Naturally occurring coumarins inhibit 7,12-dimethylbenz[a]anthracene DNA adduct formation in mouse mammary gland

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Naturally occurring coumarins (NOCs) are anti-carcinogenic in the mouse skin model. To characterize the chemopreventive potential of NOCs against breast cancer, we first examined their effects on 7,12-dimethylbenz[a]anthracene (DMBA)–DNA adduct formation in mouse mammary gland. We hypothesized that those NOCs that both inhibited cytochrome P450 (P450) and induced hepatic glutathione S-transferases (GSTs) would be the most effective in blocking DMBA–DNA adduct formation in mouse mammary gland. To address this hypothesis, simple coumarins (e.g. coumarin and limettin, which induced mouse hepatic GSTs but had little effect on P4501A1/1B1) and linear furanocoumarins (e.g. imperatorin and isopimpinellin, which induced hepatic GSTs and were potent inhibitors of P4501A1/1B1) were compared. Mice were pretreated with NOCs (150 mg/kg body wt, by gavage) prior to either a single dose of DMBA (50 μg) or multiple doses of DMBA (20 μg daily for 3 and 6 weeks). Mammary DMBA–DNA adduct formation was quantitated by the nuclease P1-enhanced 32P-postlabeling assay. With the single dose of DMBA, coumarin, limettin, imperatorin and isopimpinellin inhibited DMBA–DNA adduct formation by 50, 41, 79 and 88%, respectively. Coumarin, limettin and imperatorin blocked DMBA–DNA adduct formation by 36, 60, and 66% at 3 weeks, and by 49 and 55% at 6 weeks of DMBA dosing, respectively. In a 6 week dose–response study of select NOCs and 7,8-benzoflavone (a potent P4501 inhibitor that had little effect on GSTs), DMBA–DNA adduct formation was inhibited by 0, 43 and 24% in the limettin groups; by 26, 26 and 69% in the isopimpinellin groups; and by 80, 96 and 97% in the 7,8-benzoflavone groups at 35, 70 and 150 mg/kg, respectively. Taken together, these results suggest that linear furanocoumarins had a greater inhibitory effect on DMBA–DNA adduct formation in mouse mammary glands compared with simple coumarins, and that the predominant effect may be P4501 inhibition.

Introduction

The study of dietary factors, to which humans are routinely exposed, has been an appealing approach for the prevention of cancer. Naturally occurring coumarins (NOCs) represent one of the largest classes of phytochemicals, yet their anti-carcinogenic potential has not been fully evaluated. These compounds are found in many plants, including grasses, orchids, citrus fruits and legumes (1,2). Furthermore, umbelliferous plants used in Chinese herbal medicine contain furanocoumarins. For example, isoimperatorin, imperatorin and oxypeucedanin have been isolated from the active fraction of the crude drug ‘Tang-Bai’Zhi’ (3). NOCs have previously been shown to block benzo[a]pyrene (B[a]P) and/or 7,12-dimethylbenz[a]anthracene (DMBA) mouse skin tumor initiation (4) in a well-established model of multistage carcinogenesis (5). In the current study, we investigated the abilities of NOCs to block DMBA–DNA adduct formation in mouse mammary gland. The two major classes of coumarins investigated in this study are simple coumarins (e.g. coumarin and limettin) and linear furanocoumarins (e.g. imperatorin and isopimpinellin) (see Figure 1). In addition to these two classes of coumarins, angular furanocoumarins (e.g. angelicin) and pyranocoumarins of the linear (e.g. xanthyletin) or angular (e.g. seselin) type also occur naturally (2). Less is known about the effects of the latter coumarins on carcinogenesis, as their availability has been limited. However, studies are in place to further examine the anticarcinogenic effects of these coumarins as well.

Breast cancer is a major cause of death in women under the age of 56 (6). Although genetic susceptibility accounts for ~10% of human breast cancer (7), the etiology of the majority of breast cancers is unclear. There is conflicting evidence for the role of dietary fat, obesity and smoking in breast cancer. Of interest is the role of environmental chemicals on human breast cancer. Polycyclic aromatic hydrocarbons (PAHs), the ubiquitous environmental pollutants found in cigarette smoke, charbroiled meats, smoked foods, air pollution and other environmental sources such as coal tar, have been implicated in causing breast cancer (8–11). Cigarette smoke contains over 61 carcinogens, including B[a]P (8). Lipophilic compounds such as PAH (12,13) are likely to distribute to fatty tissues including the mammary gland. Evidence demonstrates that cigarette components reach breast tissue (8). In rodent models, the mammary gland is a primary target for PAH carcinogenesis (8,14,15). DMBA is used as a model PAH to induce carcinogenesis in rodent mammary models (16,17). Levels of aromatic/lipophilic DNA adducts have been shown to be significantly higher in normal adjacent breast tissues of breast cancer patients than in non-cancer controls (9–11). 32P-postlabeling and co-chromatography techniques using authentic standards have demonstrated that a major bulky DNA adduct detected in human breast tissue is related to PAH exposure (9).

Abbreviations: 7,8-BF, 7,8-benzoflavone; B[a]P, benzo[a]pyrene; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; DMBA, 7,12-dimethylbenz[a]anthracene; EA, ethanolic acid; EROD, ethoxyresorufin O-dealkylase; GST, glutathione S-transferase; NOCs, naturally occurring coumarins; NQO, NAD(P)H quinone oxidoreductase; PAH, polycyclic aromatic hydrocarbon; P450, cytochrome P450, TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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These observations suggest a possible role for environmental PAH exposure and a risk of breast cancer.

PAHs such as DMBA require bioactivation by the mixed function oxidase system to form reactive bay region diol-epoxides [reviewed in Ref. (5)]. P4501A1 is an extrahepatic, aryl hydrocarbon-inducible P450 and is responsible for the bioactivation of PAHs including B[a]P (5). P4501B1 is inducible and constitutively expressed in steroidogenic and hormonally responsive tissues, including adrenal, uterus and ovary (18). P4501A1 and 1B1 were also detected semiquantitatively by RT–PCR in human mammary epithelial cells (19). Recent evidence has supported a role for P4501B1 in the bioactivation of sterically hindered PAH, such as DMBA and dibenz[a,l]pyrene (20–28). Phase II detoxification of PAHs is facilitated by glutathione S-transferases (GSTs) (29). Conjugation of catechol estrogens to GSH may serve as detoxification mechanisms as well (30). GSTs have also been detected in rat mammary gland and are inducible by chemopreventive phenylenebis(methylene)selenocyanate isomers (31). NAD(P)H quinone oxidoreductases (NQO) reduce semiquinones back to hydroquinones, thus protecting cells from oxidative stress (32). Mice deficient in NQO1 showed greater susceptibility to DMBA-induced skin carcinogenesis (33). Also, dietary selenite inhibited mammary DMBA–DNA adduct formation and increase biliary excretion of DMBA glutathione conjugates in rats (34). Furthermore, dietary butylated hydroxytoluene, which inhibited DMBA-induced mammary tumorigenesis (35,36), was shown to induce hepatic GST and NQO activities in rats, increase urinary excretion of DMBA and block formation of mammary DMBA–DNA adducts (37). Polymorphisms in modifier genes such as CYPs and GSTs may influence breast cancer risk (38). In this regard, smokers with a combined CYP1A1*/1* or 2*2/2 genotypes and GSTM1 null genotypes had a higher level of bulky DNA adducts than those with either CYP1A1 or GSTM1 polymorphisms (38). Thus, inhibition of P450s 1A1/1B1 and induction of GSTs and NQO are possible strategies of cancer chemoprevention.

Previous studies showed that the ability of certain naturally occurring coumarins (NOCs, e.g. imperatorin and bergamotin) to inhibit PAH tumor initiation in mouse skin was due to their ability to inhibit certain P450s involved in metabolic activation of PAH (4,39). Furthermore, those NOCs with the ability to inhibit P450 1B1 > P450 1A1 were capable of inhibiting skin tumor initiation by both DMBA and B[a]P (40).

In the current study, we tested the hypothesis that NOCs with the ability to block both P450s 1A1 and 1B1, and induce hepatic GSTs, will be the most effective at inhibiting PAH mammary carcinogenesis when both PAHs and NOCs are administered orally.

**Materials and methods**

**Caution**

DMBA is carcinogenic and mutagenic and should be handled with extreme caution using the guidelines for carcinogenic materials developed by the National Cancer Institute. The toxicity and/or carcinogenicity of coumarins have not been fully evaluated. Therefore, coumarins should be handled with extreme care.

**Materials**

1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), DMBA, 7-ethoxyresoru tin, calf spleen phosphodiesterase, micrococcal nuclease, proteinase K and apyrase were purchased from Sigma Chemical Co. (St Louis, MO). 3′,5′-[3H]ATP (Specific Activity 6000–7000 Ci/mmol), coumarin (1,2-benzopyrone), collagenase, ethacrynic acid (EA), hyaluronidase and RNase A were obtained from ICN Biomedicals Inc. (Aurora, OH). 3 prime-T4-poly nucleotide kinase was supplied by Takara (Tokyo, Japan). Macherey-Nagel poly(ethyleneimine-cellulose) TLC sheets were supplied by Alttech Associates, Inc. (Deerfield, IL). Limettin (5,7-dimethoxycoumarin) and 2,6-dichloroiridophenol were obtained from Aldrich (Milwaukee, WI), and 7,8-benzoflavone (7,8-BF, also called a-naphthoflavone) was supplied by Acros Organics (Pittsburgh, PA). Imperatorin and isopimpinellin were purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ). Anti-GST antibodies were purchased from Oxford Biomedicals, Inc. (Oxford, MI). Goat anti-rabbit IgG (horseradish peroxidase labeled) Supersignal West Pico-enhanced chemiluminescent reagent and wide-range molecular weight markers were supplied by Pierce (Rockford, IL). All experiments using coumarins or DMBA were conducted under subdued lighting.

**HPLC analysis**

Purity of the coumarins and 7,8-BF were determined by reverse-phase HPLC with UV detection at 254 nm. Briefly, test compounds were dissolved in acetone and diluted in 70% methanol/30% water for injection onto a Shimadzu HPLC 10ATVP system with SCL-10AVP autoinjector and EZStart V7.25Pl chromatography software. Compounds were eluted on an Alttech Prevail C-18 column (5 μm, 4.6 mm i.d. × 150 mm, l) at a flow rate of 1 ml/min in methanol (B pump); ultrapure water (A pump) as follows: linear gradient 50% B to 70% B over 10 min, hold at 70% B for 5 min, increase to 100% B over 5 min and hold at 100% B for 5 min. Under these conditions, the retention times and purity of coumarin, limettin, imperatorin, isopimpinellin and 7,8-BF were 5.76, 97%; 9.85, 96.5%; 14.44’, 98.2%; 9.81’, 99.6%; and 22.02’, 97%, respectively.
IC₅₀ experiments
In order to compare the abilities of NOCs to inhibit P450s, IC₅₀ experiments were performed. Human cDNA-expressed P4501A1 and 1B1 (Gentest Corp., Woburn, MA) enzyme activities were assessed by the method of Burke (41) and as described previously (42). Briefly, increasing concentrations of inhibitors (5 × 10⁻⁸ M to 5 × 10⁻⁶ M) were incubated with 5 pmol of P450 enzyme, an NADPH generating system, and the substrate (5 μM 7-ethoxycoumarin) for 10 min (P4501A1) or 20 min (P4501B1) at 37°C in a total volume of 1 ml of 0.05 M Tris buffer (pH 7.5). The product (resorufin) was quantitated by fluorescence detection at an excitation of 540 nm and an emission of 590 nm. Experimental values were compared with a standard curve. The concentration of inhibitors that blocked 50% of the enzyme activity (IC₅₀) were determined by interpolation.

Animals
Female SENCAR mice were purchased from the National Cancer Institute (Frederick, MD) and were housed in a temperature- and humidity-controlled AAALAC facility with a 12 h light/dark cycle. All procedures were approved by the LSUHSC Institutional Animal Care and Use Committee in accordance with NIH guidelines. Mice were maintained on AIN-76A diet (Dyets, Bethlehem, PA), and allowed access to food and water ad libitum. At 6 weeks of age, 3–4 mice per group were used for experiments (described below).

Animal experiments
Table I summarizes the experimental design for the three different experiments that were performed. Experiment I investigated the effects of orally administered NOCs (coumarin, limettin, imperatorin, isopimpinellin, 150 mg/kg body wt) on mammary DMBA-DNA adduct formation following a single dose of DMBA. Experiment II investigated the effects of orally administered NOCs (coumarin, limettin, imperatorin, 150 mg/kg body wt) on mammary DMBA-DNA adduct formation following either 3 weeks or 6 weeks of DMBA dosing. Experiment III compared the effects of limettin, isopimpinellin and 7,8-BF (35, 70 and 150 mg/kg body wt) on mammary DMBA-DNA adduct formation following 6 weeks of DMBA dosing. For all three experiments, all mice were pretreated by gavage with NOCs, 7,8-BF (35–150 mg/kg body wt, suspended in 0.1 ml/25 g body wt of corn oil) or corn oil (vehicle control) for 3 consecutive days prior to the first dose of DMBA. Based on previous studies, orally administered NOCs effectively induced hepatic GST in mice using this protocol (43). For Experiment I, mice were treated by gavage with a single dose of DMBA (50 μg suspended in 0.1 ml corn oil) 1 h after the last pretreatment dose. For Experiments II and III, mice were treated with DMBA (20 μg, in 0.1 ml corn oil, by gavage) once daily 5 days/week for the duration of the study. In addition, in Experiments II and III, mice were pretreated with NOCs or 7,8-BF 1 h prior to every DMBA dose (i.e. once daily 5 days/week for the duration of the study). Control mice received 0.1 ml corn oil (vehicle) instead of DMBA or NOCs or 7,8-BF. Body weights were recorded weekly. Mice were sacrificed by cervical dislocation 24 h after the last DMBA dose, and livers and abdominal–inguinal mammary glands were processed as described below. Endpoints included the formation of mammary DMBA-DNA adducts, hepatic GST and NQO activities and GST protein expression.

Phase II enzyme assays
To determine the effects of NOCs on hepatic Phase II enzyme activities, tissues from the DMBA-DNA adduct studies were collected and analyzed as follows: livers were removed, and cytosol was isolated by differential centrifugation as described previously (42). Liver cytosolic GST activities were performed. Human cDNA-expressed P4501A1 and 1B1 (Gentest Corp., Woburn, MA) enzyme activities were assessed by the method of Burke (41) and as described previously (42). Briefly, increasing concentrations of inhibitors (5 × 10⁻⁸ M to 5 × 10⁻⁶ M) were incubated with 5 pmol of P450 enzyme, an NADPH generating system, and the substrate (5 μM 7-ethoxycoumarin) for 10 min (P4501A1) or 20 min (P4501B1) at 37°C in a total volume of 1 ml of 0.05 M Tris buffer (pH 7.5). The product (resorufin) was quantitated by fluorescence detection at an excitation of 540 nm and an emission of 590 nm. Experimental values were compared with a standard curve. The concentration of inhibitors that blocked 50% of the enzyme activity (IC₅₀) were determined by interpolation.

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Phase II enzyme assays
To determine the effects of NOCs on hepatic Phase II enzyme activities, tissues from the DMBA-DNA adduct studies were collected and analyzed as follows: livers were removed, and cytosol was isolated by differential centrifugation as described previously (42). Liver cytosolic GST activities were assessed spectrophotometrically (Shimadzu, Columbia, MD, kinetic mode) at 340, 345 and 270 nm, using CDNB, DCNB and EA as substrates, respectively. Activities were calculated using extinction coefficients of 9.6 mM⁻¹/cm⁻¹, 8.5 mM⁻¹/cm⁻¹ and 5.0 mM⁻¹/cm⁻¹, respectively, as described previously (44). NQO activity was assayed spectrophotometrically at 600 nm using 2,6-dichloroindophenol as a substrate and an extinction coefficient of 21 mM⁻¹/cm⁻¹ as described previously (45).

Western blot
To assess the effects of NOCs on protein expression of different GSTs, western blots were performed. Liver cytosolic proteins (40 μg) were loaded onto 12% SDS–PAGE gels (46) and immunoblotted onto PVDF membranes (47). Blots were incubated with anti-GSTs, α and μ-specific antibodies (1:1000 dilution), and either anti-goat or anti-rabbit IgG (H+L, peroxidase conjugated, 1:20 000 dilution) and visualized using enhanced chemiluminescence using methods similar to those described previously (48). Images were analyzed using ImageJ software (http://rsb.info.nih.gov). Background was subtracted from the integrated density of each sample.

Results
Effects of NOCs on human cDNA-expressed P450 activity
We have previously shown that imperatorin and isopimpinellin were potent inhibitors of human cDNA-expressed P450s 1A1 and 1B1 (53). We have extended these studies to include coumarin and limettin, and used 7,8-BF as a positive control to compare with the previous studies (Table II). Similar to the previously reported results [(53) and Table II], 7,8-BF inhibited P4501A1 and 1B1 with IC₅₀ values of 0.11 and 0.012 μM, respectively. Coumarin and limettin inhibited human cDNA expressed P450 1A1 with IC₅₀ values of >500 and 357 μM, respectively. Furthermore, coumarin and limettin inhibited human cDNA-expressed P450 1B1 with IC₅₀ values of >500 and 161 μM, respectively. Thus, coumarin had virtually no inhibitory activity against P450 1A1 or 1B1, and limettin had only moderate activity against these enzymes. Taken together, these results supported previous findings (42) that, as a class, the linear furanocoumarins were more potent inhibitors of P450s 1A1/1B1 compared with the simple coumarins.

Table I. Experimental design for DMBA–DNA adduct study in mouse mammary glands

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Pretreatment dose</th>
<th>Pretreatment time</th>
<th>Carcinogen dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td>Corn oil, coumarin, limettin, imperatorin, isopimpinellin (150 mg/kg)</td>
<td>Once daily for 3 days prior to first dose of DMBA; last dose 1 h prior to DMBA.</td>
<td>Single dose of DMBA (50 μg)</td>
</tr>
<tr>
<td>Experiment II</td>
<td>Corn oil, coumarin, limettin, imperatorin (150 mg/kg)</td>
<td>Once daily for 3 days prior to first dose of DMBA; then 1 h prior to each DMBA dose for 3 or 6 weeks</td>
<td>3 and 6 weeks of DMBA (20 μg)</td>
</tr>
<tr>
<td>Experiment III</td>
<td>Corn oil, limettin, isopimpinellin, 7,8-BF (35, 70, 150 mg/kg)</td>
<td>Once daily for 3 days prior to first dose of DMBA; then 1 h prior to each DMBA dose for 6 weeks</td>
<td>6 weeks of DMBA (20 μg)</td>
</tr>
</tbody>
</table>
Effects of NOCs on phase II enzyme activities

Oral administration of NOCs to mice, in combination with DMBA (6 weeks), induced liver cytosolic NQO and GST activities compared with the corn oil only treated mice (Figure 2). In contrast, treatment of mice with DMBA did not significantly increase NQO or GST activities (Figure 2). As shown in Figure 2, isopimpinellin produced a modest increase in NQO activities by ~50–60% higher than the corn oil/corn oil control. This effect did not appear to be dose-dependent at the doses used in this study. Increasing doses of isopimpinellin significantly increased liver cytosolic GST activities ~2-fold using CDNB, DCNB and EA as substrates (Figure 2). CDNB is a general GST substrate, whereas GSTM1 subunits (and to a lesser extent, GSTA subunits) display higher catalytic activity toward DCNB, and the GSTP1 subunit has higher catalytic activity for EA [reviewed in Ref. (29)].Limettin also increased liver cytosolic GST activities (using CDNB and DCNB as substrates) as a function of the dose, but did not have any significant effects on GST activities when EA was used as a substrate. Coumarin and imperatorin also significantly increased GST activities (using CDNB and DCNB as substrates) in DMBA-treated mice (data not shown). Interestingly, at the higher doses, 7,8-BF significantly increased NQO and GST activity when CDNB was used as a substrate, but not with the DCNB or EA substrates.

Effects of NOCs on GST protein expression

To further characterize which GSTs were induced by NOCs, western blot analyses were performed (Figure 3). Expression of GSTα and GSTπ was barely detectable in the corn oil only treated mice and was not increased much further by DMBA treatment. Compared with the mice treated with DMBA and pre-treated with corn oil, densitometric analysis showed that limettin increased GSTα protein expression by 3-fold at the 150 mg/kg dose, and isopimpinellin increased GSTα protein expression by ~2-, 4- and 11-fold at 35, 70 and 150 mg/kg, respectively (Figure 3B). There was no effect of 7,8-BF on GSTα protein expression at any of the doses. GSTπ protein expression was increased by ~6-fold at 35 and 70 mg/kg of limettin, and by 15-fold at 150 mg/kg limettin, whereas it was increased by 15–18-fold at all doses of isopimpinellin. There was only a modest (2–3-fold) increase in GSTπ expression in the 7,8-BF treated mice compared with the corn oil-pretreated DMBA-treated mice, and no difference compared with the corn oil only treated mice. Constitutive expression of GSTμ

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Table II. IC₅₀ values (µM) for selected NOCs and 7,8-BF against cDNA-expressed human P450s

<table>
<thead>
<tr>
<th>P450</th>
<th>7,8-BF</th>
<th>Coumarin</th>
<th>Limettin</th>
<th>Imperatorin</th>
<th>Isopimpinellin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>0.11 ± 0.02</td>
<td>&gt;500</td>
<td>357 ± 53 2.76 ± 0.64 (53)</td>
<td>3.67 ± 0.49 (53)</td>
<td></td>
</tr>
<tr>
<td>1B1</td>
<td>0.012 ± 0.002</td>
<td>&gt;500</td>
<td>161 ± 65 0.71 ± 0.17 (53)</td>
<td>3.72 ± 0.69 (53)</td>
<td></td>
</tr>
</tbody>
</table>

*IC₅₀ values (µM) were determined by interpolation. Values represent means ± SE (n = 3).

*Control activities (pmol/min/pmol P450) for P450 1A1 and 1B1 were 37.4 ± 10.6 and 5.90 ± 0.39, respectively (means ± SE).

*Previously reported in Ref. (53) and shown here for comparison.

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Fig. 2. Effects of orally administered NOCs on hepatic cytosolic GST and NQO activities following 6 weeks of dosing. NQO activity (A) was assayed using 2,6-dichloroindophenol as a substrate. GST activities were assayed using CDNB (B), DCNB (C) and EA (D) as substrates. Data were expressed as percentage of corn oil/corn oil control mice. Control enzyme activities in A, B, C and D were 15.8 ± 1.1, 1.54 ± 0.06, 0.034 ± 0.004 and 0.017 ± 0.004 µmol/min/mg protein, respectively. Figures represent means ± SE of 3–4 mice (except the limettin 70 mg/kg group, n = 2, mean ± range). *P < 0.05, **P < 0.01 (ANOVA, Fisher’s PLSD test).
appeared to be higher than the other GSTs, and only the highest dose of isopimpinellin increased it any further (by \( \frac{1.6}{C_24} \) fold).

Coumarin and imperatorin also increased GST protein expression (data not shown). These results were fairly consistent with the GST functional assays and suggested that overall, isopimpinellin and limettin induced GSTs with predominant effects on GST\( \alpha \) and GST\( \pi \), whereas 7,8-BF had little effect on GST induction.

**Effects of NOCs DMBA–DNA adduct formation in mouse mammary glands**

Administration of DMBA to mice resulted in the formation of several anti- and syn-DMBADE dAdo and dGuo adducts in the mammary glands at 24 h, 3 weeks and 6 weeks of dosing. A representative radiochromatogram showing the effects of NOCs on DMBA–DNA adduct formation at the 24 h time-point is shown in Figure 4. The inhibitory effects of NOCs on DMBA–DNA adduct formation at different time points are summarized in Table III. At 24 h, imperatorin and isopimpinellin had the greatest effects on DMBA–DNA adduct formation (79–88% inhibition), whereas coumarin and limettin had intermediate effects (41–50% inhibition). Coumarin had a mild inhibitory effect on DMBA–DNA adduct formation at 3 weeks, but not at 6 weeks. In fact, at 6 weeks, the adduct levels were higher in the coumarin-treated mice. In contrast, both limettin and imperatorin continued to inhibit DMBA–DNA adduct formation at both 3 and 6 weeks of dosing. In these studies, the mammary glands from each group were
pooled together; so, statistical analysis was not possible. In Experiment III, DNA from individual mice was analyzed. As shown in Figure 5, limettin inhibited DMBA–DNA adduct formation only at the 70 mg/kg dose; not at the 150 mg/kg dose. In contrast, isopimpinellin showed a dose response in the inhibition of DMBA–DNA adducts, reaching a maximum of 69% inhibition at the highest dose. Overall, 7,8-BF was the most effective inhibitor and blocked DMBA–DNA adduct formation by 80, 96, and 97% at 35, 70 and 150 mg/kg, respectively. In all three experiments, the inhibitory effects on DMBA–DNA adduct formation did not appear to be selective for a specific adduct; rather, both syn- and anti-DMBA diol-epoxide derived adducts were suppressed to a similar extent.

Discussion

The results from the current study confirmed that the linear furanocoumarins, imperatorin and isopimpinellin were more potent inhibitors of human cDNA expressed P450 1A1 and 1B1 activity than the simple coumarins, coumarin and limettin (Table II). Both simple coumarins and linear furanocoumarins induced hepatic GST activities when administered orally to mice (Figure 2). This corresponded to an increase in hepatic protein expression of mainly GSTα and GSTπ (Figure 3). Although in the current study we did not investigate the effects of NOCs on GST expression in the absence of DMBA treatment, the induction of GSTs by NOCs alone

![Fig. 4.](image-url) Effects of orally administered NOCs on DMBA–DNA adduct formation in SENCAR mouse mammary gland. Mice were treated with a single dose of DMBA (50 μg, gavage). DNA was pooled from three mice in each group. DNA adducts were detected using nuclease-P1 enhanced 32P-postlabeling. Based on comparisons with authentic standards and previous studies (51), DNA adduct spots were identified as follows: D1 and D5 were anti-DMBADE-dGuo adducts; D2 and D7 were syn-DMBADE-dAdo adducts, D3 and D6 were anti-DMBADE-dAdo adducts, and D9 was an anti-DMBADE adduct.

![Table III.](table-url) Effects of NOCs on DMBA–DNA adduct formation in mouse mammary gland.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Single dose</th>
<th>3 Weeks</th>
<th>6 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>3.27</td>
<td>3.98</td>
<td>6.89</td>
</tr>
<tr>
<td>Coumarin</td>
<td>1.64 (50%)</td>
<td>2.54 (36%)</td>
<td>10.2 (−148%)</td>
</tr>
<tr>
<td>Limettin</td>
<td>1.93 (41%)</td>
<td>1.59 (60%)</td>
<td>3.54 (49%)</td>
</tr>
<tr>
<td>Imperatorin</td>
<td>0.69 (79%)</td>
<td>1.35 (66%)</td>
<td>3.10 (55%)</td>
</tr>
<tr>
<td>Isopimpinellin</td>
<td>0.40 (88%)</td>
<td>Not done</td>
<td>Not done</td>
</tr>
</tbody>
</table>

*Mice (three per group) were pretreated with the test compounds (150 mg/kg, p.o.) once a day for 3 consecutive days, and then were pretreated 1 h prior to every DMBA dose. DMBA was administered as a single dose, or once daily 5 days a week for 3 or 6 weeks. Animals were sacrificed 24 h after the final dose, and mammary DNA was isolated for 32P-postlabeling analysis. Numerals represent number of adducts per 10⁹ nucleotides (numbers in parentheses represent % inhibition).
has been reported previously (43,54). We hypothesized that those coumarins (e.g. the furanocoumarins, imperatorin and/or isopimpinellin) that both inhibited P4501 family members, and induced GSTs, would be the most effective coumarins at blocking DMBA-DNA adduct formation in mouse mammary glands. Overall, the 24 h, 3 and 6 week experiments supported this hypothesis, as imperatorin and/or isopimpinellin appeared to be more effective at blocking DMBA-DNA adduct formation than coumarin or limettin. To confirm and extend these findings, 7,8-BF was used for comparison, and we examined three different doses at the 6-week time-point. 7,8-BF is a potent P4501 family inhibitor (53,55,56) and had little effect on GST activity or GST protein expression (Figures 2–3). All the coumarins we have tested did induce GST (data not shown), so it was not possible to examine the effect of a coumarin that inhibited P450 and did not induce GST. The results from the dose–response study showed that 7,8-BF was the most effective at blocking DMBA–DNA adduct formation in mouse mammary gland, followed by isopimpinellin, whereas limettin appeared to be the least effective. These observations suggested that P4501 family inhibition was the predominant mechanism. Interestingly, imperatorin also increased NQO activity, whereas limettin did not, so the effect of NQO induction cannot be ruled out. In fact, NQO1−/− knockout mice have been shown to be more sensitive to DMBA-induced skin carcinogenesis (33).

Although 7,8-BF was the most effective at blocking DMBA-DNA adduct formation, it had little effect on skin carcinogenesis induced by B[a]P (57) and was significantly more potent at inhibiting P4501B1 compared with P4501A1 (40,56). We selected DMBA as the model carcinogen in our study because its bioactivation involves both P4501A1 and 1B1 (20–28). This study design utilizes lower doses of DMBA than historical protocols, which typically use 1 mg of DMBA, once a week for 5 weeks (62). In the current study, 7,8-BF was used for comparison, and we examined three different doses at the 6-week time-point. This could establish that quinones and semiquinones can redox cycle and generate oxidative stress, and covalently bind to tissue macromolecules by Michael addition (30). Thus, a role for catechol estrogens in breast cancer has been proposed, due to their ability to form reactive oxygen species, oxidized DNA bases as well as deaminating adducts (61). It will be interesting to determine whether furanocoumarins suppress mammary carcinogenesis by this mechanism.

Several other interesting observations were made in the current study. The DMBA–DNA adduct levels in the corn oil-pretreated, DMBA treated mice were 3.27, 3.98, 6.89 and 5.54 adducts per 10^9 nucleotides in the 24 h, 3 week, 6 week and 6 week dose–response studies, respectively. The single dose 24 h study utilized a higher dose (50 μg), to ensure that adducts would be detectable. The 3 week and 6 week studies utilized a 20 μg dose, once a day, 5 days a week for 6 weeks. This study design utilizes lower doses of DMBA than the more historical protocols, which typically use 1 mg of DMBA, once a week for 5 weeks (62). In the current study, total DMBA–DNA adduct levels were higher at the 6 week time-point than the 3 week or 24 h time-points. This could

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**Fig. 5.** Summary of the effects of orally administered NOCs and 7,8-BF on DMBA–DNA adduct formation in mouse mammary gland at 6 weeks. Data are expressed as the percentage of the corn oil-pretreated DMBA treated mice and represent means ± SE of 3–4 mice except the limettin 70 mg/kg group, n = 2, mean ± range). DMBA–DNA adduct levels were 5.54 ± 0.87 adduct per 10^9 nucleotides in the corn oil-pretreated DMBA treated mice. *P < 0.05; **P < 0.01 (ANOVA, Fischer’s PLSD test).
Coumarins and mammary carcinogenesis

reflect a balance between accumulations of DNA adducts and DNA repair. Interestingly, in the study by Lin and co-workers (62), the levels of total DMBA–DNA adducts decreased from the 2 week time-point (244 adducts/10^9 nucleotides) to the 5 week time-point (125 adducts/10^9 nucleotides). However, the doses used in their study were nearly 10 times higher than the doses used in our study (e.g. 1 mg × 5 weeks = 5 mg versus 0.02 mg × 5 days/week × 6 weeks = 0.6 mg). Thus, the DMBA–DNA adduct levels reported in our study were ~20 times lower than those reported in their study at similar time-points (6 weeks versus 5 weeks, respectively). Thus, the differences in the levels of DMBA–DNA adducts over time could also be due to a difference in the treatment protocol. To our knowledge, this is the first time DMBA–DNA adduct formation has been reported using the protocol described by Qing and co-workers (17).

There have been limited studies on the effects of NOCs on mammary carcinogenesis. Oral administration of coumarin and 4-methyl-coumarin moderately inhibited DMBA-induced mammary tumors in the rat, whereas limettin was less effective at blocking DMBA-induced mammary tumors (63). Interestingly, in our study, coumarin was less effective at blocking DMBA–DNA adduct formation than limettin, and was a less potent inhibitor of P4501A1 and 1B1. This could reflect a potential species difference (rat versus mouse) in the metabolism of coumarins. We performed our studies in mice based on our previous reports that NOCs modulated carcinogen-metabolizing enzymes and PAH–DNA adduct formation in a range of tissues when administered orally (43). Alternatively, in the Wattenberg study, coumarin may have had other activities against tumorigenesis, besides inhibition of DNA adduct formation. We have previously reported that isopimpinellin (70 mg/kg body wt by gavage) inhibited [3H]DMBA–DNA adduct formation in mouse mammary glands by 28% (43). The results from the current study are consistent with this previous report. Thus, two different methods ([3H] labeled carcinogen and 32P-postlabeling) have demonstrated similar results. We have also shown that imperatorin, isopimpinellin and 7,8-BF inhibited P4501 activity and blocked B[α]P- and DMBA–DNA adduct formation in human MCF-7 breast cancer cells (53). As with the current study, 7,8-BF was the most potent at blocking DMBA–DNA adduct formation, which corresponded to its ability to inhibit P4501 activity (53).

The results from the current study support a role for P4501 family inhibition and GST induction in the suppression of DMBA–DNA adduct formation in mouse mammary gland in vivo. Furthermore, the data suggest that the linear furanocoumarins (imperatorin and isopimpinellin) were more effective than the simple coumarins (coumarin and limettin) at blocking DMBA–DNA adduct formation in mouse mammary glands. However, the greatest suppression of DMBA–DNA adduct formation in mouse mammary gland was accomplished by 7,8-BF, which was also the most potent P4501 family inhibitor used in the study. This suggests that P4501 suppression played a predominant role in the suppression of DMBA–DNA adduct formation, and GST induction played a moderate role. The possibility remains, however, that other effects of 7,8-BF may have contributed to its ability to block DMBA–DNA adduct formation.

In conclusion, we showed that both simple coumarins and linear furanocoumarins blocked DMBA–DNA adduct formation in mouse mammary gland. Many cancer chemopreventive agents that block DNA adduct formation also block tumorigenesis in a variety of models [reviewed in (64)]. In rodent mammary, dibenzoylmethane and butylated hydroxytoluene have both been shown to suppress DMBA–DNA adduct formation and tumorigenesis (62,65). The results from the current study raise the interesting possibility that a combination of both P4501 inhibition and GST induction may lead to more effective inhibition of PAH mammary carcinogenesis.

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