Suppression of lung cancer tumor growth in a nude mouse model by the Ras inhibitor salirasib (farnesylthiosalicylic acid)

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Abstract

Aberrant Ras pathway functions contribute to the malignant phenotype of lung cancers. Inhibitors of Ras might therefore be considered as potential drugs for lung cancer therapy. Here, we show that the Ras inhibitor farnesylthiosalicylic acid (salirasib) inhibits proliferation of human lung cancer cells harboring a mutated K-ras gene (A549, H23, or HTB54) or overexpressing a growth factor receptor (H1299 or HTB58) and enhances the cytotoxic effect of the chemotherapeutic drug gemcitabine. Salirasib inhibited active K-Ras in A549 cells, reversed their transformed morphology, and inhibited their anchorage-independent growth in vitro. Tumor growth in A549 and HTB58 cell nude mouse models was inhibited by i.p. administration of salirasib. P.o. formulated salirasib also inhibited A549 cell tumor growth. Our results suggest that p.o. salirasib may be considered as a potential treatment for lung cancer therapy. [Mol Cancer Ther 2007;6(6):1765–73]

Introduction

Lung cancer is the leading cause of cancer-related deaths (1). Despite advances in surgery, chemotherapy, and radiotherapy, survival rates have hardly changed in the last decade, and long-term survival rates remain extremely poor. It has been established that lung cancer arises as a consequence of the accumulation of multiple genetic changes involving critical genes that control cell motility, proliferation, differentiation, and apoptosis (2). Point mutations of the K-ras gene and overexpression of cyclin D1 are thought to be early events during carcinogenesis of some types of lung cancer (3, 4). Most lung neoplasms are bronchogenic in origin. The four typical subgroups of these lung cancers are squamous cell carcinoma, large cell carcinoma, small-cell carcinoma, and adenocarcinoma (5). Non–small cell lung carcinoma (NSCLC) represents 85% of cases of lung cancer (6).

The notion that Ras signaling contributes to the maintenance of the transformed phenotype of lung cancer cells (7–9) is supported by the high incidence of growth factor receptor (e.g., epidermal growth factor receptor) expression and, as mentioned above, the significant incidence of Ras mutations and up-regulated cyclin D1 in these tumor cells. It is therefore reasonable to suggest that treatment of lung cancer with Ras inhibitors could be beneficial. Our objective in this study was to develop a novel rational strategy for the treatment of lung cancer by using the Ras inhibitor salirasib [farnesylthiosalicylic acid (FTS); refs. 10, 11]. FTS (salirasib) is a potent Ras inhibitor that acts in a rather specific manner on the active, GTP-bound forms of H-Ras, N-Ras, and K-Ras proteins (10, 11). It competes with Ras-GTP for binding to specific saturable binding sites (12) in the plasma membrane, resulting in mislocalization of active Ras, and, in some but not all tumor cell lines, it facilitates Ras degradation (12). This competition with FTS (salirasib) prevents active Ras from interacting with its prominent downstream effectors and results in reversal of the transformed phenotype in transformed cells that harbor activated Ras (11). As a consequence, Ras-dependent cell growth and transforming activities, both in vitro and in vivo, are strongly inhibited (10, 11).

Whether FTS can inhibit growth of lung cancer cells and tumor growth was not known. This is an important question that we attempted to answer in the present study. Its importance must be viewed in light of the extremely poor long-term survival of lung cancer patients and in light of lack of any efficient nontoxic drug for the treatment of lung cancer. The commonly used treatments of lung cancer patients include cytotoxic drugs and their combinations (13). These treatments are restricted by dose-limiting side effects (13, 14). Animal studies (11, 15, 16) and initial human trials1 showed that FTS is a relatively safe compound with no adverse side effects. Our objectives were then to examine whether FTS can exert anti-Ras activity in lung cancer cell lines and inhibit their growth in vitro and in animals. Here, we show for the first time that p.o. FTS (salirasib) inhibited A549 lung cancer cell tumor growth.

1 http://www.concordiapharma.com/index.htm
Materials and Methods

Cell Lines
All cell lines were obtained from American Type Culture Collection (ATCC) and grown in medium containing 10% FCS and antibiotics as recommended by ATCC. The following cell lines were used: A549 cells, NSCLC (CCL, ATCC); HTB54 lung carcinoma cells; HTB58 (SK-MES-1, ATCC), a human lung squamous cell carcinoma cell line; H23 (NCI-H23, ATCC), a human non–small cell lung adenocarcinoma cell line; and H1299 (NCI-H1299, ATCC), a NSCLC cell line. The cells were plated in 24-well plates in 1 mL medium at a density of 5,000 cells per well (or 2,500 cells per well, HTB54) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Cells were treated with the indicated concentrations of FTS (Concordia Pharmaceuticals) or with 0.1% Me2SO4 (vehicle) 24 h after plating and counted 5 days later. Dead cells were counted after addition of Hoechst 33258 dye (1 μg/mL; Sigma-Aldrich) to vehicle-treated control cultures or to cultures treated for 24 or 48 h with 75 μmol/L FTS. Fluorescence images were collected 5 min after the dye was added. In drug combination experiments, cells were grown for 2 days in the absence or in the presence of 40 μmol/L FTS and then treated for 4 h with gemcitabine (100 or 200 nmol/L), cisplatin (50 or 100 nmol/L), doxorubicin (50 or 100 nmol/L), or paclitaxel (2.5 or 5 nmol/L). Live cells were counted after a further 3 days of incubation with or without FTS. Experiments were done twice in quadruplicate.

Data obtained in these and in all subsequent experiments were subjected to statistical analysis (t test). Synergism was determined by calculating the percentage of growth inhibition by a single drug treatment and that of growth inhibition by the combined treatment. The sum of percentage inhibition observed in each of the single drug treatments was compared with that of the combined treatment. Statistically significant higher levels in the latter were considered as synergism.

Bromodeoxyuridine Incorporation into DNA
A549 cells were plated on glass coverslips (1.2 × 105 cells per well in six-well plates) and incubated for 24 h in medium containing 5% FCS. The cells were then incubated for 24 h with or without 75 μmol/L FTS and then for 24 h with bromodeoxyuridine (BrdUrd; Zymed BrdUrd labeling kit; 1:100 dilution). Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS (5 min), then washed for 5 min with UB buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.6), 0.2% sodium azide in PBS], and blocked with 2% bovine serum albumin in UB (UBB, 5 min). The fixed cells were incubated successively with naïve goat IgG for 30 min (200 μg/mL; Jackson ImmunoResearch Laboratories), anti-vinculin antibody for 1 h (1:400; Sigma-Aldrich), goat anti-mouse Cy2-conjugated antibody for 1 h (1:200; Jackson ImmunoResearch Laboratories), and rhodamine-labeled phalloidin for 1 h (1:1,000; Sigma-Aldrich). Between each of the above steps, the cells were washed for 30 min with UBB. Last, the coverslips were washed with UB, dried, and mounted onto the slides with Muviol. F-actin (red) and vinculin (green) were visualized with a Zeiss LSM 510 confocal microscope fitted with nonleaking green and red fluorescence filters. Colocalization was assessed using the colocalization function of the LSM 510 software. To assess the extent of the effect of FTS on stress fibers, we did a statistical analysis as follows. Fluorescent images of three separate experiments were collected (altogether 23 fields of control and of FTS-treated cultures). The total number of cells and the number of cells exhibiting strong stress fibers were then double-blind scored. The means ± SD ratios of stress fiber containing cells/total number of cells were then calculated and subjected to statistical analysis (t test).

Anchorage-Independent Colony Formation Assay in Soft Agar
Noble agar (2% and 0.6%; Difco) was prepared in water and autoclaved. The 2% agar was melted in a microwave oven, mixed 1:1 with medium (×2 Kaighn’s modification of Ham’s F-12 medium with 20% FCS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin), and poured onto 96-well plates (50 μL/well) to provide the 1% base agar. The 0.6% agar (5 mL) was mixed with 5 mL medium (×2), containing 8 × 105 A549 cells, and the mixture (50 μL) was plated on top of the base agar. The cells were incubated for 19 days at 37°C with or without the indicated concentrations of FTS (six wells for the control and for each treatment) and colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL for 4 h). The colonies were then visualized by light microscopy, imaged, and counted using the ImagePro software.

Fluorescence-Activated Cell Sorting Analysis
A549 cells were plated (9 × 105 cells) in 10-cm plates, incubated for 24 h in medium containing 5% FCS, and then incubated for 24 or 48 h with or without 75 μmol/L FTS. The cells were collected, resuspended in PBS containing propidium iodide (50 μg/mL; Sigma-Aldrich) and 0.05% Triton X-100, and subjected to analysis by a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson).

Immunofluorescence and Confocal Microscopy
A549 cells were plated on glass coverslips (2 × 104 cells per well in six-well plates), incubated for 24 h in medium containing 5% FCS, and then incubated for 48 h with or without 75 μmol/L FTS. The cells were fixed and permeabilized at room temperature by successive incubations with 3.7% formaldehyde (20 min) and 0.2% Triton X-100 in PBS (5 min), then washed for 5 min with UB buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.6), 0.2% sodium azide in PBS], and blocked with 2% bovine serum albumin in UB (UBB, 5 min). The fixed cells were incubated successively with naïve goat IgG for 30 min (200 μg/mL; Jackson ImmunoResearch Laboratories), anti-vinculin antibody for 1 h (1:400; Sigma-Aldrich), goat anti-mouse Cy2-conjugated antibody for 1 h (1:200; Jackson ImmunoResearch Laboratories), and rhodamine-labeled phalloidin for 1 h (1:1,000; Sigma-Aldrich). Between each of the above steps, the cells were washed for 30 min with UBB. Last, the coverslips were washed with UB, dried, and mounted onto the slides with Muviol. F-actin (red) and vinculin (green) were visualized with a Zeiss LSM 510 confocal microscope fitted with nonleaking green and red fluorescence filters. Colocalization was assessed using the colocalization function of the LSM 510 software. To assess the extent of the effect of FTS on stress fibers, we did a statistical analysis as follows. Fluorescent images of three separate experiments were collected (altogether 23 fields of control and of FTS-treated cultures). The total number of cells and the number of cells exhibiting strong stress fibers were then double-blind scored. The means ± SD ratios of stress fiber containing cells/total number of cells were then calculated and subjected to statistical analysis (t test).

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Ras, Rac, and Rho Pull-Down Assays and Immunoblotting Procedures

A549 cells were incubated for 24 h with or without FTS, as described above, and then lysed with lysis buffer as described previously (17). The apparent amounts of K-Ras-GTP in 0.5 mg protein of total cell lysates were determined by the glutathione S-transferase–Ras-binding domain of Raf pull-down assay, as described elsewhere (18, 19). The apparent amounts of Rac1-GTP and of RhoA-GTP, each in 2 mg protein of total cell lysates, were determined, respectively, by pull-down assays with glutathione S-transferase–Rac1-binding domain of PAK1–conjugated beads and glutathione S-transferase–Rho-binding domain of Rhokin–conjugated beads (20, 21). The pulled-down GTPases were subjected to SDS-PAGE followed by immunoblotting with the appropriate antibodies: anti K-Ras (1:30; Calbiochem), anti Rac1 (1:2,500; Santa Cruz Biotechnology), or anti RhoA (1:700; Upstate Biotechnology). Immunoblots were exposed to 1:2,500 peroxidase–goat anti-mouse IgG. Levels of phospho–extracellular signal-regulated kinase (ERK) and phospho–Akt were determined by immunoblotting as described earlier (12) using rabbit anti–phospho-ERK1/2 antibody (Santa Cruz Biotechnology) and rabbit anti–phospho–Akt antibody (Cell Signaling). Protein bands were visualized by enhanced chemiluminescence and quantified by densitometry using ImageJ computer software (NIH).

Animal Studies

Athymic nude mice (6 weeks old) were housed in barrier facilities on a 12-h light/dark cycle. Food and water were supplied ad libitum. On day 0, A549 or HTB58 cells (5 × 10^6 cells in 0.1 mL PBS) were implanted s.c. just above the right femoral joint. After 5 or 11 days, the mice were separated randomly into control groups that had received only the vehicle and salirasib (FTS)–treated groups. Daily salirasib (FTS) treatments were given either i.p. or p.o. Tumor volumes or weights were determined as described previously (16). Gemcitabine treatment (36 mg/kg, i.p.) was given every 4 days.

Results

FTS (Salirasib) Inhibits Growth of Lung Cancer Cells

We first examined the effect of FTS on the proliferation of A549 cells that harbor activated K-ras gene mutated in codon 12 (22). Incubation of the cells with 75 𝛀mol/L FTS for 48 h inhibited the incorporation of BrdUrd into their DNA by 56.7 ± 17.4% relative to vehicle-treated control cells (*P < 0.05; Fig. 1A). Notably, the treatment also caused...
a significant change in cell morphology; the treated cells were large and flat, an appearance typical of transformed cells treated with FTS (15, 16). Direct counting of A549 cells grown for 6 days in the presence of FTS showed that the drug had induced a dose-dependent decrease in their number, with a decrease of 50% at 40 μmol/L FTS (Fig. 1B). This decrease could be attributed mainly to inhibition of cell proliferation because under the conditions used in these experiments, cell death (indicated by Hoechst staining), both in the absence and in the presence of FTS, was very low (between 2% and 6%; data not shown). Fluorescence-activated cell sorting analysis confirmed these observations by showing that the apoptotic (sub-G1) cell population was 3.8% at 24 h and 8.2% at 48 h in cells treated with 75 μmol/L FTS compared with 1.0% and 2.8%, respectively, in control cells (Fig. 1C). The fluorescence-activated cell sorting analysis also showed that FTS caused a reduction in the population of G1 but not of G2-M cells. Cells treated with FTS for 24 and 48 h showed reductions in G1 of 2.2% and 7.7%, respectively (Fig. 1C). Thus, FTS evidently induced cell cycle arrest in A549 cells, resulting in inhibition of cell growth with no significant effect on cell death.

The growth-inhibitory effects of FTS were not limited to the A549 lung cancer cells. This was evident from experiments in which we examined the effects of FTS on growth of other lung cancer cell lines (i.e., H23, HTB54, H1299, and HTB58; SK-MES-1). The first two, but not the last two, are known to harbor oncogenic K-Ras. H1299 and HTB58, however, express relatively large amounts of Ras, K-Ras-GTP, phospho-ERK, and phospho-Akt levels. Inhibited in A549 cells and, if so, whether the anchorage-dependent growth of the cells is affected as well. We considered the possibility that the observed FTS-induced change in A549 cell morphology is associated with the known effects of FTS on cytoskeleton organization (15, 16). That this was indeed the case was evident in A549 cells that were incubated with or without 75 μmol/L FTS for 48 h and then stained with rhodamine-labeled phalloidin (which labels polymeric F-actin) and with anti-vinculin antibody (which labels focal adhesions). Typical fluorescence images of control and of FTS-treated cells are shown in Fig. 3. The untreated cells exhibited short, thin actin stress fibers (red fluorescence) and relatively few focal adhesions (green fluorescence), whereas the FTS-treated cells exhibited long, thick stress fibers and a relatively large number of focal adhesions that looked larger than those observed in the control cells. Statistical analysis (see Materials and Methods) indicated that <80% of the cells in the FTS-treated cultures had undergone changes in cell morphology (n = 23; P < 0.0001). These results and the growth-inhibitory effects of FTS observed in the lung cancer cell lines suggested that the Ras inhibitor had at least partially reversed the transformed phenotype of the cells.

We thus examined whether active K-Ras-GTP and its prominent downstream signals to ERK and Akt are inhibited in A549 cells and, if so, whether the anchorage-dependent growth of the cells is affected as well. We incubated A549 cells in the absence and in the presence of various concentrations of FTS and then measured their total Ras, K-Ras-GTP, phospho-ERK, and phospho-Akt levels. In agreement with previous studies (11), FTS induced a small but significant decrease in the amount of total Ras (10 ± 3% reduction at 75 μmol/L; P < 0.005; Fig. 4A). FTS also reduced the amount of K-Ras-GTP in a dose-dependent manner (Fig. 4A). The reduction in K-Ras-GTP (mean ± SD) was 23 ± 15.3%, 37 ± 3.7% (P < 0.01), and 46 ± 1.9% (P < 0.002), respectively, in cells treated with 25, 50, and 75 μmol/L FTS. The effective concentration range (25–75 μmol/L) for the reduction in K-Ras-GTP (Fig. 4A) was similar to that required for the inhibition of cell growth (Fig. 1B). FTS also reduced the levels of phospho-ERK and phospho-Akt causing 33 ± 2% and 58 ± 6% inhibition, respectively (Fig. 1A). The effect of FTS seemed to be specific to the Ras protein because it had no effect on the amount of the prenylated active Rac1-GTP protein as determined by a specific Rac1-GTP pull-down assay (Fig. 4B). Moreover, using a specific pull-down assay for prenylated active RhoA-GTP, we found that FTS induced a significant increase of 2 ± 0.2-fold (P < 0.002) in RhoA-GTP (Fig. 4C). Thus, although FTS did not reduce the total amounts of the two GTPases (Rac1 and RhoA), it clearly had a selective inhibitory effect on active K-Ras. The observed increase
in RhoA-GTP can probably be attributed to the relief of the known Ras-associated negative control of RhoA activation (25) and is consistent with the observed increase in stress fiber formation and focal adhesion assembly (Fig. 3) that are controlled by active RhoA (26).

FTS inhibition of Ras and active ERK was not limited to the A549 lung cancer cells. This was evident from experiments in which we examined the effects of FTS on the levels of Ras, Ras-GTP, and phospho-ERK in H1299 and in HTB54 cells. The first does not harbor oncogenic Ras and the second harbors oncogenic K-Ras. FTS induced 14.7 ± 3.3% and 16 ± 3.3% reduction in Ras-GTP, respectively, 51.6 ± 9.6% and 64.6 ± 12.6% reduction in total Ras, and 38.9 ± 5.2% and 62.2 ± 13.9% reduction in phospho-ERK, respectively, in H1299 and HTB54 cells (Fig. 4D).

Next, using the soft agar assay, we examined the effect of FTS on the anchorage-independent growth of A549 cells. The results of these experiments are shown in Fig. 5. As shown, FTS inhibited A549 cell growth in soft agar by 27 ± 5.5% and 58 ± 21% at 50 and 100 μmol/L, respectively.

**Effect of FTS (Salirasib) Combined with a Cytotoxic Drug on A549 Cell Death**

Because active Ras is known to endow tumor cells with resistance to apoptosis, we next examined whether treatment with FTS can increase the sensitivity of A549 cells to cytotoxic drugs. A549 cells were treated with 40 μmol/L FTS for 48 h and then for 4 h with gemcitabine, paclitaxel, cisplatin, or doxorubicin. The cells were then washed, replenished with 40 μmol/L FTS, incubated for 3 days with FTS, and counted. The numbers of cells in the drug-treated cultures, expressed as percentages of the numbers in the vehicle-treated control, are shown in Fig. 6.

The effects of FTS and gemcitabine were found to be synergistic (Fig. 6A). As shown, FTS alone caused a reduction in cell numbers (mean ± SD) of 25 ± 6.3%, gemcitabine at 100 and 200 nmol/L had no effect (<11%), and the combinations of FTS and gemcitabine at 100 and at 200 nmol/L caused, respectively, reductions of 45 ± 5.3% and 60 ± 5.7%. The combined effect of FTS and cisplatin was additive (Fig. 6B), with reductions in cell numbers of 33 ± 9.5% for FTS alone, reductions of 11 ± 11% and 30 ± 12.9%, respectively, for cisplatin alone at 5 and at 10 μmol/L, and reductions of 47 ± 6.9% and 63 ± 12.7% for the respective combinations of FTS and cisplatin at 5 and 10 μmol/L. As with cisplatin, the observed effects of the combinations of doxorubicin and FTS (Fig. 6C) and of paclitaxel and FTS were additive (Fig. 6D). Thus, of the four cytotoxic drugs examined, it seems that FTS enhanced the cytotoxic action of gemcitabine only.

**Salirasib (FTS) Inhibits Tumor Growth in a Lung Cancer Cell Nude Mouse Model**

We next examined the *in vivo* effect of salirasib using a nude mouse model. The lung cancer cells were implanted s.c. above the right femoral joint and the mice were then treated with salirasib. First, we examined the effect of i.p. administration of salirasib on tumor growth in A549 cells, a procedure that has proven effective in other nude mouse tumor models (10, 11, 27). Treatment was started 5 days after cell implantation, by which time tumor volumes were 0.15 ± 0.06 and 0.1 ± 0.03 cm³ in the control and the drug-treated groups. Tumor volumes were determined 24 days after implantation in two groups of mice (*n* = 8) that had received daily i.p. administration of either the vehicle (control) or 10 mg/kg salirasib. Significant inhibition of tumor growth relative to the control (53.8%; *P* < 0.05) was recorded in the salirasib-treated group (Fig. 7A). Whereas in the control group the fold increase in tumor volume was 4.3, in the salirasib-treated group, it was 2.5. In a similar experiment carried out with mice implanted s.c. with HTB55 cells (*n* = 7 per group), significant inhibition of tumor growth (76.4 ± 48.8%) was observed in the group treated daily with 10 mg/kg salirasib i.p. (Fig. 7B). The initial tumor volumes were 0.015 ± 0.010 cm³ compared with 0.02 ± 0.03 cm³, respectively, in the control group treated daily with the vehicle (control) or 10 mg/kg salirasib. Significant inhibition of tumor growth relative to the control (53.8%; *P* < 0.05) was recorded in the salirasib-treated group (Fig. 7A). Whereas in the control group the fold increase in tumor volume was 4.3, in the salirasib-treated group, it was 2.5. In a similar experiment carried out with mice implanted s.c. with HTB55 cells (*n* = 7 per group), significant inhibition of tumor growth (76.4 ± 48.8%) was observed in the group treated daily with 10 mg/kg salirasib i.p. (Fig. 7B). The initial tumor volumes were 0.015 ± 0.010 cm³ compared with 0.02 ± 0.03 cm³, respectively, in the control group treated daily with the vehicle (control) or 10 mg/kg salirasib.

**Figure 3.** FTS (salirasib) induces stress fiber formation and focal adhesion assembly in A549 cells. A549 cells were plated on glass coverslips, grown for 24 h with 0.1% Me2SO4 (control) or 75 μmol/L FTS, and labeled with phalloidin-rhodamine (red fluorescence) and anti-vinculin antibody and then Cy2-labeled antibody (green fluorescence) as described in Materials and Methods. Typical fluorescence images (*×100 objective*) of cells in one of three experiments with similar results. Bar, 10 μm.
Tumor volume measured 14 days after cell implantation in salirasib-treated group was 0.02 ± 0.045 cm³ (a 1.3-fold increase relative to day 0) compared with 0.09 ± 0.08 cm³ (a 4.5-fold increase relative to day 0) in the vehicle-treated controls (P < 0.05).

We next used the A549 cell–implanted nude mouse model to examine the effect of salirasib given p.o. on tumor growth. In two separate experiments (Fig. 7C, experiments 1 and 2), cells were implanted as described above and daily p.o. treatment with salirasib (50 mg/kg) was started 11 days later. The initial tumor volumes in the control (n = 5) and in the FTS-treated (n = 6) groups were, respectively, 0.72 ± 0.21 and 0.75 ± 0.24 cm³ in experiment 1 and 0.66 ± 0.3 cm³ in both the control group (n = 8) and the FTS-treated group (n = 8) in experiment 2. As shown in Fig. 7C (experiment 1), after 16 days of treatment, the tumor weights (mean ± SD) in salirasib-treated and control mice were 0.4 ± 0.19 and 0.9 ± 0.39 g, respectively, representing a significant inhibition of 53.7 ± 19.1% in tumor growth (P < 0.025) in the salirasib-treated mice. Similarly, after 11 days of treatment (Fig. 7C, experiment 2), the tumor weights (mean ± SD) in salirasib-treated and control mice were 0.33 ± 0.17 and 0.58 ± 0.15 g, respectively, representing a significant inhibition of 43.4 ± 19.1% in tumor growth (P < 0.03) in the salirasib-treated mice.

Next, we examined the effects of p.o. salirasib, alone or in combination with gemcitabine, on A549 cell tumor growth (Fig. 7D). Six days after cell implantation, mice were divided into four groups (n = 8 per group) and treated p.o. with vehicle alone (control), FTS alone (60 mg/kg), vehicle and gemcitabine (36 mg/kg, i.p. every 4 days), or salirasib and gemcitabine. The initial tumor volumes in the four groups were 0.26 ± 0.049, 0.34 ± 0.073, 0.26 ± 0.063, and 0.35 ± 0.085 cm³, respectively. Treatments with gemcitabine began 1 week after salirasib treatment was started. In agreement with the results of the first experiment (Fig. 7C), p.o. salirasib treatment caused a significant inhibition in tumor growth; tumor weights in the mice treated with vehicle only (control) and with salirasib only (mean ± SD) were 0.90 ± 0.40 and 0.49 ± 0.15 g, respectively (46.2 ±
16.3% inhibition; \( P < 0.02 \); Fig. 7D). A significant reduction in tumor weight (\( P < 0.015 \)) was also observed in a fourth group of mice treated with gemcitabine alone (Fig. 7D). The combined effect of gemcitabine and salirasib treatments was somewhat more effective than the effect of each treatment alone, although the difference in tumor weight between the gemcitabine treatment and the gemcitabine plus salirasib treatment was not significant (Fig. 7D). The most significant result obtained in this experiment was clearly the difference between the effect of treatment with the vehicle alone (control) and of combined treatment with salirasib and gemcitabine (\( P < 0.006 \)).

**Discussion**

In the present work, we show that the Ras inhibitor FTS (salirasib) inhibited the anchorage-dependent (Figs. 1 and 2) and anchorage-independent (Fig. 5) growth of lung cancer cells in vitro and inhibited tumor growth in nude mouse lung cancer models given either i.p. or p.o. (Fig. 7). Consistent with the known anti-Ras activity of salirasib, we show here that salirasib reduced the levels of Ras and its downstream targets phospho-ERK and phospho-Akt in lung cancer cells (Fig. 4). As expected, salirasib inhibited active K-Ras-GTP (Fig. 4). This finding accords with the reported mode of action of salirasib as shown in many tumor cell lines that harbor activated Ras, including PANC-1 pancreatic cancer, SW480 colon carcinomas, and 518A2 melanomas (10, 11, 27). Moreover, in agreement with reports that salirasib can inhibit the growth of tumor cells that do not harbor \( ras \) gene mutations (28), we found here that salirasib inhibits the growth of H1299 and HTB58 cell lines (Fig. 2), neither of which harbors mutated \( ras \), and the growth of A549, HTB54, and H23, which all harbor activated K-Ras (Figs. 1 and 2). In other words, salirasib inhibits the growth of tumor cells whether they harbor mutated \( ras \) genes or not. The lack of correlation between \( ras \) gene mutations and growth-inhibitory effects of salirasib is explained by the known chronic activation of wild-type Ras proteins in many human tumors owing to the presence of highly active growth factor receptor signaling (29, 30).

Salirasib affects all Ras proteins, mainly their active form (12), as also observed here for K-Ras in A549 cells (Fig. 4). Because of this selectivity of salirasib toward active Ras, the growth of cells with chronically active Ras pathways [such as glioblastoma (28), neuroblastoma with amplified \( myc-N \) gene (31), and melanomas (27)] is inhibited by salirasib. Strong support for this notion comes from recent gene profiling analyses of human primary mammary epithelial cells that were transformed by human activated H-Ras, or c-Src, or activated \( \beta \)-catenin or E2F3 (32). In these studies, the pattern of the deregulated oncogenic pathway in each...

**Figure 5.** FTS (salirasib) inhibits anchorage-independent growth of A549 cells. A549 cells (8 \( \times \) 10³ cells per well) were seeded in 96-well plates in soft agar in the presence of the vehicle (0.1% Me₂SO₄; control) or the indicated concentrations of FTS as described in Materials and Methods. After 19 d, the colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and imaged. A, typical images. B, the numbers of colonies per well in control and FTS-treated cultures were counted as described in Materials and Methods. Columns, mean of two independent experiments, each repeated six times; bars, SD. *, \( P < 0.005 \), compared with vehicle-treated control.

**Figure 6.** Effect of FTS (salirasib) in combination with different cytotoxic drugs on A549 cell growth and viability. A549 cells were incubated for 48 h with 0.1% Me₂SO₄ (control) or 40 \( \mu \)mol/L FTS and then for 4 h with gemcitabine, cisplatin, doxorubicin, or paclitaxel at the indicated concentrations and for a further 72 h with 0.1% Me₂SO₄ or 40 \( \mu \)mol/L FTS only. Live cells were collected and counted. The numbers of cells in the drug-treated cultures are expressed as percentages of the number recorded in the zero drug (vehicle-treated) controls. Columns, mean of eight counts; bars, SD. *, \( P < 0.05 \); **, \( P < 0.01 \), compared with vehicle-treated control.
cell line was mapped by microarray-based gene expression profiling. When they examined the sensitivity of specific oncogenic pathways to known drugs, the authors showed that for salirasib, those cell lines showed close concordance and correlation between the probability of Ras pathway deregulation based on the gene expression prediction and the extent of the cell proliferation inhibition by salirasib.

Another important finding of the present study is the marked effect of salirasib on the actin cytoskeleton, focal adhesions, and cell morphology (Figs. 1A and 3). The observed flattening of the cells and formation of actin stress fibers and focal adhesions is typical of the inhibition of active Ras by Ras inhibitors, including farnesyltransferase inhibitors (33) and salirasib (15). These changes are likely to occur, at least in part, by a relief of Ras inhibition of the activation of RhoA. That is because active RhoA-GTP controls the formation of stress fibers and focal adhesions (26), and we found that salirasib induces an increase in RhoA-GTP in A549 cells (Fig. 4). Our experiments also show that salirasib tilts the balance between active Rho and active Rac in A549 cells (Fig. 4), which would contribute to the inhibition of cell growth and migration.

Altogether, these results point to the possibility that salirasib may be considered as a potential drug for lung cancer treatment. This suggestion must be viewed in light of the knowledge that small-cell lung carcinoma and non-NSCLC are both responsive to first-line chemotherapy, but most patients relapse and die from their disease, with 5-year survival rates of ~15% (34). Given this poor survival rates of small-cell lung carcinoma and NSCLC patients, the introduction of new targeted drugs is then of utmost importance. Indeed, many attempts to develop such drugs for lung cancer therapy have been made. Important groups of drugs used in the clinic or in clinical trials include epidermal growth factor receptor tyrosinekinase inhibitors, including gefitinib (Iressa), erlotinib (Tarceva), and cetuximab (Erbitux; ref. 35), hormones (e.g., tamoxifen, combined with chemotherapy; ref. 36), and farnesyltransferase inhibitors, which were originally designed as Ras inhibitors (37). Most of the drugs do not succeed in increasing markedly survival rates, adding a few months at best (14, 36, 37). For example, tamoxifen combined with ifosfamide, epirubicin, and cisplatin in relapsed NSCLC had a response rate of 20% and a median overall survival of about 8 months (36). A combination of tamoxifen, cisplatin, and etoposide led to a response of 37% and overall survival of more than 11 months (36). Another example is given by lack of clinically meaningful survival benefit when gemcitabine was combined to the farnesyltransferase inhibitor tipifarnib (38). In addition to the poor outcome of such
treatments, combination therapies of lung cancer patients are restricted by dose-limiting side effects (13). For example, grade 2 or higher treatment-related toxicities, including rash, diarrhea, and fatigue, were observed in patients treated with erlotinib with greater than additive cytotoxic effects in combination with the farnesyltransferase inhibitor tipifarnib (Zarnestra; ref. 39). These studies emphasize the need for new targeted nontoxic drugs for the treatment of lung cancer. Our results suggest that salirasib, which proved to be nontoxic in animals (11, 15, 16) and has no reported adverse side effects in humans,3 seems to be such a candidate drug for lung cancer.

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Suppression of lung cancer tumor growth in a nude mouse model by the Ras inhibitor salirasib (farnesylthiosalicylic acid)

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