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Identification of metabolites of the antitumor agent *d*-limonene capable of inhibiting protein isoprenylation and cell growth

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Summary. Limonene has been shown to be an effective, nontoxic chemopreventive and chemotherapeutic agent in chemically induced rat mammary-cancer models. The present study characterized circulating metabolites of limonene in female rats and determined their effects on cell growth. Metabolism of limonene was analyzed in plasma extracts by gas chromatography. Rapid conversion of limonene to two major metabolites was detected. These metabolites comprised more than 80% of the circulating limonene-derived material at 1 h after administration and thereafter, whereas limonene itself accounted for only 15%. The metabolites were characterized by mass spectroscopy and infrared spectroscopy. The probable structures were synthesized, and identities were confirmed by comparison of retention times and mass spectra. The two major circulating metabolites of limonene were found to be perillic acid and dihydroperillic acid. We have previously reported that limonene, perillic acid, and dihydroperillic acid inhibit the posttranslational isoprenylation of p21^{ras} and other 21- to 26-kDa cell-growth-associated proteins in NIH3T3 cells and in mammary epithelial cells. In the present study, perillic acid was found to inhibit cell growth in a dose-dependent manner. Thus, perillic acid and dihydroperillic acid, the two major circulating metabolites of limonene in the rat, are more potent inhibitors of protein isoprenylation than is limonene, and perillic acid is also a more potent inhibitor of cell growth. These data raise the possibility that the antitumor effects of limonene in vivo may be mediated via perillic acid and, perhaps, other metabolites.

Introduction

The naturally occurring monoterpene *d*-limonene exerts significant chemopreventive and chemotherapeutic activity in association with low toxicity in mammary and other rodent tumor models [6–8, 14, 20–22]. In the 7,12-dimethylbenz(*a*)anthracene (DMBA) rat mammary-cancer model, for example, limonene's antitumor activity is evident in rats fed as little as 0.1% (w/w) dietary limonene from -2 to 18 weeks postcarcinogenesis [6]. Limonene also shows chemopreventive activity in rats treated only during the initiation phase [8] or the promotion phase [14] of mammary carcinogenesis. For example, rats fed 5% limonene diets during the promotion phase only of *N*-nitroso-*N*-methylurea-induced mammary carcinogenesis exhibit longer latency and 5 times fewer tumors than do controls [14]. In addition to its efficacy as a chemopreventive agent, limonene holds promise as a chemotherapeutic agent for breast and possibly other cancers. Complete regression of more than 80% of chemically induced mammary tumors in association with little, if any, toxicity has been achieved in rats fed 10% limonene diets [7, 9].

We have investigated several mechanisms that may account for the antitumor effects of limonene. Since chemically induced rat mammary tumors are hormonally responsive, we first measured the effects of limonene on the endocrine system. However, the estradiol and prolactin concentrations and the duration of the estrus cycle in limonene-treated rats did not differ from those in controls [14]. More recently, we demonstrated that limonene inhibits the posttranslational isoprenylation of p21^{ras} and other 21- to 26-kDa proteins likely to play a role in cell growth control [3]. These effects have been observed in several species and cell types, including NIH3T3 murine fibroblasts, human mammary epithelial cells [3], and HT-29 human colon-carcinoma cells (Ren and Gould, unpublished data). The inhibition of 21- to 26-kDa protein isoprenylation may in part be responsible for the antitumor action of limonene, since it has been shown that *ras* oncogene mutants defective in isoprenylation are not transforming [12]. This lack of transforming capacity is attributed to the inability of the

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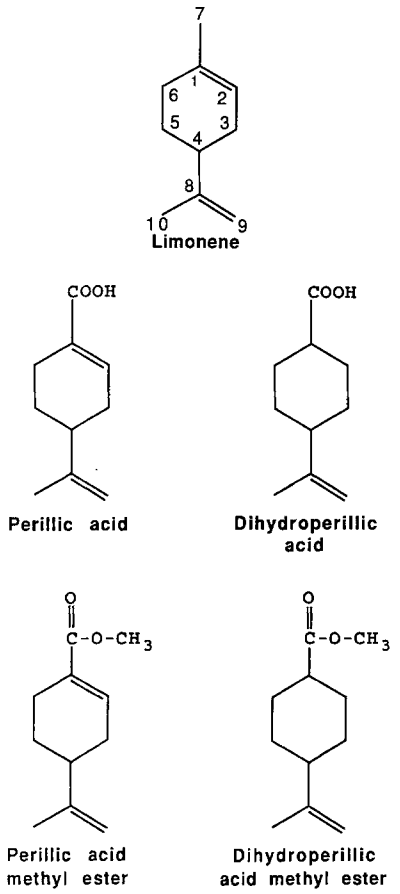

Fig. 1. Structures of limonene, perillic acid, dihydroperillic acid, perillic acid methyl ester, and dihydroperillic acid methyl ester

unprocessed p21*ras* to associate correctly with the plasma membrane, its intracellular site of action.

The metabolism and disposition of limonene in male rats, rabbits, and humans has been partially determined [11, 13, 16, 19]. Igimi et al. [11] reported that limonene (*p*-menth-1,8-diene; Fig. 1) was readily absorbed, and it distributed in every tissue tested. By 24 h after the administration of an oral dose of 600 mg/kg, 70% of the limonene-derived radioactivity had been excreted, almost exclusively via the urine. Some urinary metabolites such as uroterpenol, carveol, perillic acid, and their glycine and glucuronyl conjugates were identified.

Previous studies conducted in our laboratory have demonstrated that carveol and uroterpenol are more potent than limonene itself in the prevention of DMBA-induced rat mammary cancer during the initiation stage [4]. Thus, it is possible that a metabolite of limonene is the active antitumor agent in vivo. However, in rats undergoing chemoprevention or chemotherapy with limonene, mammary carcinomas are likely to be exposed to circulating rather than urinary metabolites. Nonurinary metabolites of limonene, particularly circulating metabolites, have not been identified to date. We therefore investigated the metabolism of limonene by female rats, placing particular emphasis on the identification of metabolites in plasma.

Materials and methods

Animal care. Female Wistar-Furth rats (Harlan Sprague-Dawley, Madison, Wis.) were kept in wire-mesh cages in a room equipped to provide a 12-h light cycle and controlled temperature and humidity. On arrival, rats were fed a purified AIN diet (Teklad). After acclimating to the diet for at least 2 weeks, rats aged 55–65 days were given a single oral dose of limonene (1 g/kg) mixed 1 : 1 (v/v) in corn oil. Control animals received corn oil alone. At the indicated time points, rats were anesthetized with ether and decapitated, and blood was collected into heparinized tubes. Heparin (Sigma, grade II) was prepared at 20 mg/ml in 0.9% saline. Several commercial solutions of heparin were found to be unsuitable, since they contained preservatives (e. g., methyl paraben, benzyl alcohol) that interfered with the terpene peaks of interest in the capillary gas chromatography analysis. Plasma was obtained after centrifugation and was stored at –20° C until further analysis.

Limonene distribution and excretion. Rats were given an oral dose of 1 g/kg [8-^{14}C]-*d*-limonene (2.9 μCi/mmol). They were then killed by ether inhalation, and blood and tissue samples were removed. The liver, kidney, lung, mammary gland, and intrascapular fat pads were placed in saline, blotted, and weighed, and 0.1 g tissue was dissolved in 1 ml Protosol at 55° C for 4 h. Samples were neutralized with 30 μl glacial acetic acid and then mixed with 10 ml BioSafe scintillation cocktail. Samples were stored at 4° C for 24 h in a dark environment to reduce chemiluminescence. Scintillation counting was done at 4° C by the internal-standard method on a Beckman 6000 scintillation counter.

Extraction of limonene and its metabolites from plasma. Limonene and its metabolites were extracted from plasma by the method of McClean et al. [15]. Plasma was mixed with an equal volume of *n*-butanol: acetonitrile (1 : 1, v/v), after which another volume of saturated potassium phosphate was added. After vortexing, the samples were centrifuged for 1 min in a microfuge, resulting in two layers with a precipitate at the interface. The organic layer was removed, concentrated under a stream of nitrogen, and then analyzed by gas chromatography. More than 99% of the limonene-derived radioactivity in plasma could be recovered in the organic phase (data not shown).

Gas chromatography. Two capillary gas-chromatography systems were used in the identification of limonene metabolites. The first consisted of a Hewlett-Packard 5891 gas chromatograph equipped with a flame-ionization detector and a 30-m J & W Scientific DB-5 column. The second system consisted of an Ultra 2 column on a 5890 chromatograph equipped with a 5971A mass-selective detector and/or a 5965B infrared detector (all from Hewlett-Packard). In all cases, the temperature program was 90° C for 4 min, increasing by 10° C/min, followed by 15 min at 275° C. The split ratio was 1 : 10 and the linear velocity was 1.1 ml/min.

Standard curves were constructed by adding 5 μl of a 10-mM solution of perillaldehyde (internal standard) and 0–7.5 μl of a mixture of limonene, perillic acid, and dihydroperillic acid (10 mM each) to 50 μl plasma. Samples were then extracted and analyzed by capillary gas chromatography with flame-ionization detection. For limonene and each of its metabolites, the ratio of the monoterpene peak area/perillaldehyde peak area versus the concentration of monoterpene in the sample was plotted. The slope of this line was used to determine the concentration of monoterpene in samples from rats fed limonene.

Monoterpene synthesis. (-)-(4S)-[2-Propenyl]-1-cyclohexane-1-carboxylic acid (perillic acid) was synthesized from (-)-perillaldehyde (Aldrich) by the method of Bal et al. [1], resulting in a 77% yield of colorless crystals [melting point (mp), 129°–131°C (10); 200-MHz nuclear magnetic resonance (NMR; $CDCl_3$, ppm): d 7.01–7.15 (br, 1H), 4.97 (s, 1H), 4.93 (s, 1H), 1.19–2.53 (m, 10H)]. 4[2-Propenyl]-1-cyclohexane-1-carboxylic acid (dihydroperillic acid) was synthesized from perillic acid by the method of Caine [2]. Recrystallization from ethanol-water resulted in a 60% yield of pure dihydroperillic acid as a low-mp solid in a 1 : 1 (v/v) mixture of diastereomers [200-MHz NMR ($CDCl_3$, ppm): d 9.11 (br, 1H), 4.65 (br, 2H), 1.04–2.35 (m, 13H)].

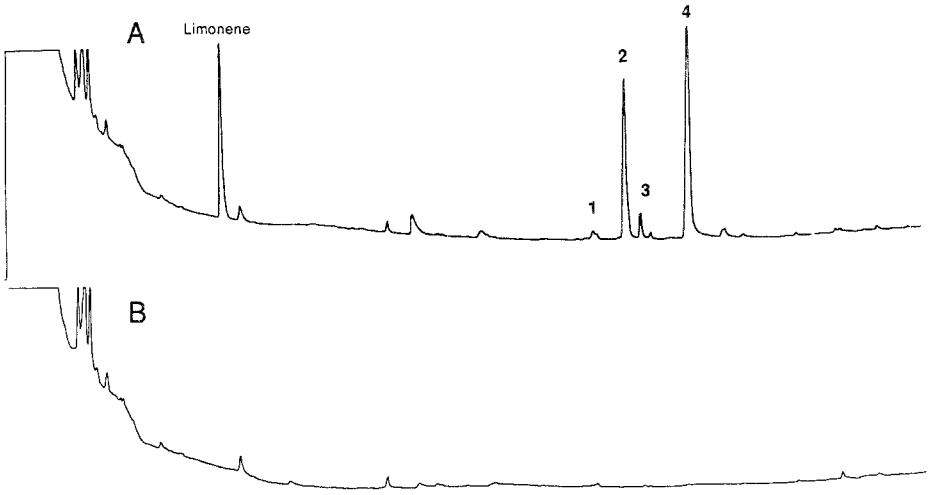

Fig. 2. Gas chromatograms obtained using flame-ionization detection in plasma extracts from female rats fed limonene: corn oil (*A*) or corn oil alone (*B*). Peaks corresponding to limonene and unknowns 1–4 are indicated

The methyl esters of perillic acid and dihydroperillic acid were synthesized by the addition of methyl iodide to the respective acids in anhydrous K_2CO_3 and acetone [23]. The residues were purified by flash chromatography on silica gel in hexane/ethyl acetate (95 : 5, v/v). Perillic acid methyl ester exhibited an R_f value of 0.5 and a yield of 75% [200-MHz NMR ($CDCl_3$, ppm): d 6.96–7.04 (m, 1H), 4.75 (m, 1H) 4.70 (m, 1H), 3.73 (s, 3H), 1.24–2.54 (m, 10H)]. The spectral data matched those previously described by Hortmann and Ong [10]. Dihydroperillic acid methyl ester was recovered as a mixture of diastereomers exhibiting R*f* values of 0.23 and 0.27 and a yield of 81% [200-MHz NMR ($CDCl_3$, ppm): d 4.68 (br, 1H), 3.69, 3.67 (ss, 3H), 1.12–2.68 (m, 3H)].

Cell culture. Cell cultures and growth assays were carried out as described elsewhere [3].

Materials. Unless otherwise indicated, all monoterpenes were obtained from Aldrich. Limonene was >99% pure as determined by capillary gas chromatography. 8-[^{14}C]-*d*-Limonene (50 mCi/mmol) was custom-synthesized by Amersham according to the method described by Igimi et al. [11]. Protosol was suppled by Amersham, and BioSafe was obtained from Research Products International.

Results

The disposition of limonene in female rats was established first to ensure that any unique compartmentation of the drug in these animals could be determined and that subsequent analyses could be done at a time point corresponding to maximal concentrations. Rats were given a single oral dose of 8-[^{14}C]-*d*-limonene. Within 1 h, radioactivity was detectable in plasma and in tissues (Table 1). Maximal concentrations ranging from 1.5 mM in plasma to 9 μmol/g in adipose tissue were detected at between 4 and 12 h; by 24 h, the amount of detectable radioactivity had begun declining. The time course in the lung was unique in that maximal concentrations were detected at 2 h, after which they declined. At 4, 12, and 24 h, limonene-derived radioactivity was greater in intrascapular adipose and mammary tissues than in less fatty tissues or serum. Cumulative urinary excretion of limonene was linear over the first 24 h, by which time 30% of the radioactivity from limonene had been excreted (data not shown).

We then began characterizing plasma metabolites of limonene in the rat by gas chromatography (GC). Plasma was used for these analyses since most of the limonene-derived material in blood accumulates in the plasma fraction rather than in blood cells [11]. Five unique peaks were consistently observed on chromatograms obtained from limonene-treated rats as opposed to controls (Fig. 2). Similar results were obtained in male rats or in female rats fed an equal quantity of orange-peel oil (Sunkist, 95% limonene; data not shown). The first peak co-migrated with *d*-limonene. The others, hereafter referred to as unknowns 1, 2, 3, and 4, respectively, in order of their retention time, did not precisely co-migrate with any available analogs of limonene. Several other peaks were sometimes detected, each representing less than 2% of the limonene-derived material. Since they were of low abundance, their infrared

Table 1. Distribution of limonene and its metabolites in plasma and tissues[a]

Time (h)	Plasma (mM)	Liver (μmol/g)	Kidney (μmol/g)	Lung (μmol/g)	Adipose (μmol/g)	Mammary (μmol/g)
1	0.62±0.16	1.76±0.45	1.09±0.27	0.48±0.16	2.14±0.54	1.10±0.22
2	1.41±0.60	3.00±0.85	1.81±0.44	2.57±0.90	4.80±1.53	2.78±0.72
4	1.45±0.10	4.09±0.48	2.16±0.25	2.31±1.17	10.37±1.05	6.80±0.51
12	1.57±0.48	3.42±1.15	2.01±0.47	1.03±0.33	9.27±4.00	7.85±3.00
24	0.67±0.23	1.84±0.59	1.33±0.39	1.03±0.33	4.60±0.84	3.58±0.70

[a] Rats were given a single oral dose of 1 g/kg 8-[^{14}C]-*d*-limonene (2.9 μCi/mmol). At the indicated time points, the rats were killed and blood and tissues were removed, dissolved, and analyzed by scintillation counting. The concentration of limonene and/or limonene-derived material was calculated by dividing the tissue radioactivity (in dpm/g tissue) by the specific radioactivity of the 8-[^{14}C]-*d*-limonene dose delivered. Data represent mean values ± SEM ($n = 5$)

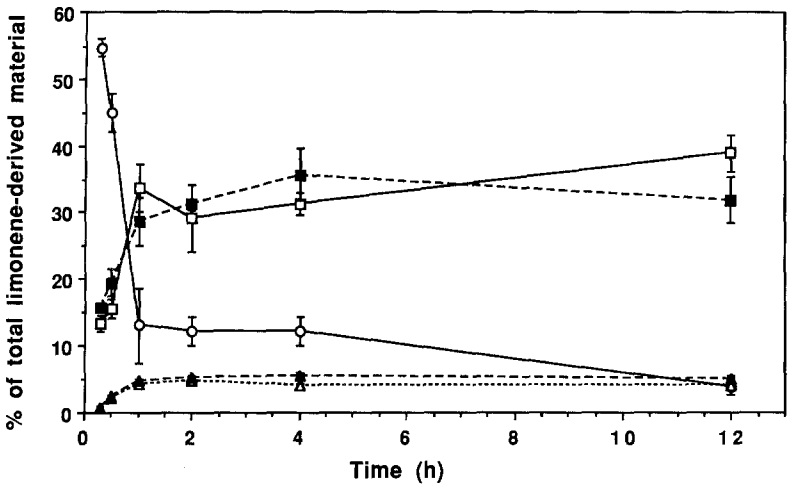

Fig. 3. Relative concentrations of limonene and unknowns 1–4 over time. A total of 24 rats were given a single dose of 1 g/kg limonene. At the indicated time points, 4 animals were killed and plasma was collected. Limonene-derived material was extracted and analyzed by capillary GC with flame-ionization detection as described in Materials and methods. The peak areas of limonene and unknowns 1–4 were summed, and the area of each peak is given as a percentage of this total. Each point represents the mean value ±SEM for 4 samples. ○, Limonene; △, unknown; □, unknown 2; ▲, unknown 3; ■, unknown 4

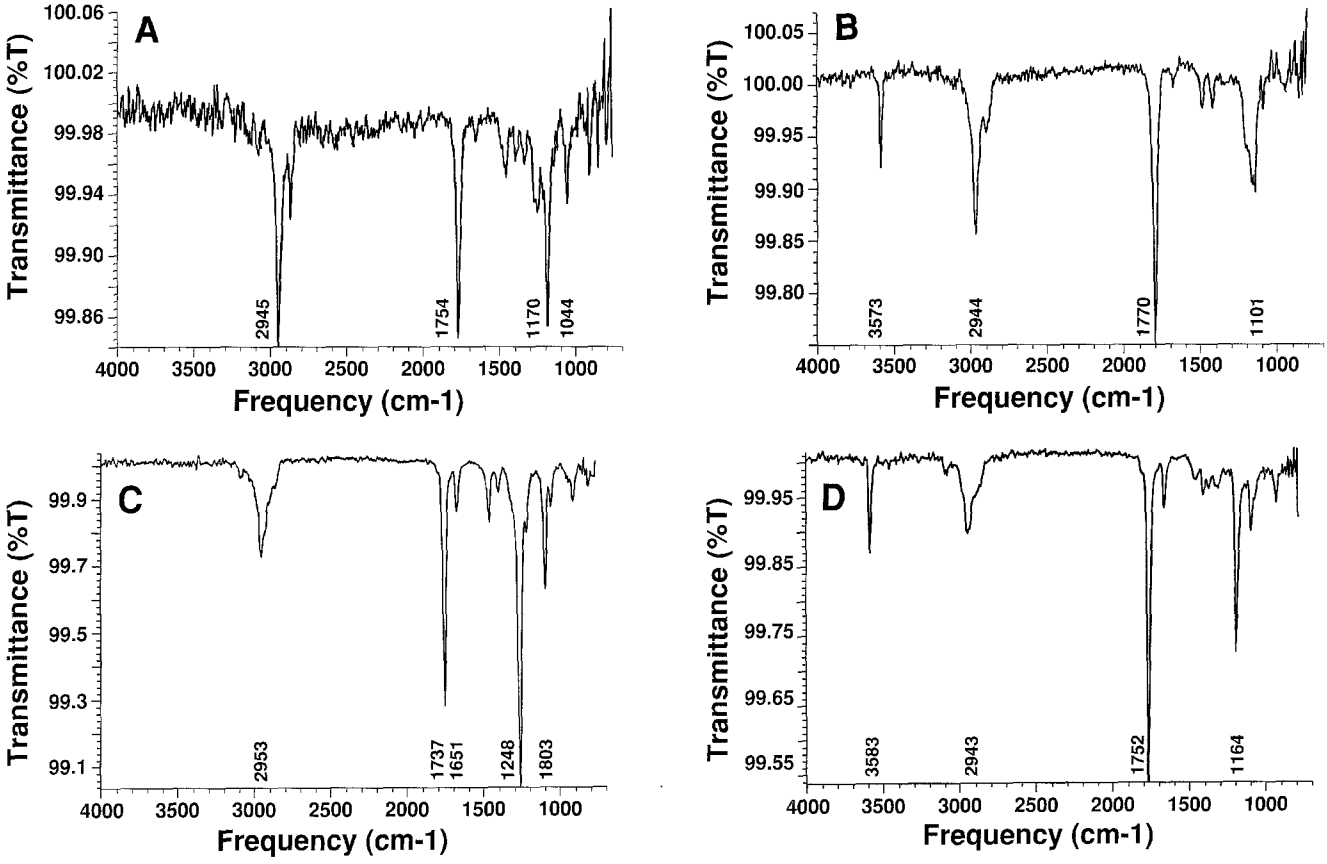

Fig. 4 A–D. Vapor-phase IR spectra of unknowns **A** 1, **B** 2, **C** 3, and **D** 4

spectra could not be obtained (see below) and they were therefore not further characterized.

Next, we examined the time course of the apparent conversion of limonene to unknowns 1–4 (Fig. 3). Within the 1st h after administration, the limonene concentration, expressed as a percentage of the circulating limonene-derived material, decreased rapidly, whereas the concentrations of unknowns 1–4 concominantly increased. At between 1 and 12 h, limonene comprised only ~10%–15% of the total limonene-derived material, unknowns 2 and 4 represented ~35% each, and unknowns 1 and 3 represented ~5% each. Similar profiles were observed in 24-h samples (data not shown). Samples obtained at 4 h were used in subsequent analyses, since the profile of apparent metabolites was representative of that observed at between 2 and 24 h and maximal or nearly maximal concentrations were present.

Unknowns 1–4 were then analyzed in organic extracts of plasma from limonene-treated rats by GC with on-line infrared (IR) detection. Unknown 1 absorbed strongly at 1,754 (C = O) and 1,170 cm^{-1}, which is characteristic of an ester (Fig. 4). The spectrum of unknown 3 was very simi-

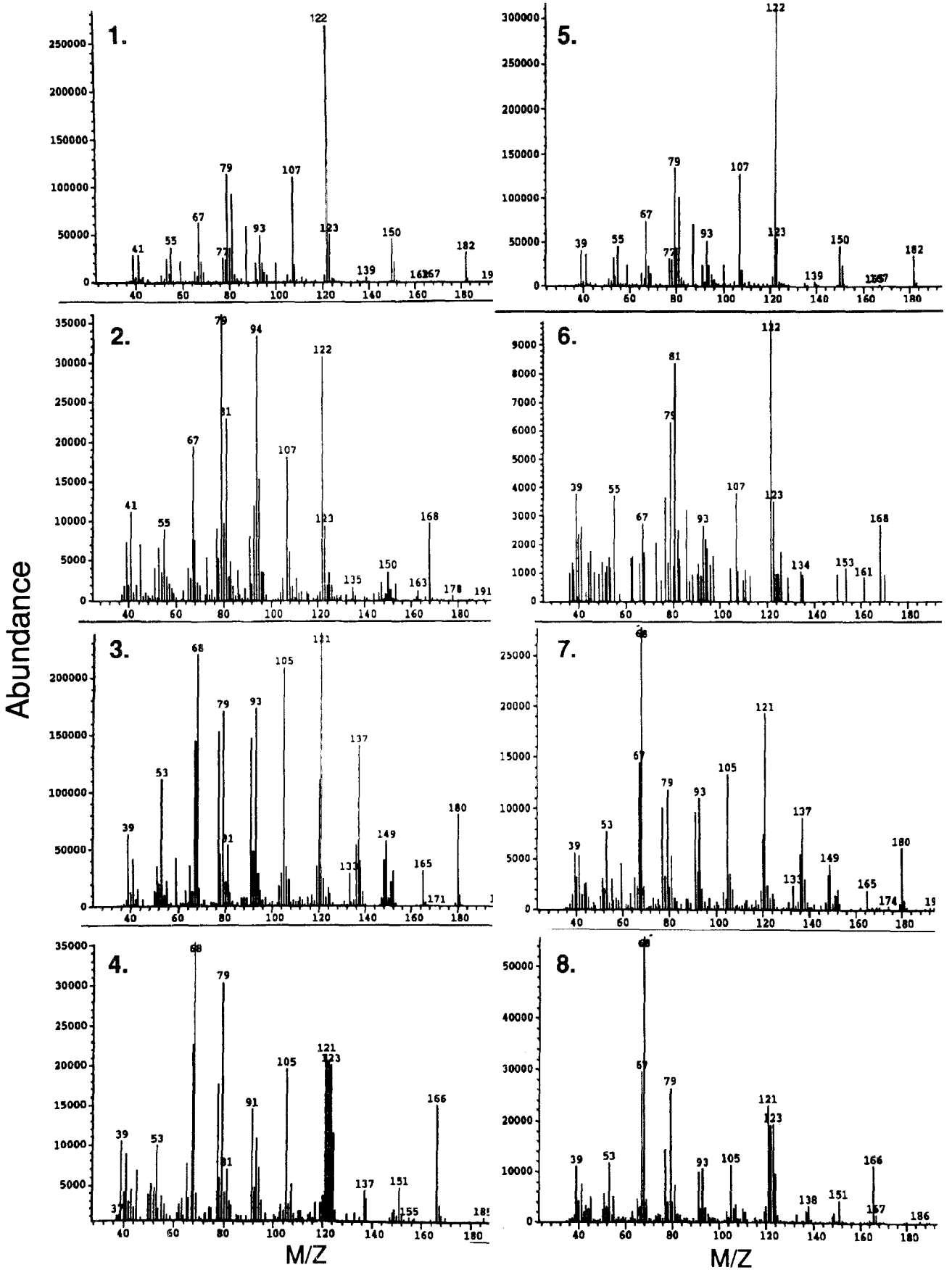

Fig. 5. Mass spectra of unknowns 1, 2, 3, and 4 (*panels 1–4*, respectively); synthetic dihydroperillic acid methyl ester (*panel 5*); dihydroperillic acid (*panel 6*); perillic acid methyl ester (*panel 7*); and perillic acid (*panel 8*). The spectra of the unknowns and the synthetic compounds were obtained on the same GC/MS system

lar, indicating strong absorbance at 1,737 and 1,240 cm^{-1}. Unknown 2 absorbed strongly at 1,770 cm^{-1} and moderately at 3,573 cm^{-1}. Similarly, unknown 4 absorbed strongly at 1,752 cm^{-1} and moderately at 3,583 cm^{-1}. Taken together, these values are characteristic of an acid in very dilute solution or in the vapor phase [17]. To corroborate this, the IR spectrum of *p*-menth-1-ene-8,9-dihydroxy-7-carboxylic acid was measured on the same GC/IR system and yielded a strong signal at 1,752 cm^{-1} and a moderate signal at 3,585 cm^{-1}.

Next, unknowns 1–4 were further characterized by GC using an on-line mass-selective detector. The mass spectra of unknowns 1–4 are given in Fig. 5. The mass spectrum of limonene contains a molecular ion peak of 136; a series of peaks of 121, 107, 93, and 79 (C_nH_{2n-1}), which is characteristic of terpenes; and a base peak of 68, which is formed from retro-Diels-Alder cleavage to yield two isoprene units of 68 [18]. The mass spectra of all four unknowns contained this series of terpene fragments as well. The mass spectrum of unknown 1 included a base peak of 122 and an apparent molecular ion peak of 182. The two most abundant peaks of unknown 2 occurred at an m/e of 79 and 122, respectively, and the apparent molecular ion peak occurred at 168. The spectrum of unknown 3 included a base peak of 68 and an apparent molecular ion peak of 180. The spectrum of unknown 4 also contained a base peak of 68, but the apparent molecular ion peak occurred at 166.

On the basis of the IR and MS data, we proposed the following structures. Unknown 1 was tentatively identified as dihydroperillic acid methyl ester. The IR spectrum indicated an ester, and a methyl ester was consistent with the apparent molecular ion peak in the mass spectrum. The 182 (M^+), 167 (M^+-CH_3), 151 (M^+-OCH_3), 123 (M^+-$COOCH_3$), and 59 ($COOCH_3$) peaks were suggestive of the methyl ester. Similarly, unknown 3 was tentatively identified as perillic acid methyl ester. The 180 (M^+), 165 (M^+-CH_3), 149 (M^+-OCH_3), 121 (M^+-$COOCH_3$), and 59 ($COOCH_3$) peaks again indicated a methyl ester. The small 68 peak of unknown 1 as compared with the base peak of 68 for limonene suggested modification of the cyclohexene ring. The carbonyl absorption of unknowns 1 and 3 differed by 20 cm^{-1}, suggesting a difference in saturation at the α-carbon. We therefore hypothesized that the methyl ester was located at carbon 7 and that the difference in saturation occurred at the 1,2 double bond.

Unknowns 2 and 4 were tentatively identified as dihydroperillic acid and perillic acid, respectively. The IR spectra were strongly suggestive of acids, and the apparent molecular ions from the mass spectra matched these formulas. Unknown 4 absorbed in the carbonyl region in a manner virtually identical to that shown by the *p*-menth-1-ene-8,9-diol-7-carboxylic acid standard, suggesting that it was perillic acid. The difference of 20 cm^{-1} in the carbonyl absorption in the IR spectra of unknowns 2 and 4 was again attributed to a difference in saturation at the 1,2 double bond, leading to the conclusion that unknown 2 was dihydroperillic acid. The 168 (M^+), 153 (M^+-CH_3), 151 (M^+-OH), 123 (M^+-COOH), and 45 (COOH) fragments of unknown 2 supported the conclusion that it was dihydroperillic acid. Similarly, the mass spectrum of unknown 4 contained fragments of 166 (M^+), 151 (M^+-CH_3), 149 (M^+-OH), 121 (M^+-COOH), and 45 (COOH).

Table 2. Concentrations of limonene and its metabolites in plasma[a]

Monoterpene	Concentration (mM)
Limonene	0.137 ± 0.012
Dihydroperillic acid	0.286 ± 0.040
Perillic acid	0.400 ± 0.053
Total	0.823 ± 0.105

[a] Rats were given 1 g/kg limonene p.o. and were killed 4 h later. Samples were extracted and analyzed by GC with flame-ionization detection as described in Materials and methods. Data represent mean values ± SEM ($n = 3$)

Dihydroperillic acid methyl ester, perillic acid methyl ester, dihydroperillic acid, and perillic acid were then synthesized. The identities of each synthetic compound were confirmed by NMR. Each compound was >98% pure as determined by capillary GC and NMR. In each case, the GC retention time (data not shown) and mass spectrum (Fig. 5) of the synthetic terpene matched that of the unknown, confirming the identities proposed above.

Using the synthetic compounds as standards, we determined the concentration of limonene and each of its plasma metabolites by capillary GC. Perillaldehyde was used as the internal standard since it was structurally related to limonene, and it migrated betwen limonene and dihydroperillic acid. The standard curves for limonene, perillic acid, and dihydroperillic acid were linear (data not shown). In rats given a single dose of 1 g/kg limonene, plasma monoterpene concentrations ranged from 0.137 mM for limonene to 0.4 mM for perillic acid (Table 2). The total concentration of limonene plus its metabolites, 0.82 ± 0.10 mM, was comparable with the 1.45 ± 0.10 mM value obtained in a separate experiment by calculations based on the specific radioactivity of the [^{14}C]-limonene given to the rats (Table 1).

It should be noted that a slight degree of methylation of perillic acid and dihydroperillic acid occurred in vitro following their addition to rat serum, extraction, and analysis by GC. The acids themselves were >98% pure and, specifically, free of methyl esters when analyzed in the absence of serum. Methylation did not occur when perillic acid was added to phosphate-buffered saline in place of serum. Inactivation of the serum by heating at 60° C for 30 min did not eliminate the methylation. Thus, the present methods did not allow us to determine whether the methyl esters of perillic acid and dihydroperillic acid, detected by GC in serum extracts of limonene-fed rats, were present in vivo or whether they were formed from the acids in vitro during the subsequent GC analysis.

We then measured the effects of limonene and perillic acid on NIH3T3 cell growth. Dihydroperillic acid was not tested since quantities of the synthetic compound sufficient for the calculation of growth curves were not available. NIH3T3 cells apparently do not metabolize limonene to perillic acid or dihydroperillic acid, since limonene was the only monoterpene detected by GC/MS analysis of conditioned medium (data not shown). At concentrations of

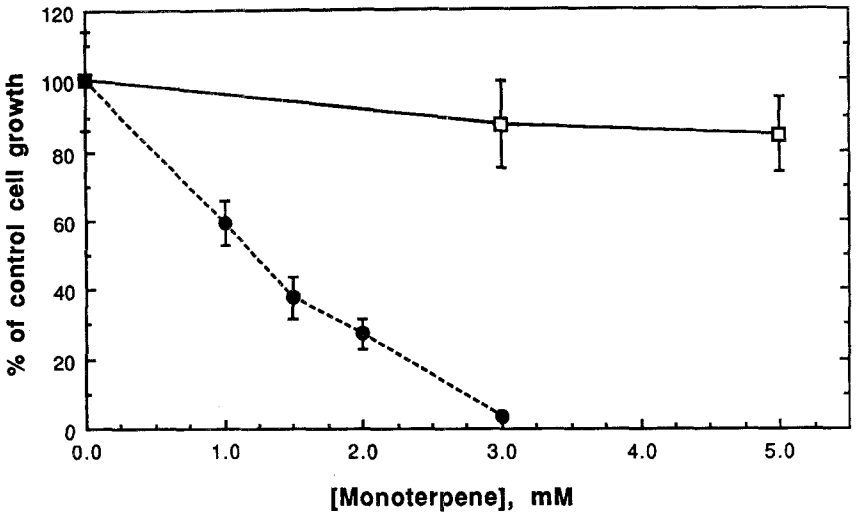

Fig. 6. Effects of limonene (□) and perillic acid (●) on NIH3T3 cell growth. Cells were seeded at a density of 1×10^5/plate and cultured in medium containing 0–5 mM limonene or 0–3 mM perillic acid for 4 days. Fresh medium was added each day. On day 4, cells were harvested, diluted, and counted on a hemocytometer

1–5 mM, limonene did not affect the rate of cell growth (Fig. 6). Perillic acid, however, caused a dose-dependent decrease in cell growth [50% inhibitory concentration (IC_{50}), 1.3 mM]; 15% inhibition was observed at a perillic acid concentration of 500 μM, and complete arrest of cell growth was attained at 3 mM.

Discussion

In the present study, we first investigated the absorption, distribution, and excretion of *d*-limonene in female rats. Limonene and/or its metabolites distributed in plasma and in every tissue tested. Our results were similar to those previously obtained in male rats [11]. One new finding of this study was the greater compartmentation of limonene and/or its metabolites in the intrascapular fat pad and mammary gland, most likely due to the lipophilic nature of the terpenes.

Circulating metabolites of limonene were characterized and identified. Limonene is rapidly and extensively metabolized by the rat. Within 20 min of ingestion, metabolism of limonene is evident in the serum. At 1 h after ingestion and thereafter, limonene itself represents more than 15% of the total limonene-derived material in the circulation. The major circulating species, perillic acid and dihydroperillic acid, are present in approximately equal proportions. In rats chronically fed limonene diets, the same metabolites are present, but limonene itself is not detectable (Crowell et al., unpublished data). Thus, in rats, the major circulating forms of limonene are perillic acid and dihydroperillic acid rather than limonene itself.

Perillic acid has previously been identified as a urinary metabolite of limonene [13, 16]. This is the first demonstration that dihydroperillic acid is a metabolite of limonene in a mammalian species. Although no mammalian species has previously been shown to metabolize limonene to dihydroperillic acid, some bacteria are known to possess this capability [5].

With this knowledge of the identity of both circulating and urinary metabolites of limonene, it will be possible to address the issue of whether limonene or one of its metabolites is the active antitumor agent. Two comparative studies of the chemopreventive or growth-inhibitory properties of limonene and its metabolites have been carried out to date. First, the chemopreventive activity of limonene was compared with that of carveol and uroterpenol, two hydroxylated urinary metabolites, and that of sobrerol, a related diol [4]. The monoterpenes were given in the diet during the initiation stage only of DMBA-induced rat mammary carcinogenesis. In all cases, rats treated with the hydroxylated monoterpenes exhibited longer tumor latency and fewer tumors than did either controls or rats treated with an equal dose of limonene. Thus, two hydroxylated urinary metabolites as well as a related nonmetabolite were more potent than limonene itself in the chemoprevention of mammary cancer.

Second, limonene has been shown to inhibit the post-translational isoprenylation of $p21^{ras}$ and other 21- to 26-kDa proteins, most if not all of which are likely to be members of the *ras* superfamily [3]. In the same assay, perillic acid and dihydroperillic acid at concentrations of 1 mM were as effective as 5 mM limonene in the inhibition of 21- to 26-kDa protein isoprenylation. Concentrations approaching these values, namely 0.4 mM for perillic acid and 0.29 mM for dihydroperillic acid, were attained in the present study in rats given 1 g/kg limonene. Thus, the two most abundant plasma metabolites of limonene are more potent than limonene in inhibiting protein isoprenylation. In the present study, perillic acid was also more potent than limonene in inhibiting cell growth at concentrations that effectively inhibit 21- to 26-kDa protein isoprenylation [3]. Taken together, these data suggest that the antiproliferative activity of limonene may actually reside within its more abundant and more potent metabolites, perillic acid and dihydroperillic acid.

The inhibition of 21- to 26-kDa protein isoprenylation by limonene and perillic acid is one possible mechanism of action of limonene, and, although highly suggestive, requires further investigation before a direct cause-and-effect relationship can be established. The antitumor activity of limonene may be due to other mechanisms as well, possibly involving remodeling or redifferentiation of mam-

mary tissue [9]. Future experiments will assess the antitumor activity of perillic acid and other limonene metabolites in mammary and, possibly, other tumor model systems. These monoterpenes may in themselves be effective chemopreventive and/or chemotherapeutic agents.

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