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INHIBITION OF TUMOR GROWTH BY S-3-1, A SYNTHETIC INTERMEDIATE OF SALVIANOLIC ACID A

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Salvianolic acid A (**1**) is one of the active components from *Salvia miltiorrhiza*, which was found to suppress the growth of mouse tumors. S-3-1 (a 2-allyl-3,4-dihydroxybenzaldehyde, **2**) is a synthetic intermediate of a salvianolic acid A derivative with strong inhibitory effects on the growth of cancer cells *in vitro*. The inhibitory effects of **2** on tumor growth and its molecular targets were studied. **2** significantly suppressed the growth of mouse Lewis lung carcinoma, S180 sarcoma and H22 hepatic carcinoma in a dose-dependent manner. With a simple scrape-loading dye transfer method, 20 $\mu\text{g/ml}$ of **2** was found to significantly enhance gap junction intercellular communication (GJIC) in human pancreatic adenocarcinoma PaCa Cells, human lung epithelial carcinoma W₁-38 cells and human lung adenocarcinoma A549 cells, but **2** had no marked effect on GJIC in human colon cancer CACO₂ cells. With Northern blot analysis, **2** was found to inhibit the expression of c-myc gene in A549 cells and have no marked effect on H-ras oncogene expression, and increase the cellular P53 mRNA contents, though it did not affect the expression of RB tumor suppressor gene. **2** also suppressed the P46 (JNK/SAPK) expression in A549 cells. Western blot analysis was applied to visualize the P21^{ras} protein. Results shows that **2** at concentrations ranging from 10 to 20 $\mu\text{g/ml}$ decreases the contents of the membranous P21^{ras} and total P21^{ras} and increases the contents of cytosolic P21^{ras} protein in a time-dependent manner. However, **2** had no significant effects on farnesyl protein transferase activities at the concentrations that could efficiently decrease the membranous P21^{ras} content. This suggested that **2** might suppress tumor growth partly through enhancement of GJIC and reversion of the transformed phenotypes. The other mechanisms may be that **2** can suppress the overexpression of c-myc oncogene, inhibit the function of Ras oncoprotein, increase the expression of P53 tumor suppressor gene and interrupt P46-associated mitogen-activated pathway other than farnesylation of Ras protein.

Keywords: Salvianolic acid intermediate; Tumor growth; Inhibition; P21^{ras} protein; Oncogene

INTRODUCTION

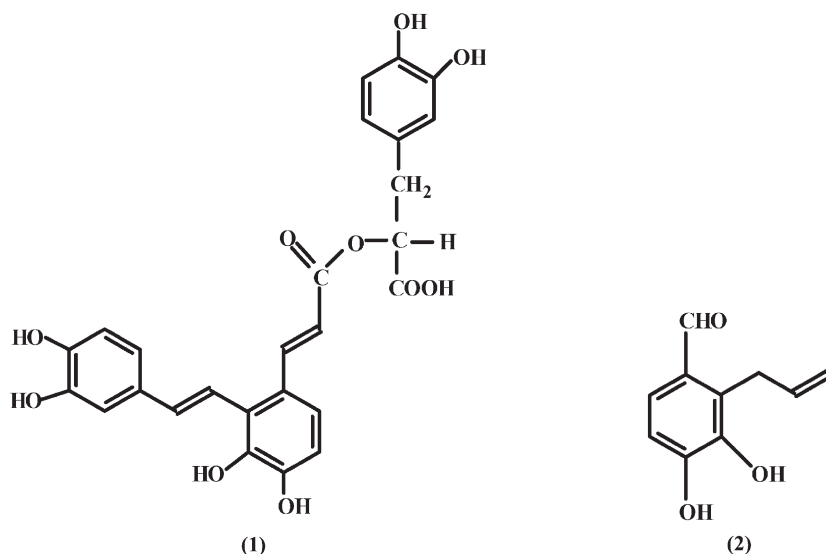
Efficiency of chemotherapy is limited by at least two factors, i.e. metastasis and drug resistance of tumor cells. After long-term administration of an anti-cancer drug, tumor cells may develop complicated resistance machinery to escape the toxicity action of this drug.

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It is believed that development and administration of drugs with novel mechanisms to kill or prevent the growth of tumor cells is one of the best strategies to increase the efficiency of chemotherapy. Some of the new targets of anti-cancer drugs include gap junction, telomerase, signal transduction elements and farnesyl protein transferase (FPTase). Compounds derived from plants, such as Chinese medicinal plants or herbs, may be candidates for development of novel anti-cancer drugs or leading compounds with high efficiency of killing tumor cells.

As a traditional Chinese medicine, *Salvia miltiorrhiza* has been widely used for the treatment of angina pectoris and coronary diseases. In recent years, the anti-tumor effects of *Salvia miltiorrhiza* have been well documented. This medicine was reported to inhibit cancer cell growth and induce apoptosis in human hepatoma and leukemia cell lines [1,2].

Salvianolic acid A (**1**) is one of the active components derived from *Salvia miltiorrhiza*, which was found to suppress the growth of mouse Lewis lung carcinoma, B16 melanoma and S-180 sarcoma. During the synthetic studies of this compound, a series of intermediates and their analogs were screened for their activities; among them **2** showed strong inhibitory effects on the growth of cancer cells *in vitro*. After extensive investigation of the pharmacological characteristics of this compound, we found that **2** may be useful to prevent the outgrowth of tumors [3]. In this paper, we report the inhibitory effects of **2** on tumor growth, and try to explore the molecular targets of this compound.



RESULTS AND DISCUSSION

Inhibitory Effect on the Growth of Cancer Cells

The effects of **2** on the growth of cancer cells were examined by MTT assay. **2** inhibited the growth of a large variety of cancer cells that include those derived from small cell lung

TABLE I Inhibitory effect of **2** on the growth of cancer cells

Cell lines	IC ₅₀ (mol/l)
NIH-3T3	3.0×10^{-4}
CHL	2.48×10^{-4}
SCLC	1.36×10^{-4}
A549	1.55×10^{-4}
KB	1.80×10^{-4}
HCT-8	1.99×10^{-4}
PLA-C	6.80×10^{-5}
BGC	5.00×10^{-5}
W ₁ -38	4.2×10^{-5}
CACO ₂	3.8×10^{-5}
PaCa	7.0×10^{-5}

carcinoma, lung adenocarcinoma, oral squamous carcinoma, colon cancer, gastric cancer, and pancreatic cancer (Table I). The half inhibitory concentrations (IC₅₀) ranged from 3.8×10^{-5} to 2.0×10^{-4} (mol/l). Similarly, **2** also inhibits the growth of mouse or hamster fibroblasts, but the IC₅₀ was relatively higher than those of cancer cells.

Inhibition of Tumor Growth

At dosages of 20 and 40 mg/kg, **2** significantly suppressed the growth of Lewis lung carcinoma (Table II). The inhibitory rates were 33.8 and 42.8%, respectively. On the other hand, **2** was found to suppress the growth of S-180 sarcoma and H22 hepatic carcinoma in a dose-dependent manner. At 40 mg/kg, the inhibitory rates were 41.6 and 34.7% for S-180 sarcoma and H22 hepatoma, respectively. At 80 mg/kg, the inhibitory rate reached about 44% (Tables III and IV).

Enhancement of Gap Junction Intercellular Communication of Human Cancer Cells

Gap junction intercellular communication (GJIC) is one of the major ways for message transfer between connective cells. Transformed cells are always characterized by a lack of GJIC or low efficiency of GJIC, which might suppress the transfer of proliferation control message between connective cells, and lead to lack of contact inhibition of growth in cancer cells. Given that **2** could inhibit the proliferation of cancer cells and outgrowth of solid

TABLE II Inhibition of **2** on mouse Lewis lung carcinoma growth

Groups	Dose (mg/kg)	n	Tumor weight (g) ($\bar{x} \pm s$)	Inhibition rate (%)
Control		10	2.69 ± 0.55	
2	10	10	1.98 ± 0.49	26.39
2	20	10	$1.78 \pm 0.36^*$	33.83
2	40	10	$1.54 \pm 0.48^*$	42.75

**P* < 0.05 vs. control.

TABLE III Inhibition of **2** on mouse S-180 sarcoma growth

Groups	Dose (mg/kg)	n	Tumor weight (g) ($\bar{x} \pm s$)	Inhibition rate (%)
Control		10	1.97 \pm 0.39	
CTX	100	10	0.067 \pm 0.047	96.60
2	20	10	1.46 \pm 0.60	25.90
2	40	10	1.15 \pm 0.51*	41.60
2	80	10	1.09 \pm 0.51*	44.70

* $P < 0.05$ vs. control.

tumors, we decided to investigate the effects of **2** on GJIC of human cancer cells. With a simple scrape-loading dye transfer method, 20 $\mu\text{g/ml}$ **2** was found to significantly enhance GJIC in human pancreatic adenocarcinoma PaCa cells (Table V). At the same concentration, **2** also enhanced GJIC in human lung squamous carcinoma W1-38 cells and human lung adenocarcinoma A549 cells, though the efficiency is relatively lower. However, **2** had no marked effect on GJIC in human colon cancer CACO₂ cells. These results suggested that **2** might suppress tumor growth partly through enhancement of GJIC and reversion of the transformed phenotypes.

Effects on Expression of c-myc, H-ras Oncogenes and P53 and RB Tumor Suppressor Gene in A549 Cells

Overexpression of oncogenes such as c-myc and H-ras is associated with aggressive phenotype of cancer cells. With Northern blot analysis, **2** was found to inhibit the expression of c-myc gene in A549 cells. However, **2** had no marked effect on H-ras gene expression (Fig. 1). On the other hand, **2** significantly increase the cellular P53 mRNA contents, though it did not affect the expression of the RB tumor suppressor gene. These results suggested different roles of individual oncogene or suppressor gene in the outgrowth of specific cancer cells, and **2** might inhibit the growth of cancer cells through suppression of overexpression of the c-myc oncogene and increase the expression of P53 genes.

Suppression of P46 (JNK/SAPK) Expression in A549 Cells

P46 is one of the isoforms of c-jun NH₂-terminal kinase (JNK). Mitogens, such as insulin and TNF- α , may stimulate cells to proliferate via the signal transduction pathway associated with the P46 kinase function. After immunoblotting onto the PVDF membrane, P46 was visualized by peroxidase-catalyzed AEC reaction. Figure 2 shows that the cellular P46 contents decreased dramatically after treatment with **2**, suggesting **2** might interrupt P46-associated mitogen-activated pathway to inhibit the proliferation of cancer cells.

TABLE IV Effect of **2** on mouse H22 hepatic carcinoma growth

Groups	Dose (mg/kg)	n	Tumor weight (g) ($\bar{x} \pm s$)	Inhibition rate (%)
Control		10	1.21 \pm 0.22	
CTX	100	10	0.31 \pm 0.09	74.40
2	20	10	0.92 \pm 0.33	24.00
2	40	10	0.79 \pm 0.44	34.70
2	80	10	0.68 \pm 0.28*	43.80

* $P < 0.05$ vs. control.

TABLE V Enhancement of **2** on gap junction intercellular communication of human cancer cells

Groups	Dose ($\mu\text{g/ml}$)	Cells of fluorescence transfer			
		W ₁ -38	PaCa	A549	CACO ₂
Control		38.0 \pm 5.45	3.00 \pm 0.4	27.0 \pm 5.06	20.7 \pm 3.97
2	20	54.2 \pm 4.78*	21.0 \pm 3.56**	38.5 \pm 7.56*	27.9 \pm 2.78

* $P < 0.05$ vs. control, ** $P < 0.01$ vs. control.

Effect on Cytosolic and Membranous P21^{ras} Contents in A549 Cells

Ras oncoprotein is believed to play roles in the pathophysiology of cell transformation and the pathogenesis of human cancer. Because Ras can function as the mediator of mitogen-associated signal transductions only after it is localized on the cytoplasmic face of the plasma membrane, we tried to investigate the changes of cytosolic and membranous P21^{ras} contents after treatment with **2**. Following isolation of cytosolic and plasma membrane-enriched fractions by centrifugation, Western blot analysis was applied to visualize the P21^{ras} protein. Figure 3 shows that **2** at concentrations ranging from 10 to 20 $\mu\text{g/ml}$ decreased the contents of the membranous P21^{ras} and total P21^{ras}. Consistent with the decrease of the membranous P21^{ras} content, the content of cytosolic P21^{ras} was increased (Fig. 3). Moreover, the increase of cytosolic P21^{ras} content was found to occur in a time-dependent manner. These results suggested that the *Salvia miltiorrhiza* derivative **2** might suppress the function of Ras oncoprotein.

Effects on Farnesyl Protein Transferase Activity

Localization of P21^{ras} on plasma membrane from cytoplasm involves a series of post-translational modification of this protein. The first and obligatory step in this series is the addition of a farnesyl moiety to the cysteine residue of the COOH-terminal of Ras in a reaction catalyzed by FPTase. Given that **2** could decrease the membranous P21^{ras} content, we further investigated the effects of **2** on FPTase activity. The FPTase was partially purified from lysates of porcine kidney epithelial-like LLC-PK cells, human lung adenocarcinoma A549 cells and human pancreatic carcinoma PaCa cells. Regardless of how the FPTase had been derived, Table VI shows that **2** had no significant effects on its activities at concentrations that could efficiently decrease the membranous P21^{ras} content. Therefore, **2** might affect the Ras function through a pathway other than farnesylation of Ras protein.

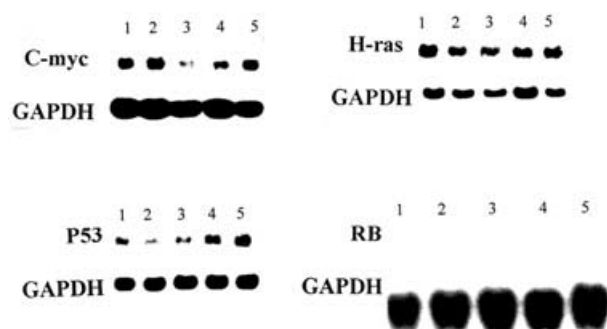


FIGURE 1 Effect of S-3-1 on the expressions of oncogenes c-myc and H-ras, suppressor tumor genes RB and P53 in human adenocarcinoma cell line A549: (1) Control; (2) S-3-1 1 $\mu\text{g/ml}$; (3) S-3-1 5 $\mu\text{g/ml}$; (4) S-3-1 10 $\mu\text{g/ml}$; (5) S-3-1 20 $\mu\text{g/ml}$.

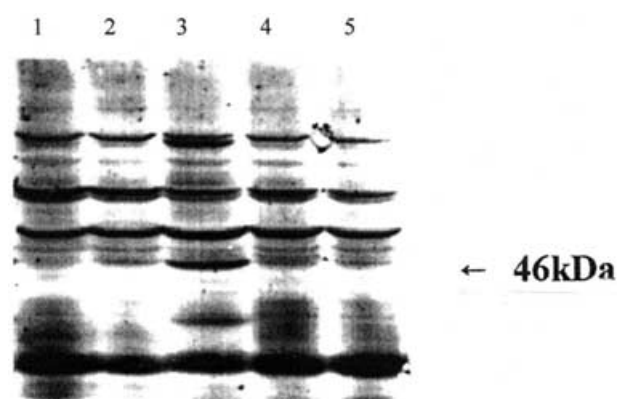


FIGURE 2 Effect of S-3-1 on the p46 tyrosine phosphorylation in human adenocarcinoma A549 cell line: (1) S-3-1 10 $\mu\text{g/ml}$; (2) S-3-1 20 $\mu\text{g/ml}$; (3) Control; (4) S-3-1 20 $\mu\text{g/ml}$ for 12 h; (5) S-3-1 20 $\mu\text{g/ml}$ for 48 h.

Compound **2** is a synthetic intermediate of the active component from salvianolic acid A. Because of its simple structure and small molecular mass, **2** can be easily synthesized on a large scale. Our previous study has suggested that this compound may be useful in preventing the development of cancer. In this study, we tried to explore its direct effects on tumor growth. As shown in the results of this study, **2** exerted inhibitory effects on the proliferation of a large variety of cancer cells, while it suppressed the growth of solid tumors such as Lewis lung carcinoma, H22 hepatic carcinoma and S-180 sarcoma. After intensive investigation of its actions on cancer cells, we found that **2** may exert its anti-cancer activities through relatively novel mechanisms such as enhancement of GJIC, and repression of the functions of Ras as a signaling mediator. This compound was also found to increase the expression of the P53 tumor suppressor gene and to decrease the expression of c-myc oncogene. This compound can be completely dissolved in water and be efficiently administered via p.o. Therefore, we concluded from this study that **2** may be valuable for development as a novel anti-cancer drug.

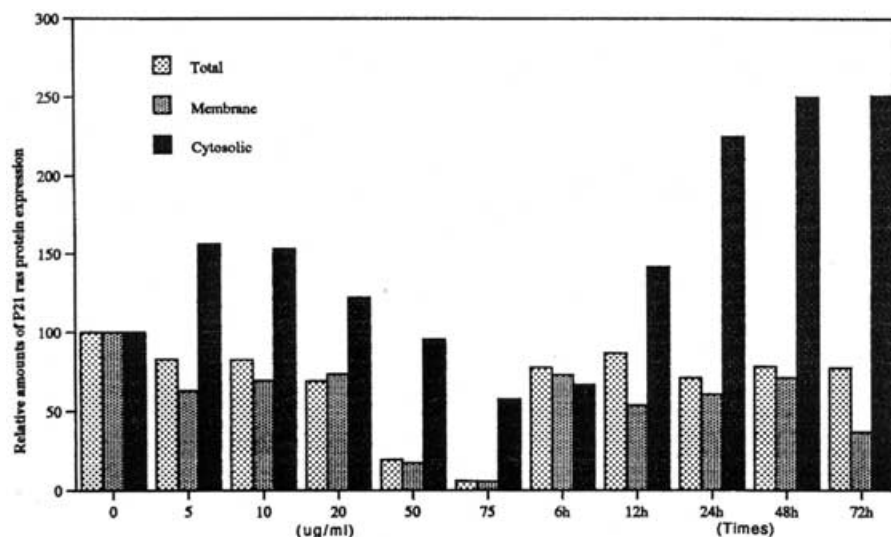


FIGURE 3 Dose-activity and time-efficiency relationship of S-3-1 on the expression of P21^{ras} total protein, membrane protein and cytosolic protein in human adenocarcinoma A549 cell line.

TABLE VI Effect of **2** on farnesyl protein transferase activity

Groups Cell lines	Dose ($\mu\text{g/ml}$)	Amount of isoprenylated P21 ^{H-ras} formation (pmol/h/mg protein)		
		LLC-PK2	A549	PaCa
Control		25.52 \pm 1.02	38.83 \pm 0.59	30.79 \pm 1.19
2	5	23.04 \pm 2.35	35.24 \pm 2.15	29.78 \pm 0.83
	10	23.00 \pm 4.17	33.08 \pm 0.85	29.43 \pm 0.45
	20	21.42 \pm 3.28	32.25 \pm 0.44	27.25 \pm 1.26

Although the factors that cause tumor cells to proliferate in an uncontrolled fashion may be different in different tumors, certain common cellular and biochemical changes do happen. For example, lack of contact inhibition or abnormality of mitogen signaling [4]. When transformed, the cells may lack contact inhibition of growth, partly due to the lack or abnormality of GJIC. In fact, GJIC may be one of the ways for message (including the growth inhibitory message) transfer between connective cells [5]. In this study, **2** was found to enhance GJIC in W1-38, PaCa and A549 cells at relatively low concentrations. The human pancreatic cancer PaCa lost GJIC almost completely, and **2** relatively strongly enhanced the GJIC of this cell line. Enhancement of GJIC may be one of the reasons that **2** suppressed the proliferation of cancer cells.

Mitogen signaling involves certain elements including some of the oncoproteins and protein kinases. Protein tyrosine kinases have aroused extensive interest recently because of protein tyrosine phosphorylation during cellular transformation and the pivotal roles in a large variety of mitogen signaling [6,7]. P45 JNK is one of these protein tyrosine kinases that belong to the mitogen-activated protein kinase family. It can phosphorylate the NH₂-terminal of c-jun and subsequently activate the transcription activity of this protein. P45 has been suggested to be related to the stimulation activity of insulin, TNF- α and epinephrine. This kinase can be activated or inactivated by drugs such as tamoxifen [8]. It is exciting that **2** could decrease the expression of this protein in cancer cells. The decrease of the cellular content of this protein may bring about a decrease of the total cellular activity of this kinase, leading to interruption of the transduction of extracellular mitogenic signals.

Ras is one of the oncoproteins that plays a role in mitogen signaling and in the uncontrolled proliferation of cancer cells. This protein and its gene have been the subject of intense investigation for nearly two decades. Mutated forms of cellular ras genes have been found to be among the most common genetic abnormalities associated with human cancers. Targeting the functions of Ras is reasonably assumed to be one of the strategies for developing anti-cancer drugs. One attempt is to disrupt the post-translational modifications of Ras. Ras is a membrane-bound GTP-binding protein though it is synthesized as a cytosolic precursor that is ultimately localized on the cytoplasmic face of the plasma membrane after a series of post-translational modifications. The first and rate-limiting step in the mature process of Ras is the addition of a farnesyl moiety to the cysteine residue of the COOH-terminal by FPTase. By Western blot analysis, we found in this study that the content of membrane-bound P21^{ras} was decreased while the cytosolic P21^{ras} content was increased after the cells had been treated with **2**, though **2** had little effect on the activity of FPTase. This result implied that **2** might dissociate Ras protein from the plasma membrane. The same effect was observed in the immunohistochemistry staining of P21^{ras}. Regardless of the cause, the decrease of membrane-bound Ras protein suggested that **2** might reverse the abnormal signaling mediated by Ras. We believe this may be another reason that **2** inhibits tumor growth.

EXPERIMENTAL SECTION

Chemicals

Salvianolic acid intermediate **2** was provided by the Department of Chemical Synthesis, Institute of Materia Medica (CAMS); Lucifer Yellow CH was obtained from Sigma Chemical Company; Rhodamine-Dextran was purchased from Molecular Probe; H-ras cDNA fragment was provided by Health Science Research Resources Bank; c-myc, P53 and RB cDNA fragments were obtained from Sino-America Biological Company (Beijing, China); Anti-P21^{ras} and anti-P46 JNK mouse monoclonal antibodies were obtained from Calbiochem (Cambridge, MA); Biotin-conjugated anti-mouse IgG antibody was purchased from Vector Laboratories, Inc. (Burlingame, CA.); [α -³²P]dCTP (3000 Ci/mol) was purchased from Amersham. Farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Company, [³H]FPP (22.5 Ci/mmol) and [³H]GGPP (19.3 Ci/mmol) were purchased from NEN/DuPont (New England Nuclear); 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 NIH-3T3 mouse fibroblast, CHL Chinese hamster lung fibroblast, human small cell lung carcinoma SCLC cells, human lung adenocarcinoma A549 cells, human oral squamous carcinoma KB cells, human colon cancer HCT-8 cells, human lung squamous carcinoma PLA-C cells, human gastric cancer BGC cells, LLC-PK1 porcine kidney epithelial-like cells, human lung squamous carcinoma W1-38 cells, human pancreatic cancer PaCa cells and human colon cancer CACO₂ cells. SCLC cells, A549 cells, KB cells, HCT-8 cells, PLA-C cells and BGC cells were normally cultivated in RPMI 1640 medium supplemented with 10% calf serum. LLC-PK1 cells were maintained in 199 medium containing 10% calf serum. W1-38 cells and PaCa cells were maintained in DMEM medium supplemented with 10% calf serum and 1% non-essential amino acids. CACO₂ cells were cultivated in MEM medium containing 20% calf serum. The cells were subcultured every three days.

MTT Assay

1200 cells were plated into each well of 96-well plates. Compound **2** was dissolved in water and added into the wells the next day. After four-days' incubation, 10 μ l MTT solution (5 mg/ml) was added to each well. Following 4 h incubation, formazan crystals were dissolved in 100 μ l DMSO. The absorbance values at 570 nm were determined with a microplate spectrophotometer to evaluate the rate of survived cells.

Transplantable Tumor Inhibition Assay

Under sterile conditions, the ascites from animals with S-180 sarcoma or H22 hepatic tumor, or ground Lewis lung carcinoma were diluted with sterile normal saline (1:3) followed by subcutaneous inoculation of the mouse armpit at 0.2 ml per mouse. **2** was administered p.o. for 10 days once daily. On day 11, the animals were executed and the tumors were weighed.

Scrape-loading/dye Transfer Assay

Prior to assay, monolayers of tumor cells (W1-38, PaCa, A549 and CACO₂ cells) in 35 mm dishes were treated with **2** or water for 48 h. After rinsing with PBS, Lucifer Yellow CH and Rhodamine-dextran (both 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. By contrast, in GJIC-incompetent cells, dye remains in the dye-loaded cells. Three minutes after scraping, the cells were washed with PBS, re-fed with fresh PBS, and photographed under both the phase-contrast microscope and the epifluorescence microscope.

Northern Blot Analysis

Cells were harvested and lysed in 4 M guanidine thiocyanate lysis buffer, and total RNA was extracted by the method described by Chomczynski and Sacchi [9]. After determination of the concentration of each sample, RNA (20 μ g/lane) was electrophoresed in a 1% agarose gel containing 6% formaldehyde and transferred onto a hybond-N membrane. The membrane was UV cross-linked, pre-hybridized at 42°C for 2 h, and then hybridized to the ³²P-labelled c-myc, H-ras, p53 or RB cDNA probe overnight at 42°C. 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 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 an intensifying screen at -70°C for 96 h. After stripping, the same membrane was rehybridized to ³²P-labeled GAPDH cDNA to normalize the RNA content of each sample.

Western Blot Analysis

For total cellular P21^{ras} and P46 analysis, the cells were harvested with a rubber policeman and lysed on ice for 10 min in solution containing 1% Triton X-100, 0.1% SDS, 10 mM phosphate buffer (pH 7.2), 50 mM Tris-HCl (pH 7.6), 2 mM PMSF and 250 μ g/ml aprotinin). After centrifugation at 40,000g for 30 min, the supernatant was analysed for its protein content by a modified Bradford method (Bio-Rad kit). For Western blot analysis of cytosolic and membranous P21^{ras}, the cell pellets were suspended in PBS buffer (pH 7.2) containing 2 mM PMSF and sonicated followed by centrifugation at 100,000g for 30 min. The supernatants (cytosolic fraction) were saved, while the pellets (membranous fractions) were solubilized in lysis solution as described above. 20 μ g of protein was loaded on each lane and electrophoresed on 15% polyacrylamide-SDS minigels in SDS sample buffer. The proteins were then electrophoretically transferred to the PVDF membrane. After being blocked for 2 h in a solution containing 3% BSA and 0.25% normal horse serum, the membranes were then incubated in solutions containing anti-P21^{ras} or anti-P46, and then the biotin-conjugated anti-mouse IgG and avidin-conjugated horseradish peroxidase. The labeled proteins were visualized in AEC solution (0.8% aminoethylcarbazole and 0.1 M acetate, pH 5.2).

Assay for Farnesyl Protein Transferase Activity

Partially purified FPTase was prepared from confluent LLC-PK, A549 and PaCa cells, respectively as described [10]. For FPTase activity assay, each 25 μ l reaction mixture contained 50 mM Tris-HCl (pH 7.5), 25 mM MgCl₂, 20 mM KCl, 1 mM DTT, 2.5–5 μ M

P21^{ras}, 10–15 pmol [³H]FFP(25,000–30,000 dpm/mol) or [³H]GGPP and 6–7 μg of partially purified FPTase, and **2**. After incubation for 1 h at 37°C in borosilicate tube in the dark, the reaction was stopped by the 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 followed by the addition of 2 ml of 6% TCA and 2% SDS. 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^{ras} protein. This blank value was subtracted before calculating the percent of control values. Protein content was determined using the Bio-Rad Protein Assay.

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