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STRUCTURE–ACTIVITY RELATIONSHIPS AMONG MONOTERPENE INHIBITORS OF PROTEIN ISOPRENYLATION AND CELL PROLIFERATION

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Abstract—The monoterpene *d*-limonene inhibits the post-translational isoprenylation of p21ras and other small G proteins, a mechanism that may contribute to its efficacy in the chemoprevention and therapy of chemically induced rodent cancers. In the present study, the relative abilities of 26 limonene-like monoterpenes to inhibit protein isoprenylation and cell proliferation were determined. Many monoterpenes were found to be more potent than limonene as inhibitors of small G protein isoprenylation and cell proliferation. The relative potency of limonene-derived monoterpenes was found to be: monohydroxyl = ester = aldehyde> thiol > acid = diol = epoxide > triol = unsubstituted. All monoterpenes that inhibited protein isoprenylation did so in a selective manner, such that 21–26 kDa proteins were preferentially affected. Perillyl alcohol, one of the most potent terpenes, reduced 21–26 kDa protein isoprenylation to 50% of the control level at a concentration of 1 mM, but had no effect on the isoprenylation of 67, 47 or 17 kDa proteins. In particular, p21ras farnesylation was inhibited 40% by 1 mM perillyl alcohol. At the same concentration, perillyl alcohol completely inhibited the proliferation of human HT-29 colon carcinoma cells. The structure–activity relationships observed among the monoterpene isoprenylation inhibitors support a role for small G proteins in cell proliferation, and suggest that many limonene-derived monoterpenes warrant further investigation as antitumor agents.

Key words: terpene; small G protein; limonene; perillyl alcohol; perilla alcohol

Many cellular proteins, including Ras-like small G proteins, heterotrimeric G proteins, cyclic GMP phosphodiesterase, and the nuclear lamins undergo post-translational isoprenylation via the covalent addition of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl moiety to a cysteine residue at or near the carboxy terminus [1, 2]. Subsequent modifications may include proteolysis of three C-terminal amino acids (leaving isoprenylated Cys at the C terminus), carboxylmethylation of the C terminal Cys, and palmitoylation of upstream Cys residues. These lipophilic modifications facilitate the association of isoprenylated proteins with an intracellular membrane or protein, which is a functional requirement. Farnesylation of Ki-Ras, for example, enhances its association with its GTP/GDP exchange factor [3]. Farnesylation of Ras also greatly enhances its ability to stimulate downstream signal-transducing enzymes, including mitogen-activated protein kinase in mammalian cells [4], and adenylyl cyclase in yeast [5]. Unfarnesylated Ras proteins do not associate with the plasma membrane and, unlike their farnesylated counterparts, are incapable of cellular transformation [6, 7]. Thus, there is great interest currently in pharmacological agents that inhibit protein isoprenylation, since they may have usefulness as cancer chemotherapeutic drugs.

d-Limonene (*p*-mentha-1,8-diene), a geranyl pyrophosphate-derived monoterpene found in orange peel and other plant essential oils [8], is one of the few compounds known to both inhibit protein isoprenylation [9] and have *in vivo* antitumor activity [10]. Limonene has chemopreventive activity against chemically induced rodent mammary [11–13], lung, forestomach [14, 15], and liver [16] tumors, and against v-Ha-*ras*-induced rat mammary tumors [17] at doses that cause no toxicity to the host. In addition, limonene is efficacious as a chemotherapeutic agent, causing >80% of chemically induced rat mammary carcinomas to regress completely [18]. Limonene-treated, regressing mammary carcinomas acquire a redifferentiated state resembling that of a benign fibroadenoma [18]. The antitumor activity of limonene may be due, in part, to its ability to inhibit the isoprenylation of cell growth-associated small G proteins such as Ras [9]. Interestingly, limonene selectively inhibits the isoprenylation of 21–26 kDa small G proteins without affecting the isoprenylation of other cellular proteins. This selectivity may be responsible, in part, for the high therapeutic index of limonene *in vivo*.

Limonene is metabolized extensively by rats and by humans [10]. The rat circulating metabolites are

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perillic acid, dihydroperillic acid, and their methyl esters [19]. Urinary metabolites include uroterpenol, perillic acid, and their glycine and glucuronyl conjugates [20]. Perillic acid, a circulating limonene metabolite is a more potent inhibitor of protein isoprenylation [9] and cell proliferation [19] than is limonene. Similarly, two urinary metabolites of limonene, carveol and uroterpenol, are more potent chemopreventive agents than limonene itself [21]. These structure–activity relationships suggest that metabolites of limonene may be the most active agents *in vivo*. In an effort to better understand the relative effects of limonene and its metabolites on protein isoprenylation and cell proliferation, and to further explore the biochemical basis for the inhibition of 21–26 kDa protein isoprenylation by limonene, the studies described below address the chemical requirements for the inhibition of protein isoprenylation by monoterpenes through an examination of structure–activity relationships. In addition, the effects of some of the active isoprenylation inhibitors on the proliferation of nontransformed and transformed cells are described.

MATERIALS AND METHODS

Monoterpene synthesis. The synthesis of perillic acid (compound M17, Fig. 1), dihydroperillic acid (M22), and their respective methyl esters (M1 and M12), as well as uroterpenol (M23), has been described elsewhere [19, 21]. Analytical thin-layer chromatography was performed on precoated silica gel plates purchased from Merck (Rahway, NJ). High resolution mass spectra were recorded on a Kratos MS-80RFA mass spectrometer.

7-Methyl perillyl alcohol (M2) was prepared as a mixture of two diastereomers from (*S*)-(-)-perillaldehyde and methylmagnesium bromide in >90% yield according to a standard procedure [22], b.p. 93° (0.7 mm Hg); $R_f = 0.58$ on silica gel, hexane; ethyl acetate, 1:1. 200 MHz NMR ($CDCl_3$, ppm) δ 4.65–4.80 (m, 1H), 4.72 (br s, 2H), 4.1–4.3 (m, 1H), 1.3–2.3 (m, 11H), 1.27 (d, $J = 8$ Hz, 3H); *m/e* calculated for $C_{10}H_{18}O$ (M^+) 166.1358, found 166.1358. 7-Ethyl perillyl alcohol (M3) was prepared in a similar way from (*S*)-(−)-perillaldehyde and ethylmagnesium chloride as a mixture of diastereomers, b.p. 102° (0.7 mm Hg); $R_f = 0.63$ on silica gel, hexane: ethyl acetate, 1:1. 200 MHz NMR ($CDCl_3$, ppm) δ 5.62–5.75 (m, 1H), 4.70 (br s, 2H), 3.92 (br s, 1H), 1.2–2.3 (m, 13H), 0.88–0.89 (two t, $J = 9.8$ Hz, 3H); *m/e* calculated for $C_{12}H_{20}O$ (M^+) 180.1514, found 180.1514.

Perillyl thiol (M14) was synthesized from (*S*)-(−)-perillyl alcohol according to the general method of Urquhart *et al.* [23]. Thus, perillyl alcohol (15.2 g), triethylamine (17 mL), and thionyl chloride (8 mL) were reacted at −10° in methylene chloride (150 mL) for 30 min. After aqueous workup and solvent removal (by aspirator), the crude chloride was distilled (b.p. 39–44°, 0.2 mm Hg) to give a mixture of chloride regioisomers. The chloride (5 g) was reacted with thiourea (2.2 g) in 95% ethanol (150 mL) as described [23]. Removal of most of the ethanol allowed crystallization of the thiouronium salt (2.5 g), which was cleaved by alkaline hydrolysis with NaOH (1 g) in water (10 mL). After acidification with dilute sulfuric acid, the product was extracted with ether, dried ($MgSO_4$), and concentrated (by aspirator). Vacuum distillation provided the product thiol, b.p. 69.5° (0.35 mm Hg), which was kept under N_2 to avoid oxidation; 200 MHz NMR ($CDCl_3$, ppm) δ 5.55–5.70 (m, 1H), 4.65–4.78 (m, 2H), 3.12 (br d, $J = 8$ Hz, 2H), 1.1–2.8 (m, 11H); *m/e* calculated for $C_{10}H_{16}S$ (M^+) 168.0973, found 168.1010; for $C_{10}H_{15}$ ($M^+ - 33$) 135.1174, found 135.1182.

p-Menth-8-ene-1,2-diol (limonene-1,2-diol, M19) is a known compound [24, 25]. It was obtained by the hydrolysis of (+)-limonene oxide [24, 25] by reaction in 0.15 M aqueous NaOH: *tert*-BuOH (7:1, v/v) at 70° for 56 hr. After aqueous workup followed by flash chromatography over silica gel, the diol was obtained in 72% yield as an oil that solidified over time; $R_f = 0.40$, hexane: ethyl acetate, 1:3. 200 MHz NMR ($CDCl_3$, ppm) δ 4.67 (br s, 2H), 3.6–3.7 (m, 1H), 1.4–2.4 (m, 12H), 1.25 (s, 3H).

p-Menth-8-ene-1,2,7-triol (M24) was obtained from (*S*)-(−)-perillyl alcohol by catalytic osmylation [26] followed by flash chromatography purification. $R_f = 0.16$ on silica gel, ethyl acetate. 200 MHz NMR ($CDCl_3$, ppm) δ 4.65–4.70 (m, 2H), 3.5–4.0 (m, 3H), 1.1–2.9 (m, 13H).

Perillyl acetate (M6) was prepared from (*S*-(−)-perillyl alcohol using the standard pyridine/acetic anhydride method [27] as a yellow oil, b.p. 125° (4.5 mm Hg); published b.p. 72–78° (0.05 mm Hg) [27]. $R_f = 0.77$ on silica gel, hexane: ethyl acetate, 1:1. 200 MHz NMR ($CDCl_3$, ppm) δ 5.65–5.75 (m, 1H), 4.65–4.80 (m, 2H), 4.46 (br s, 2H), 1.2–2.4 (m, 13H). The above compound was treated with Pb_3O_4 in acetic acid as described [28]. Flash chromatography purification afforded *p*-menth-1-ene-7,8,9-triol-7,9-diacetate (M26) as an oil; $R_f = 0.37$ on silica gel, hexane: ethyl acetate, 1:1. 200 MHz NMR ($CDCl_3$, ppm) δ 5.65–5.85 (m, 1H), 4.46 (br s, 2H), 3.9–4.2 (m, 2H), 1.1–2.3 (m, 17H). Saponification of the diacetate (0.82 g) in methanolic K_2CO_3 (2.3 g in 100 mL) for 1 hr at room temperature gave an oily-solid residue after solvent removal. The mixture was extracted with ethyl acetate (100 mL) and then filtered, and the solvent was removed to give *p*-menth-1-ene-7,8,9-triol (M25) as a thick oil; $R_f = 0.61$ on silica gel, ethyl acetate: ethanol, 2:1 (v/v). 200 MHz NMR (DMSO, ppm) δ 5.45–5.60 (m, 1H), 3.76 (br s, 2H), 3.15–3.25 (m, 2H), 0.9–2.1 (m, 13H); *m/e* calculated for $C_{10}H_{16}O_2$ ($M^+ - 18$) 168.1150, found 168.1127; for $C_{10}H_{14}O$ ($M^+ - 36$) 150.1045, found 150.1040.

All other monoterpenes were purchased from the Aldrich Chemical Co. (Milwaukee, WI) at the highest chemical purity available. The purity of perillyl alcohol (perilla alcohol), Aldrich Flavor and Fragrance Catalog No. W26641-8, was >99% by capillary gas chromatography.

Cell culture. NIH3T3 and HT-29 cells were obtained from ATCC, and M600B cells were obtained from Dr. Martha Stamfer. NIH3T3 and M600B cells were cultured as described [9]. HT-29 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. MCF-10A cells were obtained from the Michigan Cancer Foundation and were cultured as described by Soule *et al.* [29]. All media

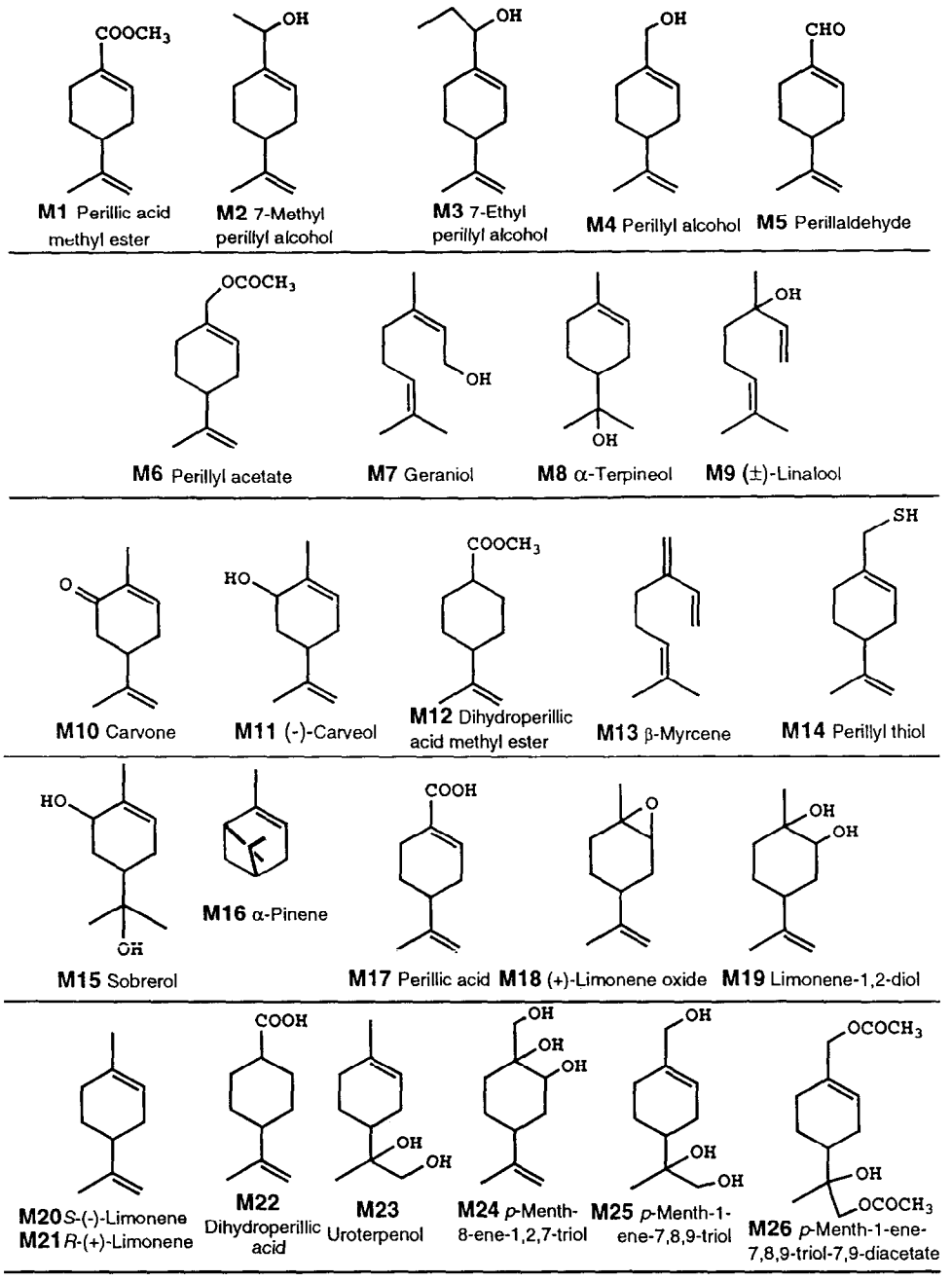

Fig. 1. Monoterpene structures. The monoterpene numbers (M1, M2, etc.) correspond to the numbers in Table 1 and in the text.

and supplements were obtained from Gibco (Grand Island, NY). Cell proliferation assays were carried out as described [9]. During proliferation assays, the cell culture medium was changed daily to counteract potential losses of the volatile monoterpenes by evaporation.

Protein isoprenylation. The monoterpenes used in these studies are somewhat difficult to solubilize in aqueous medium. To aid in solubilization, monoterpenes were mixed with prewarmed (37°) cell culture medium (containing 10% serum) immediately before addition to cells. Protein isoprenylation assays were carried out as described previously [9]. Briefly, cells were pretreated for 18 hr with 20 μM lovastatin to reduce endogenous mevalonate synthesis. Cells were then labeled with [2-^{14}C]mevalonate (15 μCi/mL final concentration), which is metabolized to the isoprenoid groups that are, in turn, attached to proteins, in the absence and presence of 1–3 mM monoterpenes. Protein

isoprenylation was assessed by measuring protein-associated radioactivity by SDS–PAGE and fluorography. Detection and quantitation of protein isoprenylation were performed on a PhosphorImager from Molecular Dynamics. A dried gel containing ^{14}C-labeled proteins was wrapped in 8 μm Mylar and exposed to a phosphor screen in an exposing cassette for 18–24 hr. The screen was then scanned by the PhosphorImager, and the data were quantitated using the ImageQuant program. Ras farnesylation was measured as described previously [9] by labeling cells with [2-^{14}C]mevalonate in the absence and presence of 1 mM perillyl alcohol, immunoprecipitating Ras with the monoclonal antibody Y13-259, and measuring isoprenylation by SDS–PAGE and fluorography.

RESULTS

Inhibition of protein isoprenylation by monoterpenes. Previous studies by our laboratory have demonstrated that the monoterpene *d*-limonene [*p*-mentha-1,8-diene, structure (M20), Fig. 1] inhibits protein isoprenylation in NIH3T3 and human mammary epithelial cells [9]. Furthermore, perillic acid [*p*-mentha-1,8-diene-7-carboxylic acid, compound (M17), Fig. 1], a circulating metabolite of limonene, was found to be a more potent inhibitor than limonene of isoprenylation [9] and cell proliferation [19]. Based on this observation, we hypothesized that other monoterpenes might also be potent inhibitors of protein isoprenylation. We therefore carried out an extensive structure–activity study among limonene-derived monoterpenes with emphasis on substitutions at carbon 7. Many of the known circulating and urinary metabolites of limonene, such as perillic acid, dihydroperillic acid, uroterpenol and carveol, were included in the study [19, 20]. Limonene-derived plant monoterpenes, such as carveol, carvone, α-pinene, α-terpineol, and perillyl alcohol [8], were included in the screen as well.

The relative inhibition of 21–26 kDa protein isoprenylation by limonene-like monoterpenes is listed in Table 1, and the monoterpene structures are presented in Fig. 1. A representative protein isoprenylation experiment is depicted in Fig. 2. In comparison to control NIH3T3 cells (lane A), cells treated for 3 hr with 1 mM perillyl alcohol (lane B) incorporated less [2-^{14}C]mevalonate into 21–26 kDa proteins than did controls. Similar effects were observed in HT-29 colon carcinoma cells treated with 0, 1, or 2 mM perillyl alcohol (lanes C, D, and E, respectively). However, the isoprenylation of 72, 70, 67, and 14 kDa proteins was equal in perillyl alcohol-treated cells and controls. This inhibition occurred progressively over the first 3 hr, and then remained at 50% over the next 10 hr (data not shown). Ras farnesylation was inhibited 40% by 1 mM perillyl alcohol in NIH3T3 cells (data not shown). Thus, 1 mM perillyl alcohol, like 5 mM limonene [9], selectively inhibits the isoprenylation of 21–26 kDa proteins, including Ras, in intact mammalian cells. All other monoterpenes that inhibited isoprenylation also did so selectively such that 21–26 kDa protein isoprenylation was decreased, whereas the isoprenylation of higher molecular mass proteins was affected to a much less extent or not at all (data not shown).

Table 1. Relative inhibition of 21–26 kDa protein isoprenylation by monoterpenes*

	Monoterpene	% Inhibition of 21–26 kDa protein isoprenylation 1 mN	3 mM	5 mM
M1.	Perillic acid methyl ester	56.5		
M2.	7-Methyl perillyl alcohol	58		
M3.	7-Ethyl perillyl alcohol	55.5		
M4.	Perillyl alcohol	50		
M5.	Perillaldehyde	50.5		
M6.	Perillyl acetate	48		
M7.	Geraniol	48		
M8.	α-Terpincol	43.5	58	
M9.	(±)-Linalool	42.5		
M10.	Carvone	38.5		
M11.	(−)-Carveol	33	58	
M12.	Dihydroperillic acid methyl ester	29.5		
M13.	β-Myrcene	20	18	
M14.	Perillyl thiol	11		
M15.	Sobrerol	0	50	
M16.	α-Pinene	0	24	
M17.	Perillic acid	0	22	
M18.	(+)-Limonene oxide	2	21	
M19.	Limonene-1,2-diol	0	18	
M20.	*S*-(−)-Limonene	0	0	50
M21.	*R*-(+)-Limonene	0	0	50
M22.	Dihydroperillic acid	0	0	40
M23.	Uroterpenol†	+/−		
M24.	*p*-Menth-8-ene-1,2,7-triol	0	0	
M25.	*p*-Menth-1-ene-7,8,9-triol	0	0	
M26.	*p*-Menth-1-ene-7,8,9-triol-7,9-diacetate	0	0	

* Protein isoprenylation was measured as described in Materials and Methods. Monoterpenes were included at 1, 3 or 5 mM. The degree of 21–26 kDa protein isoprenylation inhibition (the group of proteins most affected by the drug treatment) was calculated based on the intensity of the signal relative to that of a no monoterpene control. The data are representative of 3 independent experiments. The monoterpene numbers (M1, M2, etc.) correspond to the numbers in Fig. 1 and in the text.

† A 20% decrease in protein isoprenylation by 1 mM uroterpenol was observed in only 2 of 4 experiments (data not shown).

Several structure–activity relationships are apparent from the data in Table 1. The most striking observation is that many monohydroxylated monoterpenes are effective inhibitors of 21–26 kDa protein isoprenylation in intact cells. Every monohydroxylated monoterpene tested inhibited 21–26 kDa protein isoprenylation more effectively than did limonene. The position of the hydroxyl group on the limonene framework affected the inhibition of protein isoprenylation to some extent. For example, perillyl alcohol (M4), 7-methyl perillyl alcohol (M2), and 7-ethyl perillyl alcohol (M3) were among the most active isoprenylation inhibitors.

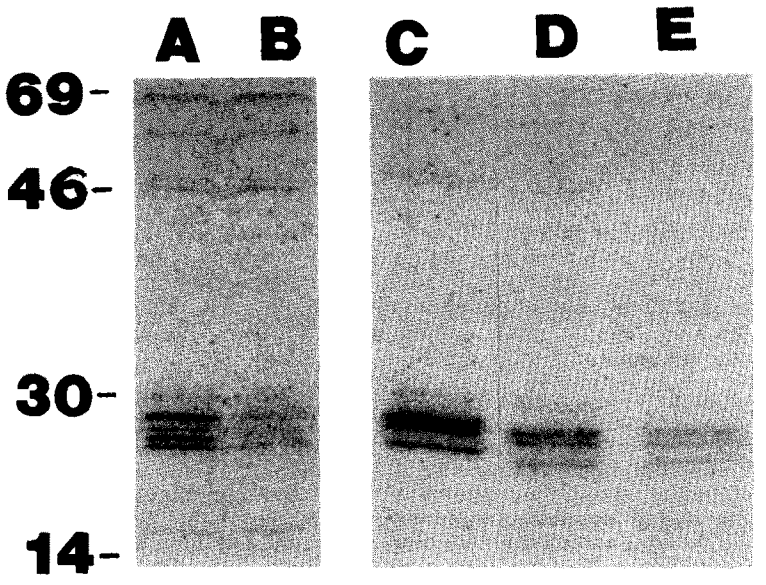

Fig. 2. Effects of perillyl alcohol on protein isoprenylation in NIH3T3 and HT-29 cells. Protein isoprenylation was carried out as described in Materials and Methods. NIH3T3 cells were treated with no monoterpene (lane A) or 1 mM perillyl alcohol (lane B). HT-29 cells were similarly analyzed in the presence of no monoterpene (lane C) or 1 (lane D) or 2 (lane E) mM perillyl alcohol. The migration of molecular weight markers (in kDa) is indicated on the left.

Perillyl alcohol was slightly more active than α-terpineol (M8), with an 8-hydroxy group, which in turn was more effective than (-)-carveol (M11), with a 6-hydroxy group. The isomeric pair of acyclic hydroxylated monoterpenes, geraniol (M7) and (±)-linalool (M9), were equally effective with α-terpineol (M8). Thus, the addition of a single hydroxyl group greatly increased the ability of a monoterpene to inhibit protein isoprenylation, with slight variation with the position of the hydroxyl group.

Monoterpenes with two or three hydroxyl groups were less effective than those with a single hydroxyl group. For example, the diol sobrerol (M15) was less active than (−)-carveol (M11) or α-terpineol (M8), both of which contain one hydroxyl group in the same position as one of those of sobrerol. Limonene 1,2-diol (M19) had slightly less inhibitory activity than sobrerol (M15). Increasing the number of hydroxyl groups to three rendered the monoterpene even less effective. The triols (M24) and (M25) were less effective than the diols sobrerol (M15) and limonene-1,2-diol (M19). Uroterpenol (M23), another diol, was slightly more active than the triols (data not shown). Thus, the number of hydroxyl groups on the monoterpene greatly affects its inhibition of protein isoprenylation. One hydroxyl group was found to be superior to two, which in turn was more effective than zero or three.

A comparison of different chemical substitutions at carbon 7 indicated that hydroxyl, aldehyde, and methyl ester substitutions yield the most active inhibitors of 21–26 kDa protein isoprenylation. Perillyl alcohol (M4), perillic acid methyl ester (M1), and perillaldehyde (M5) were among the most potent of all of the monoterpenes tested. The relative activities of these three substituted monoterpenes were not statistically different. A thiol substitution (M14) for the hydroxyl group of perillyl alcohol (M4) decreased the isoprenylation inhibition activity somewhat, although the thiol was still moderately effective. Perillic acid (M17) was less effective than the thiol (M14) or the alcohol (M4), but more effective than limonene (M20), the unsubstituted monoterpene. Similarly, the alcohol geraniol (M7) was more potent than β-myrcene (M13), its corresponding unsubstituted monoterpene. Nearly equal inhibition of isoprenylation occurred in the presence of the alcohol carveol (M11) and its ketone analog carvone (M10). Thus, the most active isoprenylation inhibitors in this series (alcohol, aldehyde, methyl ester) had intermediate polarity which differed from that of the acid or the unsubstituted compound.

Saturation at the 1,2 bond was found to affect the isoprenylation inhibitory activity of a monoterpene. Perillic acid methyl ester (M1) was more potent than the saturated analog dihydroperillic acid methyl ester (M12). Similarly, perillic acid (M17) was somewhat more potent than dihydroperillic acid (M22). Thus, the 1,2-unsaturated compounds were more potent than the corresponding saturated compounds. Carbon extensions at carbon 7 of perillyl alcohol increased the potency somewhat. The methyl (M2) and ethyl (M3) extensions were equally effective. These compounds, together with perillic acid methyl ester, were found to be the most potent inhibitors of 21–26 kDa protein isoprenylation among the compounds tested in the present study.

A comparison of acyclic, bicyclic, and monocyclic monoterpenes indicated some differences based on

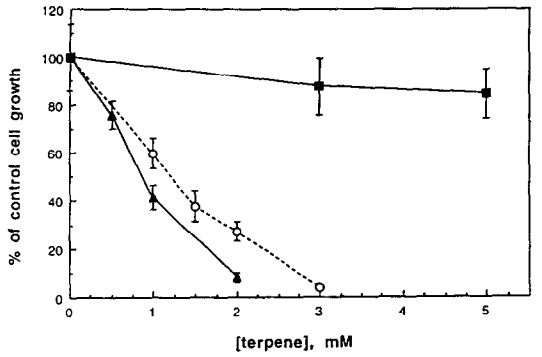

Fig. 3. Effects of limonene, perillic acid, and perillyl alcohol on the proliferation of NIH3T3 cells. NIH3T3 cells were grown for 4 days in the absence or presence of *d*-limonene (■), perillic acid (○), or perillyl alcohol (▲). The rate of cell proliferation, determined by counting cells on a hemocytometer, is expressed as a percentage of the no monoterpene control (4.5×10^5 cells/well/day). The data points represent means ± SEM, N = 4.

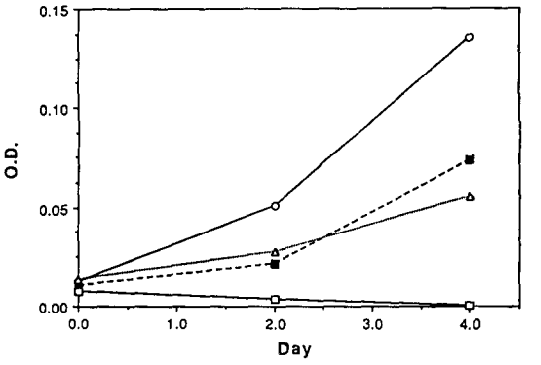

Fig. 4. Effects of dihydroperillic acid methyl ester, carvone, and α-pinene on the proliferation of NIH3T3 cells. Cell proliferation was determined by the MTT assay (Cell Titer, Promega) over 4 days in the absence (○), or presence of 1 mM dihydroperillic acid methyl ester (■), carvone (△), or α-pinene (□). The O.D. per well is plotted versus the day. The data points represent means ± SEM, N = 3.

ring structure for unsubstituted monoterpenes, but little among the hydroxylated monoterpenes. β-Myrcene (M13), an acyclic unsubstituted monoterpene, was more potent at 1 mM than either α-pinene (M16), a bicyclic monoterpene, or limonene (M20), a monocyclic monoterpene. However, among the acyclic and monocyclic hydroxylated monoterpenes, perillyl alcohol (M4), α-terpineol (M8), geraniol (M7), and linalool (M9) were similarly active, with perillyl alcohol slightly superior to the others. The alcohol substitution on perillyl alcohol would appear to outweigh any advantage of the unsubstituted acyclic over the monocyclic monoterpenes.

Inhibition of cell proliferation by monoterpenes. Monoterpenes such as perillyl alcohol and the others described above inhibit the isoprenylation of 21–26 kDa proteins, a family of isoprenylated proteins consisting of the Ras superfamily of small G proteins [30, 31]. The cell growth-signalling roles of wild-type Ras proteins in normal cells [32, 33] and that of oncogenic Ras in neoplastic cells [34] are well established. Recent evidence indicates that other isoprenylated small G proteins may be involved in cell growth regulation as well [35–37]. We therefore tested the hypothesis that monoterpene inhibitors of small G protein isoprenylation would inhibit cell proliferation and would follow the same structure–activity relationships.

The relative effects of limonene (M20), perillic acid (M17), and perillyl alcohol (M4) on NIH3T3 cell proliferation were compared. Of the three monoterpenes, perillyl alcohol was the most potent inhibitor of cell proliferation. In NIH3T3 cells, 1 mM perillyl alcohol, which reduced 21–26 kDa protein isoprenylation by 50% (Table 1), reduced cell proliferation to 42% of the control (Fig. 3). Perillic acid was a less potent inhibitor of cell proliferation than perillyl alcohol, but it was more potent than limonene. Thus, the same structure–activity relationship was observed among perillyl alcohol, perillic acid, and limonene for inhibition of 21–26 kDa protein isoprenylation and inhibition of cell proliferation. Similar antiproliferative effects of 1 mM perillyl alcohol on M600B (95% inhibition) and MCF10A (82% inhibition) human mammary epithelial cells were observed. The relative effects of three additional monoterpene isoprenylation inhibitors on NIH3T3 cell proliferation were then determined. As shown in Fig. 4 dihydroperillic acid methyl ester (M12), carvone (M10), and α-pinene (M16) significantly inhibited the proliferation of NIH3T3 cells. Thus, five of five monoterpenes that inhibited 21–26 kDa protein isoprenylation also inhibited cell proliferation at the same doses.

The cell proliferation studies were then extended to a human carcinoma cell line to determine whether there was an association of decreased tumor cell proliferation with decreased small G protein isoprenylation. Perillyl alcohol (Fig. 2) and limonene (data not shown) inhibited 21–26 kDa protein isoprenylation in HT-29 cells in a manner similar to that observed in NIH3T3 cells. We determined the anti-proliferative effects of perillyl alcohol and other monoterpenes on HT-29 human colon carcinoma cells (Fig. 5). Perillic acid methyl ester (M1) and perillyl alcohol (M4) were the most potent inhibitors of HT-29 tumor cell proliferation, each with an IC_{50} value of 50 μM (Fig. 5). Perillaldehyde (M5), perillic acid (M17), and limonene (M20) also significantly inhibited HT-29 cell proliferation (Fig. 5) at concentrations that have an effect of a similar magnitude on protein isoprenylation (Fig. 2; Tables 1 and 2). A significant rank order correlation coefficient was obtained from a comparison of inhibition of small G protein isoprenylation and inhibition of HT-29 cell proliferation. Thus, among the limonene-derived monoterpenes tested, the same structure–activity relationship was found for inhibition of protein isoprenylation and inhibition of tumor cell proliferation. Interestingly, inhibition of

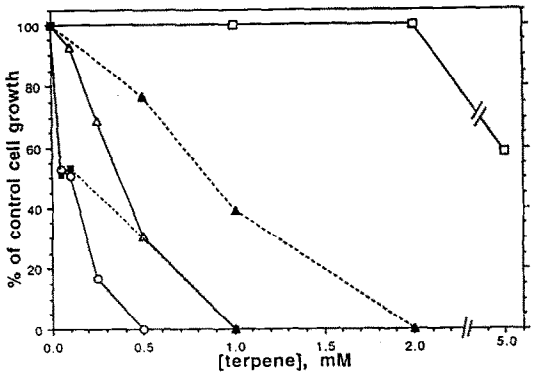

Fig. 5. Effects of monoterpenes on the proliferation of HT-29 human colon carcinoma cells. Cell proliferation was determined as described in the legend to Fig. 3. HT-29 cells were treated for 4 days with no monoterpene or with perillic acid methyl ester (■), perillyl alcohol (○), perillaldehyde (△), perillic acid (▲), or *d*-limonene (□). The control rate of cell proliferation was 1.2×10^5 cells/well/day. The data points represent means ± SEM, N = 4.

HT-29 colon carcinoma cell proliferation (Fig. 5) was attained at lower monoterpene concentrations than those required to inhibit the proliferation of nontumorigenic lines such as NIH3T3 (Figs. 3 and 4).

DISCUSSION

We have presented evidence that many limonene-like monoterpenes inhibit the post-translational isoprenylation of proteins. Many monoterpenes differing in ring structure or degree of oxidation had a similar effect on protein isoprenylation in cultured cells. In all cases in which a monoterpene inhibited protein isoprenylation, there was preferential inhibition of 21–26 kDa protein isoprenylation over the isoprenylation of other cellular proteins such as the 67–72 kDa nuclear lamins. These data suggest that common mechanisms are involved in the inhibition of protein isoprenylation by a large number of monoterpenes. One likely mechanism by which monoterpenes inhibit protein isoprenylation is through inhibition of one or more prenyltransferases [1, 2]. Both 5 mM limonene [9] and 1 mM perillyl alcohol inhibit Ras farnesylation. Farnesyl-protein transferase is therefore one prenyltransferase which is likely to be inhibited by monoterpenes.

The basis for the selective inhibition of some prenylated proteins over others by monoterpenes could be due to several factors. First, since at least three prenyl-protein transferases exist, the selective inhibition of 21–26 kDa proteins by monoterpenes could be a result of preferential inhibition of one prenyl-protein transferase(s) over the others. Second, it is possible that different prenyl-protein transferases, or isozymes thereof, reside in different compartments of the cell, and that the monoterpenes partition differentially among the compartments. The nuclear lamins are farnesylated and further processed within the nucleus [39], whereas farnesyl-protein transferase activity has thus far been detected only in cytosol [40]. It is not yet clear whether lamin farnesylation occurs via a novel nuclear farnesyl-protein transferase or whether it is accomplished by a nuclear isozyme of the known farnesyl-protein transferase. Monoterpenes such as limonene [9] or perillyl alcohol (Fig. 2) have little or no effect on the prenylation of nuclear lamins in NIH3T3 cells, raising the possibility that these compounds do not distribute in the nucleus, where the prenylation of lamins takes place.

Within the series of monoterpenes tested in these experiments, from the hydrophobic limonene to the more polar acids or triols, the most potent inhibitors of protein isoprenylation and cell proliferation had intermediate polarity. Perillyl alcohol, perillic acid methyl ester, and perillaldehyde were found to be three of the most potent inhibitors. Monoterpenes that are either less polar, such as limonene and myrcene, or more polar, such as perillic acid or diols or triols, were less effective inhibitors of protein isoprenylation and cell proliferation. Altering the structure of the monoterpene through carbon extension did not decrease its ability to inhibit protein isoprenylation. Thus, within the series of

Table 2. Rank order comparison of protein isoprenylation inhibition and cell proliferation inhibition by monoterpenes

Monoterpene	Rank order*	
	Inhibition of small G protein isoprenylation	Inhibition of HT-29 tumor cell proliferation
Perillic acid methyl ester	1	1
Perillyl alcohol	2	1
Perillaldehyde	2	3
Perillic acid	4	4
d-Limonene	5	5

* The data from Table 1 and Fig. 5 are ranked from 1 (greatest inhibition) to 5 (least inhibition). The rank correlation coefficient is calculated to be 0.900, which is significant at the 5% level [38].

limonene-derived monoterpenes tested, the relative polarity of the drug, rather than ring or other structure, would seem to be the most important factor in determining its relative ability to inhibit protein isoprenylation in cells. The correlation between polarity and inhibition of protein isoprenylation could be due in part to the relative ability of these compounds to traverse cellular membranes.

Among the monoterpene structure–activity comparisons made were those of limonene and its more polar plasma (compounds M1, 12, 17, 19, and 22) and urinary (compounds M4, 11, 17, 19, 22, and 23) metabolites [10, 19, 20]. Every limonene metabolite tested was more potent than limonene in inhibiting protein isoprenylation and cell proliferation. Such was the case for all of the known plasma metabolites of limonene, including perillic acid, dihydroperillic acid, the respective methyl esters of these acids, and limonene-1,2-diol [10, 19, 41]. Thus, the metabolites of limonene may be the most active antitumor agents *in vivo*. The concentrations at which these effects occur (1 mM or less) may be attainable *in vivo*. Rats given 1 g/kg body wt, limonene p.o. suffer no adverse side-effects and have a circulating perillic acid concentration of 0.4 mM [19]. We previously reported that, at 1 mM, perillic acid and dihydroperillic acid are more potent inhibitors of protein isoprenylation than limonene [9], and those results were confirmed in the present series of experiments. However, our initial data (from the same experiment) indicated that neither 1 mM perillic acid methyl ester nor 1 mM dihydroperillic acid methyl ester affected protein isoprenylation [9]. In the present paper, we report that these methyl esters significantly inhibited protein isoprenylation at a concentration of 1 mM (Table 1). We attribute this difference to the low solubility of the methyl esters in aqueous solution. In the previous experiments, the monoterpenes were mixed with cell culture medium at room temperature, whereas in the present experiments, monoterpenes were thoroughly mixed with prewarmed (37°) cell culture medium. Furthermore, perillic acid methyl ester concentrations in the cell culture medium were confirmed by gas chromatography in the present series of experiments (data not shown). The degree of partitioning of monoterpenes between cells and medium is not yet known, nor has subcellular distribution of monoterpenes been addressed. Thus, since the true cytosolic monoterpene concentrations are not known, the data in Table 1 can be interpreted as the degree of prenylation inhibition in cultured cells, but not as true inhibitory concentrations for target enzymes.

Our data demonstrate an association of decreased cell proliferation with decreased 21–26 kDa protein isoprenylation. For example, concentrations of perillyl alcohol, perillic acid, carvone, dihydroperillic acid methyl ester, and geraniol which result in inhibition of 21–26 kDa small G protein isoprenylation (Table 1) also result in a similar degree of inhibition of cell proliferation (Figs. 3 and 4; Ref. 42). Many isoprenylated small G proteins are involved in cell growth-associated processes. Which, if any, of these isoprenylated small G proteins are involved in the inhibition of cell proliferation by monoterpenes is not yet known. Ras and heterotrimeric G proteins are components of growth factor-induced signal transducing pathways [32, 33]. Other small G protein members of the Ras superfamily are involved in growth-factor-mediated responses as well. For example, G25K (cdc42H) is phosphorylated by the epidermal growth factor receptor [43]; rac induces membrane ruffling in response to growth factors [36]; and rho [35] and perhaps other [37] geranylgeranylated small G proteins play a role in growth-factor-induced cytoskeletal stress fiber formation. The similar structure–activity relationships observed for inhibition of protein isoprenylation and inhibition of cell proliferation by monoterpenes argue against nonspecific processes, such as disruption of membranes, as the cause of the decreased cell proliferation. Most notably, the most lipophilic monoterpene, *d*-limonene, did not inhibit cell proliferation at concentrations below 5 mM. Instead, the structure–activity relationships observed argue that inhibition of small G protein isoprenylation may be at least partly responsible for the antiproliferative effects of monoterpenes.

In both NIH3T3 cells (Table 1, Figs. 3 and 4) and HT-29 cells (Fig. 5), structure–activity comparisons indicated a positive correlation between inhibition of protein isoprenylation and inhibition of cell proliferation (Table 2). For example, among perillyl alcohol, perillic acid, and limonene, perillyl alcohol was the most potent inhibitor and limonene was the least potent inhibitor of both protein isoprenylation and cell proliferation. This relationship was consistent in all cell lines tested, including NIH3T3 (Table 1, Figs. 3 and 4), HT-29 (Figs. 2 and 5), and M600B human mammary epithelial cells (data not shown). In some cell lines (e.g. NIH3T3), the ratio between degree of inhibition of protein isoprenylation and degree of inhibition of cell proliferation by monoterpenes was approximately 1:1. In HT-29 cells, however, this ratio was much lower. Thus, HT-29 human colon carcinoma cells appear to be more sensitive to a decrease in protein isoprenylation than the nontransformed cell line NIH3T3. Furthermore, the similar structure–activity relationships obtained for small G protein isoprenylation and tumor cell proliferation suggest that inhibition of small G protein isoprenylation is a suitable endpoint for screening of potential antitumor agents.

It is well established that a metabolic product of mevalonate is essential to cell growth [44]. One or more isoprenylated proteins may be the mevalonate product(s) essential for cell growth. Our data, which demonstrate the selective inhibition of 21–26 kDa protein isoprenylation by monoterpenes that also inhibit cell proliferation, along with the burgeoning evidence for distinct roles for small isoprenylated G proteins in cell growth signalling [36, 43] and cytokinesis [37], suggest that a growth-requiring product of mevalonate may be a ras-related small G protein. DeClue *et al.* [45] have presented evidence that argues against p21ras fulfilling this role.

In addition to limonene-like monoterpenes, several other types of protein isoprenylation inhibitors have been identified recently. One type includes agents that indirectly inhibit the biosynthesis of farne-

sylpyrophosphate. This group includes HMG CoA reductase inhibitors such as lovastatin [46] and dehydroepiandrosterone [47], as well as fluoromevalonate [46], an inhibitor of isopentenylpyrophosphate biosynthesis. Both lovastatin [48–50] and dehydroepiandrosterone [51] have antitumor activity *in vivo*. However, these compounds also inhibit the biosynthesis of other mevalonate products such as cholesterol and other sterols [48], and thus may possess undesirable toxicity. A second group of isoprenylation inhibitors consists of short peptides corresponding in sequence to the C termini of farnesylated proteins. These compounds are potent and selective inhibitors of farnesyl-protein transferase *in vitro* [40, 52–56]. Two peptidomimetics have been shown to inhibit the growth of *ras*-transformed cells but not that of cells transformed by other oncogenes [55, 56]. One of these compounds also inhibits the growth of *ras*-transformed cells at concentrations that do not affect the growth of untransformed cells [55]. While selective *in vitro*, the *in vivo* antitumor efficacy of these tetrapeptide-based inhibitors remains to be established. Other *in vitro* farnesyl-protein transferase inhibitors include gliotoxin, a disulfide-containing antimicrobial agent [57], farnesyl analogs such as 4-hydroxyfarnesoic acid [58, 59] and the antibiotic maunomycin, which harbors an isoprenoid-like side chain [60]. Among these compounds, only maunomycin has been shown to inhibit tumor growth *in vivo* [60]. Limonene and perillyl alcohol, while less potent *in vitro* inhibitors of protein isoprenylation than the compounds described above, at present appear to be the most efficacious antitumor agents *in vivo*. Orally administered limonene (7.5% of the diet) [18] or perillyl alcohol (2% of the diet) [61] cause complete regression of >80% of established rat mammary carcinomas with no toxicity to normal host tissues. The antitumor activity of limonene appears to be cytostatic as evidenced by the fact that approximately half of limonene-treated tumors reappear after limonene treatment is withdrawn [18]. The only apparent side-effect of a 7.5% limonene diet is reduced weight gain [18]. Limonene also has chemopreventive efficacy when administered as 5% of the diet during the promotion/progression phase only [10]. In addition, the monoterpene geraniol inhibits small G protein isoprenylation (Table 1) and proliferation of cultured tumor cells, and it has antitumor activity *in vivo* [42]. All of the monoterpenes described above, which have antitumor activity *in vivo*, are efficacious at doses that result in little or no toxicity to the host. Thus, limonene-like monoterpenes and possibly other protein isoprenylation inhibitors [55, 60] hold promise for chemotherapy and chemoprevention of human cancers with low toxicity.

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