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# Inhibition of tumor cell growth by monoterpenes *in vitro*: evidence of a Ras-independent mechanism of action

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**(+)-Limonene (*d*-limonene) and related monoterpenes show chemopreventive activity against rodent mammary carcinoma and inhibit the growth of cancer cells *in vitro*. One suggested mechanism for the anti-tumorigenic effect of (+)-limonene is inhibition of the post-translational isoprenylation of growth controlling Ras oncoproteins. We have here examined the growth inhibitory effects of (+)-limonene and other related monoterpenes on PANC-1 pancreas carcinoma cells (carrying a K-*ras* mutation) and on 12V-H-*ras*-transformed rat fibroblasts. (+)- and (−)-perillyl alcohol, 7-methyl-perillyl alcohol, (+)-limonene oxide and (+)-perillic acid methyl ester were all found to efficiently inhibit cell growth at 1 mM, whereas (+)-limonene caused an approximately 50% growth reduction at 5 mM. Whereas BZA-5B, an inhibitor of Ras farnesyl transferase, was found to induce morphological reversion of 12V-H-*ras*-transformed cells, (+)-perillyl alcohol and (+)-limonene did not induce reversion. Furthermore, monoterpenes did not decrease MAP kinase enzyme activity or collagenase promoter activity in PANC-1 cells, two functions known to be down-stream from Ras. We conclude that although effective in inhibiting the growth of tumor cells harboring activated *ras* oncogenes, limonene and (+)-perillyl alcohol are unlikely to act by inhibiting Ras function.**

*Key words*: *d*-limonene, isoprenylation, MAP kinase, monoterpene, Ras.

## Introduction

Pancreatic carcinoma is current the fourth leading cause of cancer-related death in western society and represents a significant health problem. The disease is almost invariably fatal and current treatment modalities are not satisfactory. Mutated cellular *ras* genes are one of the most common genetic abnormalities associated with human malignancies. Ras mutations are found in more than 50% of colonic and more than 90% of pancreatic carcinomas.[1] Several lines of evidence suggest that these mutations have a critical influence on carcinogenesis and tumor progression.[2] Strategies aimed at developing specific inhibition of oncogenic Ras thus appear to offer a therapeutic potential for malignancies carrying these mutations.

Increasing knowledge of Ras function has identified novel routes to modulate Ras activity. The discovery that Ras transforming activity is dependent on post-translational modification by a farnesyl isoprenoid suggests that Ras farnesylation provides an excellent target for anti-cancer drugs. Several inhibitors of Ras farnesylation have been described recent years.[3–6]

Limonene (1-cyclohexene-1-methyl-4-isopropenyl) is one of the most abundant natural occurring monocyclic monoterpenes. The (+)-enantiomer of limonene is a major constituent of the oils of citrus fruit peel. (+)-Limonene has chemopreventive activity against spontanteous and chemically induced rodent tumors[7] (for a review see Crowell and Gould[8]). This activity is observed both at initiation and promotion.[9] A number of mechanisms of limonene action have been suggested including induction of carcinogen metabolizing enzymes,[10] growth factor/growth factor receptor expression,[11] inhibition of 3-hydroxy-3-methylglutaryl CoA reductase and inhibition of Ras protein farnesylation.[12] Limonene is rapidly and extensively metabolized in mammals and is non-toxic.[8] Investigations of structure–activity relationships among monoterpenes have revealed that several limonene metabolites, perillic acid and hydroxylated compounds such as perillyl alcohol, and methylated derivates have greater antiproliferative activity than limonene *in vitro*.[13]

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In the original study by Crowell *et al.*[12] of inhibition of Ras farnesylation by limonene, NIH 3T3 cells and a human mammary epithelial cell line were used. In a later study, Ruch and Sigler[14] did not observe an increase in cytosolic Ras following exposure of rat liver epithelial tumor cells to a number of different monoterpenes. Furthermore, Hohl and Lewis[15] recently compared the effects of limonene and perillyl alcohol on Ras processing in two human-derived leukemia cell lines, and did not observe any effect of the ratio of farnesylated/unfarnesylated Ras protein. This was in contrast to HMG-CoA inhibition with lovastatin, thus indicating a mechanism of inhibition of Ras farnesylation distinct from farnesyl transferase inhibition.

Since it is difficult to rule out that small changes in Ras farnesylation may cause growth inhibition, we have here studied whether monoterpenes affect functions down-stream from Ras We report that this does not appear to be the case. Furthermore, we report that, while inhibiting cell growth monoterpenes do not cause morphological reversion of 12V-H-*ras*-transformed fibroblasts. It therefore appears unlikely that (+)-limonene and related monoterpenes inhibit cell growth via effects on Ras function.

## Materials and methods

### Chemicals

The following chemicals were purchased from Aldrich (Steinheim, Germany): (−)-limonene (chemical purity 99%, ee 98%), (+)-limonene (chemical purity 99%, ee 98%), (−)-limonene oxide (**1**) (mixture of *cis* and *trans*, chemical purity 97%, ee 99%). (+)-Limonene oxide (**2**) (mixture of *cis* and *trans*, chemical purity 97%, ee 98%). (*S*)-(−)-Perillyl alcohol (**4**) (chemical purity 96%, $[a]D$ −88° ($c=1$, MeOH, 22°C)). (*R*)-(+)-Perillyl alcohol (**3**) (chemical purity 99%, $[a]D$ +109° (neat, 20°C)). (*S*)-(−)-Perillaldehyde (**5**) (mixture of *cis* and *trans*, chemical purity 97%, ee 98%). (*S*)-(−)-Perillic acid (**7**) (chemical purity 97%, $[a]D$ −102° ($c=2$ MeOH, 21°C)) was purchased from Acros Chimica

### Synthesis

*7-Methyl perillyl alcohol* (**6**). (S)-(−)-Perillaldehyde (**5**) was reacted with methyllihium in diethyl ether at room temperature. After standard workup procedures the product was subjected to silica gel liquid chromatography (LC). The yield of the two diastereomers was 86%. The chemical purity was 90% MS: 166(M,10), 43(100) 93(51), 79(48), 45(45), 123(45) 67(42), 108(40) 68(38), 148(15) 151 (7).

*Perillic acid methyl ester* (**8**). (S)-(−)-Perillic acid (**7**) was reacted with methyl iodide under phase transfer conditions (sodium carbonate in water/tetrabutyl ammonium hydrogen sulfate in methylene chloride) yielding the methyl ester of (*S*)-(−)-perillic acid The purity was 95% after LC. The yield of the reaction was 67%. MS 180 (M, 30), 68(100), 121(90), 67(58), 105(50), 93(48), 79(45), 137(38), 149(24), 59(18).

Mass spectra were recorded on a Finnigan SSQ 7000 GC-MS instrument. A two-dimensional GC instrument was used for the determination of enantiomeric compositions of the monoterpenoids.[16]

The benzodiazepine peptidomimetic farnesyltransferase inhibitor BZA-5B was geneously supplied from Genentech (San Francisco, CA). BZA-5B stock solution 100 mM (100% DMSO) was diluted into 10 mM DTT/PBS and diluted in DTT/Dulbecco's MEM (DMEM) with 5% FBS to final concentrations: BZA-5B (50 μM) and DTT (0.5 mM) and DMSO (0.05%) for cell growth inhibition assays.

### Cell culture

Cells were maintained as monolayer cultures in DMEM supplemented with 5% fetal bovine serum (Flow, Irvine, UK) in humidified athmosphere (7% $CO_2$/93% air) PANC-1 cells were obtained from American Type Culture Collection (Rockville, MD). A18 is a 12V-H-*ras*-transformed rat fibroblast cell line.[17]

For cell growth experiments cells were plated at a density of 5000 cells/dish in triplicate. All cells were collected by trypsinization 5 days later and counted in a hemocytometer using phase contrast optics. Monoterpenes were added directly to the growth medium from stock solutions in ethanol. The final concentration of ethanol was 0.1%. Control dishes were treated with vehicle only. A18 cells were plated at a higher density (50 000 cells).

### Transient transfection assays

−517/+63-CAT contains the human collagenase promoter in pBL-CAT3. Raf-C4 is a dominant negative mutant of Raf.[18] Cells were pre-treated with limonene or perillyl alcohol and transfected by the

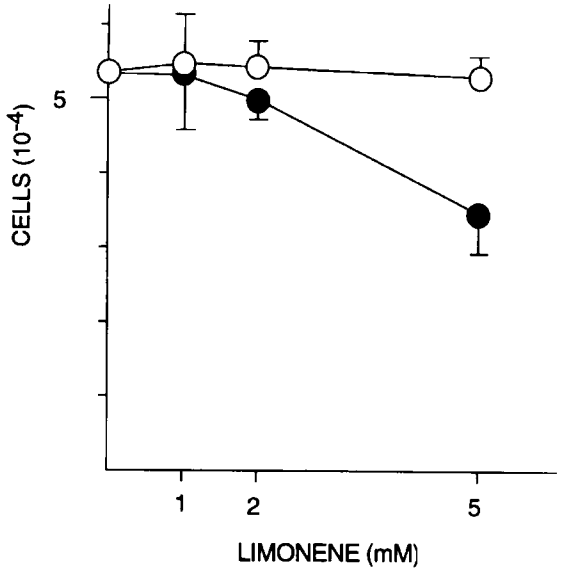



**Figure 1.** Effect of (+)-limonene and (−)-limonene on PANC-1 cell growth. PANC-1 cells ($5 \times 10^3$) were plated in different concentrations of (+)-limonene or (−)-limonene and the number of cells were counted after 5 days.

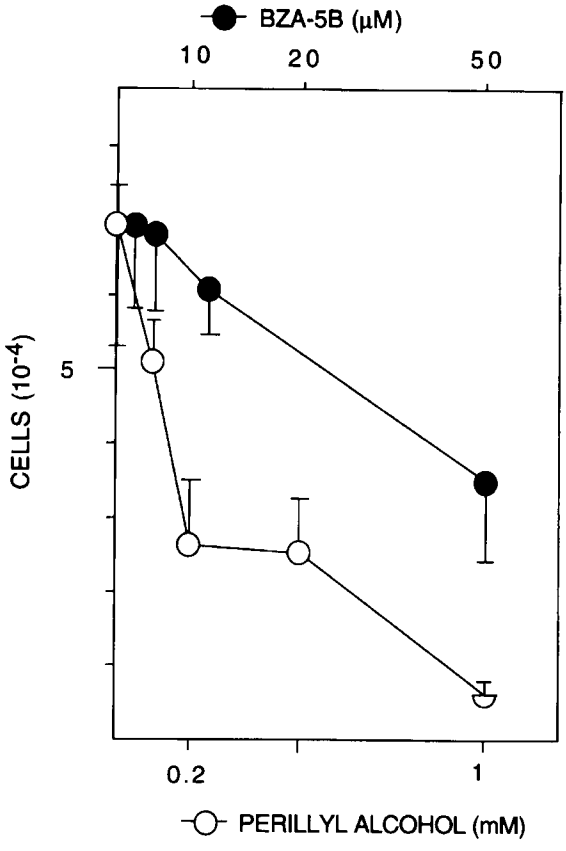



**Figure 2.** Effect of (+)-perillyl alcohol and BZA-5B, an inhibitor of Ras farnesyl transferase, on PANC-1 cell growth. PANC-1 cells were incubated in different concentrations of (+)-perillyl alcohol and BZA-5B, and the number of cells were counted after 5 days.

calcium phosphate precipitation technique (using 2 μg reporter and 1 μg Raf-C4; vector DNA was added to 5 μg). Cell were grown in the absence or presence of monoterpenes and extracts were prepared 44 h after transfection. Chloramphenicol acetyl transferase (CAT) activity was measured using standard procedures[19] and standardized for the amount of protein in each extract.

### MAP kinase assay

MAP kinase was assayed using the Amersham p42/p44 MAP kinase enzyme assay (RPN 84) as recommended by the manufacturer. All experiments were performed at least twice. To ensure that the assay was specific for MAP kinase, p44/ERK2 in lysates was immunoprecipitated with antibody C-14 (Santa Cruz Biotechnology, Santa Cruz, CA) and then subjected to the enzymatic assay.

## Results

### Inhibition of PANC-1 cell growth by monoterpenes

PANC-1 cells were used as a model for pancreas carcinoma cells. PANC-1 is a human tumor cell line derived from a carcinoma in exocrine pancreas.[20] A mutation in Ki-*ras* codon 12 has been documented in this cell line.[21] The effect of (+)-limonene and (−)-limonene on PANC-1 cell growth is shown in Figure 1. An approximately 50% reduction in cell growth was observed at high concentrations of (+)-limonene, whereas (−)-limonene did not affect cell growth at any of the concentrations tested. Limonene is metabolized *in vivo*, although not in cultured cells. A hydroxylated urinary metabolite, (+)-perillyl alcohol has been found to be more potent than (+)-limonene in a number of studies. As shown in Figure 2, (+)-perillyl alcohol was considerably more potent than (+)-limonene in inhibiting PANC-1 cell growth, showing strong growth inhibition at 0.2 mM. The effect of perillyl alcohol was compared to that of BZA-5B, an inhibitor of Ras-farnesyl transferase.[4] At a concentration of 50 μM, BZA-5B inhibited PANC-1 cell growth to about 50%. Neither perillyl alcohol nor BZA-5B induced any changes in PANC-1 cell morphology (not shown).

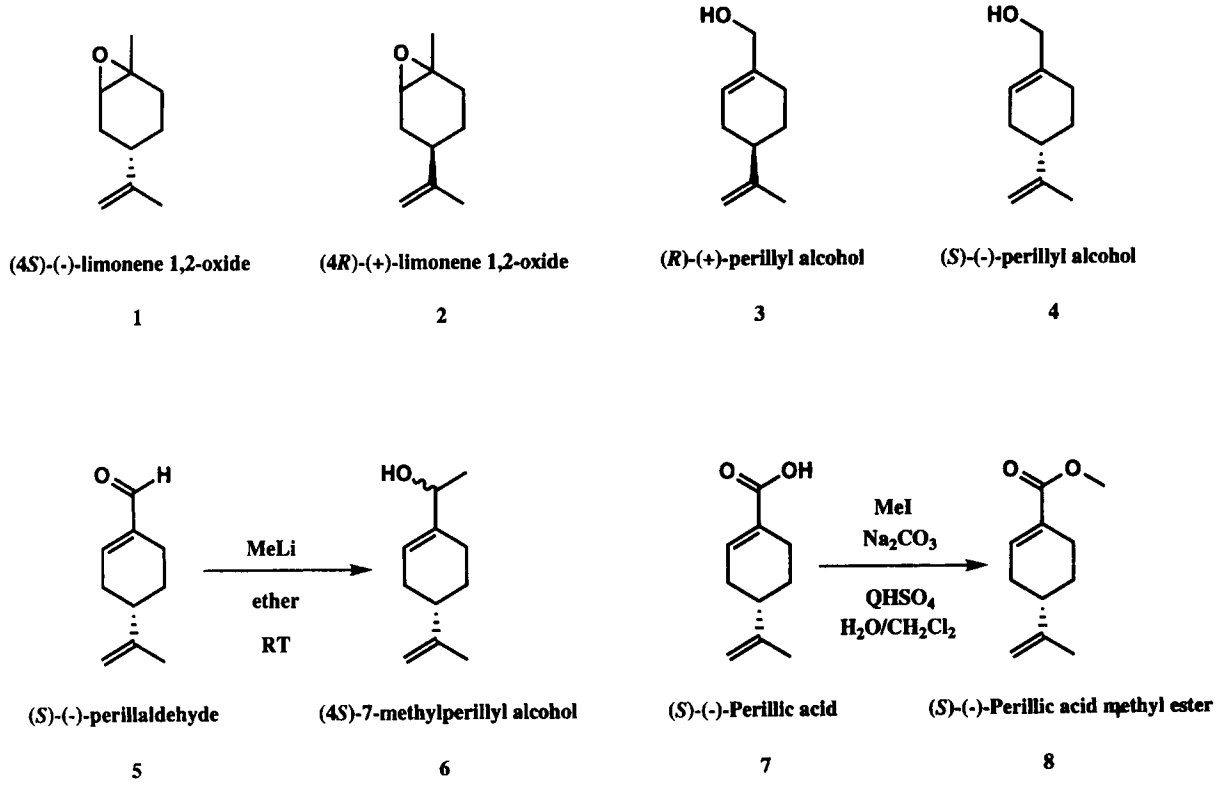



**Figure 3.** Chemical structures and synthetic routes of the oxygenated monoterpenes used in this study.

A number of monoterpenoids related to limonene were tested with regard to growth inhibitory activity. The structures of these compounds are shown in Figure 3. As shown in Figure 4, (+)-limonene oxide, (−)-perillic acid methyl ester and the racemate of 7-methyl perillyl alcohol were found to inhibit cell growth at 1 mM, whereas (−)-limonene oxide was less effective The (−)-form of perillyl alcohol did show anti-proliferative activity however. Only weak (less than 50% inhibition) effects were observed at 0.1 mM using any of these substances (not shown) PANC-1 cells were observed to lyse within a couple of hours after exposure to (−)-perillaldehyde, suggesting that this compound was acutely toxic.

## Effect of monoterpenes and BZA-5B on 12V-H-*ras*-transformed fibroblasts

A18 is a 12V-H-*ras*-transformed rat fibroblast cell line. (+)- and (−)-Perillyl alcohol and (+)-limonene oxide inhibited the growth of A18 cells (Figure 5). Growth inhibition by these monoterpenes was observed using two other 12V-H-*ras*-transformed fibroblast cell lines (not shown). The growth of A18 cells was inhibited by the farnesylation inhibitor BZA-5B (Figure 5).

Treatment of 12V-H-*ras*-transformed fibroblasts with BZA-5B was observed to induce a normal, flat morphology (reminishent of untransformed fibroblasts). In contrast, treatment with monoterpenes did not affect cell morphology. Microphotographs of cells treated with BZA-5B and (+)-perillyl alcolhol are presented in Figure 6.

## MAP kinase and collagenase promoter activity is not affected by limonene or perillyl alcolhol

Ras induces the activity of intracellular signal pathways. Inhibition of Ras function is therefore expected to lead to decreases in the activity of Ser/Thr kinases down-stream from Ras and in the activity of Ras-induced transcription factors such as AP-1 and Ets.

The activity of MAP kinase was examined in PANC-1 cells grown in the presence of perillyl alcohol or vehicle. No significant difference in MAP kinase activity was observed (Figure 7). PANC-1 cells were transiently transfected with a plasmid containing the human collagenase promoter fused

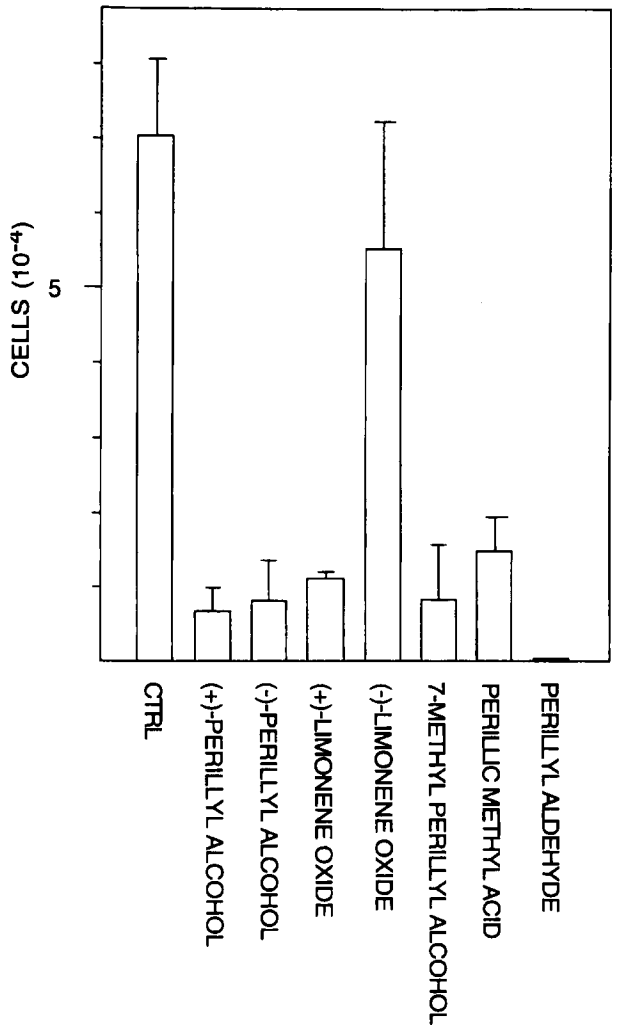



**Figure 4.** Effects of various monoterpenoids on PANC-1 cell growth. Cells were grown for 5 days in the presence of various compounds and the number of cells counted.

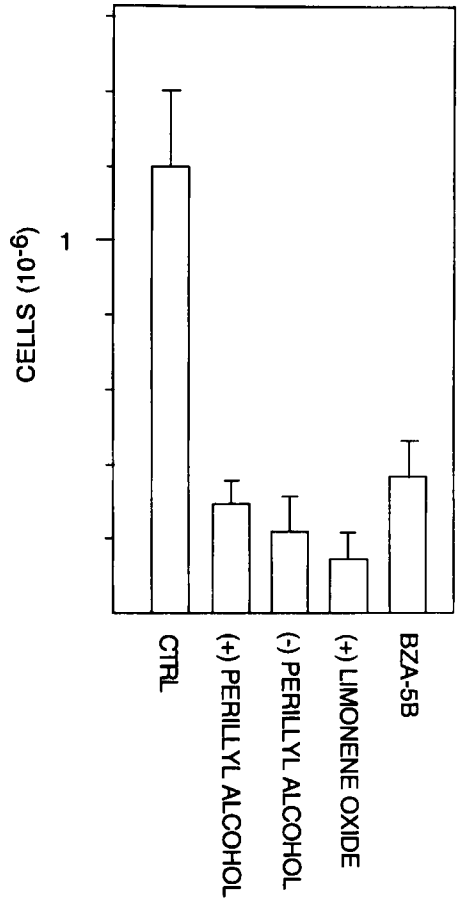


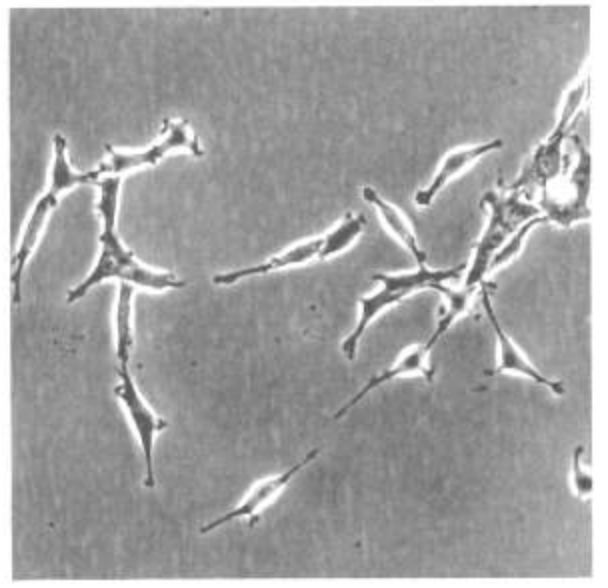

**Figure 5.** Effect of (+)- and (−)-perillyl alcohol, (+)-limonene oxide and BZA-5B on the growth of 12V-H-ras-transformed rat fibroblasts (A18 cells). Cells were grown in the presence of inhibitor for 5 days.

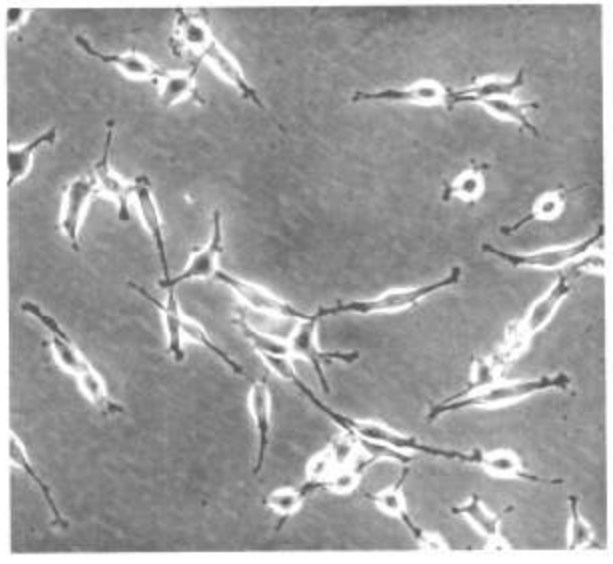
to the bacterial CAT gene (−517/+63-CAT). The collagenase promoter is known to be activated by Ras and phorbol esters.[22] As shown in Figure 8, coll-CAT activity decreased by transfection of a dominant negative Raf mutant, showing that blocking the Raf → MAP kinase signal pathway decreased coll-CAT activity. In contrast, (+)-limonene or (+)-perillyl alcohol did not decrease coll-CAT activity.

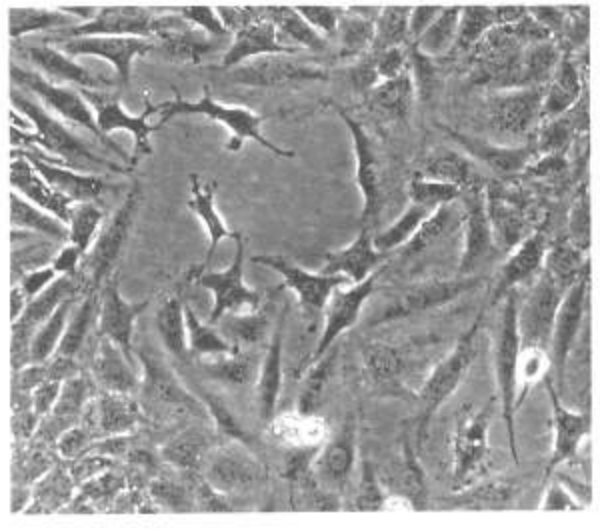
## Discussion

The (+)-enantiomer of limonene is widely distributed in the plant kingdom and a main constituent in the peel oil of citrus fruits, also found in spices and herbs. (+)-Limonene has been used in doses up to 20 g per day for the dissolution of gall stones.[8] The low toxicity of (+)-limonene, combined with its anti-tumorogenic activities, makes it a promising anti-cancer agent. Crowell *et al.*[12] reported that limonene inhibits Ras protein farnesylation. This observation raises the possibility that (+)-limonene can be used for treatment of malignancies such as pancreas and colon cancer characterized by high frequencies of *ras* oncogene mutation.

Limonene was originally reported to cause a 50% inhibition of Ras farnesylation.[12] In a later study, Ruch and Sigler[14] did not observe any effect of limonene on the distribution of Ras between membrane and cytosolic fractions. Whether these apparently conflicting data reflect differences between cell types or whether small decreases in Ras farnesylation may still affect cell growth is not clear. In the present study, we chose to examine the effects of monoterpenes on cellular phenotype. We found that although (+)-limonene and (+)-perillyl alcohol inhibited cell growth, these compounds did not







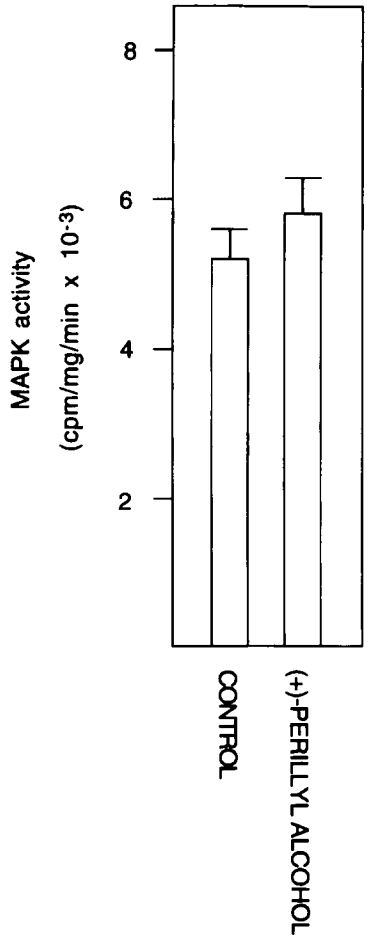



**Figure 7.** MAP kinase of PANC-1 cells grown for 6 days in the presence of peryllic alcohol or vehicle. Activity is expressed as $^{32}P$ transferred to a substrate peptide/mg extract/min.

induce morphological reversion of 12V-H-*ras*-transformed fibroblasts. As a control, we used the Ras farnesylation inhibitor BZA-5B which was found to cause morphological reversion. BZA-5B did not excert a strong growth inhibitory effect on PANC-1 cells and did not affect the morphology of these cells. BZA-5B is most effective on farnesylation of H-Ras protein and inhibits K-Ras farnesylation only weakly.[23]

(+)-Limonene and (+)-perillyl alcohol did not decrease MAP kinase activity or collagenase promoter activity in PANC-1 cells at a concentration where

**Figure 6.** Effect of peryllyl alcolhol and BZA-5B on the morphology of 12VH-*ras*-transformed fibroblasts. (A) Untreated cells, (B) perillyl alcohol (1 mM; 5 days)-treated and (C) BZA-5B (50 $\mu$M; 5 days)-treated cells. Note the flat morphology of BZA-5B-treated fibroblasts.

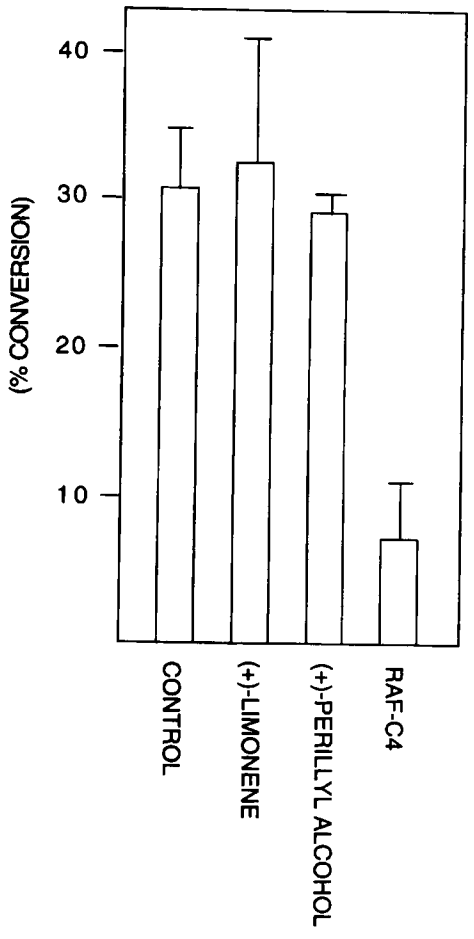



**Figure 8.** PANC-1 cells were transiently transfected with a human collagenase promoter–CAT fusion construct (−517/+63 -CAT), either alone or together with plasmid Raf-C4 [a dominant negative mutant of Raf kinase]. Cells were grown in the presence of 5 mM (+)-limonene or 1 mM (+)-perillyl alcohol as indicated. CAT activity was measured in cell extracts 2 days after transfection. Shown is the ratio of acetylated/non-acetylated [$^{14}$C]chloramphenicol. Note that monoterpenes did not affect coll-CAT activity.

growth inhibition was observed. Due to the inefficiacy of BZA-5B, we used a dominant negative mutant of Raf as a control for the collagenase promoter experiments. In contrast to the inefficiency of (+)-limonene and (+)-perillyl alcohol, co-transfection of this mutant resulted in a decrease in collagenase promoter activity.

The (+)-enantiomers of limonene and *cis/trans*-limonene oxides were found to inhibit cell growth more than their (−)-enantiomers. Thus, the chirality of the active compounds must be considered in future studies. The less specificity observed for the enantiomers of perillyl alcohol and the activity of the methylated derivatives might be explained by the free rotation of the functional groups at C1, compared with the more stiff construction of the epoxides and the limonene enantiomers.

Based on their inability to cause morphological reversion and to inhibit MAP kinase and collagenase promoter activity, it appears unlikely that monoterpenes have significant effects on Ras-mediated cell transformation and signal transduction. Nevertheless, some enantiomers of monoterpenes were found to have strong growth inhibitory activity on pancreas carcinoma cells. Irrespective of mechanism, such inhibitory activity by natural occurring untoxic compounds is promising for future clinical use, where in addition, the enantiomeric composition of the compounds must be considered.

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