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A Novel Ras Antagonist Regulates Both Oncogenic Ras and the Tumor Suppressor p53 in Colon Cancer Cells

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Abstract

Background: In colon cancer, K-Ras oncogenes, which appear to be linked to chemoresistance and poor prognosis, are activated in more than 50% of cases, whereas the tumor suppressor gene p53 is mutationally altered in about 70% of all cases. The transcription factor p53, which is frequently mutated at codon 273, maintains wild-type configuration and possibly carries out residual functions. Although blocking of activated K-Ras may constitute a rational therapeutic concept for this treatment-resistant malignancy, a strategy influencing both oncogenic Ras and the tumor suppressor p53 may be even more promising.

Materials and Methods: We evaluated the effects of S-trans, trans-farnesyl-thiosalicylic acid (FTS), a novel Ras antagonist on human SW480 and HT-29 colon cancer cells, which both harbor a p53 His273 mutation but express activated K-Ras and wild-type, but overexpressed, H-Ras, respectively. Besides cell growth and morphology, levels of cellular Ras proteins, regulation of p53 and $p21^{(waf1/cip1)}$ expression were analyzed by immunoblotting. The cell cycle arresting potential of FTS was quantified by flow cytometry.

Results: We demonstrate that FTS treatment alters the morphology and blocks the growth of SW480 and HT-29 colon cancer cells by both reducing the total amount of Ras and up-regulating the tumor suppressor p53. Furthermore, FTS caused an up-regulation of the cyclin-cyclin-dependent kinase (CDK) inhibitor $p21^{(waf1/cip1)}$ and blocked the cell cycle. p53 antisense oligonucleotides not only reduced the level of p53 proteins but correspondingly also blocked the expression of $p21^{(waf1/cip1)}$ in FTS-treated colon cancer cells.

Conclusions: FTS, a unique compound capable of regulating both oncogenic Ras and the tumor suppressor p53 may prove particularly useful for the therapy of colon cancer and other treatment-resistant malignancies where Ras is altered and p53 is either wild-type or mutated in positions that allow residual p53 functions.

Introduction

Colon cancer is the second most common cause of cancer death in the United States (1). This malignancy does not only bear oncogenic K-Ras but also p53 tumor suppressor gene mutations in the majority of cases. Both alterations are involved in colonic oncogenesis and tumor progression (2–6); activating point mutations in the K-Ras oncogene occur at a rate of 59% in primary tumors of the colon and 76% in advancing metastases (6,7). p53 gene mutations are present in up to 70% of colorectal cancers and occur mainly at specific conserved regions in the exons 6–8 (3,4,8–11).

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p53, the founding member of a new class of tumor suppressor genes, has been shown to be a transcriptional regulator that blocks entry of cells into S-phase in response to DNA damage by inducing the expression of a set of genes related to the control of cell proliferation (12–15). The $p21^{(waf1/cip1)}$ gene, for instance, which is directly regulated by p53, is located on chromosome 6p and encodes the $p21^{(waf1/cip1)}$ protein. The $p21^{(waf1/cip1)}$ protein functions as a potent inhibitor of CDK activity. CDK family members function as key positive regulators of the cell cycle and therefore of cell proliferation. $p21^{(waf1/cip1)}$ induces G1 arrest and blocks entry into S-phase by inhibiting CDKs (16,17) or by binding to the proliferating cell nuclear antigen (PCNA), resulting in a DNA replication block (18,19). As a downstream target effector of p53, $p21^{(waf1/cip1)}$ is transcriptionally activated (20,21). p53 proteins can accumulate as a consequence of DNA damage and are capable of binding to specific sites on the $p21^{(waf1/cip1)}$ promotor, resulting in induction of $p21^{(waf1/cip1)}$ expression (20).

Point mutations, deletions, or inversions of p53—which can either trigger a loss of function or an altered activity of p53—in highly conserved regions of the p53 gene are very frequent events in colorectal cancers (22). However, p53 activity is not necessarily completely lost. Recent studies demonstrate a residual transactivation and tetramerization activity for select mutations either in the DNA-binding or tetramerization domains, respectively (23,24). Examples of such alterations are point mutations at positions 273 (His 273) and 175 (Gly 175), both within the DNA-binding domain, or at positions 333 (Ala 333) as well as 326 (Ala 326) in the tetramerization domain. The p53 gene in SW480 and HT-29 cells is mutated at the position 273 (Arg → His) (25), within a highly conserved region of the specific DNA-binding domain (3). Saintigny et al. (23) demonstrated that p53 with a His 273 mutation still shows wild-type conformation and maintains transactivation activity.

Very recent data confirm Ras oncogenes and the tumor suppressor p53 as key players of oncogenesis and treatment resistance. Hahn et al. (26) showed that the ectopic expression of the telomerase catalytic subunit (hTERT) in combination with two oncogenes, the simian virus 40 (SV40) large-T oncoprotein, which blocks the p53 as well as the retinoblastoma pathway and an oncogenic allele of H-Ras, results in direct tumorigenic conversion of normal epithelial and fibroblast cells supporting their central role in the malignant transformation of human cells.

In the present study we investigated the effects of S-trans, trans-farnesylthiosalicylic acid (FTS), a novel Ras antagonist (27–32) on the human colon cancer cell lines SW480 and HT-29. These cell lines share the same functionally active p53 mutation (273Arg → His) (25) but differ in their Ras status; SW480 cells harbor a mutated K-Ras gene (33) whereas HT-29 cells have wild-type, but overexpressed, H-Ras (34–36). The therapeutic mechanisms of action of FTS are in clear contrast to farnesyl transferase inhibitors (FTIs), which effectively target H-Ras but fail to inhibit the processing of K-Ras4B and N-Ras altogether or lead to alternatively geranylated Ras proteins that remain active (37,38). Because K-Ras4B and N-Ras are the most commonly mutated forms of Ras in human tumors, the ability of FTS to target both H-, N-, and K-Ras may be a finding of great clinical relevance.

Here we demonstrate a pronounced growth inhibition and morphological alteration of SW480 human colon cancer cells induced by FTS, which is preceded by a reduction of total cellular Ras protein. To better understand the mechanism that arrests the growth of cancer cells triggered by FTS, we examined the cell cycle regulating factors p53 and $p21^{(waf1/cip1)}$ and detected an up-regulation in both instances. Flow cytometric analysis of the cell cycle revealed an arrest in G0/G1 in FTS-treated SW480 cells. Reduction of the p53 protein level via p53 antisense phosphorothioate oligonucleotides additionally resulted in a corresponding $p21^{(waf1/cip1)}$ protein decrease during treatment with FTS, clearly supporting the idea that mutated p53 in SW480 cells is still capable of $p21^{(waf1/cip1)}$ activation. To support the general nature of our findings we included HT-29, a second human colon cancer cell line, into our analysis of FTS effects. In HT-29, like in SW480 cells, FTS reduced the amount of total cellular Ras proteins and induced an up-regulation of p53 expression, resulting in a pronounced growth inhibition.

Our finding that FTS is capable of regulating both the oncogene Ras and the mutated but functionally active tumor suppressor p53 links two therapeutic targets that may contribute to tumorigenesis and tumor progression from opposite ends of the cancer biology spectrum.

Materials and Methods

Ras Antagonist

A 0.1-M stock solution of the competitive Ras antagonist FTS (27–32,39) was prepared in chloroform (0.1 M) and stored at −70°C. The chloroform was removed by evaporation under sterile conditions prior to use and FTS was then dissolved in DMSO (Sigma, St. Louis, MO). The FTS/DMSO solution was first diluted 1:10 with RPMI 1640/10% FCS (both from GIBCO BRL, Gaithersburg, MD) and finally adjusted to the working concentration of 100 μM. The DMSO content of this solution was 0.4%; this carrier solution was used as a control in all experiments.

Cell Culture Procedures and Growth Inhibition

The human colon carcinoma cell lines SW480 and HT-29 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were routinely grown in RPMI 1640 with 10% heat inactivated fetal calf serum and 1% antibiotic/antimycotic mix (all from GIBCO BRL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. To determine the growth inhibitory potential of FTS, 4×10^3 cells were plated in 12-well plates and incubated for 7 days in media supplemented with either 0.4% DMSO plus 100 μM FTS or 0.4% DMSO alone. Pilot experiments revealed that an FTS concentration of 100 μM was most effective at dislodging Ras proteins, so all other experiments were also conducted under these conditions. However, in dose-dependent studies, FTS concentrations below 50 μM did not have any impact on SW480 cells, and concentrations beyond 100 μM (up to 150 μM) did not further enhance the compound's activity. Fresh media containing FTS and/or DMSO was added daily to guarantee constant drug concentrations over the incubation period. Cells were harvested, from day 2 to day 7 and counted with a Coulter Z1 counter (Coulter, Luton, Beds., UK). Three wells were evaluated for FTS and control samples at each time point and results are shown as mean ± standard deviation (SD).

Ras Dislodgment and Western Blotting of Ras, p53, and p21$^{(waf1/cip1)}$

To study the reduction of total cellular Ras we plated 5×10^5 cells (SW480 or HT-29) for both the FTS and control groups. Cells were incubated for 4 days with a daily change of media. To obtain total cell extracts, cells were washed once with PBS, 500 μl of lysis buffer (0.14 M NaCl, 0.2 M triethanolamine, 1% NP-40, 0.2% Na-deoxycholate, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin; pH 7.4) was added and the cells were mechanically removed from the dishes. All following procedures were carried out at 4°C. The lysate was shock frozen twice in liquid nitrogen and insoluble material was pelleted through centrifugation at 15000 rpm for 15 min. To detect cytosolic Ras proteins, we immunoprecipitated both membrane and cytosolic cell fractions as described elsewhere (29). Protein concentrations were determined by Bradford analysis (Biorad, Hercules, CA). Equal amounts of total cellular proteins (10–20 μg) were separated by 10–12% sodium dodecylsulfate polyacryl amide gel electrophoresis (SDS-PAGE) and blotted onto PVDF membranes (Millipore Corp., Bedford, MA). Immunoblotting was performed with the Western Light Chemiluminescence Detection System (TROPIX, Bedford, MA). For specific protein detection we used the following antibodies: Oncogene monoclonal pan-Ras (Calbiochem, San Diego, CA), monoclonal p53 and polyclonal p21$^{(waf1/cip1)}$ (Santa Cruz, Santa Cruz, CA), and alkaline phosphatase conjugated secondary antibodies (TROPIX, Bedford, MA). Bands were visualized by ECL and quantified by densitometry on a Pharmacia LKB UltroScan XL (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Cell Morphology

SW480 cells were plated at a density of 2×10^5 cells/10-cm dish and incubated in either FTS (100 μM) or control solvent. Photomicrographs (original magnification ×100) were taken after 5 days of treatment on a Olympus (IMT-2) microscope (Olympus, Lake Success, NY) with black and white 400 ASA films.

Cell Cycle Analysis

Cells were plated at a density of 1×10^5 cells/10-cm dish, either in the presence of 0.4% DMSO with FTS (100 μM) or control solvent. The treatment period was 4 days with daily change of media. Single-cell suspensions were collected and cells were processed following the CycleTest Plus DNA Reagent Kit (Becton Dickinson, San Jose, CA). Briefly, cells were washed twice in buffer solution and adjusted to 1×10^6 cells/ml. For the assay, 0.5 ml of that solution was used, where cells were trypsinized,

RNAse treated, and finally stained with propidium iodide. Samples were analyzed on a FacsCalibur (Becton Dickinson). Data were evaluated using ModFit software (Verity Software House, Topsham, ME).

p53 Antisense Oligonucleotide Treatment

Antisense oligonucleotides and controls directed to human p53 have been designed and manufactured by Biognostik (Göttingen, Germany). The antisense (AS) (Seq-ID: 3.03689) as well as the control (C) (CG-matched randomized-sequence; Seq-ID: 1.02799) oligonucleotides have phosphorothioate chemistry, which increases the resistance to exo- and endonucleases. SW480 cells (1.5×10^5 cells/well; 6-well plate) were incubated with 400 nM oligonucleotides (AS or C) in the presence of 10 μl lipofectin (GIBCO BRL) for 4 hr. The transfection was performed according to the procedure recommended by GIBCO BRL. Cells were harvested and proteins extracted 20 hr after transfection. To coincubate SW480 cells with both FTS and the p53 oligonucleotides (AS and C), transfection was performed following a two-step protocol. Cells were plated in a density of 4×10^5 cells/6-cm dish and first incubated with 200 nM oligonucleotides and 10 μl lipofectin. Oligonucleotides as well as lipofectin were preincubated separately with serum-free RPMI media (200 μl) for 45 min at room temperature, then mixed and incubated for another 15 min. Each transfection batch was mixed with 1.6 ml of RPMI supplemented with 6% FCS and overlayed onto the cells. Cells were incubated for 4 hr at 37°C in a CO_2 incubator; the lipofectin–DNA complex was then removed and the cells were incubated either with FTS (100 μM) or DMSO (0.4%) containing growth media. After 48 hr, a second oligonucleotide boost was performed, this time with 400-nM DNA (10 μl lipofectin) and the use of serum-free media only. Fresh growth media containing FTS or DMSO was added after the lipofectin–DNA incubation period and 24 hr later cells were harvested and protein extracts obtained. Protein levels of p53 and $p21^{(waf1/cip1)}$ were evaluated by Western blotting.

Results

To determine whether FTS may be able to affect growth of human colon cancer, we used SW480 colon cancer cells that harbor activated K-Ras genes and p53 mutated at position 273, typical for this type of treatment-resistant malignancy (33). SW480 cells were grown for 7 consecutive days in media containing 100 μM FTS or control solvent. 48 hr after the initiation of treatment, a change in cell morphology and cell growth could already be seen in the FTS group. Cell growth was retarded and SW480 cells grew preferentially as single cells rather than 5–10 cell clusters, a growth pattern seen in the control (data not shown). Figure 1 shows the pronounced change in cell morphology following FTS treatment. SW480 cells lost their spherical appearance and changed to long, rod-shaped cells with thin pseudopodia. We confirmed that this morphological appearance does not simply apply to the low confluency caused by the changed growth pattern of cells in the FTS experiments. Even at very low cell densities (less than 10% confluency), untreated cells never show such alterations (data not shown).

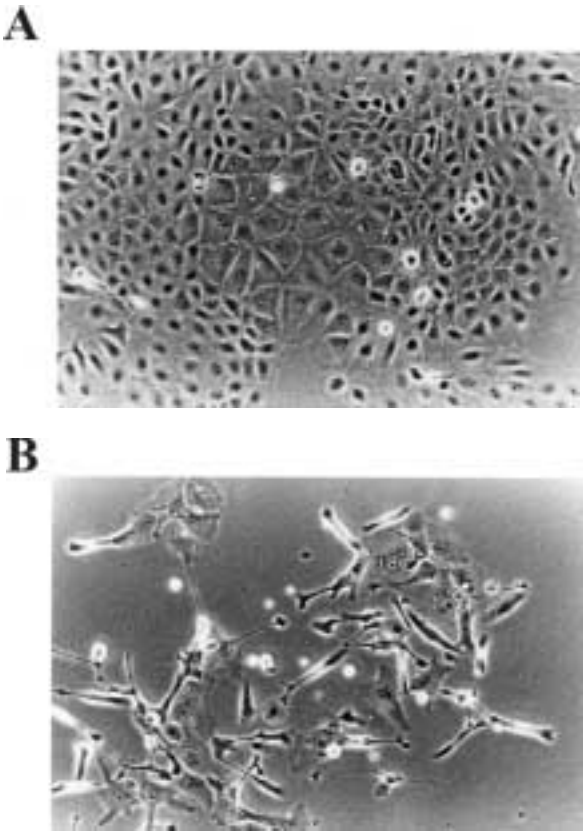


Fig. 1. Cell morphology of FTS treated cells. Typical photomicrographs (×100) of human SW480 colon cancer cells grown in the absence (A) and the presence (B) of FTS (100 μM). Cells were plated at a density of 2×10^5 cell/10-cm dish and grown for 5 days.

To examine the growth inhibitory effects of FTS on SW480 and HT-29, an additional colon cancer cell line (with p53 mutated at position 273 and overexpressed wild-type K-Ras) in detail, cells were grown either in the presence or in the absence of FTS (100 μM) and counted daily starting day 2. As shown in Fig. 2A, SW480 growth inhibition caused by FTS was 48 $\pm$10% on day 2. These antiproliferative influences of FTS continued during the complete treatment period, finally resulting in a 92 $\pm$12% growth reduction on day 7. The growth inhibitory response of HT-29 cells due to FTS was, compared to SW480 cells, somewhat delayed but finally resulted in a 85 $\pm$ 3% growth reduction on day 7 (Fig. 2B). These results

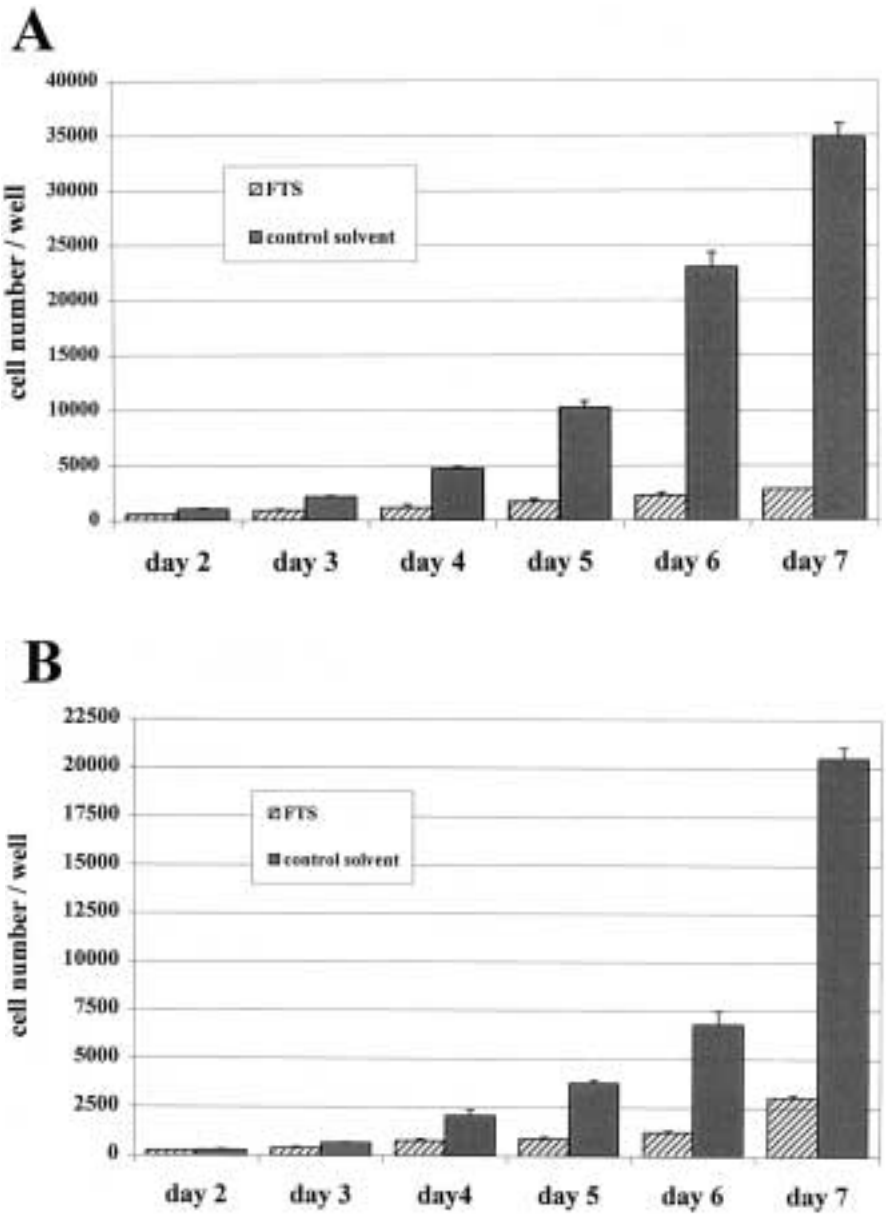


Fig. 2. FTS inhibits the cell growth of SW480 and HT-29 cells. Cells were plated at a density of 4×10^3 in a 12-well plate and grown for 7 days in the absence or presence of 100 μM of FTS. From day 2 to day 7, cells were harvested and counted. Assays were performed in triplicate and the data (mean $\pm$ SD) are presented as total cell numbers per wells. For SW480 cells (A) the growth inhibition experiment showed on day 2 a 48 $\pm$ 10% reduction of cell growth. This growth-arresting influence of FTS continued during the complete treatment period, finally resulting in a 92 $\pm$ 12% growth reduction on day 7. Growth inhibition for HT-29 cells (B): 38 $\pm$ 10% on day 3 and 85 $\pm$ 3% on day 7.

emphasize the potential of FTS to inhibit cell growth of colon cancer cells. Notably, additional experiments demonstrated that SW480 cells do not develop resistance mechanisms to FTS treatment. SW480 cells remain responsive to FTS treatment even if this treatment has been interrupted and reapplied (data not shown).

We also evaluated the ability of the Ras antagonist FTS to dislodge Ras from the inner membrane of SW480 cells. Analysis of the total cellular Ras content following treatment with FTS (100 μM) for 96 hr revealed a decrease of 54 $\pm$ 12% in the FTS group when compared to treatment with solvent alone (Fig. 3). Results were obtained from three separate experiments and Ras protein expression was evaluated relative to actin protein expression. Actin expression in three independent experiments varied by no more than a mean of 11 $\pm$ 3%. Attempts to detect cytosolic Ras by immunoprecipitation (see Methods section) failed, which suggests that Ras is degraded very rapidly in SW480 cells when removed from its membrane anchorage domain (data not shown). These results demonstrate that FTS can reduce the amount of membrane-bound, active Ras and therefore inhibit, Ras-dependent signaling in SW480 cells.

Given that in SW480 cells and many cases of colon cancers, not only K-Ras but also p53, is mutated (273; Arg $\rightarrow$ His), we investigated the influence of FTS on the cell cycle, knowing that changes in the distribution of cell-cycle phases and the regulation of p53 downstream effectors could be based on residual p53 function or mediated by p53-independent mechanisms. Protein extracts of SW480 cells treated with FTS (100 μM) or solvent for 96 hr were analyzed by immunoblotting. p53 gene products in the drug-treated group increased by 1.6-fold relative to control (Fig. 4). HT-29 cells, mutated in the p53 gene and wild-type for Ras, responded to FTS treatment with a 1.5-fold reduction of total cellular Ras and with a 3-fold higher level of p53 protein compared to control (Fig. 5). Activation was calculated by dividing results obtained by densitometry of FTS and control relative to actin protein expression.

Based on these findings, we additionally immunoblotted protein extracts of SW480 cells to obtain information about the regulation of

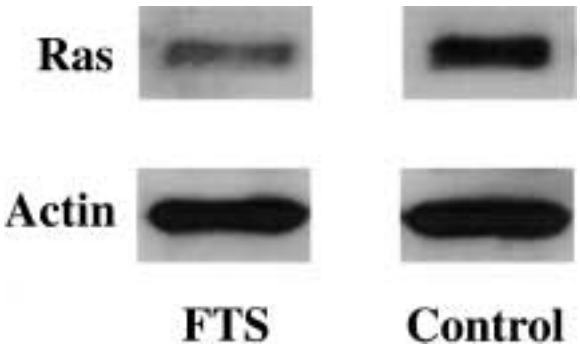


Fig. 3. Reduction in the amount of cellular Ras in SW480 cells by FTS. 5×10^5 cells were diluted directly in media containing either 100 μM of FTS or control solvent and plated in 10-cm dishes. After 4 days of treatment, with fresh media and drug added every 24 hr, cells were harvested and analyzed for Ras content by Western blotting. Actin served as an internal control to demonstrate equal protein amounts in the respective lanes. Densitometric analysis of three separate experiments showed a 2-fold reduction of total cellular Ras in FTS-treated cells; one of three representative experiments is shown.

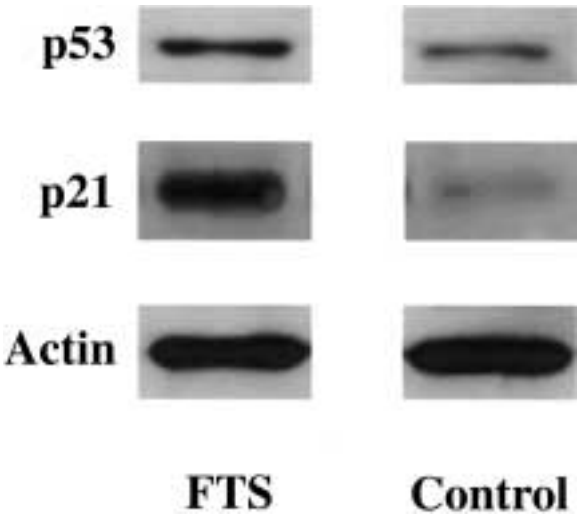


Fig. 4. Induction of the tumor suppressor p53 and cyclin-CDK inhibitor $p21^{(waf1/cip1)}$ expression by FTS. 5×10^5 SW480 cells were plated on a 10-cm dish. After 20 hr and thereafter daily, media containing FTS (100 μM) or control solvent was added. Cells were harvested and immunoblotted for p53 and $p21^{(waf1/cip1)}$ protein levels after 4 days of treatment. Actin served as an internal control to demonstrate equal protein amounts in the respective lanes. Densitometric analysis showed a 1.6-fold induction of p53 expression and a 3.7-fold increase of $p21^{(waf1/cip1)}$ protein in FTS-treated cells.

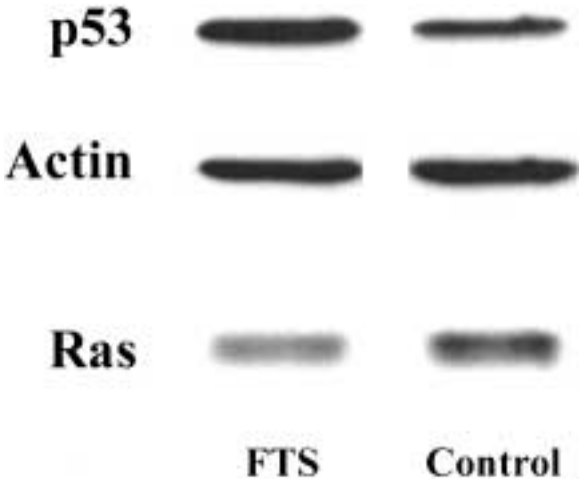


Fig. 5. Regulation of Ras and p53 in HT-29 cells. 5×10^5 cells were diluted directly in media containing either 100 μM of FTS or control solvent and plated in 10-cm dishes. After 4 days of treatment, with fresh media and drug added every 24 hr, cells were harvested and analyzed for Ras and p53 content by Western blotting. Actin served as an internal control to demonstrate equal protein amounts in the respective lanes. Densitometric analysis showed a 1.5-fold reduction of total cellular Ras and a 3-fold induction of p53 expression in FTS-treated cells. A representative experiment is shown.

cell-cycle arrest and the apoptosis pathway. Our results provide evidence that FTS caused a significant 3.7-fold increase in $p21^{(waf1/cip1)}$ protein (see Fig. 4). We could not detect FTS-induced apoptosis in SW480 cells; FTS treatment did not alter apoptosis-promoting factors, such as bax, bcl-xs, caspase 3, or caspase 8 (data not shown).

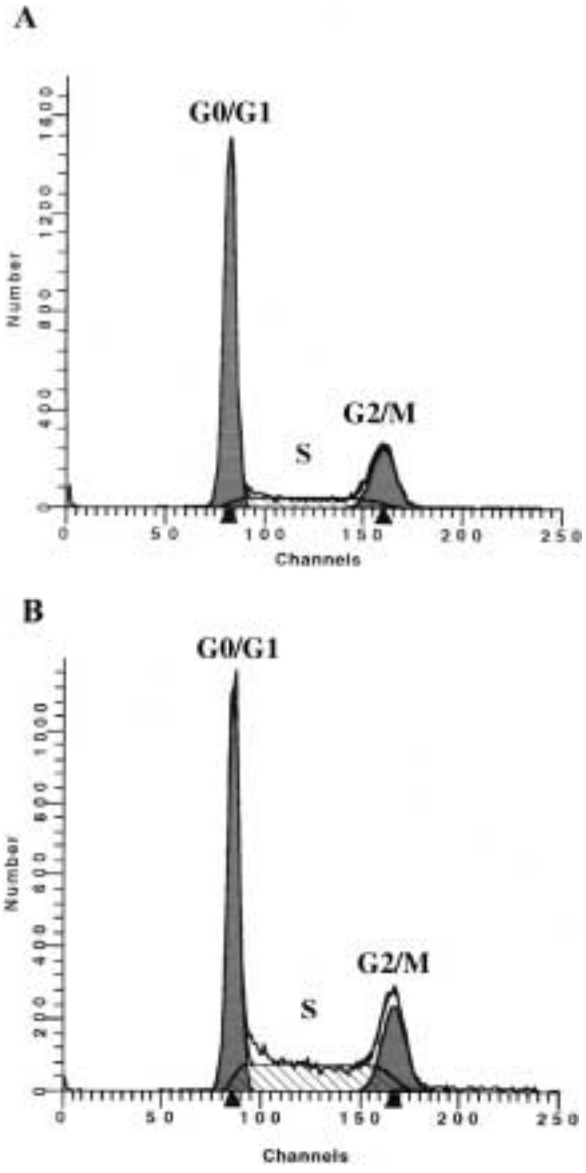
To confirm that FTS induces $p21^{(waf1/cip1)}$ expression and leads to cell-cycle arrest, we performed flow cytometric cell cycle analyses of SW480 cells (Fig. 6). In a representative experiment, 62% of an exponentially growing cell population arrested in G0/G1 after FTS treatment (4 days), whereas only 46% of the control population stayed in G0/G1. Even more clear-cut was the comparison of the S-phases, which showed 34% and 17% S-Phase cells in the control or in the FTS group, respectively (Fig. 6). Thus, FTS reduced the numbers of cells synthesizing DNA by half, while similar percentages of FTS treated or control cells were in G2/M (21% and 20%, respectively). These findings support the notion that activation of the MAPK pathway and repression of $p21^{(waf1/cip1)}$ expression is required for cells to pass the G1 restriction point.

Knowledge of the p53 genotype in SW480 colon cancer cells—point mutation at position 273 (His 273)—leads to the question of whether this mutated p53 is really capable of regulating/activating $p21^{(waf1/cip1)}$. We addressed this critical point by regulating p53 expression levels via p53 antisense phosphorothioate oligonucleotides. Treatment with 400 nM (together with a lipid uptake enhancer) of either antisense or control oligonucleotides resulted in a 2-fold reduction of p53 proteins in the antisense group, whereas the Ras level remained unaffected (Fig. 7A). Levels of p53 and $p21^{(waf1/cip1)}$ are elevated in cells treated with FTS, as shown in Fig. 4. Additional treatment of these cells with p53 antisense oligonucleotides resulted in a 3-fold reduction of p53 as well as of $p21^{(waf1/cip1)}$ protein levels (Fig. 7B).

Discussion

Previous studies have shown that FTS can act as a selective Ras antagonist in rodent fibroblasts (28,29,31,32). Its mechanism of action appears to be associated primarily with the dislodgment of mature Ras from anchorage domains in the cell membrane, which facilitates Ras degradation (29). The effects of FTS in H-Ras (V12) (29,32) transformed fibroblasts manifest as a decrease in the total amount of cellular Ras, with all its consequences on cell growth and proliferation. Importantly, FTS does not inhibit Ras farnesylation or Ras methylation at the effective concentration range (32). FTS also has no effect on the amount of N-myristylated Ras (nonfarnesylated) or on the amount of the prenylated G$\beta\gamma$ subunits of heterotrimeric G-proteins in Rat-1 cells (29). These data indicate that FTS and some of its analogs (28,31) are functional Ras antagonists and therefore may serve as a new class of compounds with potential clinical use, even under conditions where FTIs lack clinical potential owing to alternative geranylation of, for instance, K- or N-Ras (40,41).

This concept is supported by the results of the present study, which demonstrate that FTS can reduce the amount of Ras in SW480 and HT-29 human colon cancer cells, both of which have a mutated but functionally active p53 gene. SW480 cells additionally harbor an activated K-Ras gene (3,25,33). Such alterations can frequently be found in clinical colon cancer samples (4,42,43), supporting the value of these



C

	% G0/G1	% S	% G2/M
Control	46	34	20
FTS	62	17	21

Fig. 6. Cell-cycle analysis of SW480 cells by flow cytometry. Cells were plated at a density of 1×10^5/10-cm dish. Treatment with 100 μM of FTS (A) or control solvent (B) was carried out for 4 days with new media plus drug changed every 24 hr. Nuclei were stained with propidium iodide and the analysis was performed on a FacsCalibur (Becton Dickinson). Data were processed using ModFit software. The percentage of cells in each phase of the cell cycle is shown in the histograms (C).

cell lines as model systems. Disruption of biochemical pathways involving both Ras and p53 are key events in the malignant transformation of normal mesenchymal and epithelial precursors. However, additional events, such as up-regulated telomerase activity, are essential for the formation of human tumor cells (26).

Important consequences of the effects of FTS on Ras in Ras-transformed rodent fibroblasts in human Panc-1 cells (27) and human melanoma cells (44) are the inhibition of ERK activity and altered cell morphology (30,32). Both the Raf-1–MEK–ERK and the Rac/Rho cytoskeleton pathways associated with cell shape and morphology are known to be activated by oncogenic Ras proteins. Moreover, the Ras-dependent Raf-1–ERK and Rac/Rho pathways were shown to contribute synergistically to cell transformation (45–48). Thus, our observation of growth inhibition (see Fig. 2) and alterations of cell morphology in SW480 cells (see Fig. 1) lend additional support to the direct effects of FTS on Ras, which consequently appears to mediate inhibition of cell growth. FTS was shown to effect all Ras isoforms, with no specificity toward one isoform (27,29,44, 49). Accordingly, also in our systems, no preferential loss of any of the Ras isoforms—mutated or wild-type—could be observed. In addition to these not unexpected effects on Ras, we could demonstrate that FTS leads to an induction of p53 expression (see Figs. 4 and 5) and cell-cycle arrest (Fig. 6). Proliferation signals originating in oncogenic Ras are reduced by FTS. Thus, the cell-cycle arresting machinery appears to be reestablished. Although the p53 regulation initiated by FTS is clearly demonstrated in our two cell lines, the pattern of activation, either via a transcriptional or a posttranslational event, remains to be clarified in detail. One of the most common mutations of the p53 gene, G → A transition in codon 273 resulting in an Arg → His substitution, can be found in both SW480 and HT-29 cells (3,25). This mutation is located within the central, sequence-specific DNA-binding domain, necessary for the transcriptional transactivation of p53 downstream effector genes. Codon 273 mutations, resulting in different amino acid substitutions, are the most common p53 gene alteration found in human tumors in general and in colon cancer in particular, with an incidence of about 50% (8,9,50,51). Recent data (23,24) support the concept that the

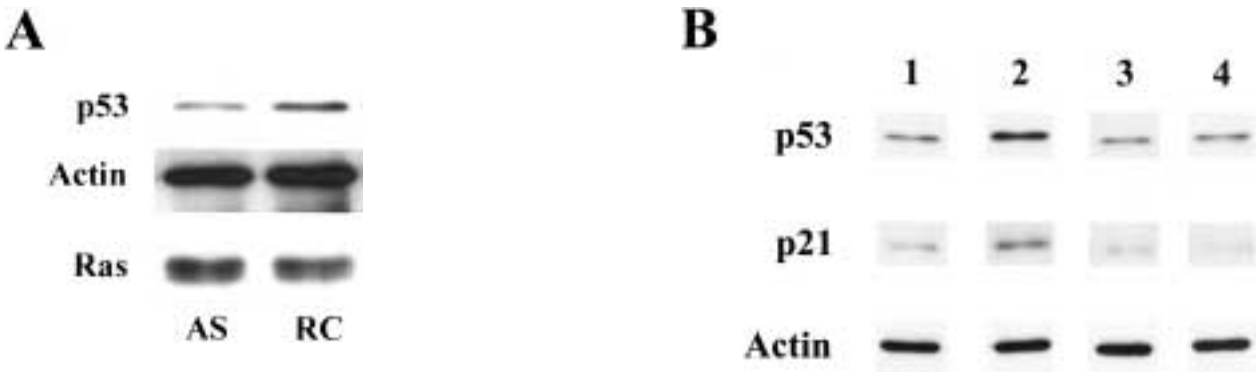


Fig. 7. Antisense oligonucleotide-mediated regulation of p53. (A) Down-regulation of p53 proteins by treatment of SW480 cells with 400 nM of p53 AS oligonucleotides leads to a 2-fold reduction of total cellular p53 proteins, compared to a control (RC) oligonucleotide. As expected, the Ras level remained unaffected by the oligonucleotide treatment. (B) Protein levels of p53 and $p21^{(waf1/cip1)}$ under combined treatment of FTS and antisense (lane 1); FTS and control (lane 2); DMSO and antisense (lane 3); and DMSO and control (lane 4). Antisense treatment of SW480 cells not only reduced the level of p53 but also the level of $p21^{(waf1/cip1)}$ 3-fold, compared to a control oligonucleotide (lanes 1 and 2). Lane 4 shows the baseline for p53 and $p21^{(waf1/cip1)}$ in those cells, and lane 3 shows a slightly reduced level of p53 and an unaffected $p21^{(waf1/cip1)}$ level. Actin served as an internal control to demonstrate equal protein amounts in the respective lanes. One of three representative experiments is shown.

impact of p53 mutations on p53 function strongly depends on the site as well as on the type of the genetic alteration. Saintigny et al. (23) demonstrated that, at position 273, the replacement of the amino acid arginine by a histidine has no effect on homologous recombination. Additionally, this gene product retains residual transactivation activity of the $p21^{(waf1/cip1)}$ promotor and is capable of G1 arrest after γ-ray exposure. However, substitution by a proline triggers homologous recombination and this protein lacks $p21^{(waf1/cip1)}$ transactivation activity. This result could be associated with the fact that the His273 protein keeps a wild-type conformation whereas the Pro273 protein adopts a mutant form (52).

With p53 antisense experiments, we could confirm that mutated p53 in SW480 cells is truly capable of activating $p21^{(waf1/cip1)}$ (Fig. 7B). Simultaneous down-regulation of p53 and $p21^{(waf1/cip1)}$ under the treatment of p53 antisense oligonucleotides stresses the causal relationship between these two regulatory factors, even if p53 has a mutated genotype at position 273. This is strong evidence supporting the notion that His273 mutated p53 has residual activity and is definitely capable of transcriptionally activating $p21^{(waf1/cip1)}$. Despite the point mutation, p53 still seems to be a key player in the signal transduction of SW480 cells and is likely to be able to regulate/activate additional downstream effector molecules, other than $p21^{(waf1/cip1)}$. Based on the above findings we postulate a residual activity of p53 in SW 480 and HT-29 colon cancer cells capable of inducing $p21^{(waf1/cip1)}$ expression. This FTS triggered $p21^{(waf1/cip1)}$ induction leads to cell cycle arrest and growth reduction. Notably, Merkel cell carcinomas (MCC), with wild type p53, grown in SCID mice, also show pronounced up-regulation of p53 after FTStreatment (53).

The orderly progression of cells through the cell cycle depends on a finely tuned balance between levels of activated cyclins and CDKs that provide positive growth signals, and kinase inhibitors that suppress these effects. One of those, $p21^{(waf1/cip1)}$, inhibits the catalytic activity of all CDKs, although with variable efficiency (16,17). Moreover, $p21^{(waf1/cip1)}$ can inhibit DNA replication by blocking the ability of PCNA to activate DNA polymerase-δ (18,54). In our experiments, induction of $p21^{(waf1/cip1)}$ expression (see Fig. 4) reduced cells passing through S-phase by half (see Fig. 6).

Because recent evidence suggests that $p21^{(waf1/cip1)}$ may protect against p53-mediated

(55) and p53-independent (56) apoptosis, we examined the expression of apoptosis-promoting (bax, bcl-xs) or executing factors (caspase 3, caspase 8) in our system. Western blot analysis did not show regulation or activation of any of those factors (data not shown), indicating that apoptosis is not directly initiated by FTS and plays, if any, a minor role in the response of SW480 or HT-29 cells to FTS.

Recently, FTIs have been reported to be able to induce wild-type but not mutated p53 (57). While we regulated mutated, but functionally active, p53 with FTS (a Ras antagonist), Sepp-Lorenzino and Rosen (57) showed in their work that wild-type p53 was up-regulated because of FTI treatment. These two very different ways of influencing Ras protein levels lead to the same response—induction of p53 expression, up-regulation of $p21^{(waf1/cip1)}$ and GO/G1 cell-cycle arrest. This strongly supports the hypothesis that p53 is up-regulated in a Ras-dependent fashion. It is still possible that some farnesylated protein, which functions in the p53 sensor pathway, is influenced by both agents FTS and FTIs and leads to this p53 effect. However, there is no evidence to support this rather unlikely notion. Although preliminary, these pieces of evidence suggest that FTS, in contrast to FTIs, may be able to up-regulate both wild-type and mutated but active p53.

The ability of FTS to influence both Ras oncogenes and the tumor suppressor p53 makes it an interesting compound for targeting a variety of cancers where mutations in Ras as well as p53 genes contribute to oncogenesis and tumor progression.

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