekly. For the complete carcinogenesis experiments, groups of 30 mice received weekly applications of two dose levels of imperatorin 5 min prior to applications of DMBA (50 nmol per mouse). The control mice were treated with acetone 5 min prior to each weekly application of the hydrocarbon. A group of 30 mice was treated weekly with imperatorin only (800 nmol per mouse) as the compound control. The incidence and multiplicity of both papillomas and carcinomas were recorded weekly. Carcinomas were initially recorded grossly as downward invading lesions and later verified histologically.

Results

Inhibitory effect of coumarins on covalent binding of B[a]P and DMBA to epidermal DNA

Initially, several coumarins were tested for their effects on the covalent binding of [3H]B[a]P (200 nmol) and [3H]DMBA (10 nmol) to DNA as shown in Table I. A dose of 400 nmol per mouse of each coumarin was chosen for these initial experiments. Topical application of 400 nmol bergamottin, coriandrin, ostruthin, imperatorin, or isopimpinellin given 5 min prior to the hydrocarbons, inhibited the covalent binding of B[a]P to DNA. Bergamottin was the most effective inhibitor of the formation of total covalent B[a]P–DNA adducts. In contrast, bergamottin and coriandrin did not significantly inhibit covalent binding of DMBA to epidermal DNA. Imperatorin, isopimpinellin, and ostruthin, however, significantly inhibited covalent binding of DMBA to epidermal DNA. Notably, imperatorin (67% inhibition at the 400 nmol dose) was the most effective inhibitor of the formation of DMBA–DNA adducts but was less effective at inhibiting covalent binding of B[a]P.

More detailed studies with bergamottin and imperatorin were performed by examining a wider range of dose levels given 5 min prior to topical application of 200 nmol [3H]B[a]P or 10 nmol [3H]DMBA. As shown in Figure 1, bergamottin caused a dose-dependent decrease of the covalent binding of B[a]P to epidermal DNA. Doses of 20 and 50 nmol/mouse of bergamottin were sufficient to decrease the covalent binding of B[a]P to DNA by 24% and 50%, respectively. At dose levels of 200 nmol, 400 nmol, and 800 nmol, bergamottin dramatically inhibited covalent binding of B[a]P to DNA by ~70%. In contrast, bergamottin did not inhibit covalent binding

Table I. Inhibition of covalent binding of B[a]P and DMBA to mouse epidermal DNA *in vivo* by naturally occurring coumarins^a

Treatment	Dose (nmol/mouse)	[3H] B[a]P (200 nmol, 200 μ Ci/mouse) Covalent binding (pmol/mg of DNA)	[3H] DMBA (10 nmol, 100 μ Ci/mouse) Covalent binding (pmol/mg of DNA)
Control	–	32 \pm 3 (100)	6.1 \pm 0.8 (100)
Bergamottin	400	9 \pm 2 (28) ^b	5.6 \pm 0.8 (92)
Coriandrin	400	13 \pm 2 (41) ^b	5.4 \pm 0.4 (89)
Ostruthin	400	19 \pm 1 (59) ^b	4.1 \pm 0.2 (67) ^b
Imperatorin	400	21 \pm 5 (66) ^b	2.0 \pm 0.8 (33) ^b
Isopimpinellin	400	19 \pm 3 (59) ^b	2.9 \pm 0.6 (48) ^b

^aGroups of 3 mice each were treated with coumarins 5 min prior to B[a]P or DMBA treatment. Twenty four hours after B[a]P and DMBA treatment, DNA was isolated and DNA binding was determined as described in Materials and methods. The data are the average value from three separate experiments. Numbers in parentheses represent the pmol/mg DNA expressed as a percent of the control value.

^bSignificantly different than the control values as analyzed by Mann-Whitney *U*-test with *P* < 0.01.

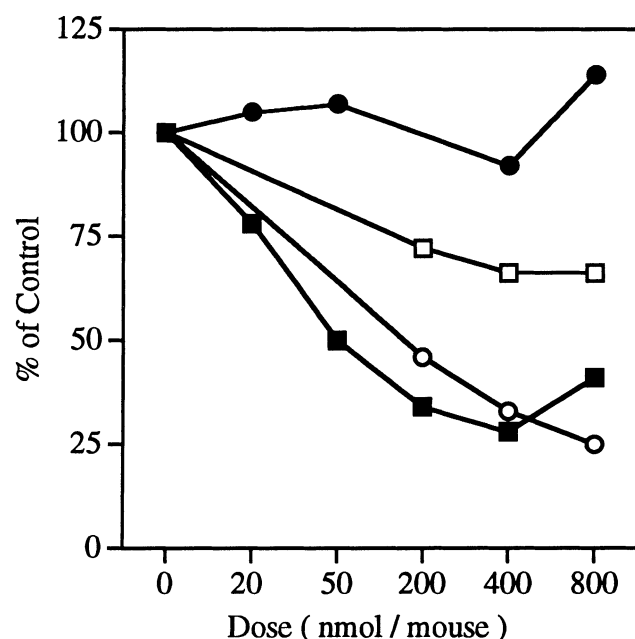


Fig. 1. Effect of bergamottin and imperatorin on the covalent binding of B[a]P and DMBA to mouse epidermal DNA. The determination of DNA binding is described in Materials and methods. Coumarins were applied 5 min prior to the [3H]PAH. The data are expressed as percentage of control, which was 32 \pm 3 pmol/mg DNA for B[a]P or 6.1 \pm 0.8 pmol/mg of DNA for DMBA. Control mice received acetone 5 min prior to 3H-labeled PAH. ●, bergamottin + DMBA; □, imperatorin + B[a]P; ○, imperatorin + DMBA; ■, bergamottin + B[a]P

of DMBA to DNA at doses from 20 nmol to 800 nmol per mouse. On the other hand, imperatorin inhibited covalent binding of both B[a]P and DMBA to DNA in a dose-dependent manner. As observed in the initial studies with a single dose of imperatorin (Table I), a differential inhibitory effect was also observed with this compound depending on whether B[a]P or DMBA was used. In this regard, at all doses tested imperatorin reduced covalent binding of DMBA to a greater extent than that of B[a]P.

Effect of coumarins on the formation of individual DNA adducts derived from B[a]P and DMBA

Additional experiments examined the effect of bergamottin, coriandrin, and ostruthin on formation of specific B[a]P DNA adducts in mouse epidermis. For these experiments, DNA adduct samples were obtained from mice treated with 400 nmol of the specific coumarins. Bergamottin, coriandrin, and ostruthin significantly decreased formation of the (+) anti-

Table II. Inhibitory effect of coumarins on formation of specific DNA adducts derived from B[a]P in mouse epidermis *in vivo*^a

Groups	9-OH-4,5-oxide BP-dGuo (pmol/mg of DNA)	Anti-BPDE-dGuo (pmol/mg of DNA)
Acetone	1.66 \pm 0.19	22.12 \pm 2.54
Bergamottin	0.17 \pm 0.05 (10)	4.4 \pm 1.23 (20)
Coriandrin	1.0 \pm 0.01 (60)	10.0 \pm 1.03 (45)
Ostruthin	0.84 \pm 0.01 (51)	12.9 \pm 0.07 (58)

^aGroups of 4–5 mice each were treated with coumarins (400 nmol/mouse) on the dorsal skin 5 min prior to B[a]P treatment (200 nmol, 200 μ Ci/mouse). Twenty-four hours after treatment B[a]P DNA was isolated and DNA adducts were analysed as described in Materials and methods. The data represents an average of three separate experiments \pm SD. The numbers in parentheses represent the pmol/mg DNA expressed as a percent of control values.

BPDE-N2-dGuo adduct and a DNA adduct that cochromatographed with a marker adduct derived from further metabolism of 9-OH-B[a]P. This adduct was tentatively identified as a 9-OH-4,5-oxide-B[a]P-dGuo adduct (Table II). Bergamottin inhibited formation of (+) anti-BPDE-N2-dGuo by 80% and inhibited formation of 9-OH-4,5-oxide B[a]P-dGuo by 90%. Bergamottin was the most effective inhibitor of individual B[a]P DNA adducts which correlated with its inhibitory effect on total covalent binding of B[a]P to DNA. The effect of bergamottin, coriandrin, and imperatorin on the formation of individual DMBA–DNA adducts in mouse epidermis is shown in Table III. Bergamottin and coriandrin, neither of which significantly inhibited total DMBA–DNA adducts at a dose of 400 nmol, did lower the level of both the anti-DMBADE-dGuo and dAdo adducts by ~40%. However, the level of the major syn-DMBADE-dAdo adduct was enhanced by pretreatment with bergamottin (48%) or coriandrin (18%). Imperatorin, which efficiently inhibited total DMBA–DNA adducts at a dose of 400 nmol per mouse, significantly decreased formation of all three major DMBA–DNA adducts.

Effect of coumarins on skin tumor initiation by B[a]P and DMBA

For tumor initiation experiments, groups of 25 or 30 mice each were treated with coumarins 5 min prior to application of 200 nmol B[a]P or 10 nmol DMBA. Control mice were treated with acetone 5 min prior to the hydrocarbon treatment. Two weeks later, mice were treated with twice-weekly application of TPA (1.7 nmol). Tumors were recorded weekly starting in week 6 after initiation (i.e. week 4 of promotion). The effects of bergamottin, coriandrin, ostruthin, and imperatorin

Table III. Effects of coumarins on the formation of the major anti and syn DMBADE DNA adducts in mouse epidermis *in vivo*^a

Coumarins	Anti-dGuo (pmol/mg of DNA)	Syn-dAdo (pmol/mg of DNA)	Anti-dAdo (pmol/mg of DNA)
Control	1.81 ± 0.12	1.15 ± 0.29	1.07 ± 0.16
Bergamottin (400 nmol/mouse)	1.13 ± 0.02 (63) ^b	1.70 ± 0.04 (148)	0.64 ± 0.01 (60)
Coriandrin (400 nmol/mouse)	1.14 ± 0.17 (63)	1.36 ± 0.05 (118)	0.66 ± 0.08 (62)
Imperatorin (400 nmol/mouse)	0.62 ± 0.20 (34)	0.66 ± 0.21 (57)	0.34 ± 0.11 (32)

^aThe data represent the means ± SD from three separate experiments.^bThe data in parentheses are the percentage of control values.**Table IV.** Effects of coumarins on skin tumor initiation by B[a]P^a

Coumarins	Dose (nmol/mouse)	% of mice with papillomas	Papillomas per mouse	% of Control
Experiment I				
Acetone	–	91	7.05	–
Bergamottin	20	100	5.67	80
Bergamottin	100	87	5.65	80
Bergamottin	400	92	3.92 ^b	56
Bergamottin	800	82	1.77 ^b	25
Coriandrin	400	91	5.26	75
Ostruthin	400	91	6.57	93
Experiment II				
Acetone	–	92	5.5	–
Imperatorin	800	92	5.0	91
Imperatorin	1600	85	3.5 ^b	63
Ostruthin	1200	93	4.3	78

^aGroups of 25 mice each were treated with coumarins 5 min prior to initiation with 200 nmol B[a]P. Two weeks after initiation, mice were treated with 1.7 nmol TPA twice a week. Promotion was stopped after week 25 when all groups had reached a plateau. Control mice that received acetone, bergamottin (800 nmol), ostruthin (1200 nmol), or imperatorin (1600 nmol) only at initiation had 0.17 (10%), 0.17 (14%), 0.23 (13%), or 0.11 (11%), respectively, papillomas per mouse (numbers in parentheses represent papilloma incidence).

^bSignificantly less than the control receiving acetone in the place of coumarins based on Wilcoxon rank sum test with $P < 0.05$.

on tumor initiation by B[a]P are summarized in Table IV. As shown in Table IV, bergamottin inhibited tumor initiation by B[a]P in a dose-dependent manner. Even the lowest dose of bergamottin (20 nmol) reduced the average number of papillomas per mouse at plateau (Table IV) in mice treated with B[a]P (~20%) although this reduction was not statistically significant ($P > 0.05$). In the group of mice treated with 800 nmol bergamottin, an ~75% reduction in the average number of B[a]P-initiated papillomas was achieved (Table IV). Table IV shows the effect of a 400 nmol dose of either coriandrin or ostruthin on papilloma formation in mice initiated with 200 nmol B[a]P (Experiment I). At this dose, coriandrin caused a slight reduction in papilloma formation (25%) compared with the control group, however, this reduction was not statistically significant ($P > 0.05$). At 400 nmol, ostruthin did not produce any inhibitory effect on tumor initiation by B[a]P. In a second experiment, the effect of imperatorin on tumor initiation by B[a]P was examined. In addition, a higher dose of ostruthin was also tested in this experiment. As shown in Table IV, imperatorin at a dose of 1600 nmol produced a 37% inhibition of tumor initiation by B[a]P that was statistically significant ($P < 0.05$) while at 800 nmol had little or no effect. In addition, ostruthin at 1200 nmol inhibited papilloma formation by 22% but this effect was not statistically significant.

The effects of imperatorin, bergamottin, coriandrin, and ostruthin on tumor initiation by DMBA are summarized in

Table V. Effects of coumarins on skin tumor initiation by DMBA^a

Coumarins	Dose (nmol/mouse)	% of mice with papillomas	Papillomas per mouse	% of Control
Experiment I				
Acetone	–	96	7.7	–
Bergamottin	400	100	13.6 ^b	177
Bergamottin	800	100	13.8 ^b	180
Coriandrin	400	96	10.7 ^b	139
Ostruthin	400	96	8.9	116
Experiment II				
Acetone	–	93	9.3	–
Bergamottin	800	96	12.9 ^b	139
Ostruthin	1200	96	7.3	78
Imperatorin	400	93	5.6 ^b	60
Imperatorin	800	75	2.9 ^b	31

^aGroups of 25 mice each were treated with coumarins 5 min prior to initiation with 10 nmol DMBA. Two weeks after initiation, mice were treated with 1.7 nmol TPA twice a week. Promotion was stopped after week 25 when all groups had reached a plateau. Data are recorded at week 25. Control mice that received acetone, bergamottin (800 nmol), ostruthin (1200 nmol), or imperatorin (1600 nmol) only at initiation had 0.17 (10%), 0.17 (14%), 0.23 (13%), or 0.11 (11%), respectively, papillomas per mouse (numbers in parentheses represent papilloma incidence).

^bSignificantly less than the control receiving acetone in the place of coumarins based on Wilcoxon rank sum test with $P < 0.05$.

Table V. In the first experiment shown in Table V, none of these coumarins inhibited tumor initiation by DMBA; however bergamottin, significantly enhanced tumor initiation at both 400 nmol (177% of control, $P < 0.05$) and 800 nmol (180% of control, $P < 0.05$). In a second experiment (Table V), imperatorin effectively inhibited tumor initiation (as indicated by a reduction in the average number of papillomas per mouse at plateau) by DMBA at both 400 nmol (40%) and 800 nmol (69%) doses (Table V). In addition, ostruthin at a dose of 1200 nmol also produced a slight inhibition of tumor initiation by DMBA (Table V, 22%) although this effect was not statistically significant ($P > 0.05$). Finally, bergamottin enhanced tumor initiation by DMBA (Table V, 39%) which was again statistically significant ($P < 0.05$).

Effect of imperatorin on complete carcinogenesis by DMBA

As part of the current study, we also examined the effect of imperatorin on complete carcinogenesis by DMBA. For these experiments, groups of 30 mice were each treated with either DMBA alone (50 nmol) or two doses of imperatorin (400 and 800 nmol) 5 min prior to topical application of 50 nmol DMBA. All treatments were given once weekly. Imperatorin was chosen for these experiments because it inhibited formation of covalent DNA adducts by both B[a]P and DMBA and it also inhibited tumor initiation by both hydrocarbons. Starting at week nine, the number of tumors per mouse was recorded each week. As shown in Figure 2A, the cumulative number

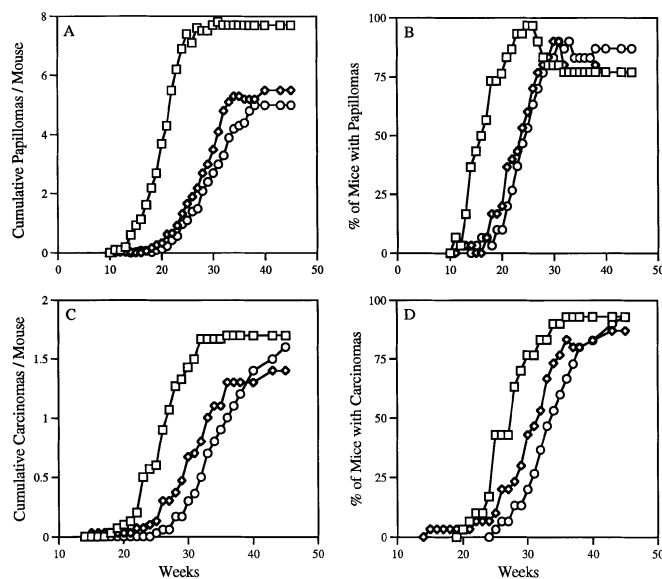


Fig. 2. Effect of imperatorin on complete carcinogenesis by DMBA. Groups of 30 mice each received once-weekly applications of DMBA (50 nmol). Imperatorin was given 5 min prior to each application of DMBA. Panels **A** and **B** represent cumulative papilloma yield and incidence, respectively. Panels **C** and **D** represent cumulative carcinoma yield and incidence, respectively. \square , acetone 0.2 ml + DMBA; \diamond , imperatorin 400 nmol + DMBA; and \circ , imperatorin 800 nmol + DMBA.

of papillomas in the control group of mice treated with acetone and DMBA reached a plateau by the 25th week. Imperatorin at dose levels of 400 nmol and 800 nmol significantly inhibited DMBA-induced papilloma formation by 76% and 85%, respectively, at this time. The appearance of papillomas was delayed 9 weeks by pretreatment with imperatorin compared with the control group. Figure 2B shows the percentage of mice with papillomas as a function of treatment week and also indicates the delay in appearance in groups pretreated with the coumarin.

Figure 2C shows that the number of carcinomas reached a maximum yield (1.6 carcinomas/mouse) at the 32nd week in the DMBA only group, whereas only 0.8 carcinomas/mouse and 0.5 carcinomas/mouse were present in the imperatorin pretreated groups at 400 nmol and 800 nmol, respectively. These differences in carcinoma yield were significant ($p < 0.05$). A 5 and 7 week delay in the appearance of the first carcinoma was observed in the groups treated with 400 nmol and 800 nmol imperatorin, respectively (Figure 2D). In addition, the time to 50% carcinoma incidence in the DMBA only group was also significantly delayed by imperatorin pretreatment (27 vs 31 and 27 vs 33, at the 400 and 800 nmol doses, respectively). Pretreatment with imperatorin also dramatically improved survival. In this regard, control mice treated with DMBA alone had a TS50 of 29 weeks, while the groups of mice pretreated with 400 nmol or 800 nmol imperatorin has TS50 of 35 and 36 weeks, respectively (see Figure 3 and Table VI). These differences in survival were highly significant ($p < 0.0001$). All of the data from this experiment is summarized in Table VI.

Discussion

In the present study, several naturally occurring coumarins previously shown to be effective at inhibiting either EROD

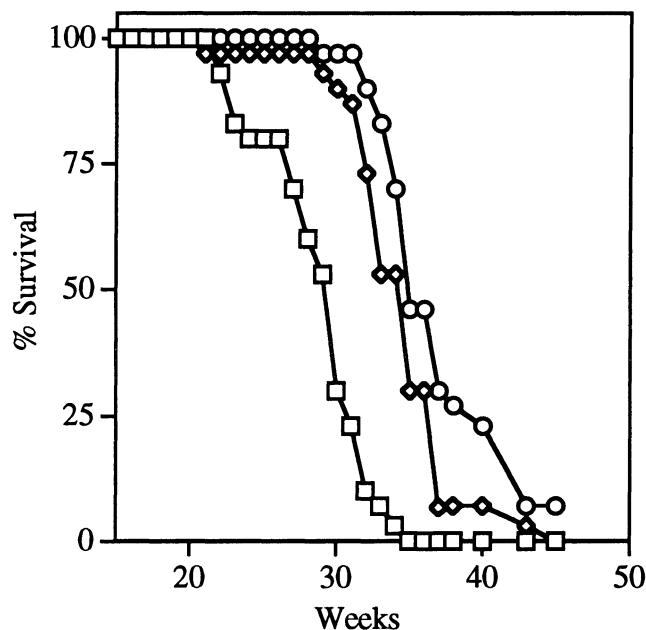


Fig. 3. Effect of imperatorin on survival rate in complete carcinogenesis by DMBA. \square , acetone 0.2 ml + DMBA; \diamond , imperatorin 400 nmol + DMBA; and \circ , imperatorin 800 nmol + DMBA.

(and in the case of coriandrin human P450 1A1) or PROD activity *in vitro* (11) were examined for their effects on the formation of DNA adducts in mouse epidermis and on skin tumorigenesis by either B[a]P or DMBA. Imperatorin and isopimpinellin were effective and more selective inhibitors of hepatic PROD activity *in vitro* (presumably mediated by P450 2b family enzymes) (11), whereas bergamottin and coriandrin were more selective inhibitors of hepatic EROD activity *in vitro* (presumably mediated by P450 1a-1) (11). Finally, ostruthin was also included in this study because, based on its activity against hepatic EROD and PROD *in vitro*, it was expected to be less effective at inhibiting tumorigenesis than the other selected coumarins. The major findings of the current study were as follows: i) the ability of this group of coumarins to inhibit covalent binding of B[a]P to DNA and to inhibit tumor initiation by B[a]P roughly correlated with their ability to inhibit hepatic EROD activity *in vitro* (bergamottin > coriandrin > ostruthin \cong imperatorin); ii) with the exception of bergamottin, the ability of this group of coumarins to inhibit covalent binding of DMBA and to inhibit tumor initiation by DMBA correlated roughly with their ability to inhibit hepatic PROD activity *in vitro* (imperatorin \cong isopimpinellin > ostruthin > coriandrin \cong bergamottin); iii) bergamottin, the compound most effective against B[a]P DNA adduct formation and tumor initiation, actually enhanced tumor initiation by DMBA, whereas imperatorin, the compound most effective against DMBA DNA adduct formation and tumor initiation, had less inhibitory activity against tumor initiation by B[a]P; and iv) bergamottin and coriandrin selectively inhibited formation of anti-diol-epoxide DNA adducts from both B[a]P and DMBA similar to our observations with these compounds in studies using cultured keratinocytes (13). Collectively, the data suggest that the major P450 systems involved in the metabolic activation of B[a]P and DMBA differ in mouse epidermis. In addition, the data indicate that certain coumarins have the ability to inhibit tumor

Table VI. Effect of imperatorin on complete carcinogenesis by DMBA^a

Groups	Dosage (nmol/mouse)	% of mice with papillomas ^b	Papillomas/mouse ^b	% of mice with carcinomas ^c	Carcinomas/mouse ^c	TS50 ^f
DMBA(50 nmol)						
+ Acetone	-	97	7.4 (100)	90	1.7 (100)	29
+ Imperatorin	400	60 ^d	1.7 (76) ^e	53 ^c	0.8 (48) ^e	34 ^g
+ Imperatorin	800	53 ^d	1.1 (85) ^e	37 ^c	0.5 (30) ^e	35 ^g

^aThirty mice were used for each experimental group. Mice received once-weekly applications of 50 nmol DMBA for the duration of the experiment (45 weeks). Imperatorin was given 5 min prior to each weekly application of DMBA. Control mice that received 800 nmol imperatorin only, given once-weekly, had no tumors at the end of the experimental period.

^bThese data are from the week when papilloma yield in the control group reached a maximum. Numbers in parentheses represent the percent inhibition.

^cThese data are summarized from the week when carcinoma yield in the control group reached maximum.

^dSignificantly less than the control receiving acetone in place of imperatorin ($P < 0.05$, χ^2 test)

^eSignificantly less than the control receiving acetone in place of imperatorin ($P < 0.01$, Wilcoxon rank sum test).

^fTS50 is based on the number of animals present at the time the first carcinoma appeared and is defined as the time (in weeks) required to reach 50% survival.

^gSignificantly greater than the acetone control group ($P < 0.0001$).

initiation by both B[a]P and DMBA and, in the case of imperatorin, inhibit complete carcinogenesis by DMBA.

Inhibition of PAH-induced tumorigenesis by either synthetic or naturally occurring compounds has been widely studied in the past (reviewed in 2 and 3). These modifying agents include: antioxidants (e.g., BHA, BHT, and selenium), inducers of certain cytochrome(s) P450 (e.g., TCDD and 5,6-BF), and inhibitors of certain cytochromes P450 (e.g., 1-EP and 7,8-BF). Most inhibitors of skin tumor initiation in the two-stage model inhibit tumor initiation by either DMBA or B[a]P, but not by both of these PAHs (also reviewed in 2 and 3). In fact, many compounds selectively inhibit tumor initiation by DMBA but only a few of them selectively inhibited tumor initiation by B[a]P (reviewed in 2 and 3). For example, 7,8-BF and benzo[e]pyrene (B[e]P) inhibit skin tumor initiation by DMBA but not B[a]P (2,3). In the current study, bergamottin significantly inhibited the formation of papillomas initiated by B[a]P but had little or no effect or, at some doses, actually enhanced tumor initiation by DMBA. In contrast, Alworth *et al.* (9) reported that 1-EP inhibited tumor initiation by both B[a]P and DMBA. 1-EP is a mechanism based inactivator of P450 1A1 and P450 1B1, being more potent toward the latter P450 (18). Compared with 1-EP, bergamottin achieved similar inhibition of papilloma formation (75%) at a dose (800 nmol) 5-fold lower than 1-EP indicating that bergamottin was a much more potent inhibitor of B[a]P tumor initiation than 1-EP. The inhibitory effect of bergamottin on tumor initiation by B[a]P correlated with its ability to inhibit formation of B[a]P-DNA adducts, supporting the conclusion that the effect of bergamottin on tumor initiation was due to inhibition of the metabolic activation of B[a]P. The inability of bergamottin to inhibit tumor initiation by DMBA was also consistent with its lack of effect on formation of total covalent DNA adducts from DMBA in mouse epidermis *in vivo*. The ability of bergamottin, coriandrin and ostruthin to inhibit covalent binding of B[a]P to DNA (Tables I and II) correlated with their effects on tumor initiation by B[a]P. One possible explanation for the observation that doses of bergamottin, coriandrin, imperatorin, and ostruthin which produced a statistically significant reduction in B[a]P-DNA adduct levels (Table I and Figure 1) did not produce a statistically significant reduction in tumor initiation by B[a]P is that there is a threshold effect. In this regard, it may be necessary for total DNA adduct levels to be reduced below a certain level in order to observe a

significant reduction in tumor yield. Future experiments will explore this possibility in more detail. Nevertheless, the overall effects of this group of coumarins on tumor initiation by B[a]P roughly followed their *in vitro* potency for inhibition of hepatic EROD activity (11) and their ability to reduce B[a]P DNA adduct levels.

Imperatorin, at 400 nmol and 800 nmol doses, significantly inhibited tumor initiation by DMBA, but was much less effective against tumor initiation by B[a]P. However, it should be noted that the ratio of imperatorin:DMBA was significantly higher (40:1 and 80:1 at the 400 and 800 nmol doses) than the ratio of imperatorin:B[a]P (4:1 and 8:1 at the 800 and 1600 nmol doses), suggesting that the difference in inhibitory effectiveness of imperatorin on B[a]P and DMBA was due, at least in part, to the lower ratio of imperatorin:B[a]P used. The results from two-stage carcinogenesis experiments showed that imperatorin, at a dose of 1600 nmol, did significantly inhibit tumor initiation by B[a]P supporting this conclusion. Thus, the ability of this series of coumarins to inhibit tumorigenesis by DMBA roughly correlated with their ability to inhibit hepatic PROD activity *in vitro* (9). The one exception to this latter correlation was bergamottin which, in addition to being the most potent inhibitor of hepatic EROD activity *in vitro* (IC₅₀ = 0.12 μ M, reference 9), was also a fairly potent inhibitor of hepatic PROD activity *in vitro* (11). A possible explanation for the lack of effect of bergamottin on DMBA tumor initiation is that this compound was a more potent and selective inhibitor of hepatic EROD. The major P450 mediating MC-induced EROD activity, P450 1A1 (or 1a-1 in mouse) appears to be involved in metabolizing DMBA to relatively nontoxic metabolites (19,20). Thus, bergamottin, by blocking P450 1a-1, may slow the major detoxication route for DMBA thus allowing more of this PAH to be available for metabolic activation by other P450s present in mouse epidermis.

An interesting observation in the current study was that bergamottin and coriandrin reduced formation of anti-DMBADE-DNA adducts while increasing formation of syn-DMBADE-DNA adducts (Table III) thereby resulting in no significant reduction of overall DMBA-DNA adducts at the dose used in these experiments. Several studies have suggested that the formation of anti-DMBADE-DNA adducts may require induction of a specific cytochrome P450 (21–23). For example, metabolism of DMBA in undifferentiated keratinocytes [low aryl hydrocarbon hydroxylase (AHH)

activity] results in the formation of primarily syn DMBADe-DNA adducts (16). However, keratinocytes maintained in high Ca^{2+} medium possess high AHH activity and a high level of anti-DMBADE DNA adducts are formed following exposure to DMBA (16). In addition, a time-dependent increase in the ratio of anti to syn-DMBADE-DNA adducts in mouse epidermis was observed when mice were exposed topically to DMBA (22). Furthermore, anti-DMBADE-DNA adduct formation following exposure of fetal mouse cells to DMBA was inhibited by actinomycin D, an RNA-synthesis inhibitor (18). Recently, Lau *et al.* (24) found that the predominant DNA adducts formed from DMBA in human MCF-7 cells were derived from the anti-DMBADE and attributed this observation to the high constitutive expression of P450 1B1 in these cells (19). The reduction of anti-DMBADE-DNA adducts caused by bergamottin and coriandrin pretreatment raises the interesting possibility that these compounds modestly inhibited an epidermal cytochrome P450 capable of converting DMBA to its anti diol-epoxide (e.g. P450 1b-1). The enhancement of syn-DMBADE-DNA adducts by bergamottin and coriandrin pretreatment could be explained by inhibition of detoxification of DMBA (through inhibition P450 1a-1) by these coumarins, resulting in accumulation of DMBA-3,4-diol and/or a shunting of DMBA metabolism toward formation of the syn diol epoxide by inhibiting formation of the anti diol epoxide (through inhibition of P450 1b-1). In either case above, more DMBA-3,4-diol would be available for conversion to the syn-DMBADE by other cytochrome(s) P450. In support of the detoxifying role of P450 1a-1 in DMBA metabolism is a report that antibody against P450 1A1 enhanced the formation of DMBA-3,4-diol in rat mammary epithelial cells (19).

In conclusion, the current data demonstrate that several naturally occurring coumarins including bergamottin, imperatorin, ostruthin, and coriandrin have the ability to modulate PAH-induced tumorigenesis by inhibition of DNA adduct formation. Bergamottin and imperatorin were potent inhibitors of tumor initiation by B[a]P and DMBA, respectively. Future studies will continue to evaluate the overall effectiveness of one or more of these coumarins on skin carcinogenesis in this model system. In addition, future studies will also evaluate the major P450 isoforms responsible for the metabolic activation of DMBA in mouse epidermis.

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