Inhibition of Farnesyl Protein Transferase, H-ras Oncogene Expression and P21[ras] Membrane Association by Natural Products in Human Solid Tumor Cell Lines

Xiao Guang Chen[a]; Otani Shuzo[b]; Yan Li[a]; Rui Han[a]

[a] Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences Peking Union Medical College, Beijing, China [b] Department of Biochemistry, Osaka City University Medical School, Osaka, Japan

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INHIBITION OF FARNESYL PROTEIN TRANSFERASE, H-ras ONCOGENE EXPRESSION AND P21^{ras} MEMBRANE ASSOCIATION BY NATURAL PRODUCTS IN HUMAN SOLID TUMOR CELL LINES

XIAO GUANG CHEN[a,*], OTANI SHUZO[b],
YAN LI[a] and RUI HAN[a]

[a]*Department of Pharmacology, Institute of Materia Medica, Chinese Academy of
Medical Sciences Peking Union Medical College, 1 Xian Nong Tan Street,
Beijing 100050, China;* [b]*Department of Biochemistry,
Osaka City University Medical School Osaka 545, Japan*

Ras proteins must be isoprenylated at a conserved cysteine residue near the carboxyl terminus (Cys-186 in mammalian Ras P^{21} proteins) in order to extend their biological activity. Previous studies indicated that an intermediate in mevalonate pathway, most likely farnesyl pyrophosphate, is the donor of this isoprenyl group, that using inhibitors of the mevalonate pathway could block the transforming properties of ras oncogene. Unfortunately, mevalonate is the precursor of various end products essential to mammalian cells, such as dolichols, ubiquinones, heme A, and cholesterol. In this study, we partially purified farnesyl protein transferase (*FPTase*) capable of catalyzing the farnesylation of unprocessed Ras P^{21} proteins *in vitro* from porcine kidney epithelial-like LLC-PK1 cells, human lung adenocarcinoma A549 cells and human pancreatic cancer MIA PaCa-2 cells. We observed the effects of the monoterpene compound, d-limonene (1); turmeric derivatives, TD-I (2) and TD-II (3); polyphenol compound, gallotannin; salviol derivative, SMD; and retinoid acid derivative, RAD on FPTase activity. We found that turmeric derivatives and gallotannin had a strong inhibition on FPTase besides d-limonene, while gallotannin was the strongest among synthetic and natural compounds tested. Saliviol and retinoid acid derivatives had no influence on FPTase activity. Our results suggest that compounds containing polyphenol hydroxyl may be a new source of FPTase inhibitors. The experiment also showed that availability of an *in vitro* FPTase assay could be useful in screening for potential inhibitors of ras oncogene function that will not interfere with other aspects of the mevalonate pathway.

Concomitantly, we also studied gap junction intercellular communication (GJIC), H-ras oncogene expression and ras oncogene product (P21^{ras} protein) expression in four human solid

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* Corresponding author. Tel.: 010-63165207. Fax: 010-63017757.

tumor cell lines, W1-38, CACO₂, A549 and PaCa (with different ras gene mutation rates), and the effects of four compounds, SMD, d-Limonene, TD-I and TD-II.

We examined the abilities of the four solid tumor cell lines to transfer dye to adjacent cells using the scrape-loading/dye transfer technique, and the H-ras oncogene expression by Northern blotting and P21^{ras} protein expression by Western blotting. The results showed the loss of intercellular coupling in PaCa cells, slight GJIC in A549 and CACO₂ cells, and a good GJIC in W1-38 cells. The four compounds used could improve the GJIC of PaCa to different extents. The amount of total and membrane associated P21^{ras} in PaCa cells were decreased after treatment with SMD, d-limonene and TD-I (2.5 µg/ml) for 48 h. At the same time, the growth of PaCa cells decreased in soft agar and had enhanced GJIC. The relative potency was found to be: d-limonene > SMD > TD-I = TD-II. There was no significant effect of the four compounds on H-ras oncogene expression. These results suggest that: (1) there was an excellent correlation between loss of lucifer Yellow dye transfer and ras gene mutation rate in the four solid tumor cell lines used (ras gene mutation rate inversely correlated with average cell number coupled, $r = 0.98$), i.e. showing that high ras gene mutation is closely correlated with loss of GJIC in these malignant human tumor cells; (2) the antitumor effect of the monoterpene d-limonene and the phenol compound, SMD, might be related to inhibition of P21^{ras} membrane association and enhancement of GJIC, whilst that of the others may be by a different mechanism; (3) the inhibition of P21^{ras} membrane association is directly related to the enhancement of GJIC.

Keywords: Farnesyl protein transferase; P21^{ras} membrane association; Natural product; Human tumor cell line

INTRODUCTION

The ras oncogenes and the proteins (Ras) encoded by these genes have been the subjects of intense investigation for nearly two decades. Much of the interest in these genes has been founded in their presumed roles in the pathophysiology of cell transformation and the pathogenesis of human cancer. Presently, mutant ras genes are routinely found in 30–40% of human lung adenocarcinoma, > 50% of human colon carcinomas and > 90% of pancreatic cancers. However, until now, attempts at blocking Ras-induced transforming activity with small molecule inhibitors have been largely unsuccessful. More recently, progress has been made in the development of inhibitors of Ras activity by exploiting one of the earliest observations concerning Ras physiology, that is, its subcellular localization to the plasma membrane [1]. This association of Ras with the outer membrane of the cell is mediated by a 15-carbon isoprenyl (farnesyl) group that is covalently linked to Ras, a post-translational modification that is obligatory for Ras cell-transforming activity.

Ras is synthesized as cytosolic precursor that ultimately localize to the cytoplasmic face of the plasma membrane after a series of post-translational modifications [2]. The first and obligatory step in this series is the addition of a farnesyl moiety to the cysteine residue of the COOH-terminal CAAX motif in a reaction catalzyed by farnesyl protein transferase

(FPTase). This modification is essential for Ras function, as demonstrated by the inability of Ras mutants lacking the COOH-terminal cysteine to be farnesylated, to localize to the plasma membrane, and to transform mammalian cells in culture [3–5]. Thus, inhibition of the Ras farnesylation reaction is a possible anticancer strategy. Several strategies have been employed to inhibit Ras farnesylation including inhibition of isoprenoid biosynthesis and inhibition of the enzyme which catalyzes the farnsylation reaction, FPTase. A more specific approach to inhibiting Ras farnesylation is to inhibit FPTase. Therefore, it is a rational approach towards the selective inhibitors of FPTase in order to block the neoplastic effect of ras oncogenes in human tumors.

Recently, many studies have been focused on the relationship between ras oncogene product and gap junction intercellular communication (GJIC) [6,7]. There has been evidence that the Ras protein serves as mediator of growth factor induction of inositol phospholipid turnover [8]. The viral v-Ki-ras oncogene has been shown to inhibit GJIC between NRK cells [9], and the activated H-ras-1 oncogene inhibited GJIC between epithelial clone rat liver cells.

Salviol derivative is a potent compound. Our previous works [10] have demonstrated that it has stronger antioxidation and antitumor activities. d-limonene (1), the predominant monoterpene in orange peel oil, has substantial chemopreventive and chemotherapeutic activity against chemically induced mammary, lung, and stomach [11] cancer in rodents. When fed during the promotion/progression stage of mammary carcinogenesis, limonene inhibits the development of tumors induced by either 7,12-dimethylbenz(α)anthracene which requires metabolic activation to its carcinogenic form, or nitrosomethylurea, a directly acting carcinogen [12]. Dietary limonene also causes the regression of rat mammary carcinomas induced by either 7,12-dimethylbenz(α)anthracene or N-nitroso-N-methylurea in a dose-dependent manner. The mechanisms of action responsible for these chemopreventive and chemotherapeutic activities of d-limonene may be due, in part, to its ability to inhibit the isoprenylation of cell-growth-associated small G proteins such as Ras [13,14]. Turmeric derivatives (TD-I and TD-II) are a series analogs of turmeric. Our previous research [15] has shown that turmeric derivative I (2) and II (3) possess antitumor activities on S180 solid type and H22 hepatoma in mice, enhance GJIC and counteract the inhibition of TPA-induced GJIC in V_{79}, Balb/c-3T3, WB and 2BS normal cells. In view of the antioxidative, antitumor-promoting and antitumor activities of Salviol derivative, d-limonene and the turmeric derivatives, we considered that these compounds may possess significant

antitumor potentials in human solid tumor cell lines. In this study, we assessed the antitumor activities of these compounds in the four human solid cell lines, viz, WI-38, CACO$_2$, A549 and PaCa cells. We examined H-ras oncogene expression and GJIC in these cell lines and the relationship between them when these cells were treated with the experimental agents to determine the mechanism of the effect produced.

Limonene (1)

TD–I (2)

TD–II (3)
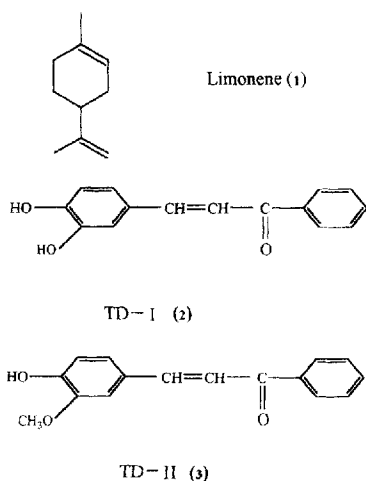
RESULTS AND DISCUSSION

Farnesylation of P21^{H-ras} protein in vitro   Recent studies have indicated that farnesyl is the isoprenyl unit linked to the carboxyl terminus of Ras proteins. Therefore, the most likely donor of this farnesyl moiety is farnesyl pyrophosphate (FPP). As a consequence, we based our assays to identify farnesyl protein transferase activity on the incorporation of radioactivity from [^{3}H]FPP into recombinant wild-type human P21^{H-ras} protein in the presence of partially purified FPTase obtained from porcine kidney and human solid tumor cell lines. Partially purified FPTase from LLC-PK1, A549 and PaCa cells catalyzed the incorporation of radioactivity from [^{3}H]FPP into TCA precipitable P21^{H-ras}. However, FPTase activity from human lung adenocarcinoma A549 cells was the highest (38.81 pmol/h/mg protein), the second, PaCa (30.79 pmol/h/mg protein), and that of LLC-PK1 was the lowest (25.51 pmol/h/mg protein) among the three cell lines. Addition of [^{3}H]FPP to the reaction mixture resulted in the labeling of a single protein of 21 kDa (Fig. 1). Labeling of this protein was dependent on the presence of both the FPTase and P21^{H-ras} protein.

FPP and GGPP as substrates   The relative effectiveness of FPP and geranylgeranyl pyrophosphate (GGPP) as substrates was tested using partially purified FPTase from three cell lines (Table I). GGPP was a poor substrate in all three cases. It is also noteworthy that enzymatic activity with endogenous acceptor ($P21^{H-ras}$-independent) using GGPP as the prenyl donor was not detectable with any of the partially purified FPTase.

Inhibition of FPTase activity   One monoterpene compound, two turmeric derivatives, two phenol compounds, a retinoid acid derivative, a S-methyl-L-cysteine and the synthetic peptide, TKCVIM, were tested as inhibitors of
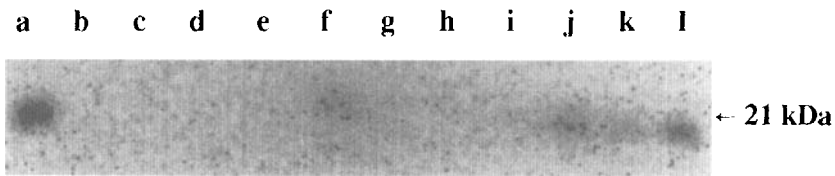

FIGURE 1   SDS-PAGE analysis of farnesylation of $P21^{H-ras}$ protein *in vitro*. Reaction mixture contained 50 mM Tris-chloride (pH 7.5), 25 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 2.5 μM $P21^{H-ras}$, 10 pmol [^{3}H] FPP and 6 μg of partially purified FPTase, and incubated at 37°C for 60 min. SDS-PAGE analysis of its product of the enzyme reaction and carried out as described in Experimental Section. (a) Complete reaction mixture; (b) without $P21^{H-ras}$ protein; (c) without partially purified FPTase; (d) [^{3}H]FPP was replaced with [^{3}H]GGPP; (e) [^{3}H]GGPP and without $P21^{H-ras}$ protein; (f) addition of SMD (0.2 mM); (g) addition of d-limonene (0.2 mM); (h) addition of gallotannin (0.15 mM); (i) TKCVIM peptide (1 μM); (j) TD-I (0.1 μM); (k) TD-II (0.1 mM), and (l) addition of RD (0.25 mM). Position of the farnesylated $P21^{H-ras}$ proteins is indicated by an arrow.

TABLE I   Effect of isopreneoid compounds on amount of isoprenylated $P21^{H-ras}$ formation in LLC-PK$_1$, A549 and PaCa cell lines

Cell lines	Isopreneoid compounds	FPTase activity (DPM/mg protein)		$P21^{H-ras}$ dependent	Amount of isoprenylated $P21^{H-ras}$ formation (pmol/h/mg protein)
		With $P21^{H-ras}$	Without $P21^{H-ras}$		
LLC-PK$_1$	[^{3}H]FPP	818020.03 ± 293771.75	200752.15 ± 54033.27	617267.87 ± 24661.52	25.51 ± 1.02
	[^{3}H]GGPP	240233.55 ± 18816.13	227310.38 ± 10272.54	12923.43 ± 8543.55	0.54 ± 0.20
A549	[^{3}H]FPP	1460540.63 ± 13959.06	162838.98 ± 5374.29	1297701.64 ± 19693.36	38.81 ± 0.59
	[^{3}H]GGPP	404161.41 ± 40261.25	376714.22 ± 4999.84	27447.19 ± 5526.48	0.98 ± 0.07
PaCa	[^{3}H]FPP	928318.35 ± 7773.51	131431.13 ± 23221.11	796887.22 ± 30994.62	30.79 ± 1.19
	[^{3}H]GGPP	271543.93 ± 17033.27	211774.31 ± 5360.46	59769.62 ± 11672.80	1.96 ± 0.17

FPTase activity using partially purified FPTase from LLC-PK1 cells. The results showed that d-limonene, TD-I, TD-II and gallotannin significantly inhibited FPTase activity in a dose-dependent manner (Fig. 2A, B, C and D) (Table II). Among these compounds, the inhibition of FPTase activity by gallotannin was the strongest. In addition, gallotannin also inhibited FPTase activities from A549 and PaCa tumor cells (Fig. 2D). The six amino acid residue, TKCVIM peptide could competitively inhibit FPTase, while S-methyl-L-cysteine appeared to have a poorer inhibition than the TKCVIM peptide (Fig. 3). SMD and RAD had no significant effect on FPTase activity.

Effect of the four test compounds on human solid tumor cells   The concentrations of SMD, d-limonene, TD-I and TD-II that inhibit the growth
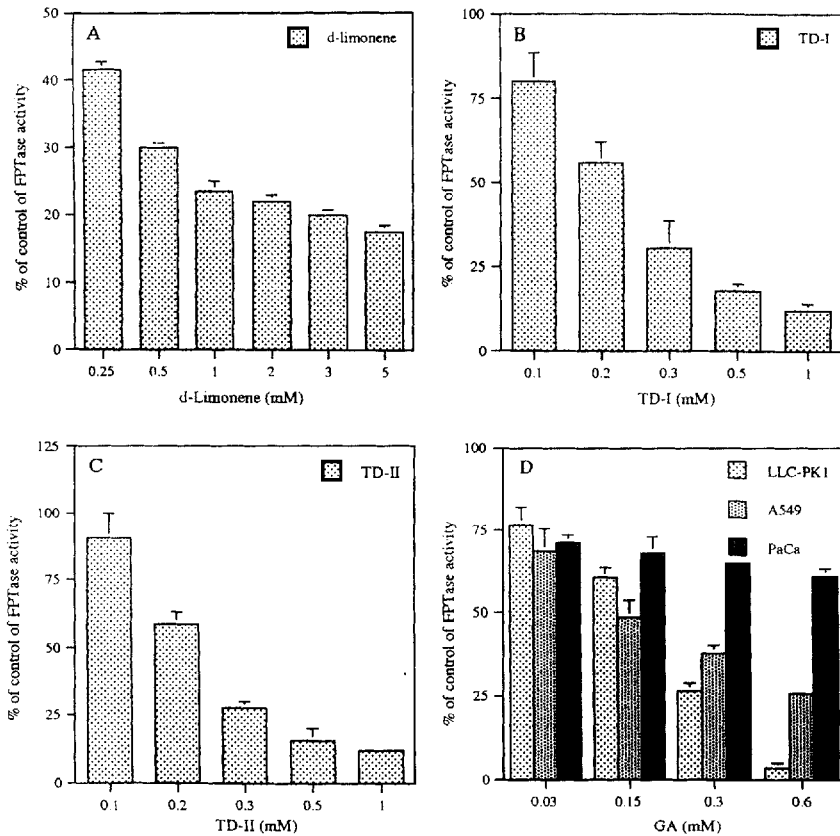

FIGURE 2   Inhibition of FPTase activity in LLC-PK1, A549 and PaCa cells by d-limonene (**1**), turmeric derivatives TD-I (**2**) and TD-II (**3**) and gallotannin.

TABLE II Comparison of IC_{50} of inhibition on FPTase and GGPTase activity by compounds

Compounds	Inhibition on FPTase activity	Inhibition on GGPTase Activity	
	LLC-PK1 IC_{50}	LLC-PK1 IC_{50}	A549
d-Limonene	0.21 mM	2.67 mM	1.99 mM
GA	0.20 mM	0.69 mM	0.76 mM
TD-I	0.25 mM	0.024 mM	0.0091 mM
TD-II	0.29 mM	0.035 mM	0.031 mM
TKCVIM peptide	1.39 µM	ND	ND
S-methyl-L-cysteine	33.51 µM	ND	ND

ND: not determined.
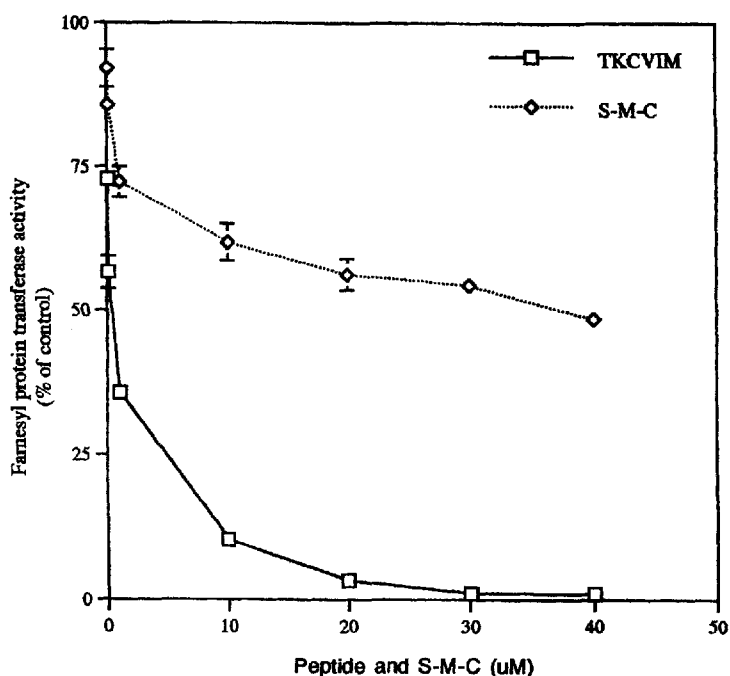
FIGURE 3 Inhibition of FPTase activity by TKCVIM peptide and S-methyl-L-cysteine.

of WI-38, CACO$_2$, A549 and PaCa cells were determined by MTT and clonal assay. SMD, d-limonene, TD-I and TD-II could significantly inhibit WI-38, CACO$_2$, A549 and PaCa cell proliferation at concentrations greater than 10 µg/ml after four days of exposure (Fig. 4), and inhibited clonal growth at concentrations greater than 5 µg/ml after nine days of exposure
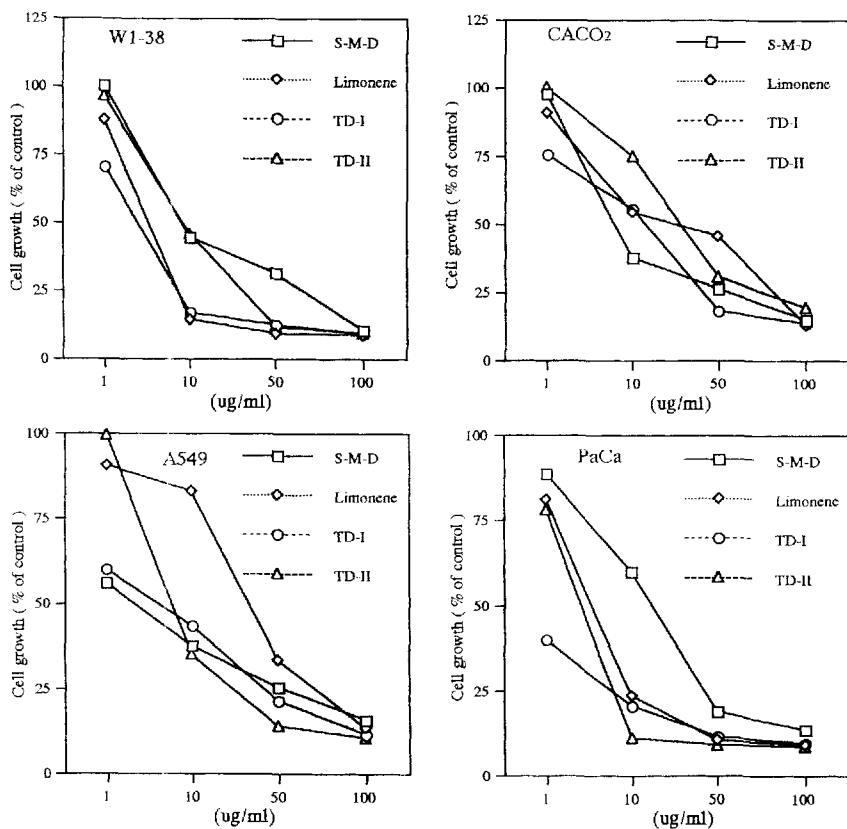
FIGURE 4   Effect of test compounds on the proliferation of WI-38, $CACO_2$, A549 and PaCa tumor cells. The control used was 0.1% DMSO. Exposure time was four days.

(Fig. 5). Colony formations of the four tumor cells were also inhibited by the four compounds dose-dependently (Fig. 6). At higher doses of the four test compounds, all four types of tumor cells rounded up and got detached from the dishes.

Enhancement of GJIC of human tumor cells by test compounds   The concentration of the four test compounds (2.5 µg/ml) used did not significantly inhibit clonal growth of four types of tumor cells (Fig. 5). Figure 7 however, shows the effects of these compounds on GJIC in the four tumor cell lines. Among these human tumor cells, WI-38 cells possessed a better GJIC (Fig. 8A,B), slightly good GJIC in A549 and $CACO_2$ cells (Fig. 7) and loss of GJIC in PaCa cells (Fig. 9A,B). SMD and d-limonene improved GJIC of A549 and $CACO_2$, and increased A549 and $CACO_2$ cell dye coupling
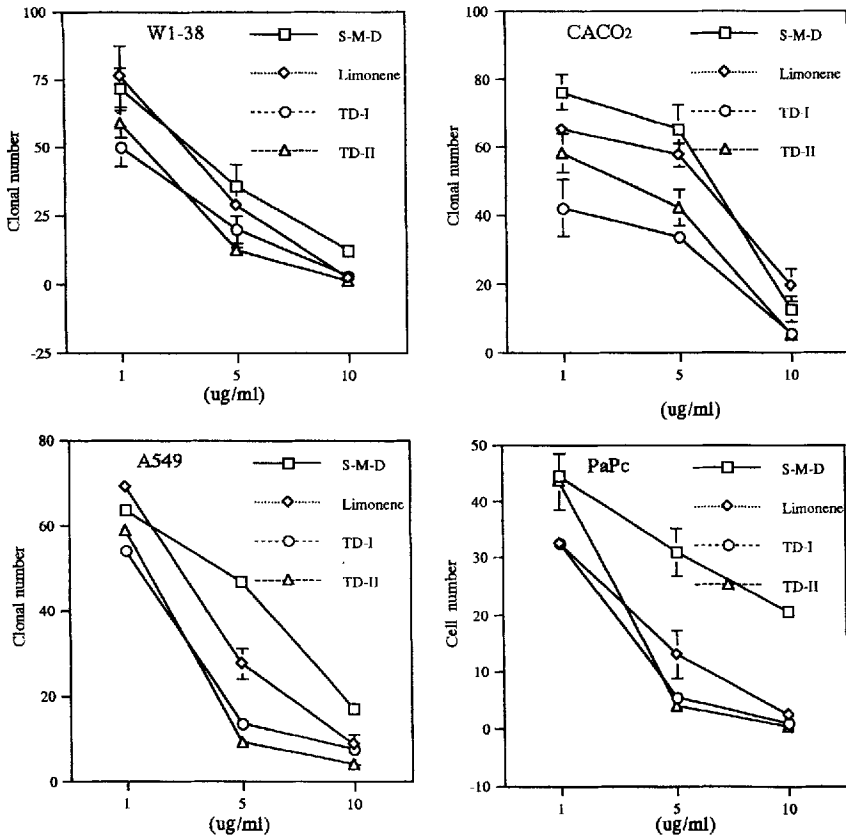
FIGURE 5 Inhibition of colonal growth by test compounds in W1-38, $CACO_2$, A549 and PaCa tumor cells after nine days of treatment. Results are mean $\pm$ SD of three cultures performed with each test compound on cells plastic dishes.

(Fig. 7). No effects were seen in W1-38 cells after 48 h exposure to the four test compounds, but significant increase was noted in PaCa cells (Fig. 7, Fig. 9C–J). The potency is d-limonene > SMD > TD-I = TD-II.

Effect of compounds on the H-ras oncogene expression Figure 10 showed that SMD, TD-I and TD-II (2.5 µg/ml for 48 h) significantly and d-limonene (2.5 µg/ml for 48 h) slightly inhibited H-ras oncogene expression in A549 cells, but no obvious effects were found in other tumor cells.

Effect of test compounds on P21^{ras} protein expression By Western-blot analysis, and increase in cytosolic P21^{ras} was evident in W1-38 cells treated for 48 h with 2.5 µg/ml SMD, d-limonene, TD-I and TD-II (Fig. 11A,B). Only TD-I and TD-II decreased total P21^{ras}, but there was no significant
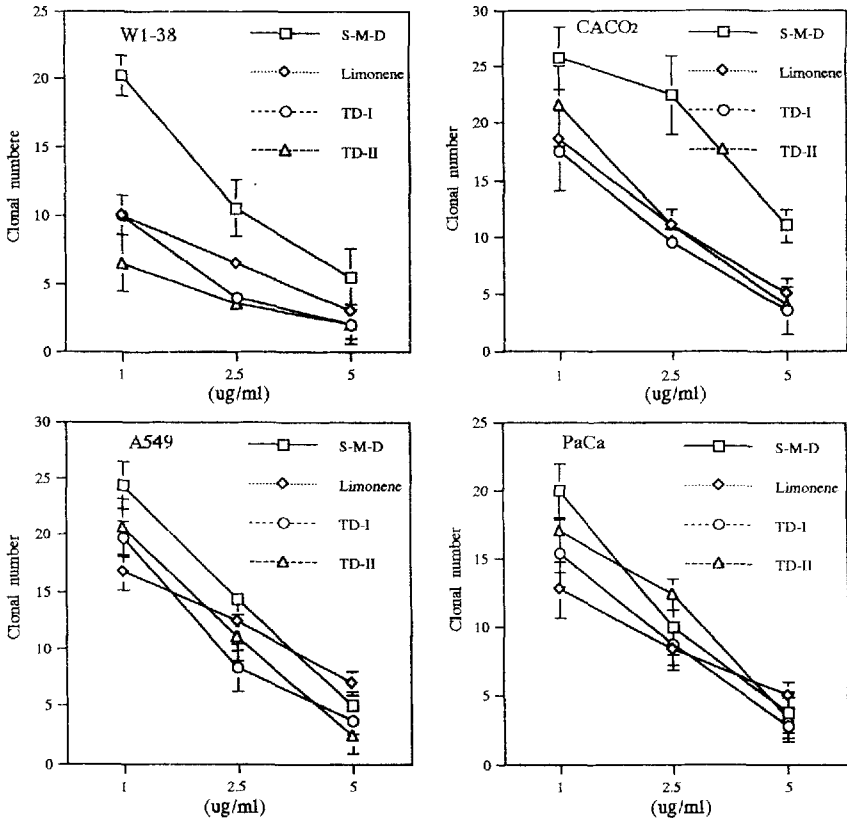
FIGURE 6   Inhibition of clonal growth by test compounds in four tumor cell lines after ten days of treatment. Results are mean ± SD of three cultures performed with each test compound on cells in soft agar.

change in membrane P21^{ras} (Fig. 11A,B). There was an increase in cytosolic P21^{ras} by SMD, TD-I and TD-II in CACO$_2$, but no evident effects were seen in total and membrane P21^{ras} (Fig. 11C,D). Only d-limonene reduced membrane P21^{ras} expression in A549 cells after 48 h of exposure of test compounds (Fig. 11E,F). The significant cytosolic accumulation of P21^{ras} and the decrease in total and membranous P21^{ras} were also noted in PaCa cells with 2.5 µg/ml SMD and d-limonene of treatment for 48 h (Fig. 11G,H). The changes in PaCa cell P21^{ras} membrane association and GJIC induced by d-limonene and SMD were accompanied by changes in morphology (data not shown) and reduced ability to grow in soft agar (Fig. 6). The inhibition of anchorage-independent growth by the test
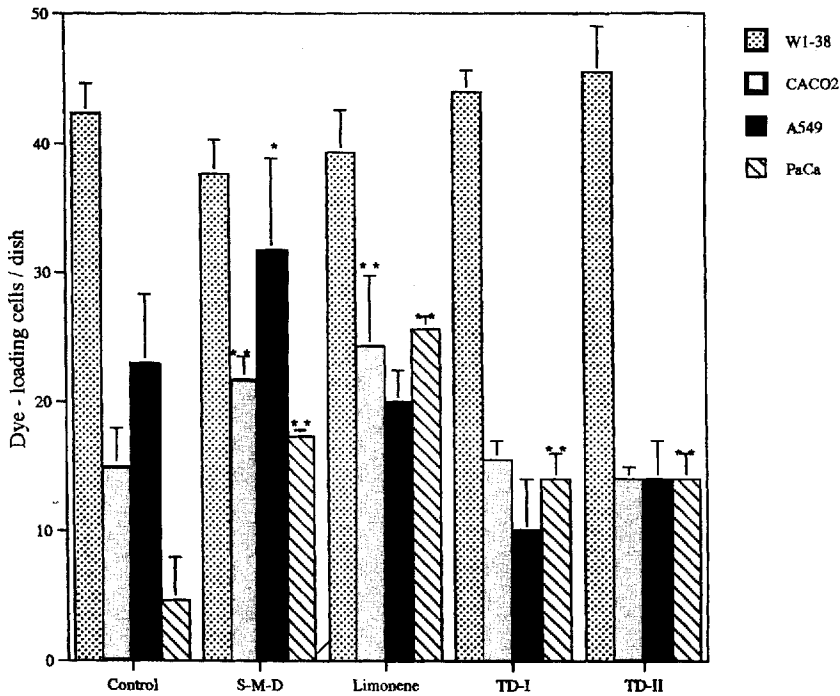
FIGURE 7   Enhancement of GJIC in WI-38, $CACO_2$, A549 and PaCa tumor cell lines by test compounds after 48 h of treatment. Results are mean ± SD of triplicate determinations. Significantly different from control; $*P < 0.05$, $**P < 0.01$.

compounds was at lower concentration than required to inhibit growth on plastic dishes.

A number of proteins have been found to be post-translationally modified by attachment of an isoprenoid group derived from mevalonic acid, and the importance of prenylation for the function of the modified protein is being intensively stuided. The present study demonstrates that porcine kidney epithelial-like LLC-PK1 cells, human lung adenocarcinoma A549 cells and human pancreatic cancer PaCa cells contain distinct FPTase and geranylgeranyl protein transferase. FPTase could catalyze the transfer of the farnesyl group of FPP to $P21^{H-ras}$ protein ending with the carboxy-terminal sequence CAAX forming a thioether linkage to the cysteine residue. A hexapeptide, TKCVIM, was found to competitively inhibit FPTase activity. This peptide blocks FPTase activity without itself being farnesylated, while with S-methyl-L-Cysteine, FPTase activity was slightly decreased. This may be because CAAX tetrapeptide comprises the minimum region required for interaction of the protein substrate with enzyme.
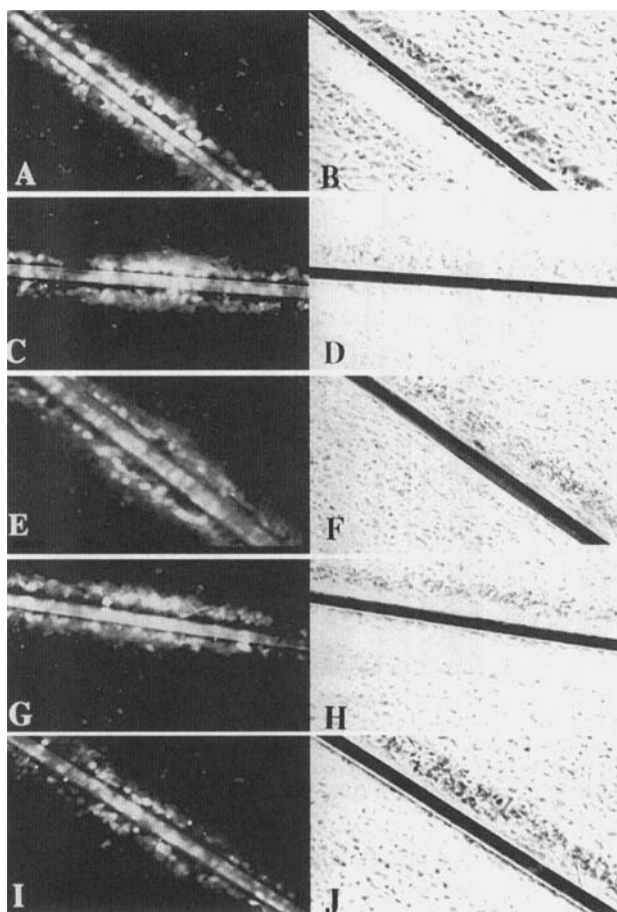
FIGURE 8   Effect of test compounds on GJIC in W1-38 tumor cells, (A, C, E, G, I) show
epifluorescent images, and (B, D, F, H, J) phase-contrast micrographs. The cells were with
0.1% DMSO (control cells, A and B), or 2.5 µg/ml SMD (C and D), 2.5 µg/ml d-limonene (E
and F), TD-I (G and H) and TD-II (I and J) for 48 h.

It is highly likely that LLC-PK1, A549 and PaCa cells also contain
GGPT-II activity and we are currently investigating these. Since this
enzyme recognizes more extensive features in the sequence or structure of
the target proteins, a different assay procedure is necessary and protein-
substrate-based assay is required to investigate the presence of such an
activity.

Turmeric derivatives are compounds containing phenolhydroxyl, having
modified turmeric structure. Turmerics have been reported to possess
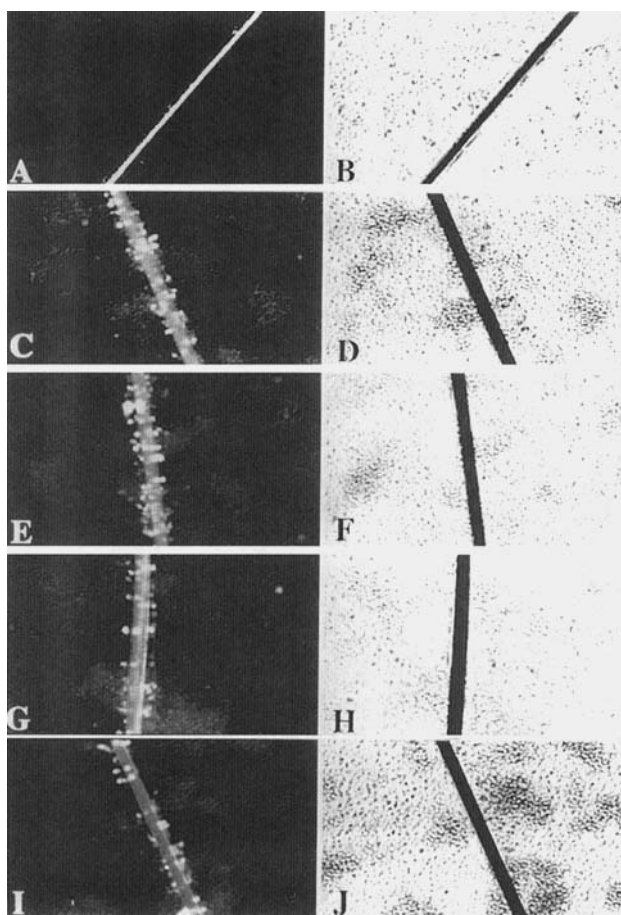
FIGURE 9   Enhancement of dye coupling PaCa by test compounds, demonstrated by scrape-loading dye transfer, (A, B, E, G, I) shows epifluorescent images, and (B, D, F, H, J) phase contrast micrographs. The cells were treated with 0.1% DMSO (control cells, A and B), or 2.5 μg/ml SMD (C and D), 2.5 μg/ml d-limonene (E and F), TD-I (G and H) and TD-II (I and J) for 48 h.

stronger cancer chemopreventive and chemotherapeutic activities [16], and enhance GJIC [17]. Our experiment found that turmeric derivatives, TD-I and TD-II, inhibit porcine kidney epithelial-like LLC-PK1 cell FPTase activity with IC_{50} of 0.25 and 0.29 mM, respectively.

Gallotannin, a naturally occurring polyphenol compound from *Rhus chinensis*, was identified as FPTase inhibitor by this FPTase assay. It not only inhibited LLC-PK1 cell FPTase, but also human lung adenocarcinoma A549 cell and human pancreatic cancer PaCa cell FPTase activities with
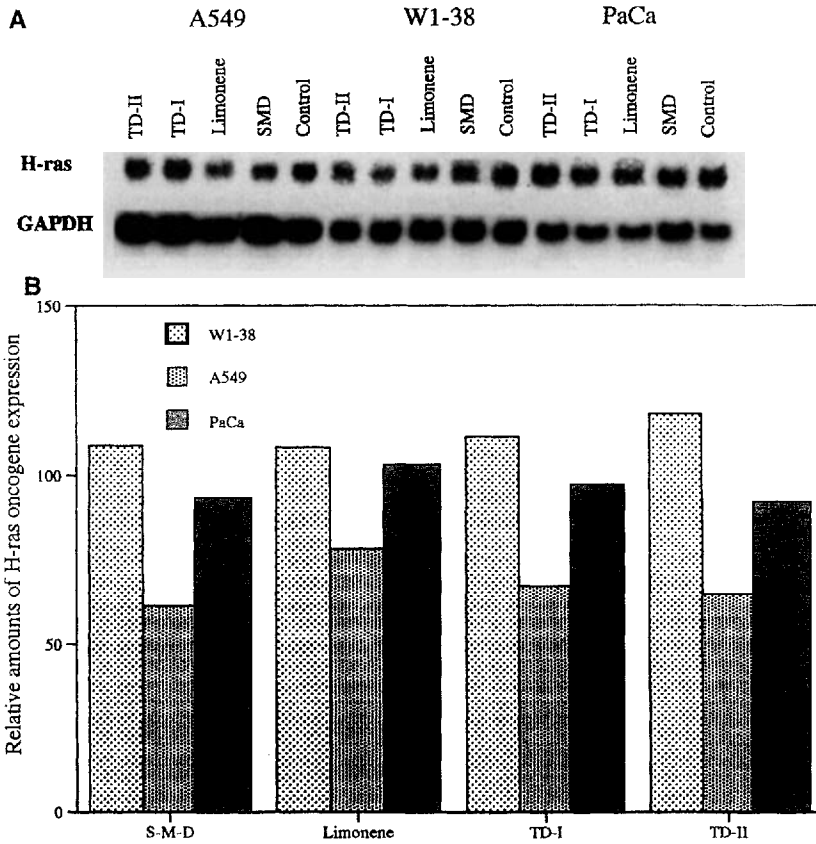
FIGURE 10   Effect of test compounds on H-ras oncogene expression from W1-38, A549 and PaCa cells. A: The total RNA was isolated. Fractionation of RNA by agarose gel electrophoresis, Northern blotting and hybridization to the ^{32}P-labeled H-ras cDNA probe are described in Experimental Section. Loading of RNA was monitored by hybridization to labeled GAPDH. B: Relative amounts of H-ras oncogene expression, as determined by laser densitometer.

IC_{50} of 0.2, 0.19 and 1.44 mM, respectively. Gallotannin did not act as far-nesyl transfer substrate nor did it act as competitive inhibitor with respect to the acceptor protein. The mechanism of inhibition remains to be deter-mined. Gallotannin possesses a broad range of effects which include the inhibition of metabolic activation of carcinogen which elicits DNA damage, the scavenge of the DNA-reactive metabolites of benzo(α) pyrene. antiinitiation, antimutagenic, antipromotion and antioxidant activities [18]. It also inhibited the growth of animal tumors. These activities appear to be distinct from the FPTase inhibition.
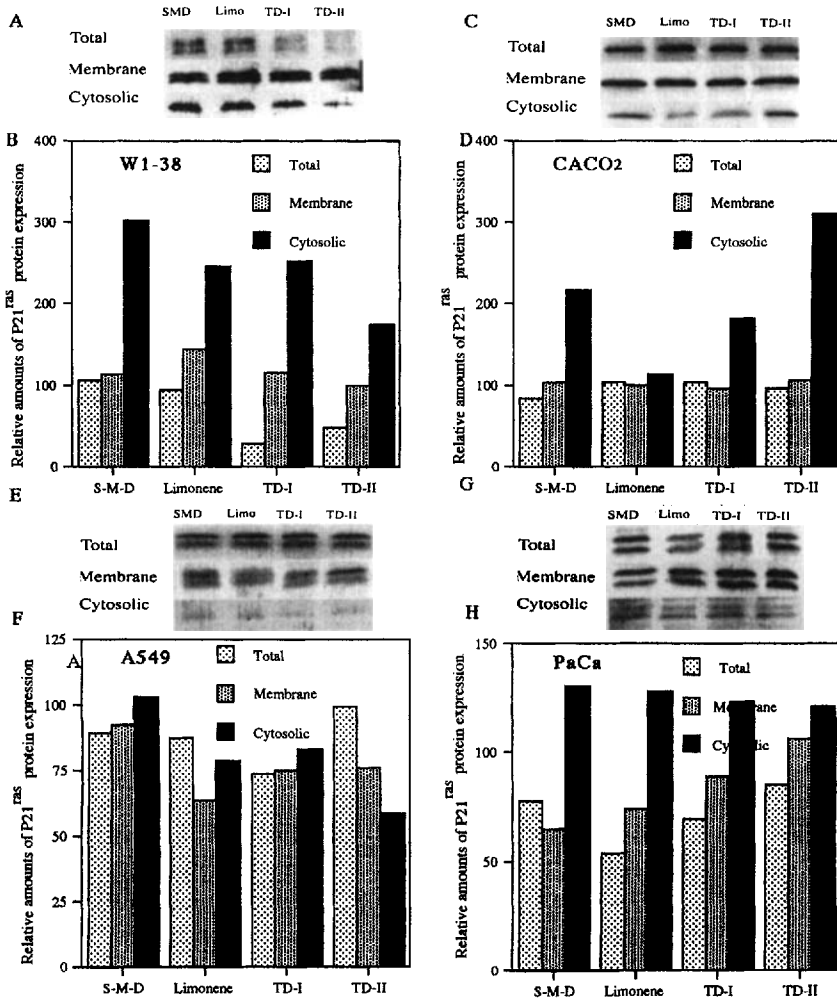
FIGURE 11 A, C, E and G: Effect of test compounds on P21^{ras} protein expression in W1-38, CACO$_2$, A549 and PaCa. The total cellular, membrane and cytosolic proteins were extracted and separated by SDS-PAGE electrophoresis. P21^{ras} proteins were detected by Western blotting with pan anti-P21^{ras} antibody as described in Experimental Section. Molecular size of protein was measured using protein molecular weight marker (Daiichi Pure Chemicals, Japan) as the standard; B, D, F and H: Densitometic anslysis of relative amounts of P21^{ras} protein expression.

d-Limonene, produced by plant cells, is one of the end-products of the mevalonate pathway. It is metabolized from geranyl pyrophosphate. This compound is of particular interest since it possesses chemopreventive activity against spontaneous and chemically induced rodent tumors [19]. The

effect appears to be at both initiation and promotion stages, as deduced from the study in the rat mammary carcinogenesis model. In addition, d-limonene has chemotherapeutic activity, causing regression of mammary tumors. Furthermore, inhibition by d-limonene of prenylation of a class of 21 26 kDa proteins, including Ras, was observed in NIH3T3 and human mammary epithelial cells [20]. Our results suggest that it inhibits FPTase activity in a dose-dependent manner by this direct FPTase assay. Its above-mentioned activities appear to be distinct from the FPTase inhibition activity. Recently, due to the therapeutic efficacy of monoterpenes in experimental model systems, clinical evaluation of this class of compounds has begun in advanced cancer patients. A Phase I trial of limonene is in progress in the UK. Efforts in the US will target perillyl alcohol for Phase I testing [21]. That limonene is used in clinical research will facilitate the screening and development of protein isoprenylation inhibitors and open a prospective future for the treatment of some solid tumors with high ras protein mutant.

The test compounds used in our study possessed stronger inhibition of proliferation on the four human solid tumor cell lines WI-38, CACO$_2$, A549 and PaCa cells *in vitro*. d-Limonene and SMD-reduced P21^{ras} membrane association and concomitantly accumulated cytosolic P21^{ras} in PaCa cells. This was manifested as enhanced GJIC, and reduced ability to grow in soft agar. These effects occurred with d-limonene and SMD at doses that did not inhibit the growth of PaCa cells on plastic dishes. These data indicated that limonene and SMD-reduced P21^{ras} membrane association and enhanced GJIC at nontoxic, and non-growth-inhibitory concentrations. Our results and those of Dotoo *et al.* [22] and Vanhamme *et al.* [23], support the hypothesis that pharmacological inhibition of P21^{ras} membrane association is correlated with enhancement of GJIC at non-growth-inhibitory drug concentrations.

Our data suggest that the effects of d-limonene and SMD on PaCa cells were due to the inhibition of P21^{ras} membrane association and the enhancement of GJIC. d-Limonene, however, also inhibited the isoprenylation of 21–26 kDa small G proteins [24] and FPTase.

In the last few years, many lines of evidence have been accumulated showing the important role of oncogene in the process of malignant progression. On the other hand, a lack of ability to communicate has been suspected to be involved in the anarchical behavior of cancer cells. It was thus all the more interesting to investigate the effects of the ras oncogene (which has been found activated in 30% of the investigated cancer cells) on GJIC. In our experiment, we found that cancer cell lines with higher ras

gene mutation rate communicate less than those with lower ras gene mutant rate, i.e., ras gene mutation rate is inversely correlated with GJIC. It suggests an important effect of the ras oncogene on GJIC.

How could the ras gene product interfere with GJIC? According to the reports of Musil *et al.* [25], Saez *et al.* [26], and Dotoo *et al.* [22], cAMP quickly increases junctional conductance and stimulates phosphorylation of the principal gap junction 26-kDa protein; GJIC might thus be dependent on cAMP-dependent phosphorylation of the principal gap junction polypeptide. Moreover, the activated ras oncogene is known to interfere with two metabolic pathways that are known to control intercellular protein phosphorylations: it down-modulates enzymatic activities of the adenylate cyclase [27,28]. In summary, our results showed tumor cell lines contained higher FPTase activity than those of normal cell line, the high ras gene mutation cells may exhibit the loss of GJIC; the inhibition of ras product is closely correlated with the enhancement of GJIC. We postulate that the effects of ras gene mutation or ras product expression may be due to interferences with the phosphorylation of the gap junction protein connexin. The compounds containing polyphenol hydroxyl may be a new source of FPTase inhibitors.

EXPERIMENTAL SECTION

General experimental procedure Salviol derivative was synthesized by Professor Lianniang Li (Institute of Materia Medica, CAMS); d-limonene was purchased from the Aldrich Chemical Co. (Milwaukee, WI) at the highest chemical purity available; turmeric derivatives were synthesized by Professor Dakuan Liu (Institute of Materia Medica, CAMS); gallotannin was extracted from *Rhus chinensis*; retiniod acid derivative was synthesized by Professor Siping Xu (Institute of Materia Medica, CAMS); S-methyl-L-cysteine was a gift from Dr. S. Fukushima (Department of Pathology, Osaka City University); six residue peptide TKCVIM was synthesized by BEX Co., Ltd. (Tokyo, Japan); recombinant wild-type human P21[H-ras] protein, farnesyl pyrophosphate, geranylgeranyl pyrophosphate and phenylmethyl sulfonyl fluoride were purchased from Sigma Chemical Co.; leupeptin hemisulfate monohydrate was obatined from Wako Pure Chemical Industries, Ltd; DEAE-Sephacel and activated CH sepharose 4B were provided by Pharmacia LKB Biotechnology; [³H]FPP (22.5 Ci/mmol) and [³H]GGPP(19.3 Ci/mmol) were purchased from NEN/DuPont (New England Nuclear); lucifer Yellow CH was obtained from Sigma Chemical Co; rhodamine dextran was purchased from Molecular Probe; H-ras

cDNA(1.02 kb) was prepared in a bacterial expression system with H-ras provided by Health Science Research Resources Bank; pan anti-P21[ras] mouse monoclonal antibody was obtained from Calbiochem (Cambridge, MA); biotin-conjugated antimouse IgG antibody was purchased from Vector Laboratories Inc. (Burlingame, CA); $[\alpha^{-32}P]dCTP$ (3000 ci/mol) was purchased from Amersham; tissue culture media and fetal calf serum were products of Gibco Laboratories (Grand Island, NY) and all other chemicals and reagents were of reagent grade or better and used without further purification.

Cell culture Cell lines used in this study included porcine kidney epithelial-like LLC-PK1 cells, human lung adenocarcinoma A549 cells, human lung squamous W1-38 cells, human colon carcinoma $CACO_2$ cells and human pancreatic cancer MIA PaCa-2 cells. LLC-PK1 cells were maintained at 37°C in 199 medium with 10% fetal calf serum. A549, W1-38 and PaCa cells were cultured in Eagle's minimal essential medium with 1% nonessential amino acid and 10% fetal calf serum. $CACO_2$ cells were cultured in MEM medium with 20% fetal calf serum. The cells were subcultured every three days.

MTT assay 1200 cells per well plated in 96-well plates. After culturing for 24 h, test compounds were added onto triplicate wells with different concentrations, and 0.1% DMSO for control. After four days of incubation, 10 μl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well, and after shaking for 1 min the plate was incubated further for 4 h. Formazan crystals were dissolved with 100 μl DMSO. The absorbance (OD) was quantitated with microplate spectrophotometer at 570 nm. Wells containing no drugs were used as blanks for the spectrophotometer. The survival of the cells were expressed as percentage of untreated control wells.

Partial purification of FPTase According to Reiss' method [29], purification steps were slightly modified. The cells grown in log phase (4×10^8) were washed with ice-cold phosphate-buffered saline (PBS) and incubated at 4°C for 30 min in lysis buffer (50 mM Tris-chloride pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM leupeptin), then sonicated on ice with 20 strokes of a tight pestle. The homogenate was centrifuged at 10,000 $\times g$ for 15 min at 4°C, then the resulting supernatant fraction was centrifuged at 10,000 $\times g$ for 60 min at 4°C. The supernatant was brought to 30% saturation with solid ammonium sulfate, stirred for 30 min on ice, and centrifuged 12,000 $\times g$ for 10 min to remove precipitated proteins. The resulting supernatant was adjusted to 50% saturation with ammonium sulfate, and the

precipitate was dissolved in a buffer of 20 mM Tris-chloride (pH 7.5) containing 1 mM DTT and 20 μM $ZnCl_2$ and dialyzed for 4 h against 2 l of the same buffer, and then 2 l of fresh buffer of the same composition for 12 h. The dialyzed material was chromatographed on DEAE-Sephacel column that had been equilibrated with 50 mM Tris-chloride (pH 7.5) containing 1 mM DTT, 20 μM $ZnCl_2$, and 0.05 M NaCl. The column was washed with the same buffer, and proteins were eluted with a linear gradient of NaCl (0.05–0.5 M) in the same buffer. Fractions (1 ml) were collected, and those containing transferase activities were pooled separately, divided into multiple aliquots, and stored at −80°C. Only the peak fraction was used for experiments.

Two milligrams of partially purified FPTase was applied to a 1 ml peptide (TKCVIM) column equilibrated in 50 mM Tris-chloride (pH 7.5) containing 0.1 mM NaCl and 1 mM DTT. The solution, which passed through the column, was cycled three times at room temperature. The column was washed with 10 ml of the same buffer. The enzyme was then eluted with 10 ml of 50 mM Tris-succinate (pH 5.0) containing 1 mM DTT, 0.1 mM NaCl. This eluate was concentrated and brought to 20 μl.

Assay for FPTase activity   FPTase activity was determined by measuring the amount of [³H]farnesyl transferred from [³H]FPP to P21[H-ras] protein. Each 25 μl reaction mixture contained 50 mM Tris-chloride (pH 7.5), 25 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 2.5–5 μM P21[H-ras], 10–15 pmol [³H]FPP (25,000–30,000 dpm/pmol) or [³H]GGPP and 6–7 μg of partially purified FPTase, and different concentration of test compounds. Additions or changes in the reaction mixture for certain experiment are indicated in the appropriate figure legends. After incubation for 1 h at 37°C in borosilicate tube in the dark, the reaction was stopped by addition of 0.5 ml of 4% SDS and then 0.5 ml of 30% TCA. The tubes were vortexed and left on ice for 1 h, after which 2 ml of 6% TCA, 2% SDS solution was added. The mixture was filtered on a 2.4 cm glass-fiber. The tubes were rinsed twice with 2 ml of the same solution, and each filter was washed five times with 2 ml of 6% TCA, dried, and counted in a scintillation counter. A blank value was determined in parallel incubation mixtures containing no enzyme or P21[H-ras] protein. This blank value was subtracted before calculating the percentage of control values. Protein content was determined using the Bio-Rad Protein Assay.

To identify farnesylation H-Ras P[21] protein, SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli [30]. The above-mentioned reactions were terminated by the addition of 5 × SDS sample buffer and analyzed by 12.5% SDS/PAGE. After electrophoresis, gels were

fixed, treated with enlightening and exposed at $-70°C$ to Fuji film in the presence of intensifier screens for 14 days. Position of the farnesylated H-Ras P^{21} protein was identified.

Clonal assay One hundred cells were plated per 35 mm plastic dish and treated for nine days with the test compounds in DMSO or 0.1% DMSO (control group). After nine days of treatment, the cells were fixed in 10% buffered formalin and stained with 0.25% crystal violet. Colonies of more than 50 cells were counted.

Anchorage-independent growth assay One hundred cells were plated in culture medium containing 0.3% agar onto a base of 0.6% agar in culture medium. The cultures were gently overlaid with the test compounds. After 10 days of treatment, colonies of more than 50 cells were counted.

Scrape-loading/dye transfer assay Four types of monolayer of tumor cells in 35 mm dishes were treated with the test compounds or 0.1% DMSO for 48 h, and then the culture medium was removed and the cells were rinsed with phosphate-buffered saline (PBS). Lucifer Yellow CH and Rhodamine dextran (both of 0.05% in PBS, 2 ml/dish) were added to each dish, and the cells were scraped with a fine surgical blade. The scraping causes a transient rupture of the cell membranes and permits entrance of the dye. In GJIC-competent cells, dye spreads from these dye-loading cells to neighboring cells; in GJIC-incompetent cells, dye remains in the dye-loaded cells. Three minutes after scraping, the cells were washed with PBS, refed with fresh PBS, and photographed (phase-contrast micrograph and epifluoresence image).

Isolation of total RNA and Northern-blot analysis Cells were harvested and lysed in 4 M guanidine thiocyanate lysis buffer, and total RNA was extracted by the method of Chomczynski and Sacchi [31] with some modification as described earlier [32]. The concentration of total RNA was determined by measuring the absorbance at 260 nm with a spectro-photometer. Total RNA (20 µg/lane) was electrophoresed through a 1% agarose gel containing 6% formaldehyde and transferred onto a Hybond-N membrane. The membrane was UV cross-linked (1000 m J/cm^2), pre-hybridized at 42°C for 2 h, and hybridized to the ^{32}P-Labelled H-ras cDNA probe overnight at 42°C. The H-ras cDNA, a fragment of about 1.02 kb, was labelled with [^{32}P]dCTP using a random DNA labelling Kit (Amersham LIFE SCIENCE, England). After hybridization, the Hybond-N membrane was washed three times for 20 min each time in $2 \times$ SSPE and 0.1% SDS buffer at 42°C, once for 30 min in $1 \times$ SSPE and 0.1% SDS at 42°C and once for 15 min in $0.1 \times$ SSPE and 0.1% SDS at room temperature. The membrane was autoradiographed using Fuji film

with intensifying screen at $-70°C$ for 96 h. After stripping, the same membrane was rehybridized to ^{32}P-labelled GAPDH cDNA to verify equal loading of RNA onto the gel. The autoradiograph was scanned with a densitometer, and the results were integrated and normalized to the value of the GAPDH.

Western blotting of P21^{ras} For total cellular P21^{ras} analysis by Western blotting, cells were harvested by scraping, pelleted by centrifugation (1500 $\times g$), and solubilized in lysis buffer (1% Triton X-100; 0.1% sodium dodecyl sulfate (SDS); 10 mM sodium phosphate buffer, pH 7.2; 50 mM Tris base, pH 7.6; 2 mM phenylmethylsulfonyl fluoride (PMSF) and 250 kU/ml aprotinin) by vortexing and incubating on ice for 10 min. The lysates were clarified by centrifugation at 100,000 $\times g$ for 30 min. For Western-blotting analysis of cytosolic and membranous P21^{ras}, the pelleted cells were solubilized in ice-cold PBS-PMSF (10 mM phosphate buffer, pH 7.2 and 2 mM PMSF) by homogenization and centrifuged at 100,000 $\times g$ for 30 min. The supernatants (cytosolic fractions) were saved, and the pellets (membranous fractions) were solubilized in lysis buffer as described above. The protein levels in the cell fractions were determined with the Bio-Rad Protein Assay using bovine serum albumin as a standard. Twenty micrograms of protein per lane was electrophoresed on 15% polyacrylamide-SDS minigels [30] in SDS sample buffer. The proteins were then transferred to PVDF membrane. After electrophoretic transfer, membrane was blocked for 2 h in blocking buffer (3% BSA and 0.25% normal horse serum). The blots were then analyzed using anti-P21^{ras} (primary antibody) and biotin-conjugated antimouse IgG (secondary antibody) and developed in AEC solution (0.8% amino-ethylcarbazole and 0.1 M acetate, pH 5.2). The pan-ras antibody recognizes both normal and oncogenic forms of P21^{ras}. P21^{ras} bands were scanned with a densitometer.

Acknowledgments

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