The Ras Inhibitor S-trans,trans-Farnesylthiosalicylic Acid
Chemosensitizes Human Tumor Cells Without
Causing Resistance

Mali Gana-Weisz, Julius Halaschek-Wiener,
Burkhard Jansen, Galit Elad, Ronit Haklai, and
Yoel Kloog
Department of Neurobiochemistry, The George S. Wise Faculty of
Life Sciences, Tel-Aviv University, 69978 Tel Aviv, Israel [M. G.-W.,
G. E., R. H., Y. K.], and Department of Clinical Pharmacology,
Section of Experimental Oncology/Molecular Pharmacology and
Department of Dermatology, Division of General Dermatology,
University of Vienna, Vienna, A-1090 Austria [J. H.-W., B. J.]

ABSTRACT
Ras transformation requires Ras membrane anchor-
age, which is promoted by a farneslycysteine carboxymethyl
ester and by additional sequences specific to each Ras iso-
form. We showed previously that S-trans,trans-farnesylthio-
salicylic acid (FTS) disrupts Ras membrane anchorage and
that this disturbance contributes to inhibition of cell transforma-
tion and tumor growth. Most tumor cells develop resistance to anticancer agents. Here we examined whether
tumor cells develop resistance to FTS and evaluated the
therapeutic potential of FTS combined with cytotoxic drugs,
because oncogenic Ras promotes antiapoptotic signals in
tumors of epithelial origin. We showed that Panc-1 pan-
creatic cancer cells, SW480 colon cancer cells, and H-ras
(EJ)-transformed Rat-1 fibroblasts exposed to FTS for prolonged
periods (>6 months) do not escape FTS-induced growth inhibition and do not develop drug resistance. These cells
transitioned to express reduced amounts of Ras, exhibit a
reversed phenotype, and show an altered response to the
cytotoxic drugs doxorubicin and gemcitabine. FTS-treated
Panc-1 or SW480 cells acquired sensitivity to the cytotoxic
drugs, whereas FTS-treated EJ cells lost sensitivity to doxo-
rubicin, reflecting the opposite effects of oncogenic Ras on
the survival of epithelial cells and fibroblasts. Treatment
with FTS led to a marked increase in sensitivity to gemcitabine of the formerly resistant SW480 cells and a 100-fold
increase in sensitivity to gemcitabine of Panc-1 cells. Such
treatment in mice with preexisting Panc-1 tumors provided a
synergistic effect of FTS and gemcitabine, leading to en-
hanced inhibition of tumor growth and a 65% increase in
survival rate.

INTRODUCTION
Ras oncogenes, commonly found in many types of hu-
mans and animals (1), contribute to deregulated cell growth, differen-
tiation, and cell death (3–8). Malignant transformation induced by Ras and maintenance of the transformed phenotype
require association of Ras with the inner surface of the cell
membrane (9–13). This association is promoted by the COOH-
terminus farnesylecysteine carboxymethyl ester common to all of
the Ras proteins and by additional signals typical of each Ras isoform (9–12, 14). When Ras farnesylation is blocked, either
by mutations of the cysteinyl residue modified by the enzyme
farnesyl transferase or by farnesyl transferase inhibitors, it re-
mains cytosolic and loses its transforming activity (10, 11).
Therefore, Ras inhibitors may be useful anticancer drugs. Far-
nesyl transferase inhibitors blocking farnesylation of H-ras
were indeed shown to inhibit tumor growth in mice xenografted
with H-ras-transfected fibroblasts and in transgenic mice that
stochastically develop mammary and salivary tumors because of
the H-ras transgene (15, 16). However, farnesyl transferase
inhibitors are not true Ras inhibitors; they do not block the
oncogenic activity of the most frequently occurring oncogene
K-ras and N-ras, because these two isoforms undergo alterna-
tive prenylation (17, 18).

We have proposed an additional way to inhibit oncogenic
Ras functions using compounds that resemble the farneslycys-
teine of Ras and directly affect the interactions of Ras with the
cell membrane (19–22). Among these compounds, FTS3 has
been the most intensively studied. We showed that FTS dis-
lodges all types of oncogenic Ras proteins from their membrane
anchorage sites and inhibits Ras transformation both in vitro and
in vivo (21–26). FTS was found to inhibit tumor growth in nude
mice xenografted with H-ras-transformed fibroblasts or with a
human pancreatic tumor cell line (Panc-1) and in SCID mice
xenografted with human melanomas (23, 24, 26). Because FTS
exhibits strong inhibitory effects on tumor growth with no
adverse toxic effects in animals (23, 24, 26), it may qualify as a
potential antitumor drug. FTS is the only compound described to

1 Supported by the SAFAHO Fund, the Friends of Tel Aviv University
in Austria, and Thyreos Inc. (to Y. K.), and by the Austrian National
Bank, the Austrian Science Fund, the Komission Onkologie, the Hans
and Blanca Moser Fund the Niarchos Foundation, and the Kamillo
Eisner Fund (to B. J.).
2 To whom requests for reprints should be addressed, at Department of
Neurobiochemistry, The George S. Wise Faculty of Life Sciences,
Tel-Aviv University, 69978 Tel-Aviv, Israel. Phone: 972-3-640-9699;
Fax: 972-3-640-7643; E-mail: kloog@post.tau.ac.il.

Received 6/15/01; revised 11/14/01; accepted 11/15/01.
The costs of publication of this article were defrayed in part by the
payment of page charges. This article must therefore be hereby marked
advertisement in accordance with 18 U.S.C. Section 1734 solely to
indicate this fact.
3 The abbreviations used are: FTS, S-trans,trans-farnesylthiosalicylic
acid; CRYSMEB, methyl cycloextrin; ERK, extracellular signal-regu-
lated kinase; MBP, myelin basic protein; RP, reversed phenotype;
TRITC, tetramethylrhodamine isothiocyanate; Ab, antibody; MDR,
multidrug resistance; CDK, cyclin-dependent kinase.

Vol. 8, 555–565, February 2002
Clinical Cancer Research 555
date that affects oncogenic K-ras and N-ras without cytotoxicity at the effective doses used.

Most tumor cells eventually develop resistance to anticancer agents (27–30). A main aim of the present study was to determine whether this phenomenon of increased drug resistance also occurs with FTS. A second aim was to determine the potential of combination therapies of FTS and cytotoxic drugs, because oncogenic Ras is known to promote antiapoptotic effects in tumors of epithelial origin (31, 32). We chose to study three transformed cell lines of different origins, all expressing an activated Ras protein. The Panc-1 and the SW 480 cells represent human tumors of epithelial origin that express activated K-Ras 4B and exhibit significant resistance to chemotherapy. The EJ cells are Rat-1 fibroblasts transformed by the activated H-Ras oncogene isolated from human bladder carcinoma. In fibroblasts, oncogenic Ras acts as a proapoptotic factor, unlike in tumor cells of epithelial origin where it exhibits antiapoptotic functions (5).

We report here that Panc-1 pancreatic cancer cells, SW 480 colon cancer cells, and H-ras (EJ)-transformed Rat-1 fibroblasts exposed to FTS for prolonged periods (>6 months) do not escape FTS-induced growth inhibition and do not develop drug resistance. These cells continued to express reduced amounts of Ras and show a clearly altered response to the cytotoxic drugs doxorubicin and gemcitabine. The FTS-treated Panc-1 or SW 480 cells acquired sensitivity to the cytotoxic drugs, whereas the FTS-treated H-ras-transformed fibroblasts lost sensitivity to doxorubicin, reflecting the opposite functions of oncogenic Ras on the apoptotic machinery of epithelial cells and fibroblasts.

MATERIALS AND METHODS

Materials. FTS was prepared, purified, and analyzed as described in detail elsewhere (19). Its purity, as assessed by TLC, ³¹H nuclear magnetic resonance, and mass spectral analysis, was >95%. Doxorubicin (Adriamycin) was purchased from Sigma Chemical Co., St. Louis, MO, and gemcitabine (Gemzar) from Eli Lilly, Indianapolis, IN. [γ-⁴³P]-ATP (3000 Ci/mmol) was purchased from Rotem Industries, Negev, Israel. FTS was formulated in CRYSMEB according to the instructions of the manufacturer (Thyreos, Newark, NJ). The stock solution of PBS containing FTS in CRYSMEB (10% w/w) was stable for the duration of the study. CRYSMEB in PBS served as a control. Solutions of FTS for in vitro experiments were prepared as described elsewhere (19). Noble agar, used for soft agar assays, was from Difco Laboratories, Detroit, MI. Tissue culture supplies were from Biological Industries, Beit Haemek, Israel, and from Life Technologies, Inc., Gaithersburg, MD. TRITC-labeled phalloidin and rabbit antitubulin Ab were from Sigma Chemical Co. Mouse anti-Ras Ab (Pan-Ras Ab 03) was from Calbiochem, La Jolla, CA, anticablevin-1 Ab (Ab 2297) was from BD Transduction Laboratories, Franklin Lakes, NJ, anti ERK Ab was from Santa Cruz Biotechnology, Santa Cruz, CA, and peroxidase-goat antimouse Ab was from Jackson ImmunoResearch, West Grove, PA.

Cells. Human H-Ras-transformed Rat-1 cells (EJ cells), Rat-1 cells, and Panc-1 human pancreatic cancer cells as described in previous studies (19, 20, 26) were routinely grown in DMEM/10% FCS, both from Biological Industries Beit Haemek, Israel, at 37°C in a humidified atmosphere of 95% air/5% CO₂. SW 480 human colon cancer cells (American Type Culture Collection Manassas, VA) were routinely grown in RPMI 1640 containing 10% FCS, both from Life Technologies, Inc.

To allow adaptation of EJ and Panc-1 cells to FTS, the cells were plated in 10-cm dishes at a density of 2 × 10⁶ cells/dish. FTS (25 μM) was added 2 h after plating, and the cultures were then split 1:5 every 3–4 days. Two weeks later the FTS concentration was increased to 50 μM, and the treatment was continued for ≥5 weeks. After 5 weeks of treatment the concentration of FTS was increased to 100 μM for an additional 5 weeks or more. Control cells were grown in the presence of 0.1% DMSO and split at the same times as the adapted cells. These controls did not show any change in their transformed phenotype in vitro and were tumorigenic in nude mice. Therefore, they were defined as naive cells. The terms RP50 and RP100 define cells that were adapted to grow in the presence of 50 μM and 100 μM FTS, respectively, and exhibit a RP.

To allow adaptation of SW 480 cells to FTS, the cells were grown in culture flasks and split twice a week. They were first grown in the presence of 50 μM for 5 weeks, then in the presence of 75 μM FTS for an additional 5 weeks, and finally in the presence of 100 μM FTS for ≥2 weeks. Control and adapted EJ, Panc-1, and SW 480 cells were saved in liquid nitrogen. The adapted cells were recovered from FTS in cultures that were grown in the absence of the drug and split every 3–5 days.

Chemosensitization. EJ or Panc-1 cells and their FTS-adapted counterparts were plated at a density of 2500 cells/well in 24-well plates and grown for 3 days in the presence of the indicated FTS concentration. The cells then received doxorubicin (0.1 or 0.25 μM) or gemcitabine (1.6–160 μM) for 4 h. The numbers of live cells were counted 3 days later. SW 480 cells and their FTS-adapted counterparts were plated at a density of 2500 cells/well in 96-well plates and grown for 3 days in the presence of 100 μM FTS with or without 1 μM doxorubicin or 3 mM gemcitabine. This procedure was used, because the SW 480 cells exhibited substantially more resistance to the cytotoxic drugs than the EJ or the Panc-1 cells. The numbers of live SW 480 cells were estimated by the Wst-1 cell proliferation assay (Boehringer Mannheim, Mannheim, Germany).

Labeling of Cells with Phalloidin–TRITC. Cells were plated on glass coverslips placed in 35-mm dishes at a density of 2500 cells/dish. After incubation for 24 h, the cells were treated for 24 h with 0.1% DMSO or FTS at the indicated concentrations. They were then fixed with 3% formaldehyde (20 min), treated with 0.2% Triton X-100 (5 min), and stained with 0.1 μg/ml phalloidin (40 min). F-actin was visualized by confocal microscopy as described (24).

Western Immunoblotting and Mitogen-activated Protein Kinase Assay. FTS-treated and control cells were plated at a density of 2–4 × 10⁶ cells/10-cm dish and grown for 24 h. The apparent amounts of Ras and caveolin-1 in the total particulate fractions of the cells (100,000 × g pellet) were then determined as described (20, 22). Briefly, 25 μg protein (from EJ or Panc-1 cells) or 100 μg protein (from SW 480 cells) were separated by 12.5% SDS-PAGE and blotted onto nitrocellulose paper. Immunoblotting with pan-Ras antibodies (1:2,000), with caveolin-1 Ab (1:1,000), or with antitubulin Ab (1:25,000) and
enhanced chemiluminescence assays were performed as described (22). Mitogen-activated protein kinase (ERK) activity was determined in ERK immunoprecipitates using MBP and [γ-32P]-ATP as substrates (33).

**Soft Agar Assays.** Cells were seeded at a density of 1 × 10^4 cells/well in six-well plates. The cells (in DMEM/10% FCS) were mixed with 0.5 ml of 0.33% Noble agar, and the mixture was poured onto a layer of 1.5 ml 0.5% Noble agar in DMEM/10% FCS (24). The upper layer of agar was covered with 250 μl of medium. All of the agar layers contained either 0.1% DMSO (control) or FTS dissolved in 0.1% DMSO. Colonies were photomicrographed on day 21.

**Animal Studies.** Nude CD1-Nu 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 zero, 2.5 × 10^6 cells in 0.1 ml of PBS were implanted s.c. just above the right femoral joint. In experiments where tumor growth of FTS-adapted RP cells was examined, the mice received EJ-RP50 cells or their naive EJ counterparts, Panc-1-RP100 cells or their naive Panc-1 counterparts, or SW480-RP100 cells or their naive counterparts. The animals received no additional treatment, and tumor volumes were then estimated. In experiments where the effects of FTS and the combination of FTS and gemcitabine were examined, naive Panc-1 cells were implanted beneath the skin of nude mice as described above. When tumor volumes reached values of 0.06–0.07 cm^3_ (day 0 of FTS treatment), the mice were randomly separated into four groups. Control mice received vehicle; FTS-treated mice received 14 mg/kg FTS (i.p. daily); gemcitabine-treated mice received 3.3 mg/kg gemcitabine on days 8, 11, and 18 after starting FTS treatment; and mice treated with FTS plus gemcitabine received both drugs as described above. Tumor volume was calculated as (length × width) × (length + width) ÷ 2. All of the animal studies were approved by the Animal Research and Care Committee of Tel-Aviv University.

**RESULTS**

Prolonged Exposure of Tumor Cells to FTS Does Not Cause Drug Resistance. To examine whether transformed cells develop resistance to FTS we exposed EJ, Panc-1, and SW480 cells to different concentrations of the Ras inhibitor for prolonged periods of time. EJ and Panc-1 were first exposed to 25 μM of FTS for 2 weeks, then to 50 μM of FTS for ≥5 weeks, and then to 100 μM of FTS for ≥5 weeks. SW 480 cells were exposed to 50, 75, and 100 μM of FTS in a similar manner. We started with a higher FTS concentration in the protocol for the SW480 cells, because these cells are known to be less sensitive than the EJ and the Panc-1 cells to FTS. We then evaluated the effects of the prolonged exposure to FTS on cell morphology and growth. The morphology of the cells exposed to FTS for a prolonged period was significantly altered (Fig. 1). Almost all of the cells under FTS exposure gained a more spindle-like, flattened appearance and remained so as long as FTS was present in the medium. It is important to note that the EJ (24), Panc-1 (26), and SW480 (34) cells all acquired a RP after 1–3 days in culture with FTS and retained it after the long-term treatment of ≥6 months (Fig. 1). These findings suggest that the tumor cells remain sensitive to FTS even when grown in its presence for long periods and do not gain resistance. The RP and lack of resistance were also apparent when the actin cytoskeleton of EJ cells was labeled with TRITC-phalloidin. EJ-RP100 cells (cells adapted to grow in the presence of 100 μM of FTS) exhibited strong actin stress fibers, whereas no such fibers were seen in control EJ cells (Fig. 2A). Both SW480 and Panc-1 cells had strong stress fibers in the absence of FTS, and this did not change with FTS treatment (not shown). Reversal of the transformed phenotype in EJ cells was also apparent by the loss of anchorage-independent growth of the EJ-RP100 cells in soft agar (Fig. 2B).

To examine whether a population of poorly transformed cells was selected by the prolonged FTS treatment, we removed FTS from EJ-RP100 cells that had been exposed for 6 months to the drug. Removal of FTS resulted in full recovery of the transformed phenotype (Fig. 2). Interestingly, the time required for recovery was relatively long (3 weeks). Similar results were obtained with Panc-1 and SW 480 cells (not shown). Our previous experiments (23, 24, 26, 34) suggested that the in vitro FTS-induced reversal of the transformed phenotype of tumor
cells expressing oncogenic Ras is rapidly reversible. The present studies show that the continued presence of FTS is required to maintain the RP of these tumor cells and that recovery after a prolonged period of exposure to FTS is relatively slow (Fig. 2).

Next we evaluated the effects of the prolonged exposure of the cells to FTS on their anchorage-dependent growth. The growth of Panc-1, SW480, and EJ cells was significantly altered by the prolonged FTS treatment as evident by the reduction in cell number in the 7-day cultures (Fig. 1). As in short-term treatment with FTS (19, 20), inhibition of anchorage-dependent cell growth was evident 2–3 days after plating and increased with time. For example, on days 2, 5, and 7 after plating, the numbers of cells in Panc-1-RP100 cultures corresponded respectively to 90 ± 12%, 60 ± 7%, and 45 ± 9% (n = 4) of the numbers in the naïve Panc-1 cultures. The multiplication times estimated at the logarithmic phase of growth of the naïve and RP cells were significantly altered. The EJ-RP50 and EJ-RP100 cells multiplied every 45 and 66 h, respectively, whereas the control EJ cells multiplied every 24 h. The multiplication time was 20 h in Panc-1-RP100 cells compared with 16 h in their controls, and 25 h in the SW480-RP100 cells compared with 19 h in their controls. Therefore, we concluded that inhibition of the anchorage-dependent growth of these tumor cells by FTS, which is apparent in short-term treatments (20, 24, 26, 34), is not lost during long-term exposure to the inhibitor. It is important to note that under the conditions used here (10% serum) FTS did not induce cell death and that the continued presence of the inhibitor was required to maintain the relatively slow growth rates of the cells. This was true for periods of exposure to FTS as long as 6 months. When FTS was removed from the RP cells they reverted to their normal growth rates, which were indistinguishable from those recorded in the untreated Panc-1, SW 480, and EJ cells. The return to normal growth rates, like the recovery from the RP, was, however, a very slow process requiring a period of at least 3 weeks.

Ras Remains Low in FTS-adapted Tumor Cells. We have shown previously that FTS treatment results in a rapid reduction in the amounts of Ras protein in EJ, Panc-1, and SW480 cells within 12–48 h (22, 26, 34). To determine whether the amounts of Ras remain low in the FTS-treated RP cells, we subjected the cells to Western immunoblotting with pan-Ras Ab. As shown in Figs. 3 and 4, the amounts of Ras were significantly lower in the RP cells than in their respective untreated controls. The apparent amounts of Ras in the Panc-1-RP100, SW480-RP100, and EJ-RP100 cells were respectively 30%, 55%, and 49% of their controls. The extent of Ras reduction in each cell line was similar to that observed after short-term FTS treatment of that cell line (22, 26, 34). The amounts of Ras in the RP cells remained low as long as the cells were exposed to the drug (Figs. 3 and 4). Thus, the reduced amounts of Ras after short-term (22, 26, 34) or long-term treatment with FTS in the

Fig. 2 FTS induces persistent actin rearrangements and persistent inhibition of anchorage-independent growth in EJ-RP100 cells. A, EJ (control) and EJ-RP100 cells grown for 6 months in the presence of FTS and EJ-RP100 cells 3 weeks after FTS washout were grown on coverslips. After 24 h the cells were fixed and labeled with phalloidin-TRITC, as described in “Materials and Methods.” The cells were then subjected to fluorescence confocal microscopy. Typical images of EJ, EJ-RP100, and recovered EJ-RP100 cells are shown. Similar results were obtained in three independent experiments. B, anchorage-independent growth of the FTS-adapted cells in soft agar. Soft agar assays were performed in triplicate, as described in “Materials and Methods” with EJ (control) cells, EJ-RP100 cells, and recovered EJ-RP100 cells. Typical photomicrographs, taken 21 days after plating, are shown. Similar results were obtained after longer periods of FTS treatment and after longer periods of recovery from FTS treatment but not after shorter periods of recovery.
RP cells correlates well with the RPs and with slow growth rates.

ERK Activity Is Down-Regulated, and Caveolin-1 Is Up-Regulated in FTS-adapted EJ Cells. The Ras-dependent activation of ERK is an essential signaling component in Ras transformation (8, 35, 36) and is down-regulated in tumor cells acutely treated with FTS (25, 26, 33). Caveolin-1, a membrane protein associated with membrane microdomains (37–39), has been linked to Ras signaling (39–42), and its expression is down-regulated by oncogenic Ras protein expression (43). Therefore, it was of interest to examine the relationships between the amounts of Ras, ERK activity, and caveolin-1 expression in the RP cells. We chose the fibroblast model for this set of experiments, because with this model we could make a controlled comparison between the parental Rat-1 cells, the H-ras-transformed Rat-1 EJ cells, and the EJ-RP100 cells before and after the removal of FTS. Typical results of these experiments are shown in Fig. 4. The amounts of Ras in EJ cells were 268% higher than the amounts of endogenous wild-type Ras in Rat-1 cells (Fig. 4). As indicated above, the amounts of Ras in the EJ–RP100 cells are low, comparable with the amounts of endogenous Ras in Rat-1 cells (Fig. 4). On removal of FTS from EJ-RP100 cells, the amounts of Ras increased to the relatively high values observed in the untreated EJ cells (105% relative to naive EJ cells; Fig. 4). Recovery was slow, requiring a period of 2–3 weeks. A similar pattern of alterations in ERK activity was observed in these cells: the ERK activity was substantially higher in EJ cells than in the untransformed parental Rat-1 cells (164%) and significantly lower in EJ–RP100 cells than in EJ cells (43%, Fig. 4), but 2 weeks after removal of FTS from the EJ-RP100 cells their ERK activity increased to the relatively high values observed in EJ cells (88%; Fig. 4).

In agreement with previous studies (43), oncogenic Ras induced a marked reduction in the expression of caveolin-1 in EJ cells compared with that in the parental Rat-1 cells (24%) of the value recorded in Rat-1 cells; Fig. 4). The amounts of caveolin-1 were significantly higher in EJ-RP100 cells than in EJ cells (201%; Fig. 4). The amounts of caveolin-1 in the latter cells were only 50% lower than in the parental Rat-1 cells. On removal of FTS from the EJ-RP100 cells, caveolin-1 declined to the relatively low values observed in EJ cells (Fig. 4). Taken together, the results obtained with the fibroblast model are consistent with the notion that down-regulation of oncogenic Ras by FTS is manifested not only as an early event of inhibition of Ras signaling (24, 26, 33) but also as a long-term effect on Ras signaling and on membrane components associated with the transformed phenotype induced by Ras. These effects of FTS on Ras, ERK, and caveolin-1 are reversible.

Chemosensitization of Human Tumor Cells by FTS. Next we examined whether the reduction in Ras and the reversal of the transformed phenotype of cells exposed to FTS are also reflected by altered sensitivities to cytotoxic agents. The agents chosen for these experiments, gemcitabine and doxorubicin, are apoptosis-inducing drugs that have been used clinically with limited success in the treatment of human pancreatic and colon cancers (44–47). First we examined the effects of these cytotoxic drugs on naive SW480 and naive Panc-1 cells and found that the former was by far the less sensitive of the two. For example, 1 μM of doxorubicin (3 days of treatment) had a relatively small cytotoxic effect on naive SW480 cells (Fig. 5), whereas under the same conditions, Panc-1 cells did not survive. To achieve a relatively small degree of cytotoxicity with this agent in Panc-1 cells comparable with that observed in SW480 cells, only a 4-h treatment with 0.25 μM of doxorubicin was required (Fig. 6). Differential sensitivities were also observed with gemcitabine: treatment of SW480 cells with 3.3 mM of gemcitabine for 3 days killed only 17% of the cells, whereas treatment of Panc-1 cells with 16 μM of gemcitabine for only 4 h was sufficient to kill 72% of the cells (Fig. 6). Accordingly, we used different protocols for cytotoxic drug treatment of the two cell lines. SW480 cells and their SW480-RP100 counterparts were incubated for 3 days in the presence of 1 μM of doxorubicin or 3.3 mM of gemcitabine and then assayed. Panc-1 cells and their Panc-1-RP counterparts were treated with the lower concentrations of the cytotoxic drugs for only 4 h and were assayed 3 days later. It is important to note that with these experimental paradigms of 3 days in culture the degree of cell growth inhibition by FTS alone (Figs. 5 and 6) was, as expected, relatively small (15–20%).
Chemosensitization by a Ras Inhibitor

in Panc-1-RP cells exposed to gemcitabine (Fig. 6B/H9262). Here too the synergistic effect of the two drugs was manifested by additional cell death 49% and 54%, respectively (Fig. 6B/H9262). Under the specified conditions, all of the naive Panc-1 cells survived after treatment with 1.6 μM of gemcitabine, the survival rate of the Panc-1-RP100 cells was only 18% (Fig. 6B/H9262). Taking into account the 15% inhibition of Panc-1-R100 cell growth attributable to FTS, the additional extent of cell death because of the combined drug treatment was 67%. This strong synergism is reflected by the ~100-fold increase in sensitivity of the Panc-1-RP100 cells to gemcitabine: whereas 82% of these cells died with 1.6 μM of doxorubicin, a similar number of the naive Panc-1 cells (87%) died with 160 μM of gemcitabine (Fig. 6B/H9262). The chemosensitization effect of FTS in Panc-1-RP100 cells, but not in SW480-RP100, was reversible; 3 weeks after the removal of FTS the cells were less sensitive to doxorubicin (Fig. 6A) or to gemcitabine (Fig. 6B/H9262) than the chronically treated Panc-1-RP100 cells.

The differences noted above in drug sensitivity between Panc-1 and SW480 cells were also apparent in another aspect of chemosensitization by FTS. In Panc-1 cells, short-term treatment with FTS was sufficient for chemosensitization to gemcitabine, though the effect was somewhat weaker than that of the long-term treatment (Fig. 6, A and B). In SW480 cells, short-term treatment had no chemosensitization effect (Fig. 5).

Interestingly, unlike the Panc-1-RP or the SW480-RP100 cells, the EJ-RP cells exhibited a lower sensitivity to doxorubicin than their naive counterparts (Fig. 7). Consistent with the apoptosis-promoting function of oncogenic Ras described in fibroblasts (5), we found that EJ cells are much more sensitive to doxorubicin than the parental untransformed Rat-1 cells (Fig. 7). Dxorubicin at concentrations of 0.1 μM and 0.25 μM did not induce death of Rat-1 cells under the specified conditions. In naive EJ cells, however, 0.1 μM of doxorubicin caused ~80% cell death (Fig. 7), and at 0.25 μM practically no viable cells remained in the culture. Thus, expression of the oncogenic H-ras in the Rat-1 fibroblasts indeed increased sensitivity to an apoptotic stimulus. FTS treatment reversed this effect, as shown for example in the EJ-RP100 cells, in which sensitivity to doxorubicin was as low as in the naive Rat-1 cells (Fig. 7).

**Panc-1-RP and EJ-RP Cells Exhibit Low Tumorigenic Potential in Nude Mice.** Because the effects of FTS in the RP cells were only slowly reversible *in vitro*, it was of interest to determine whether the reverted cells can from tumors in nude mice without any additional FTS treatment *in vivo*. Naive or RP cells were implanted beneath the skin above the femoral joint of nude mice, and tumor volumes were estimated 1 month later. Nude mice that had received naive EJ cells developed relatively large tumors (mean volume of 6.9 ± 4.5 cm³; n = 4), whereas of four mice that had received EJ-RP50 cells, two did not develop tumors at all, and two developed tumors with a mean volume of 0.83 cm³. Mice that had received naive Panc-1 cells also developed relatively large tumors 1 month after cell implantation (mean volume of 7.5 ± 3.3 cm³; n = 4), but mice that had received the Panc-1-RP100 cells developed much smaller tumors (mean volume of 2.08 ± 1.4 cm³; n = 4). A similar set of experiments, performed with naive SW480 and SW480-RP100 cells, revealed no differences in tumor volumes between the two groups. This is despite the observed inhibitory effect of FTS on SW480 tumor growth. FTS at a dose of 14 mg/kg (i.p. daily) inhibited growth of the naive SW480 cell tumors: the mean tumor volumes on day 28 of

---

**Fig. 5** Chemosensitization of SW480 cells by long-term FTS treatment. Naive SW480 or SW480-RP100 cells were plated in 96-well plates at a density of 2500 cells/well. The naive SW480 cells received 0.1% DMSO or 100 μM FTS, and the SW480-RP100 cells received 100 μM FTS. The cells were grown for 3 days in the presence or absence of doxorubicin (A) or gemcitabine (B). The numbers of live cells were then estimated as described in "Materials and Methods." The experiment was performed in quadruplicate and repeated three times. Data (means) are expressed as the number of live cells as a percentage of the apparent number of untreated naive SW480 cells; bars, ± SD.

Results of a typical experiment with naive SW480 and SW480-RP100 cells are shown in Fig. 5. The results demonstrate that FTS chemosensitized the RP cells. Under the specified conditions, doxorubicin treatment resulted in the death of 7% of SW480 cells compared with the death of 56% of SW480-RP100 cells. Taking into account the 13% reduction in growth of the SW480-RP100 cells because of FTS and the 7% cell death in SW480 cells, the additional extent of cell death attributable to the combined treatment was 36% (Fig. 5A). Similarly, the additional extent of cell death because of the combined effect of FTS and gemcitabine was 25% (Fig. 5B). However, short-term FTS treatment (3 days in culture) was not sufficient for chemosensitization of SW480 cells (Fig. 5, A and B).

As shown in Fig. 6, FTS also chemosensitized the Panc-1-RP cells. Under the specified conditions, all of the naive Panc-1 cells survived after treatment with 0.1 μM of doxorubicin, and treatment with 0.25 μM of doxorubicin resulted in only 14% cell death. In Panc-1-RP50 treated with 0.1 μM and 0.25 μM of doxorubicin, the numbers of viable cells decreased by 49% and 54%, respectively (Fig. 6A). Here too the synergistic effect of the two drugs was manifested by additional cell death of 25–40%. FTS had an even stronger chemosensitization effect in Panc-1-RP cells exposed to gemcitabine (Fig. 6B). Under the specified conditions, while 100% of the naive Panc-1 cells
treatment were 1.3 ± 1.02 cm³ (n = 5) in the control group and 0.31 ± 0.09 cm³ (n = 4) in the FTS-treated group.

Taken together, the results obtained with the EJ-RP and Panc-1-RP cells but not with the SW480-RP cells indicate that the slow reversibility of in vitro treatment with FTS is also manifested in vivo by the low tumorigenic potential of the RP cells.

**Chemosensitization of Panc-1 Tumors by FTS in Nude Mice.** The experiments described above provided a potential treatment protocol for a more realistic situation in which existing tumors may be treated first with FTS and then with a cytotoxic drug. Therefore, we examined whether early treatment with FTS would chemosensitize preexisting tumors in nude mice. For this purpose we used naive Panc-1 cells and either an FTS treatment protocol (26) or a combined treatment protocol of FTS and gemcitabine. Naive Panc-1 cells were implanted beneath the skin of nude mice. When measurable tumors were observed (tumor volumes of 0.06–0.07 cm³), the mice received first 14 mg/kg FTS (i.p., daily) for 8 days and then FTS and gemcitabine (3.3 mg/kg, i.p., on days 8, 11, and 18). The daily FTS treatment was then continued. Tumor volumes were measured 3 weeks after the start of the FTS treatment, and the survival rates of the mice were monitored. As shown in Fig. 8A, treatment with FTS alone resulted in a 62% reduction in tumor volumes compared with the control group. At the relatively low dose of gemcitabine used in these experiments, the cytotoxic drug alone did not cause a reduction in tumor volume (Fig. 8A). However, the effect of the combined treatment of FTS and gemcitabine was stronger than that of the individual drug treatments and resulted in 81% reduction in Panc-1 tumor volume (Fig. 8A). Similarly, the combined effect of the two drugs on the survival rates of the mice was stronger than the effect of FTS alone or of gemcitabine alone (Fig. 8B). The half-cumulative survival rate was 32 days in the control group, 35 days (an increase of 10%) in the FTS group, and 42 days (31% increase) in the gemcitabine group. The half-cumulative survival rate recorded in the combined treatment group was 53 days (65% increase). Thus, the synergistic effect of the combined treatment of gemcitabine and FTS was manifested by an additional increase of 24% in the survival rate.

**DISCUSSION**

Many human tumor cell lines develop drug resistance (27–30). For example, SW480 cells used in the present study develop resistance when chronically exposed to doxorubicin, cisplatin, and 5-fluorouracil (48). The development of such drug
resistance of tumor cells is associated with the MDR family of proteins (49). The results of this study showed that human epithelial cancer cells or rodent fibroblasts expressing oncogenic Ras do not develop resistance to the Ras inhibitor FTS even after prolonged and continued exposure to FTS. This may be attributed to lack of recognition by the MDR protein. Farnesylcysteine analogues with a free carboxyl group, as in FTS, are not MDR substrates (49), and the structural requirements among such analogues for the inhibition of Ras (50) and for the interactions with MDR (49) are distinct. It is also worth noting that FTS was shown to dislodge the mature active Ras protein from the cell membrane in intact cells without an effect on Ras farnesylation or methylation (20). As in cancer cells subjected to short-term treatment (21–23, 25, 26, 34), Ras protein is reduced after long-term FTS exposure (Figs. 3 and 4). The reduction of Ras protein in each of the cell lines tested was shown previously to occur at a relatively rapid rate, reaching a maximum within 12–48 h of drug treatment (21–23, 25, 26, 34). Initial effects of FTS on membrane-bound Ras are observed as early as 30 min after FTS treatment (21) and with long-term effects (21, 22) that are probably linked to alterations in gene expression of proteins that control Ras-membrane interactions. One such alteration is the expression of caveolin-1 (Fig. 4).

Caveolin-1 is a major component of caveolea or membrane rafts, which are important membrane domains associated inter alia with signal transduction (39, 40, 42, 51). Cell transformation by oncogenes such as c-myc, ErbB2, or Ras induces down-regulation of caveolin-1 and in some cases disappearance of caveolea (52). Moreover, overexpression of caveolin-1 in tumor cell lines reverses their transformed phenotype (52, 53). Other studies have pointed to interactions of Ras with caveolin-1 (51). Clearly then, long-term expression of oncogenic Ras has a profound effect on the structure of the cell membrane. In agreement with those reports (43), we showed in this study that the amounts of caveolin-1 are substantially lower in EJ cells than in their parental untransformed Rat-1 cells (Fig. 4). Moreover, removal of Ras and reversal of cell transformation by FTS resulted in an increase in caveolin-1 in the EJ-RP100 cells (Fig. 4). Therefore, we suggest that caveolin-1 in increased amounts alters the properties of the cell membrane in FTS-treated cells. This alteration is manifested in modified Ras-membrane interactions and inhibition of Ras transformation.

Another key finding of this study is that continuous treatment with FTS is capable of chemosensitizing human tumor cells originating from pancreatic and colon cancers. These tumors, which are of epithelial origin, represent examples of treatment-resistant malignancies (31, 32). Notably, FTS was found here to reduce the chemosensitivity of EJ fibroblasts. The known opposing effects of oncogenic Ras in epithelial cells (antiapoptotic) and in fibroblasts (proapoptotic; Refs. 5, 54) were, thus, also manifested in our experiments. Oncogenic K-ras, which is expressed in Panc-1 pancreatic cancer cells (1) and in SW480 colon cancer cells (55), is known to provide survival signals in epithelial cells (7). It is also known that stress and survival signals, such as those conferred by activated K-ras, change the survival threshold of epithelial cells (7, 56). When epithelial cells, for example, lose their attachment to the extracellular matrix they are highly sensitive to generic cellular damage (such as irradiation), and oncogenic Ras provides a protective signal (57). Our observations are consistent with those findings, as we showed that reduced amount of the oncogenic Ras in Panc-1-RP100 or SW480-RP100 cells enhances the induction of cell death by the cytotoxic drug doxorubicin or gemcitabine.

It is interesting that the FTS-treated RP cells recovered only very slowly after the removal of FTS and that this slow process was even apparent in vivo with EJ-RP and Panc-1-RP cells. The slow recovery of the RP cells was in contrast to the rapid effects of FTS on Ras and Ras-dependent signaling. The effects of FTS on Ras-membrane interactions are already observed after 30 min of treatment and are followed by a relatively rapid dislodgment (within 4–24 h) of Ras from the cell membrane (21). Similarly, FTS rapidly (within 24 h) inhibits Ras signaling to ERK (33), and its effects on the actin cytoskeleton are already apparent by 12–24 h after treatment (24). Therefore, the slow recovery of the RP cells may reflect a late response of the cells to the early FTS-induced inhibition of Ras and its signaling. A delayed response of cells to changes in the amount of Ras expression is not unprecedented. The previously observed premature cell senescence provoked by oncogenic Ras in primary cells occurs after a lag period (58). This response is associated with accumulation of p53 and the CDK4 inhibitor p16 ink4a (58). We showed recently that inhibition of Ras by FTS is also accompanied by an increase in p53 and in the CDK2 inhibitor p21waf in SW480 cells, which correlates with cell cycle arrest (34). These effects can be understood in light of the recent demonstration that in SW480 cells Ras activation of the Raf/mitogen-activated protein/ERK kinase/ERK cascade leads to increased expression of Mdm2 and attenuation of p53 accumulation (59). In another study it was shown that FTS restores
and leads to an increase in survival rate. The effect of FTS and gemcitabine on the inhibition of tumor growth is significant. We demonstrate that such treatment in mice provides a synergistic effect on the growth of various tumors, including pancreatic and colon cancers. With the Panc-1 model we were able to show a reduction in growth rate and increased (epithelial cell) or decreased (fibroblast) sensitivity to apoptotic stimuli. It is tempting to speculate that such mechanisms would also be associated with the regulation of Bcl-2 family members. Oncogenic Ras-induced inhibition of anoikis in epithelial cells results in part from Ras-induced down-regulation of Bak (61), a proapoptotic member of the Bcl-2 family. Also, oncogenic Ras was shown to abolish down-regulation of the antiapoptotic effector Bcl-XL, triggered by detachment of epithelial cells from the extracellular matrix (57).

The implications of this study must be viewed in light of the resistance of human pancreatic and colorectal tumors to cytotoxic drugs. Our results suggest that treatment with FTS would down-regulate oncogenic Ras and cause a marked increase in the chemosensitivity of previously resistant pancreatic and colon cancers. With the Panc-1 model we were able to demonstrate that such treatment in mice provides a synergistic effect of FTS and gemcitabine on the inhibition of tumor growth and leads to an increase in survival rate.

REFERENCES


# Clinical Cancer Research

## The Ras Inhibitor $S$-trans,$trans$-Farnesylthiosalicylic Acid Chemosensitizes Human Tumor Cells Without Causing Resistance


*Clin Cancer Res* 2002;8:555-565.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://clincancerres.aacrjournals.org/content/8/2/555">http://clincancerres.aacrjournals.org/content/8/2/555</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 61 articles, 22 of which you can access for free at: <a href="http://clincancerres.aacrjournals.org/content/8/2/555.full#ref-list-1">http://clincancerres.aacrjournals.org/content/8/2/555.full#ref-list-1</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Citing articles</th>
<th>This article has been cited by 11 HighWire-hosted articles. Access the articles at: <a href="http://clincancerres.aacrjournals.org/content/8/2/555.full#related-urls">http://clincancerres.aacrjournals.org/content/8/2/555.full#related-urls</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Reprints and Subscriptions</th>
<th>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Permissions</th>
<th>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</th>
</tr>
</thead>
</table>