Oral administration of the citrus coumarin, isopimpinellin, blocks DNA adduct formation and skin tumor initiation by 7,12-dimethylbenz[a]anthracene in SENCAR mice

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The current study was designed to evaluate the effects of oral administration of the citrus coumarin, isopimpinellin, on skin tumor initiation by topically applied benzo[a]pyrene (B[a]P) and 7,12-dimethylbenz[a]anthracene (DMBA). To evaluate the effects of orally administered isopimpinellin on skin tumor initiation by B[a]P and DMBA, its effects on DNA adduct formation were first evaluated. Female SENCAR mice were pre-treated twice with corn oil, or isopimpinellin (70 mg/kg body wt per os) at 24 h and 2 h prior to topical treatment with B[a]P or DMBA. Another citrus coumarin, imperatorin, was also included in these experiments for comparison. Orally administered isopimpinellin and imperatorin significantly inhibited B[a]P–DNA adduct formation by 37 and 26%, respectively. Imperatorin also blocked DMBA–DNA adduct formation by 43%. In a second dose–response study, orally administered isopimpinellin (35, 70 and 150 mg/kg) blocked DMBA–DNA adduct formation by 23, 56 and 69%, respectively. For the tumor study, mice were pretreated orally with corn oil or isopimpinellin at 24 and 2 h prior to initiation with DMBA, and 2 weeks later promotion began with 12-O-tetradecanoylphorbol-13-acetate (TPA). Isopimpinellin significantly reduced the mean number of papillomas per mouse by 49, 73 and 78% compared to corn oil controls at 30, 70 and 150 mg/kg body wt, respectively. Orally administered isopimpinellin also significantly reduced the percentage of mice with papillomas at the highest dose tested (150 mg/kg). The effectiveness of isopimpinellin given orally was also evaluated for comparison. As part of this study, several parameters of systemic toxicity were evaluated following oral dosing with isopimpinellin and imperatorin. Mice were treated orally with corn oil, isopimpinellin or imperatorin (35, 70 and 150 mg/kg body wt per os) once daily for four consecutive days, killed at 24 h after the last dose, and livers, lungs, and kidneys evaluated histologically. In addition, urinary parameters of nephrotoxicity, blood parameters of liver and kidney function, and thrombin clotting time were assayed. No significant changes in blood clotting, or renal or hepatic function were observed. There was, however, a significant increase in liver wt accompanied by cytoplasmic vaculation of hepatocytes. There were no histopathological changes in lungs or kidneys. Overall, these data indicate that isopimpinellin (and imperatorin) have chemopreventive effects when administered orally on skin tumor initiation by DMBA.

Introduction

Naturally occurring furanocoumarins are abundant in citrus fruits, umbelliferous vegetables including parsley, parsnips, cilantro, and celery, grasses, and in many traditional herbal medicines (1–6). For example, imperatorin (structure shown in Figure 1) is present in the root of Angelica dahurica (7); the fruits, leaves and roots of parsnip (200, 7.8 and 0.06 mg/100 g dry wt, respectively) (8); the fruits, leaves, and roots of Anmmi majus L. (94.7, 14.1, and 3.3 mg/100 g dry wt, respectively) (9); lemon and lime oils (5); the fruits of parsley (0.7–5.9 µg/g) (2); the fruits of fennel (2.80 µg/g) and possibly coriander (2); and has been isolated from the dried root of Saposhnikovia divaricate (Turcz.) Schischk (Umbelliferae) (10). Isopimpinellin (structure shown in Figure 1) is found in healthy celery (0.38 µg/g dried root tissue) (11); the fruits, leaves, and roots of parsnip (8.0, 4.8, and 6.4 mg/100 g dry wt, respectively) (8); the fruits of Anmmi majus L. (205 mg/100 g dry wt) (9); and in the rind and pulp of limes (22–53 µg/g rind, 1.7–2.9 µg/g pulp) (4). In fact, squeezed limeade may contain up to 3 µg/g isopimpinellin and lime oils may contain up to 5080 µg isopimpinellin per gram (discussed in ref. 4).

Furanocoumarins possess a variety of pharmacological activities and have been studied for their anti-inflammatory and anti-tumor promoting activities (10,12,13), and for their anti-fungal (14,15), anti-microbial (16) and anti-mutagenic properties (17–20) as well. We have previously shown that naturally occurring coumarins, including certain linear furanocoumarins (e.g. imperatorin, bergamottin, and coriandrin), inhibited cytochrome P450 (CYP)–mediated enzyme activities in vitro, in some cases by mechanism-based inactivation (21,22). Other investigators have shown that bergamottin, a component of grapefruit juice, is a potent mechanism-based CYP3A4 inhibitor (23,24). We previously reported that the linear furanocoumarins, bergamottin and coriandrin, blocked metabolism and/or DNA adduct formation by B[a]P and DMBA in cultured mouse epidermal keratinocytes (25). Several of these furanocoumarins were also found to inhibit DNA adduct formation and skin tumor initiation in mice by B[a]P.

Abbreviations: AFB₁, aflatoxin B₁; ALP, alkaline phosphatase; B[a]P, benzo[a]pyrene; BPDE-dGuo, anti-benzo[a]pyrene diol-epoxide-dGuo; BUN, blood urea nitrogen; dAdo, deoxyadenosine; CYP, cytochrome P450; dGuo, deoxyguanosine; DMBA, 7,12-dimethylbenz[a]anthracene; EROD, ethoxyresorufin O-deethylase; GGT, γ-glutamyl transpeptidase; GST, glutathione S-transferase; 5-MOP, 5-methoxypsoralen; PAH, polycyclic aromatic hydrocarbon; PROD, pentoxyresorufin O-dealkylase; PUVA, psoralen plus UVA; GOT, glutamic oxalacetic transaminase; GPT, glutamic pyruvate transaminase; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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and/or DMBA (26). Simple coumarins have also been shown to possess anti-carcinogenic activities. Coumarin (1,2-benzopyrone) administered to rats in the diet induced detoxification enzymes aflatoxin B\(1\) (AF\(B\)\(1\))-aldehyde reductase, glutathione S-transferase (GST) A5 and pi, and NAD(P)H quinone oxidoreductase in liver, and inhibited AF\(B\)\(1\) hepatocarcinogenicity (27). Coumarin is a prototypic CYP2A6 substrate (28). Since CYP2A6 is also involved in AF\(B\)\(1\) metabolic bioactivation (reviewed in refs 28,29), possible competitive inhibition of this enzyme by coumarin may also contribute to its inhibitory effects on AF\(B\)\(1\) hepatocarcinogenesis. Coumarin and related compounds limettin (5,7-dimethoxycoumarin) and 4-methylcoumarin also inhibited mammary tumorigenesis induced by DMBA in rats (30,31). Treatment of mice with coumarin (which induces GST activities in the forestomach, liver, and intestines of mice 32,33) and 6-methylcoumarin, blocked B[a]P-induced forestomach neoplasia (31). Thus, coumarins have been shown to exhibit anti-carcinogenic activity in several biological systems by modulating carcinogen metabolism.

Recently, we showed that oral administration of isopimpinellin and imperatorin to mice (70 mg/kg body weight, per os, four consecutive daily doses) significantly inhibited 7-ethoxyresoruﬁn O-deethylase (EROD) and 7-pentoxysrofuin O-dealkylase (PROD) activities in epidermis, lung, and forestomach (34). The EROD assay is selective for CYP1 family members, whereas PROD is more selective for CYP2B activities (35). At 1 h after the last of four consecutive oral doses, lung microsomal EROD and PROD activities were reduced by up to 40% and 72%, respectively. These effects were sustained over 24 h. Epidermal EROD and PROD activities were inhibited by up to 57% and 61%, respectively, at 1 h after the final dose and were still signiﬁcantly reduced at 24 h. Forestomach EROD and PROD activities were reduced by up to 24% and 48%, respectively, at 1 h after the final dose but had returned to control levels at 24 h after the final dose. In addition, these compounds modulated EROD and PROD activities in liver, and significantly elevated hepatic GST activity (34). In the liver, signiﬁcant inhibition of EROD activity was observed at 1 h after the final dose. However, a significant increase in both EROD and PROD activities in liver was observed at 24 h. These results suggested that orally administered linear furanocoumarins (especially imperatorin and isopimpinellin) could modulate carcinogen metabolizing enzymes in various organs in the body.

To date, our studies of the anti-tumor initiating properties of several linear furanocoumarins have utilized only topical application to mice (26). Since coumarins are present in dietary sources, it was of interest to determine whether oral administration of naturally occurring coumarins could also produce anti-tumorigenic effects. We hypothesized that since isopimpinellin and imperatorin blocked CYP activities in the epidermis following oral administration (34), they should also block DNA adduct formation and skin tumor initiation by PAHs (which are metabolically bioactivated by CYPs, see ref. 36). In the current study, we investigated the effects of orally administered isopimpinellin and imperatorin on DNA adduct formation by topically applied B[a]P and DMBA in mice. In addition, we compared the effects of oral or topical administration of isopimpinellin on skin tumor initiation by DMBA. Finally, we also evaluated the toxicity of orally administered isopimpinellin and imperatorin in mice. The results indicate that isopimpinellin and imperatorin can effectively inhibit DMBA skin tumor initiation when given orally and have little or no systemic toxicity in mice at relatively high doses.

Materials and methods

B[a]P was purchased from Aldrich Chemical Co. (Milwaukee, WI). DMBA was purchased from Eastman Kodak Co. (Rochester, NY). 7-[\(3^H\)]HBlue (sp. act. 66-70 Ci/\(\mu\)mol) and [\(\gamma\)-\(3^H\)]DMBA (sp. act. 55-62 Ci/\(\mu\)mol) were obtained from Amersham Co. (Arlington Heights, IL) and diluted with unlabeled B[a]P or DMBA to a specific activity of 1 or 10 Ci/\(\mu\)mol, respectively. Typical batch analysis by tritium nuclear magnetic resonance spectroscopy shows the distribution of the label as follows, although batch variation may occur: for [\(\gamma\)-\(3^H\)]HBlu, position 1, 8.4%; 2, 6%; 3, 8.5%; 4, 11%; 5, 9%; 6, 10.1%; 7, 8.1%; 8, 9.1%; 9, 10.3%; 10, 3.7%; 11, 3.7%; 12, 12.3%. For [\(\gamma\)-\(3^H\)]DMBA, distribution of label was as follows: 7-methyl position, 6%; 12-methyl position, 1.5%; 9,10 position, 27.1%; 2,3,5 positions, 43%; position 4, 17.6%; position 8, 4.9%; and, 1,6,11 positions, nil. Isopimpinellin and imperatorin were purchased from Indofine Chemical Co. (Somerville, NJ). DNase I (bovine pancreas, EC 3.1.4.1), snake venom phosphodiesterase (Crotalus atrox, EC 3.1.4.1), and alkaline phosphatase (Escherichia coli, type III, EC 3.1.3.1) were supplied from Sigma Chemical Co. (St. Louis, MO). Sep-pak (C-18) cartridges were obtained from Waters Corporation (Milford, MA). HPLC-grade acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ). All experiments using coumarins or carcinogens were conducted under subdued lighting.

HPLC analysis of imperatorin and isopimpinellin

Imperatorin and isopimpinellin were dissolved in HPLC-grade methanol and injected onto a Shimadzu HPLC-10ADVP system on a Beckman reverse-phase ODS C-18 column (5 \(\mu\)m, 4.6 mm \(\times\) 25 cm). Compounds were eluted in a linear gradient of 70% methanol in water up to 100% methanol over 30 min and monitored at 254 nm. Under these conditions, imperatorin eluted at 7.87 min and was 98.6% pure; isopimpinellin eluted at 4.87 min and was 100% pure (Figure 1).

DNA adduct studies

Female SENCAR mice (NCI, Frederick, MD) (7–9 weeks of age) were fed AIN-76A semi-purified diet (Dyets, Bethlehem, PA) for 2 weeks prior to and during the study. Mice were randomized and divided into 3–4 mice/group. Mice were shaved on the dorsal side two days prior to carcinogen treatment, and dosed by gavage with corn oil (0.1 mL), or isopimpinellin or imperatorin (35–150 mg/kg body wt, suspended in 0.1 mL corn oil) at 24 h and 2 h prior to topical treatment with [\(\gamma\)-\(3^H\)]HBlu (200 \(\mu\)mol, 1 Ci/\(\mu\)mol) or [\(\gamma\)]HDMBA (10 nmol, 10 Ci/\(\mu\)mol) (each in 0.2 mL acetone). The data from three separate experiments was averaged together. Mice were killed by cervical dislocation 24 h after PAH treatment, and the epidermis was scraped and pooled for DNA isolation and DNA adduct analysis.

Epidermal scrapings were homogenized using a Polytron PT10 in 6% (v/w) para-amino salicylate, 1% (w/v) sodium chloride, 1% (w/v) tri-isopropylpyriphethalanesulfonic acid, and 6% (v/v) sec-butanol (37). DNA was isolated by phenol extraction and RNase A digestion as described previously (37). Purity of DNA was determined spectrophotometrically and DNA content was determined using calf thymus DNA as a standard (38). To
Determine total DNA adducts, DNA was hydrolyzed with DNase I and aliquots were analyzed by liquid scintillation spectroscopy. To analyze specific DNA adducts, the DNase I DNA hydrolysates were further hydrolyzed with snake venom phosphodiesterase and alkaline phosphatase as described previously (39) and purified through Sep-pak cartridges (40). HPLC analysis of PAH-DNA adducts was conducted by a method previously described (26,40). Prior to HPLC analysis, [3H]P-9,10-diol was added to each sample as an internal UV marker. Specific activity of DNA binding was represented as pmol DMBA or B[a]P bound per mg of DNA.

**Tumor studies**

Female SENCAR mice (NCl, Frederick, MD) (7–9 weeks of age) were fed AIN-76A semi-purified diet (Dyets, Bethlehem, PA) for 2 weeks before, during, and one week after initiation with DMBA, and then returned to chow diet. Mice were shaved on the dorsal side 2 days prior to carcinogen treatment, and only those mice in the resting stage of the hair cycle were used. Mice were randomized and divided into 30 mice per group. For the oral administration of isopimpinellin, mice were dosed by gavage with corn oil (0.1 mL) or isopimpinellin (30, 70, or 150 mg/kg body wt, suspended in 0.1 mL corn oil) at 24 h and 2 h prior to topical treatment with DMBA (10 nmol, in 0.2 mL acetone). For the topical administration of isopimpinellin, mice were treated with isopimpinellin (100–3200 nmol) 5 min prior to initiation with DMBA (10 nmol). Two weeks after initiation, mice received topical applications of TPA (1.7 nmol in 0.2 mL acetone) twice a week. The promotion stage was continued in each experimental group until a maximal papilloma response was achieved. Negative control mice received acetone in place of DMBA. As a control for any coumarin related effects, groups of 30 mice each were treated with isopimpinellin (150 mg/kg body wt, per os) for the oral studies, or with 3200 nmol isopimpinellin (for the topical studies) at the initiation stage followed by twice-weekly treatment with TPA. The incidence and multiplicity of skin papillomas were recorded weekly.

**Mutation analysis**

Upon termination of the tumor study, mice were killed by cervical dislocation. Papillomas were excised quickly, frozen in liquid nitrogen, and stored at −80°C until analyzed. Ten individual tumors, randomly selected from each group of mice, were ground in liquid nitrogen with a mortar and pestle, homogenized in 0.75 M guanidine isothiocyanate and the DNA was subsequently isolated as described (40). DNA samples were analyzed spectrophotometrically at 260 and 280 nm for purity and concentration. Samples were analyzed for mutations in codon 61 of the Ha-ras gene as previously described (41).

**Toxicity tests**

Female SENCAR mice (7–8 weeks old) were divided randomly into groups of three and housed individually in Nalgene metabolism cages for at least 2 days prior to the study. Mice were allowed access to food (AIN-76A) and water ad libitum. Mice were treated with corn oil, imperatorin, or isopimpinellin (35, 70, or 150 mg/kg body wt, per os in 0.1 mL corn oil per 25 g body wt) once daily for 4 consecutive days. Body wt and consumption of water were recorded daily, and urine was collected into light-protected tubes on a mixture of ice/dry ice daily. At 24 h after the final treatment, mice were killed by carbon dioxide asphyxiation, and blood was collected by cardiac puncture. Livers, lungs, and kidneys were removed, and samples were fixed in phosphate-buffered formalin. Samples were processed, paraffin-embedded, sectioned, and stained with hematoxylin and eosin for histological examination by light-microscopy.

Plasma samples were used to assess the following biochemical parameters of hepatotoxicity: glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) (Sigma Diagnostic Kit 505). In addition, plasma was used to quantify blood urea nitrogen (BUN) as an index of renal function (Sigma Diagnostics Procedure No. 535). Urine samples were used to assess biochemical parameters of nephrotoxicity. Brush border membrane damage was assessed by measuring the urinary concentrations of alkaline phosphatase (ALP) (Sigma Diagnostic Kit 245-50) by monitoring the formation of p-nitrophenol at 405 nm, and γ-glutamyltranspeptidase (GGT) activity using γ-glutamyl-p-nitroanilide as the substrate (Sigma Technical Bulletin 545). Plasma membrane integrity (cell death) was determined by measuring the activity of GST as a cytosolic enzyme marker, by the method of Habig (42). Citrated plasma was used to assess clotting time using Sigma Diagnostic Accuclot™ Thrombin Time reagent (Procedure No. A 8713/A 4589).

**Results**

Effects of orally administered isopimpinellin and imperatorin on epidermal DNA adduct formation

The abilities of orally administered isopimpinellin and imperatorin to inhibit DNA adduct formation by topically applied B[a]P and DMBA were evaluated. Pretreatment of mice with both compounds (70 mg/kg body wt, per os) significantly inhibited epidermal B[a]P-DNA adduct formation by 57% (isopimpinellin) and 26% (imperatorin) (Figure 2A). In a separate experiment, orally administered isopimpinellin (35, 70, or 150 mg/kg body wt) blocked DMBA–DNA adduct formation by 23%, 56%, and 69%, respectively (Figure 2B). Pretreatment of mice with imperatorin (70 mg/kg body wt, per os) also significantly inhibited DMBA–DNA adduct formation by 43% (Figure 2B). HPLC analysis of individual DNA adducts revealed that inhibition of total DNA adduct formation by isopimpinellin and imperatorin corresponded to inhibition of the major diol-epoxide derived adducts formed from B[a]P (anti-B[a]P diol-epoxide-dGuo)
and DMBA (anti-DMBA diol-epoxide-dGuo, syn-DMBA diol-epoxide-dAdo, and anti-DMBA diol-epoxide dAdo) (Figures 2 and 3 respectively). The specific activity (S.A.) of anti-B[α]P diol-epoxide-dGuo adducts in the corn oil control mice treated with B[α]P (2.51 pmol/mg DNA) was reduced to 1.85 and 1.58 pmol/mg DNA in the imperatorin and isopimpinellin pretreated groups, respectively (Figure 3). The S.A. of the anti-DMBA diol-epoxide-dGuo, syn-DMBA diol-epoxide-dAdo, and anti-DMBA diol-epoxide-dAdo adducts in the corn oil control mice treated with DMBA were 0.71, 0.54, and 0.48 pmol/mg DNA, respectively (Figure 4). These adducts were reduced by 50%, 33%, and 50%, respectively, in the mice pretreated with 70 mg/kg imperatorin, and were reduced by 70%, 59%, and 69%, respectively, in the mice pretreated with 70 mg/kg isopimpinellin (Figure 4).

Effects of orally administered isopimpinellin on skin tumor initiation by DMBA

Consistent with the inhibition of DNA adduct formation by DMBA (Figure 2B), orally administered isopimpinellin also significantly inhibited skin tumor initiation by DMBA (Figure 5 and Table I). Orally administered isopimpinellin significantly (P ≤ 0.0003) reduced the mean number of papillomas per mouse (19 weeks of promotion) by 49%, 73%, and 78% compared to corn oil controls at 30, 70, and 150 mg/kg body wt, respectively. The maximal effect of isopimpinellin on the number of papillomas per mouse occurred between 70 and 150 mg/kg. Isopimpinellin pretreatment also significantly decreased (P ≤ 0.006) the percentage of mice with papillomas at the highest dose tested (150 mg/kg body wt) compared with corn oil pretreated mice (Table I). Mice that received acetone in place of DMBA did not develop any tumors in either the corn oil or the isopimpinellin (150 mg/kg body wt) pretreated groups (Figure 5).

Effects of topically applied isopimpinellin on skin tumor initiation by DMBA

The anti-tumor initiating effects of topical isopimpinellin pretreatment in mice initiated with DMBA and promoted with TPA was examined (Figure 6 and Table II). At all doses tested, there were significantly (P ≤ 0.0002) fewer papillomas per mouse after 18 weeks of promotion in mice pretreated with isopimpinellin (100–3200 nmol) compared with acetone pretreated mice (Table II). Greater effects were observed as the dose of isopimpinellin was increased, ranging from 54% inhibition (100 nmol) up to a maximum of 83% (1600 nmol) (Figure 6 and Table II). Increasing the dose to 3200 nmol did not achieve greater protection than that observed at 1600 nmol (Figure 6 and Table II). While there was less of an effect on papilloma incidence, isopimpinellin at a dose of 1600 nmol did produce a statistically significant reduction in the percentage of...
mice with tumors by 23% (P ≤ 0.002) compared with mice pretreated with acetone (Table II). The overall lack of significant effects of isopimpinellin on the percentage of mice with papillomas (tumor incidence) is not surprising and is due to several factors. Most important, in this regard, is the dose of DMBA and the fact that tumor incidence follows a very narrow dose–response window (43,44). In control mice that received acetone in place of DMBA, there was no difference in papilloma incidence or multiplicity compared to isopimpinellin (3200 nmol) pretreated mice (Figure 6 and Table II, footnote).

**Mutation analysis**
Ha-ras mutation analysis of individual tumors (10 per group) showed no difference in the mutations observed from the DMBA group or the groups that received isopimpinellin (400, 1600 nmol) at initiation had 0.25 (7%) or 0.03 (3%), respectively, papillomas per mouse (numbers in parentheses represent % of mice with papillomas). Data are recorded at week 18 and represent means ± SE.

(43,44)

### Table I. Effect of orally administered isopimpinellin on skin tumor initiation by DMBA

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Dose (mg/kg)</th>
<th>% Mice with papillomas</th>
<th>Papillomas per mouse</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>100 µl</td>
<td>100</td>
<td>11.4 ± 1.1</td>
<td>–</td>
</tr>
<tr>
<td>Isopimpinellin 30</td>
<td>97</td>
<td>5.8 ± 0.9²</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Isopimpinellin 70</td>
<td>86</td>
<td>3.1 ± 0.5²</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Isopimpinellin 150</td>
<td>78²</td>
<td>2.4 ± 0.4²</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>

(a) Groups of 30 mice each were treated with isopimpinellin (30, 70, and 150 mg/kg body wt, per os) at 24 and 2 h prior to initiation with 10 nmol DMBA. Two weeks after initiation, mice were treated with 1.7 nmol TPA twice a week for 19 weeks. Control mice, pretreated with either acetone or isopimpinellin (150 mg/kg body wt), received acetone instead of DMBA at initiation. Figures represent the mean number of papillomas per mouse (means ± SE).

(b) Significantly less than the control that received acetone in place of isopimpinellin based on Mann–Whitney U-test, P = 0.003.

**Table II. Effect of topically applied isopimpinellin on skin tumor initiation by DMBA**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Dose (nmol)</th>
<th>% Mice with papillomas</th>
<th>Papillomas per mouse</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>200 µl</td>
<td>100</td>
<td>11.1 ± 1.1</td>
<td>–</td>
</tr>
<tr>
<td>Isopimpinellin 100</td>
<td>96</td>
<td>5.1 ± 0.6²</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Isopimpinellin 400</td>
<td>93</td>
<td>4.1 ± 0.5²</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Isopimpinellin 800</td>
<td>90</td>
<td>4.3 ± 0.6²</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Isopimpinellin 1600</td>
<td>77²</td>
<td>1.9 ± 0.3²</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Isopimpinellin 3200</td>
<td>89</td>
<td>2.4 ± 0.4²</td>
<td>79</td>
<td></td>
</tr>
</tbody>
</table>

(a) Groups of 30 mice each were treated with isopimpinellin (100–200 nmol) 5 min prior to initiation with 10 nmol DMBA. Two weeks after initiation, mice were treated with 1.7 nmol TPA twice a week for 18 weeks. Control mice that received acetone or isopimpinellin (3200 nmol) instead of DMBA at initiation had 0.25 (7%) or 0.03 (3%), respectively, papillomas per mouse (numbers in parentheses represent % of mice with papillomas). Data are recorded at week 18 and represent means ± SE.

(b) Significantly less than the control that received acetone in place of isopimpinellin based on Mann–Whitney U-test, P = 0.0002.
Table III. Effects of isopimpinellin and imperatorin on liver wt in SENCAR mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver wt (g)</th>
<th>Liver wt (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>2.03 ± 0.12</td>
<td>6.35 ± 0.35</td>
</tr>
<tr>
<td>Imperatorin (35)</td>
<td>2.54 ± 0.20bc</td>
<td>7.54 ± 0.71bc</td>
</tr>
<tr>
<td>Imperatorin (70)</td>
<td>3.01 ± 0.38b</td>
<td>8.39 ± 0.29bc</td>
</tr>
<tr>
<td>Imperatorin (150)</td>
<td>3.40 ± 0.11bc</td>
<td>9.92 ± 0.18bc</td>
</tr>
<tr>
<td>Isopimpinellin (35)</td>
<td>2.53 ± 0.10b</td>
<td>8.18 ± 0.12b</td>
</tr>
<tr>
<td>Isopimpinellin (70)</td>
<td>3.30 ± 0.15b</td>
<td>9.77 ± 0.22b</td>
</tr>
<tr>
<td>Isopimpinellin (150)</td>
<td>3.29 ± 0.10b</td>
<td>10.33 ± 0.22b</td>
</tr>
</tbody>
</table>

Female SENCAR mice were treated with the indicated compounds and livers collected as described in Materials and methods. Values represent means ± SE (n = 3) of liver wt (g) or liver wt expressed as a percentage of total body wt (%). aSignificantly different from corn oil control (P < 0.05). bSignificantly different from each other (P < 0.05). cSignificantly different from each other (P < 0.05). Numbers in parentheses represent percent increase over corn oil control.

Table IV. Effects of isopimpinellin and imperatorin on blood parameters in SENCAR mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clotting time (min)</th>
<th>BUN (mg %)</th>
<th>GPT (U/L)</th>
<th>GOT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>0.41 ± 0.08</td>
<td>27.0 ± 1.9</td>
<td>97 ± 36</td>
<td>166 ± 23</td>
</tr>
<tr>
<td>Imperatorin (150 mg/kg)</td>
<td>0.43 ± 0.16</td>
<td>22.6 ± 2.6</td>
<td>83 ± 18</td>
<td>137 ± 16</td>
</tr>
<tr>
<td>Isopimpinellin (150 mg/kg)</td>
<td>0.35 ± 0.07</td>
<td>20.4 ± 2.0</td>
<td>127 ± 28</td>
<td>158 ± 22</td>
</tr>
</tbody>
</table>

Female SENCAR mice were treated with the indicated compounds and assays performed as described in Materials and methods. Values represent means ± SE (n = 5).

Table V. Effects of orally administered isopimpinellin and imperatorin on urinary GGT activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>0.27 ± 0.05</td>
<td>0.19 ± 0.05</td>
<td>0.29 ± 0.08</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Imper. 35</td>
<td>0.26 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.38 ± 0.07</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>Imper. 70</td>
<td>0.26 ± 0.03</td>
<td>0.01 ± 0.01</td>
<td>0.42 ± 0.05</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Imper. 150</td>
<td>0.10 ± 0.03b</td>
<td>0.07 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Isopimp. 35</td>
<td>0.12 ± 0.02</td>
<td>0.31 ± 0.07</td>
<td>0.20 ± 0.03</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Isopimp. 70</td>
<td>0.08 ± 0.03b</td>
<td>0.22 ± 0.05</td>
<td>0.29 ± 0.04</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Isopimp. 150</td>
<td>0.22 ± 0.08</td>
<td>0.33 ± 0.13</td>
<td>0.20 ± 0.08</td>
<td>0.25 ± 0.08</td>
</tr>
</tbody>
</table>

Female SENCAR mice were treated with imperatorin or isopimpinellin (35–150 mg/kg, per os, suspended in corn oil) once daily for 4 consecutive days, and killed at 24 h after the last dose. Urine was collected daily and assayed for GGT activity. Values represent mean units of GGT/24 h ± SE (n = 3).

Discussion

We recently reported that orally administered isopimpinellin and imperatorin modulated phase I and II enzyme activities in various tissues of SENCAR mice (34). In particular, isopimpinellin and imperatorin (four consecutive daily doses, 70 mg/kg body wt, per os) significantly inhibited EROD and PROD activity by up to 61% in epidermis compared to vehicle (corn oil) treated control mice (34). Epidermal EROD and PROD activities were still significantly suppressed 24 h after the final dose (34). As expected, isopimpinellin and imperatorin, when administered orally, also inhibited epidermal DNA adduct formation by topically applied PAH by up to 69%. Both linear furanocoumarins appeared to be somewhat more effective at blocking DNA adduct formation by DMBA than by B[a]P. Consistent with this finding, we have previously observed that topically applied imperatorin is more effective at inhibiting formation of epidermal DNA adducts by DMBA compared to B[a]P (26).

As shown in Table I and Figure 5, isopimpinellin was effective at blocking skin tumor initiation by DMBA when mice were dosed orally. By this route of administration, isopimpinellin produced a maximum inhibition of ~80% of mean papillomas per mouse in an initiation-promotion protocol. Similarly, the maximum inhibition when isopimpinellin was administered topically was also ~80% (as shown in Table II and Figure 6). The striking effectiveness of isopimpinellin on DMBA skin tumor initiation following oral administration is quite interesting. Little is known about the absorption, distribution, and metabolism of isopimpinellin, but several studies have investigated these parameters for psoralens that are used in PUVA therapy (psoralens with UVA) (45–48). Psoralens are structurally related to imperatorin and isopimpinellin. PUVA therapy is used to treat skin disorders such as psoriasis and vitiligo, and the drug is administered orally 2–3 h prior to exposure to UVA (320–400 nm) (48,49).

In humans treated orally with 5-methoxypsoralen (5-MOP), Zucchi et al. (48) determined that 5-MOP accumulated in the skin at levels ~200 times that of the plasma concentrations, representing 59% (3 h after a single dose) to 66% (3 h after multiple dosing) of the administered dose. In serial 20 µm frozen sections of skin, the concentration decreased from the stratum corneum to the dermis (48). The concentration of 5-MOP in the skin remained about the same from 3 h to 3 days after dosing (48). These studies suggest that furanocoumarins (or psoralens) accumulate in the skin, especially indicated by the reduced number of papillomas per mouse (Figure 6 and Table II).

Toxicity studies

No adverse changes were observed in body wt or water consumption over the course of the toxicity study (data not shown). However, with oral dosing of both isopimpinellin and imperatorin, there was a significant increase in liver wt (g) or liver wt expressed as a percentage of total body wt, particularly at the higher doses, compared with the corn oil controls (Table III). Despite the increase in liver wt, there were no significant changes in plasma GOT or GPT concentrations (Table IV). In addition, no significant changes in BUN or blood clotting time were observed (Table IV). There were no significant elevations in urinary GGT or GST activity by isopimpinellin or imperatorin at any of the doses tested on any of the days after treatment (Table V and data not shown). ALP activity was not significantly changed in any of the imperatorin groups on any of the days after treatment (Table VI). ALP was not elevated at the low and middle doses of isopimpinellin, but at the highest dose of isopimpinellin, there was a statistically significant elevation in ALP by 3- to 5-fold at days 2–5 (Table VI). Histopathological evaluation of the liver revealed moderate to marked diffuse hepatocellular vacuolation of the perportal and mid-zonal regions in the 70 and 150 mg/kg isopimpinellin dose and 150 mg/kg imperatorin dose groups. Less extensive vacuolation was seen in the remaining dose groups of both compounds. Neither isopimpinellin nor imperatorin induced histological lesions in the lungs or kidneys (data not shown).
the epidermis, and this may explain why they are effective for both PUVA therapy and anti-carcinogenesis in mouse epidermis when administered orally.

Mutation analysis revealed that papillomas arising in the isopimpinellin pretreated groups possessed $A^{182}T$ mutations in the 61 codon of the Ha-ras proto-oncogene that were the same as those in the acetone pretreated groups. Almost all skin papillomas in mice initiated with DMBA have $A^{182}T$ mutations at Ha-ras codon 61 (50). Therefore, since isopimpinellin pretreatment reduced the total number of papillomas per mouse, it can be concluded that there were fewer mutations induced by DMBA at Ha-ras codon 61. The results of this study, in conjunction with our previous work, support the hypothesis that orally administered isopimpinellin inhibits skin tumor initiation in mice by blocking CYP-mediated bioactivation of DMBA to its mutagenic/carcinogenic products, inhibiting DNA adduct formation, and subsequently reducing the frequency of mutational events at codon 61 of Ha-ras in target epithelial cells.

Although the toxicity and human risk assessment for coumarin (1,2-benzopyrone) has been reported (28), less is known about the toxicity of furanocoumarins. It is also widely known that coumarin derivatives (e.g. warfarin, 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-l-benzopyran-2-one) inhibit prothrombin time and thereby block blood coagulation. Furanocoumarins differ in structure from simple coumarin by the presence of the furan moiety. Certain furanocoumarins are known to exhibit phototoxicity and photocarcinogenicity (4,51). However, few reports have further evaluated the potential systemic toxicity of furanocoumarins.

The toxicity studies presented in this paper demonstrated essentially no overall adverse effects of isopimpinellin or imperatorin on lung or kidneys. Although there was an elevation of urinary ALP at the highest dose of isopimpinellin, this did not correspond to changes in other parameters of renal toxicity, such as BUN, or urinary GGT or GST, nor did it correspond to the lack of histopathology. Furthermore, although certain coumarin derivatives are known to produce anticoagulating effects, isopimpinellin and imperatorin, which are linear furanocoumarins and are therefore different in structure, did not alter prothrombin time. The effects on liver were dose-related and corresponded to an increase in liver wt. The accumulation of vacuoles within the cytoplasm of hepatocytes is believed to have contributed to the overall wt of the liver. These vacuoles could be either glycogen or lipids. Despite these effects on liver, there were no changes in the biochemical parameters of liver function (GPT, GOT). The increase in liver wt might be related to the induction of CYPs and GST previously observed (34). In rodents, liver enlargement is a typical response to enzyme inducers such as phenobarbital (52). Phenobarbital causes hepatocellular hypertrophy and increases in smooth endoplasmic reticulum, most likely due to enzyme induction and cell replication (52). Although many naturally occurring chemopreventive agents are known to induce drug metabolizing enzymes, few studies have reported whether this corresponds to changes in liver size or function. Rosemary extracts have been shown to induce CYPs, GST, and other drug metabolizing enzymes in rat liver, with a concomitant increase in relative liver wt (53). The mechanism(s) of CYP and GST induction in liver of mice following oral administration of imperatorin and isopimpinellin is currently under investigation in our laboratory.

Although prevention of carcinogen exposure is most desirable, in many cases it may not be feasible. It is therefore advantageous to characterize dietary factors which may counteract carcinogen exposure in man. By using a mechanistic approach, we have determined that orally administered isopimpinellin modulates carcinogen metabolizing activity, blocks DNA adduct formation and blocks skin tumor initiation by PAH in mice. The current results also indicate that isopimpinellin and imperatorin, which are present in the human diet, have little or no systemic (renal, hepatic or pulmonary) toxicity when administered orally to mice. In conclusion, these studies demonstrate that certain citrus coumarins, when administered orally, can produce chemopreventive effects in a well-characterized animal model of chemical carcinogenesis.

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