Novel Ras antagonist blocks human melanoma growth

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During past decades, knowledge of melanoma biology has increased considerably. Numerous therapeutic modalities based on this knowledge are currently under investigation. Advanced melanoma, nevertheless, remains a prime example of poor treatment response that may, in part, be the consequence of activated N-Ras oncoproteins. Besides oncogenic Ras, wild-type Ras gene products also play a key role in receptor tyrosine kinase growth factor signaling, known to be of importance in oncogenesis and tumor progression of a variety of human neoplasms, including malignant melanoma; therefore, it is reasonable to speculate that a pharmacological approach that curtails Ras activity may represent a sensible approach to inhibit melanoma growth. To test this concept, the antitumor activity of S-*trans*, *trans*-farnesylthiosalicylic acid (FTS), a recently discovered Ras antagonist that dislodges Ras from its membrane-anchoring sites, was evaluated. The antitumor activity of FTS was assessed both *in vitro* and *in vivo* in two independent SCID mouse xenotransplantation models of human melanoma expressing either wild-type Ras (cell line 518A2) or activated Ras (cell line 607B). We show that FTS (5–50 μM) reduces the amounts of activated N-Ras and wild-type Ras isoforms both in human melanoma cells and Rat-1 fibroblasts, interrupts the Ras-dependent extracellular signal-regulated kinase in melanoma cells, inhibits the growth of N-Ras-transformed fibroblasts and human melanoma cells *in vitro* and reverses their transformed phenotype. FTS also causes a profound and statistically significant inhibition of 518A2 (82%) and 607B (90%) human melanoma growth in SCID mice without evidence of drug-related toxicity. Our findings stress the notion that FTS may qualify as a novel and rational treatment approach for human melanoma and possibly other tumors that either carry activated *ras* genes or rely on Ras signal transduction more heavily than nonmalignant cells.

Advanced human melanoma is the most malignant type of skin cancer and remains a paradigm of poor treatment response intrinsically linked to poor prognosis (1, 2). Although a multitude of factors have been suspected to play a role in melanoma growth and progression (1–4), the most common specific gene defects identified in this tumor are activating mutations in *ras* genes. The 15% incidence of *ras* gene mutations in human melanoma represents predominantly alterations in N-*ras* at codon 61 (3–6), whereas Ha-*ras* and K-*ras* mutations are rare (3–6). The high frequency of this particular mutational hotspot (codon 61) in the N-*ras* gene basically excludes the possibility of its incidental nature and suggests the involvement of the constitutively active N-Ras protein encoded by the mutated gene in the oncogenesis of human melanoma (6). More recent studies have shown that activated N-Ras confers chemoresistance to human melanoma because expression decreases chemotherapy-induced apoptosis in melanoma xenotransplantation models (7). Because Ras proteins are regulators of multiple signaling pathways that control cell growth, differentiation, and apoptosis (8–13), the deregulation of other cellular factors may also mimic effects of aberrant Ras function even in the absence of a *ras* gene mutation. Indeed, overexpression of receptor tyrosine kinases such as ErbB2 or epidermal growth factor receptor, common in several types of human cancers (14–16), leads to cell transformation that involves the participation of Ras proteins (17). Human melanomas appear to belong to this class of tumors because their growth and tumorigenicity depend on autocrine loops, particularly on enhanced secretion of basic fibroblast growth factor that activates specific receptor tyrosine kinases (18); thus, the influence of normal and aberrant Ras function on the biology of human melanoma may be even greater than expected from the frequency of *ras* gene mutations in this tumor and highlights the potential benefits that Ras antagonists may provide in attempts to block their growth.

One important class of functional Ras antagonists is the group of synthetic FTIs that block the modification of proteins by the lipid farnesyl, which is required for Ras membrane anchorage and signaling and transforming activity (19–26). Several FTIs inhibit Ras processing, signaling, and transformation in cell lines *in vitro* and *in vivo* (23, 25). A significant limitation of FTIs as general anti-Ras agents is, however, that K-Ras and N-Ras undergo alternative lipid modification by geranylgeranyltransferases in cells treated with FTIs (27, 28). Mutated geranylgeranyl Ras is biologically active (29, 30).

A second group of functional Ras antagonists is the synthetic S-prenyl derivatives of rigid carboxylic acids (31–34), which resemble the carboxyl-terminal farnesylcysteine common to all Ras proteins. The most potent inhibitor in this group, S-*trans*, *trans*-farnesylthiosalicylic acid (FTS), inhibits the growth of Ha-Ras- (31, 32, 34) and K-Ras-transformed rodent fibroblasts (G. Elad, A. Paz, R. Haklai, D. Marciano, A. Cox, and Y.K., unpublished data) by direct effects on membrane-anchored Ras and not by inhibition of Ras processing (32, 35). FTS appears to act as a rather specific Ras antagonist. It inhibits the growth of Ras and ErbB2-transformed fibroblasts but not of v-Raf-transformed cells (32). Other data that suggest selectivity and specificity of S-prenyl analogs toward Ras include distinctive structure-function relationships (31, 34), lack of effects on membrane localization of the prenylated G/β/γ subunits of stereotrimeric G proteins, and lack of effects on the genetically engineered N-myristylated (nonfarnesylated) Ha-Ras protein (35).

The mechanism of action of FTS is associated primarily with the displacement of the mature Ras protein from membrane-localized domain structures that associate with Ras and the subsequent accelerated degradation of the diassociated Ras protein (35). These effects of FTS are manifested by a decrease in the amount of cellular Ras (35). Recent fluorescence photobleaching recovery experiments and confocal fluorescence microscopy showed that FTS has distinctive effects on membrane-bound green fluorescent protein fused to K-Ras 4B [GFP–K-Ras 4B(12V)] (36). These experiments demonstrated that FTS has a biphasic effect on

Abbreviations: FTS, S-*trans*, *trans*-farnesylthiosalicyclic acid; ERK, extracellular signal regulated kinase; FTIs, farnesyltransferase inhibitors; MAPK, mitogen-activated protein kinase.

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membrane-bound GFP–K-Ras 4B(12V). At the initial phase, FTS elevated the lateral diffusion rate of GFP–K-Ras 4B(12V) to the level of lipids, suggesting that the protein was released from some constraints on its lateral mobility and could therefore move as fast as the membrane lipids. This was followed by dislodgment of GFP–K-Ras 4B(12V) into the cytoplasm, accompanied by a reduction in the diffusion rate of the fraction of GFP–K-Ras 4B(12V) that remained associated with the plasma membrane (36). This observation suggests that in addition to the FTS-induced reduction in the amount of active Ras, FTS also disrupts the interaction of residual Ras with the membrane (36). The above-noted effects of FTS on Ras are manifested by interruption of the Ras-dependent Raf-1-MEK-extracellular signal-regulated kinase (ERK) signaling cascade (37).

Based on these observations, it seemed reasonable to assume that FTS could be a useful inhibitor of melanoma growth and thus form the basis of a novel and rational concept of melanoma therapy. The aim of the present work was to test this hypothesis by examining both the basic effects of FTS on N-Ras function as well as its potential for the therapy of human melanoma employing SCID mouse xenotransplantation models.

Materials and Methods

Materials. [γ-^{32}P]ATP (3,000 Ci/mmol) and [^{3}H]thymidine were purchased from Amersham. The anti-pan-Ras Ab (Ab-3) was purchased from Calbiochem. Anti-N-Ras Ab, anti-ERK2 Ab, anti-MEK Ab, anti-Raf-1 Ab, anti-Rac-1 Ab, and anti-Rho A Ab were purchased from Santa Cruz Biotechnology. Myelin basic protein and protein A Sepharose were purchased from Sigma. Protein A agarose and EGF were from Boehringer Mannheim and Noble agar was purchased from Difco.

FTS was prepared as detailed (31) and dissolved either in DMSO (*in vitro* experiment) or ethanol (*in vivo* experiments).

Cell Culture and Transfection Procedures. The human melanoma cell line 518A2, the N-*ras* transfectants 518A2/N-Ras (L61), and the respective vector controls 518A2/neo have been described (7). The human melanoma cell line 607B harboring a naturally occurring N-*ras* gene mutation (38) was obtained from P. I. Schrier (Univ. of Leiden, Leiden, The Netherlands). Cell lines were maintained in DMEM (GIBCO/BRL) supplemented with 10% FCS (GIBCO/BRL) in a humidified 5% CO_2/95% ambient air atmosphere at 37°C. Rat-1 fibroblasts were transfected by the lipofectamin reagent (GIBCO/BRL) either with the pCMVneo vector, pCMV (wt) N-*ras*, or by pCMV N-*ras*(V13) (39). Stable transfectants were selected in a medium containing G418 (800 μg/ml) and clones were grown routinely in DMEM containing 10% FCS and G418 (400 μg/ml).

Soft Agar Assays. Cells were seeded at a density of 1×10^4 cells per well in 6-well plates. The cells (in DMEM/10% FCS) were mixed with 0.5 ml of 0.33% Noble agar. The mixture was poured onto a layer of 1.5 ml of 0.5% Noble agar in DMEM/10% FCS. The upper layer of agar was covered with 250 μl of medium. All agar layers contained either 0.1% DMSO (control) or FTS dissolved in 0.1% DMSO.

Determination of Ras, Rac, and Rho. The total amounts of Ras, Rac-1, and Rho A were determined in cell lysates prepared as detailed (32, 35). Ras was also determined in total cell membranes (P100) and cytosol (S100) obtained by centrifugation (100,000 $\times$ *g* for 30 min at 4°C) as described (32, 35). Briefly, 25 μg of total cellular proteins were separated by SDS/PAGE (12.5% gel) and blotted onto nitrocellulose membranes. Immunoblotting with pan-Ras, N-Ras, Rac-1, or Rho A Abs, enhanced chemiluminescense assays, and densitometric analysis were then performed as stated (35).

Mitogen-Activated Protein Kinase (MAPK) Assay. The effects of FTS on MAPK activity in melanoma cells grown in 10% FCS were determined as detailed (37). Briefly, melanoma cells were plated at a density of 1×10^6 cells per 10-cm dish in DMEM/10% FCS. After 24 h, the cells received either FTS (5–25 μM) or 0.1% DMSO (control) in the medium for another 24 h. ERK activity was then assayed in the cell lysates. To each dish, 1 ml of ERK lysis buffer (50 mM β-glycerophosphate/1.5 mM EGTA/2 mM sodium orthovandate/1 mM DTT/2 μg/ml leupeptin/2 μg/ml aprotinin/1 mM benzamidine/1% Nonidet P-40) was added and the dishes were placed on ice for 10 min. Cell lysates were then cleared by centrifugation, and 5-μg samples were used for the determination of total ERK and Raf-1 by immunoblotting with a 1:2,000 dilution of the respective Ab as described (37). Lysate samples (500 μg of protein) were incubated with 5 μl of anti-ERK2 Ab and 30 μl of protein A Sepharose beads. The immunoprecipitated ERK was then incubated for 30 min at 37°C with kinase buffer containing 30 mM Tris·HCl (pH 8.0), 20 mM $MnCl_2$, 2 mM $MgCl_2$,15 μg of myelin basic protein, 10 μM ATP, and 5 μCi [γ-^{32}P]ATP (3,000 Ci/mmol). The reaction was stopped by addition of 6 μl of 6$\times$ Laemmli sample buffer and heated for 5 min at 95°C. After a short centrifugation, the supernatant was collected and proteins separated by SDS/PAGE (15% acrylamide) on minigels. Phosphorylated myelin basic protein was visualized by autoradiography of the dried gels on x-ray films and quantified by densitometry (37).

Experimental Animals, Generation of Tumors, and Therapy. Pathogen-free female C.B-17 scid/scid (SCID) mice, 4–6 weeks old, tested for leakiness, were obtained from Bomholtgard Breeding and Research Center (Ry, Denmark), and assigned to control or treatment groups (n = 5–6 mice per group). SCID mice in these groups were injected s.c. into the left lower flank with 2.5×10^7 human melanoma cells (518A2, 607B). FTS (treatment) or carrier solution (control) was administered daily for 2 weeks via the i.p. route at 5 mg/kg to the animals in the respective groups. Treatment was initiated 1 day or 5 days after cell inoculation. SCID mice were evaluated for tumor growth, organ involvement, and apoptosis after 2 weeks of FTS or control treatment. All untreated animals developed human melanomas. The immunohistochemical detection of apoptosis in paraffin-embedded and frozen tumor sections was performed by TUNEL staining (Boehringer Mannheim; ref. 9).

Statistical Analysis. Statistical significance of differences in tumor weight between treated and untreated animals in the respective groups (treated vs. untreated) and between cell numbers of the various groups *in vitro* were determined by using the Mann–Whitney U test. P values of <0.05 were considered to be of statistical significance.

Results

FTS Affects N-Ras in Rat-1 Fibroblasts and Human Melanoma Cells. Previous studies have shown that FTS reduces the amount of activated Ha-Ras in Ha-Ras-transformed Rat-1 fibroblasts, an event accompanied by inhibition of MAPK activity and inhibition of cell growth (35, 37). Because human melanomas usually do not express activated Ha-Ras, yet frequently harbor an activated N-*ras* gene (3, 6), and because activated N-Ras confers chemoresistance in human melanomas (7), we first examined whether FTS can affect N-Ras in cells that stably express N-Ras isoforms.

Two sets of cell lines were used in these experiments: (*i*) Rat-1 fibroblasts that stably overexpress wild-type N-Ras or activated N-Ras(V13) (Fig. 1*A*); (*ii*) human 518A2 melanoma cells (which do not harbor an activated *ras* gene), 518A2-transfected melanoma cells overexpressing activated N-Ras(L61), respective vector controls (7) (Fig. 1*B*), and the human melanoma cell line 607B with a naturally occurring N-*ras* gene mutation (38). The cells were treated with FTS for 24 h, a time point at which FTS exerts its maximal effects on Ras in fibroblasts (35). The total amount of Ras was then

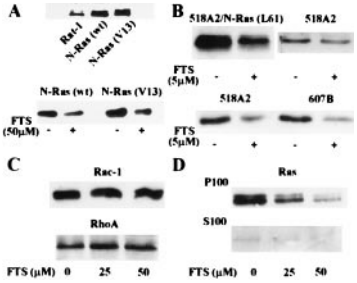

Fig. 1. FTS induces a decrease in the amount of N-Ras in Rat-1 fibroblasts and human melanoma cells without an effect on the amount of Rac-1 and Rho A. Cells were plated at a density of 1.5×10^6 cells per 10-cm dish in DMEM/10% FCS. After 24 h, the cells received either 0.1% DMSO carrier solution (control) or FTS. Ras, Rac-1, and Rho A expression were then determined in total cell lysates (10 μg of protein) by immunoblotting/enhanced chemiluminescence assays with the corresponding antibodies as detailed in *Materials and Methods*. (*A Upper*) Immunoblots with pan-Ras Ab demonstrating the amounts of Ras in untransfected Rat-1 cells and Rat-1 cells stably expressing wild-type N-Ras or activated N-Ras (V13). (*Lower*) Immunoblots with pan-Ras Ab demonstrating the reduced amount of N-Ras in N-Ras expressing Rat-1 cells after a 24-h treatment with 50 μM FTS. (*B Upper*) Typical immunoblots with pan-Ras Ab demonstrating the FTS-induced reduction of N-Ras in human 518/N-Ras (L61) melanoma cells and the drug-induced reduction in the amount of endogenous Ras in 518A2 cells. (*Lower*) Immunoblots with N-Ras Ab demonstrating the FTS-induced reduction in the amount of N-Ras in 518A2 and in 607B cells after 24 h of FTS treatment. The results shown are representative examples of three sets of experiments. (*C*) Immunoblots with Rac-1 and Rho A Abs demonstrating the lack of FTS effects on the total amounts of Rac-1 and Rho A in 518A2 cells after a 24-h treatment with FTS at the indicated concentrations. (*D*) Dislodgment of Ras by FTS from 518A2 cell membranes. Cells were treated with the indicated concentrations of FTS for 24 h and Ras was then determined in the particulate (P100) and cytosolic (S100) fractions with pan-Ras Ab as detailed in *Materials and Methods*.

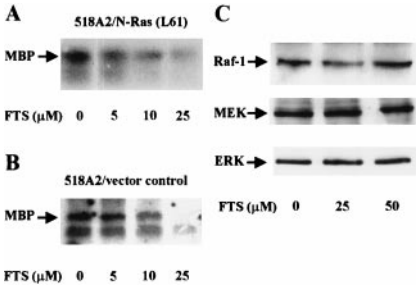

Fig. 2. FTS interrupts ERK activity in human melanoma cells. Human 518A2/N-Ras (L61) melanoma cells (*A*) and a vector-transfected control (*B* and *C*) were grown for 24 h in DMEM/10% FCS in the presence of 0.1% DMSO (zero drug control) or in the presence of the indicated concentrations of FTS. ERK activity (*A* and *B*) was then determined in cell lysates with MBP as a substrate as described in *Materials and Methods*. The phosphorylated MBP bands, visualized by autoradiography, demonstrate the dose-dependent decrease in ERK activity. Densitometric analysis (see *Materials and Methods*) of data obtained from representative experiments indicated a decrease in ERK activity of 25–32%, 39–58%, and 65–95%, respectively, in the presence of 5, 10, and 25 μM FTS either in the 518A2/N-Ras (L61) or the vector-transfected control cells. The total amount of Raf-1, MEK, ERK remain unaltered in 518A2 cells treated 24 h with FTS (C). Raf-1, MEK, and ERK in cell lysates were determined by immunoblotting with the corresponding Abs.

determined in the cell lysate by the immunoblotting/enhanced chemiluminescence assay with pan-Ras antibodies (35). Results of a typical experiment shown in Fig. 1*A* demonstrate that FTS caused a significant reduction in the amount of both wild-type N-Ras and activated N-Ras(V13) expressed by the respective Rat-1 cell lines (80% and 70% reduction, respectively). A similar FTS-induced decrease in the amount of Ras (56% $\pm$ SD = 9% reduction) was observed in 518A2 and 518A2/N-Ras(L61) cells (Fig. 1*B*). In additional experiments with 518A2 cells and 607B cells that harbor an activated N-*ras* gene, we confirmed with N-Ras-specific antibodies that FTS had indeed affected N-Ras in these cells (Fig. 1*B*). Interestingly, the melanoma cells were more sensitive to FTS than Rat-1 cells. Five micromolar FTS induced an almost 60% reduction in the amount of Ras in the 518A2/N-Ras(L61) cells (Fig. 1*B*), whereas 50 μM FTS were required to induce a similar Ras decrease in the nonhuman Rat-1 cells (Fig. 1*A*); thus, sensitivity of Ras proteins to FTS may therefore be influenced by tissue and possibly species characteristics in addition to the type of its isoform and the mutation status.

FTS also affected wild-type Ras isoforms expressed by 518A2 melanoma cells (Fig. 1*B*) and vector-transfected 518A2 melanoma cells (data not shown). 518A2 melanoma cells were as sensitive to FTS treatment as were the 518A2/N-Ras(L61) transfectants (Fig. 1*B*). Interestingly, although FTS induced a significant decrease in the total amount of Ras in 518A2 cells (Fig. 1*B*), it had no effect on the total amount of Rac-1 and Rho A (Fig. 1*C*). These results suggest some specificity of FTS toward the prenylated Ras protein. Because we know from earlier studies that FTS affects the mature membrane-bound Ras in fibroblasts (35), we examined whether this was also the case in 518A2 melanoma cells. In these cells, as in other cell lines (35, 36), most of the Ras is associated with the cell membranes (Fig. 1*D*). FTS caused a marked decrease in the amount of membrane-bound Ras (40% at 25 μM and 75% at 50 μM; Fig. 1*D*). This decrease was not accompanied by an increase in cytosolic Ras (Fig. 1*D*). It appears then that removal of Ras from its membrane anchorage sites by FTS renders the protein susceptible to proteolytic degradation.

The above notion on the sensitivity of Ras to FTS gained additional support in experiments where its effects on the Ras-dependent MAPK in melanoma cells were examined. In these experiments, cells were grown at 5–25 μM FTS for 24 h and the activity of immunoprecipitated ERK1 and ERK2 was determined in lysates as described (37) by using MBP as a substrate. Results of typical experiments performed with 518A2/N-Ras(L61) cells (Fig. 2*A*) and with vector-transfected controls (Fig. 2*B*) demonstrate dose-dependent inhibition of ERK activity in both cell lines by FTS. Inhibition of ERK activity by FTS was observed in cells treated with 5–10 μM FTS (Fig. 2), a concentration range at which FTS also affected Ras (Fig. 1). At higher concentrations, FTS induced 60–90% inhibition in ERK activity (Fig. 2) with no effect on the amount of ERK, MEK, or Raf proteins (Fig. 2). These results suggest that one important consequence of the FTS-induced reduction in the amount of Ras in human melanoma cells is the inhibition of the Ras-dependent MAPK ERK (40, 41). Consistent with the data on the effects of FTS on wild-type Ras in 518A2 cells (Fig. 1*A*) and with previous reports on the inhibition of Ras and ERK in untransformed Rat-1 cells (37), ERK activity is inhibited in melanoma cells whether or not they harbor an activated *ras* gene (Fig. 2).

Inhibition of Anchorage-Independent and -Dependent Growth of N-Ras Transformed Rat-1 Cells. Because Ras-dependent ERK is involved in the regulation of cell growth and in Ras transformation (40, 41), we also examined whether the effects of FTS on N-Ras and ERK (Figs. 1 and 2) affect cell growth and the transforming activity of N-Ras. These experiments were performed with Rat-1 fibroblast cell lines to obtain a direct comparison between untransformed cells that express wild-type N-Ras and N-Ras(V13)-transformed Rat-1 cells. Rat-1 cells that stably express activated N-Ras demonstrate a typical transformed phenotype showing anchorage-dependent growth characterized by focus formation and anchorage-independent cell growth characterized by the formation of colonies in soft agar. In contrast, Rat-1 cells overexpressing the wild-type N-Ras isoform demonstrated a normal, flattened phenotype and did not form colonies in soft agar (data not shown).

When the transfected Rat-1/N-Ras(V13) cells were grown for 5

Table 1. Inhibition of anchorage-independent growth of N-Ras(V13)-transformed Rat-1 cells by FTS

Treatment	No. of colonies ($\times 10^2$)		
	Colony size		
	<100 mm	100–200 mm	>200 mm
0.1% DMSO	5.3 ± 0.6 [100%]	1.5 ± 0.74 [100%]	0.25 ± 0.2
50 μm FTS	3.1 ± 0.4 [60%]	0.3 ± 0.2 [20%]	0
100 μm FTS	2 ± 0.3 [38%]	0	0

N-Ras(V13)-transformed Rat-1 cells were grown on soft agar with or without the indicated concentrations of FTS for 17 days. The sizes and numbers of colonies in three visual fields of duplicate wells were estimated by using a light microscope attached to an image analyzer (44). The estimated numbers of colonies (±SD) per well represent the means of three independent experiments. The numbers given in brackets represent numbers of colonies as % of the DMSO-treated control.

days in the presence of 50 μM FTS, most of the foci disappeared and cells showed the flattened morphology characteristic of untransfected Rat-1 fibroblasts. Colony formation in soft agar was also strongly inhibited by FTS (Table 1). Both the number and the size of the generated colonies were decreased by FTS (Table 1). These results suggest that FTS can inhibit the transforming activity of N-Ras(V13) in Rat-1 fibroblasts. To examine the effects of FTS on anchorage-dependent growth in N-Ras-transformed Rat-1 fibroblasts, cells were grown for 5 days in DMEM/10%FCS in the presence of FTS (2.5–50 μM) and then counted. A dose-dependent decrease in anchorage-dependent growth of Rat-1/N-Ras(V13) cells was observed with 50% inhibition at 30–40 μM FTS. In agreement with previous experiments (32), trypan blue staining showed that under these conditions, FTS did not affect the viability of transformed Rat-1 cells; moreover, FTS had no effect on anchorage-dependent growth of the untransformed Rat-1 cells that overexpress the wild-type N-Ras isoform (data not shown).

Inhibition of Human Melanoma Cell Growth by FTS. We next investigated the effects of FTS on the growth of human melanoma cells and examined the influence the *ras* gene mutation status may have on these effects. For this purpose, we used 518A2 cells that do not harbor an activated *ras* gene, the N-Ras-transfected 518A2/N-Ras(L61) cells and respective vector controls. Additionally, we studied the influence of FTS on the human melanoma cell line 607B, which has a naturally occurring N-*ras* gene mutation (38). Melanoma cells were grown for 5 days in DMEM/10% FCS in the presence of FTS and then counted and compared with controls. Trypan blue staining showed that under these conditions, FTS did not cause cell death of the melanoma cells tested.

Typical photomicrographs of 518A2 melanoma cells grown for 5 days in the presence of FTS are shown in Fig. 3. FTS treatment at 50, 100, and 125 μM caused a dose-dependent decrease in cell number relative to the control. Cell growth was inhibited by 21%, 55%, and 84%, respectively. FTS also caused a significant change in 518A2 melanoma cell morphology (Fig. 3). where cells presented a more flattened and spindle-like morphology reminiscent of normal human melanocytes. Results similar to the ones obtained with the 518A2 melanoma cells could be generated with 607B melanoma cells that have a naturally occurring N-*ras* gene mutation (data not shown). Taken together, these results suggest that FTS inhibits melanoma cell growth irrespective of the presence of a *ras* gene mutation. In these malignant human cells, however, there is a clear correlation between the FTS-induced reduction in the amount of Ras, inhibition of ERK, and the inhibition of cell growth.

Inhibition of the Growth of Human Melanoma Xenotransplants in SCID Mice. Based on the *in vitro* experiments described above, we next evaluated the antitumor efficacy of FTS in SCID mouse xenotransplantation models of 518A2 and 607B human melanomas. Melanoma cells were injected s.c. into the left lower flank and FTS treatment (5 mg/kg i.p. daily) or control treatment was initiated one day later (n = 5–6 animals per group). Two weeks after the implantation of melanoma cells, animals were killed and evaluated for tumor growth and potential toxicity of FTS treatment. As shown in Fig. 4, both 518A2 and 607B melanoma cells caused human melanomas in all the untreated animals. The mean tumor weights were 0.22 g ± SD = 0.09 g and 0.29 g ± SD = 0.11 g, respectively. In both groups, tumor growth was strongly inhibited by FTS. In mice carrying 518A2 human melanomas, FTS inhibited tumor growth by 82% (mean tumor weight 0.04 g ± SD = 0.01 g; P = 0.009; Fig. 4) and in mice carrying 607B human melanomas, FTS inhibited the tumor growth by 90% (mean tumor weight 0.03 g ± SD = 0.02 g; P = 0.003) (Fig. 4). Notably, in two of the six animals injected with 607B human melanoma cells, no tumors were detected after FTS treatment. In the 607B model harboring activated N-Ras, 2 weeks of FTS led to a statistically significant reduction in the weight of human melanomas even if well-established tumors treated 5 days after cell inoculation were targeted (P = 0.03). The mean tumor weights of FTS treated and control melanomas were 0.51 g ± SD = 0.06 g, and 0.70 g ± SD = 0.13 g, respectively. The

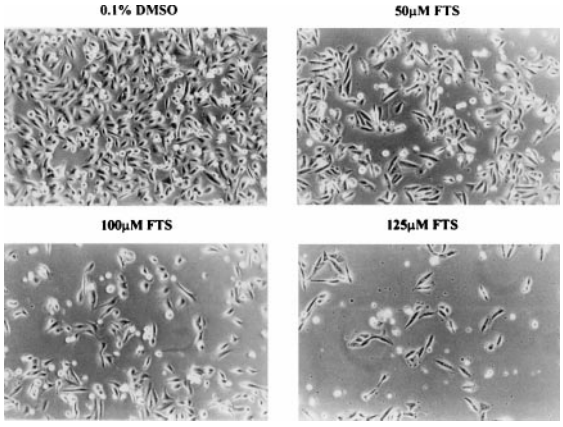

Fig. 3. FTS alters the morphology of human 518A2 melanoma cells and inhibits their growth. Human 518A2 melanoma cells were plated at a density of 1×10^4 cells per well in 6-well plates and grown for 3 days in DMEM/10% FCS in the presence of 0.1% DMSO (control) or the indicated concentrations of FTS. Representative photomicrographs taken on day 3 (original magnification ×100) show that with increasing molar concentration of FTS there is a decrease in cell number and progressive flattening and spindle cell morphology reminiscent of normal melanocytes.

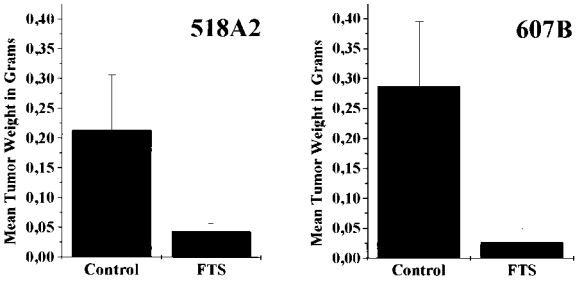

Fig. 4. Inhibition of human melanoma growth in SCID mice by FTS. Mice were xenotransplanted subcutaneously into the lower left flank with 2.5×10^7 518A2 or 2.5×10^7 607B human melanoma cells and received FTS treatment (5 mg/kg, i.p., daily) for 2 weeks. The treatment was initiated the day after cell inoculation. Control animals received injections with the carrier solution. The mean tumor weights after the 2-week treatment period (n = 5–6 animals per group) are given. All untreated mice that received 518A2 or 607B human melanoma cells developed tumors.

growth of established 518A2 melanomas not harboring activated N-Ras was also inhibited by FTS; however, the inhibition was not statistically significant.

TUNEL assays in sections of tumors excised from the animals indicated no significant differences in the rate of apoptosis between the FTS-treated and control groups (data not shown). These results suggesting that FTS does not induce apoptosis in melanomas or mouse tissue are consistent with the lack of adverse FTS toxicity both *in vitro* and *in vivo*. Indeed, histological analysis of various mouse organs including lung, liver, brain, kidney, and small intestine did not reveal any evidence of FTS-related morphological alterations.

Discussion

The high prevalence of *ras* gene mutations (10, 42) and the frequent overexpression of receptor tyrosine kinases that activate wild-type Ras proteins (14–18) in human tumors make Ras proteins important targets for drug design. The discovery that both signal transduction through Ras and Ras-transforming activity require the correct membrane localization of Ras through a farnesyl moiety (12, 29, 30) led to the development of at least two independent methods to disrupt Ras functions. One method utilizes inhibitors of the enzyme farnesyltransferase that farnesylates proteins including Ras (19–26), whereas another utilizes farnesyl derivatives which resemble the structure of the farnesylcysteine of Ras and other proteins (31–35). Potent and specific FTIs affect the functions of Ha-Ras by inhibiting farnesylation and membrane anchorage (19–26). FTIs also block farnesylation of N-Ras or K-Ras, but these proteins are then alternatively prenylated by the enzyme geranylgeranyltransferase I and remain active (27, 28). In spite of this, FTIs have been proven useful inhibitors of several activated K-Ras expressing tumors in animal models (23, 25), presumably because of the inhibition of farnesylation of yet unknown proteins (30). Farnesyl derivatives, such as FTS, that resemble the structure of the farnesylcysteine of Ras affect the functions of activated Ha-Ras by dislodging the mature protein from the cell membrane and by accelerating its degradation (35). FTS is known to inhibit the growth of ErbB2-transformed fibroblasts, and to block mitogenic stimuli of EGF and basic fibroblast growth factor in Rat-1 cells (32). These data, along with the demonstrated dislodgment of wild-type Ras isoforms from untransformed Rat-1 cell membranes, suggest that FTS would also be capable of inhibiting the growth of human tumor cells that overexpress tyrosine kinase receptors or of cells which harbor activated K- or N-*ras* genes.

Here we show that FTS reduces the amount of activated N-Ras and wild-type Ras isoforms in human melanoma cells and Rat-1 cells in culture (Fig. 1), inhibits the Ras-dependent MAPK in melanoma cells (Fig. 2), suppresses activated N-Ras-dependent growth and transforming activity in Rat-1 cells, and inhibits human melanoma cell growth *in vitro* (Fig. 3); moreover, FTS appears to reverse the transformed morphology of human melanoma cells (Fig. 3) and potently inhibit the growth of human melanomas in SCID mice (Fig. 4). These observations suggest that FTS, and perhaps some of its analogues (31, 33), may serve as a new class of functional Ras antagonists with clinically useful antimelanoma potential. At 5 mg/kg/day, FTS caused a greater than 80% inhibition of melanoma growth in our *in vivo* model systems (Fig. 4). No adverse toxic effects in SCID mice were observed at this dose level. These data, along with the relatively high LD_{50} of FTS in mice (75–100 mg/kg) (43), suggest that FTS is not only a novel but also a safe and potent inhibitor of human melanoma growth. It is interesting to note that the effective dose of FTS for inhibition of melanoma growth in mice is relatively low when compared with the drug concentrations that inhibit melanoma growth *in vitro*. This would suggest that beyond its direct inhibitory effects on Ras (Fig. 1), FTS may, depending on the system investigated, affect other mechanisms supporting tumor growth. A recent study showed, for example, that FTS can inhibit FGF-induced endothelial cell proliferation and differentiation (44).

The significance of the present results must be viewed in light of the poor prognosis of human melanomas and the almost paradigmatic resistance of human melanomas to chemotherapy and radiotherapy (1, 2). Over the last 35 years, the number of people diagnosed with melanoma has doubled and worldwide cases of melanoma are increasing at a rate of approximately 5% per year (45). Melanoma is currently the leading cause of death among skin cancer patients. It is estimated that by the year 2000, 1 in 75 people born in the United States or Europe will develop malignant melanoma in their lifetime (45). Incidence rates for Australia are estimated to be even beyond these alarming figures.

The precise mechanism behind the FTS-induced reduction in the amount of activated N-Ras and wild-type Ras in human melanomas is not yet known. The precedence of FTS-induced dislodgment of activated Ha-Ras from Rat-1 cell membranes (35) and of activated K-Ras from human Panc-1 cell membranes (43) suggested that FTS would act in a similar manner in human melanomas. Consistent with this possibility, we found that FTS induced a reduction in the amount of membrane-bound Ras in 518A2 melanoma cells that was not accompanied by an increase in cytosolic Ras (Fig. 1). We know from previous studies that FTS does not inhibit the enzyme farnesyltransferase and does not affect Ras processing in intact cells (32). We also know that unprocessed Ras has a relatively slow gel migration (46). FTS, unlike lovastatine, for example, also does not cause the accumulation of unprocessed Ha-Ras in Rat-1 fibroblasts (35). Similarly in the present study, we did not find slowly migrating Ras isoforms in melanoma cells that were treated with FTS, although the total amount of Ras protein was significantly reduced (Fig. 1). This is consistent with the concept that FTS-induced reduction in the amount of Ras in melanoma cells is because of dislodgment and accelerated degradation. In this regard, it is important to note that both activated and wild-type Ras proteins are localized in specific membrane domains such as caveoli or related domains enriched in glycosphingolipids and cholesterol (47–49). FTS may exert its actions by interfering with the interactions of Ras with specific lipids or proteins present in such domains.

The observed reduction in the amount of wild-type Ras isoforms in human melanomas that do not harbor activated *ras* genes (Fig. 1) and concomitant inhibition of cell and tumor growth (Figs. 3 and 4) is consistent with the concept that FTS also blocks autocrine (e.g., basic fibroblast growth factor-dependent) loops. Such loops are known to be important factors in melanoma growth and tumorigenicity (18). This notion is further supported by early observations that FTS blocks basic fibroblast growth factor mitogenic stimuli in Rat-1 cells (32) and endothelial cells (44).

The important consequences of the effects of FTS on Ras in Ras-transformed rodent fibroblasts (32, 35) and human melanoma cells are the inhibition of ERK activity and the altered cell morphology (Figs. 2 and 3). Both the Raf-1-MEK-ERK (40, 41) and the Rac/Rho cytoskeleton pathways associated with cell shape and morphology (50, 51) are known to be activated by oncogenic Ras proteins and several growth factors; moreover, the Ras-dependent Raf-1-ERK and Rac/Rho pathways were shown to contribute synergistically to cell transformation (51); thus, our observation on the inhibition of ERK activity (Fig. 2) and alterations in melanoma cell morphology (Fig. 3) lend additional support to the direct effects of FTS on Ras which consequently appears to induce inhibition of melanoma cell and tumor growth. Interestingly, whereas FTS affected Ras, it had no effect on the total amount of Rac-1 and Rho A in 518A2 melanomas (Fig. 1). This suggests that the effects of FTS on Rac/Rho-dependent pathways, e.g., on membrane ruffles and stress fibers (52), would be mostly because of its effects on Ras. We cannot rule out, however, the possibility that FTS may interfere with the functions of Rac/Rho or other prenylated proteins without an effect on their total amount. It should also be pointed out that inhibition of ERK activity by FTS in Rat-1 cells (37) is neither

accompanied by inhibition of cell growth nor by altered cell morphology (32). It is not yet clear why growth of untransformed cells such as Rat-1 (32) or Rat-1 expressing wild-type N-Ras is not inhibited by FTS even though their Ras levels and ERK activity are reduced. One possible explanation is that untransformed cells are less committed to Ras signaling pathways and can use alternative, Ras-independent, growth mechanisms. Another possibility is that the amount of wild-type Ras that remains in the cell membranes and the extent of ERK activation after FTS treatment are sufficient for normal cell growth. In this regard, it is important to note that, unlike overexpression of activated N-Ras that induced cell transformation, overexpression of wild-type N-Ras (Fig. 1) had no effect on cell growth and morphology and certainly did not induce cell transformation. It appears that in untransformed cells the amount of wild-type Ras may indeed vary without a significant effect on cell growth. More experiments are required to distinguish between the above and possible other explanations to the lack of FTS effect on growth of nontransformed cells. This phenomenon could, however, help explain the lack of FTS toxicity in animals and may also explain the more potent effects of FTS on established melanomas harboring activated N-Ras compared with melanomas with wild-type *ras* genes.

In summary, the poor prognosis of advanced human melanoma and the dramatic increase in the incidence of this malignancy over the past decades clearly warrant the search for new and improved treatment strategies in both adjuvant and nonadjuvant settings. FTS promises to have the potential for the development of such a strategy.

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1. Albino, A. P., Le Strange, R., Oliff, A. I., Furth, M. E. & Old, L. J. (1984) *Nature (London)* **308,** 69–72.
2. Albino, A. P. & Fountain, J. W. (1993) *Current Research and Clinical Management of Melanoma*, ed. Nathanson, L. (Kluwer, Norwell, MA), pp. 201–255.
3. Ball, N. F., Yohn, J. J., Morelli, J. G., Norris, D. A., Golitz, L. E. & Hoeffler, J. P. (1994) *J. Invest. Dermatol.* **102,** 285–290.
4. van't Veer, L. J., Burgering, B. M., Versteeg, R., Boot, A. J. M., Ruiter, D. J., Oanto, S., Schrier, P. I. & Bos, J. L. (1989) *Mol. Cell. Biol.* **9,** 3114–3116.
5. Shukla, V. K., Hughes, D. C., Hughes, L. E., McCormick, F. & Padua, R. A. (1989) *Oncog. Res.* **5,** 121–127.
6. Van Elsas, A., Zerp, S., Van Der Flier, S., Kruse, M., Aarnoudse, C., Hayward, N., Ruiter, D. & Schrier, P. I. (1996) *Am. J. Pathol.* **149,** 883–893.
7. Jansen, B., Schlagbauer-Wadl , H., Eichler, H.-G., Wolff, K., van Elsas ,A., Schrier, P. & Pehamberger, H. (1997) *Cancer Res.* **57,** 362–365.
8. Boguski, M. S. & McCormick, F. (1993) *Nature (London)* **366,** 643–654.
9. Lange-Carter, C. A. & Johnson, G. L. (1994) *Science* **265,** 1458–1461.
10. Bos, J. L. (1997) *Biochim. Biophys. Acta* **1333** (2)**,** M19–M31.
11. Lowy, D. R. & Wilumsen, B. M. (1993) *Annu. Rev. Biochem.* **62,** 851–891.
12. Marshall, C. J. (1996) *Curr. Opin. Cell Biol.* **8,** 197–204.
13. Khosravi-Far, R. & Der, C. J. (1994) *Cancer Metastasis Rev.* **13,** 67–89.
14. Kolibaba, K. S. & Druker, B. J. (1997) *Biochim. Biophys. Acta* **1333,** F217–F248.
15. Huang, H. J., Nagane, M., Klingbell, C.K., Lin, H., Nishikawa, R., Ji, X.-D., Huang, C.-M., Gill, G. N., Wiley, H. S. & Cavenee, W. K. (1997) *J. Biol. Chem.* **272,** 2927–2935.
16. Smith, J. J., Derynck, R. & Korc, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7567–7570.
17. Pazin, M. J. & Williams, L. T. (1992) *Trends Biochem. Sci.* **17,** 374–378.
18. Rodeck ,U., Becker, D. & Herlyn, M. (1991) *Cancer Cells* **3,** 308–311.
19. James, G. L., Goldstein, J. L., Rawson, T. E., Somers, T. C., McDowell, R. S., Crowley, C. W., Lucas, B. K., Levinson, A. D. & Masters, C. J., Jr. (1993) *Science* **260,** 1937–1942.
20. Kohl, N. E., Mosser, S. D., DeSolms, S. J., Giuliani, E. A., Pompliano, D. L., Graham, S. L., Smith, R. L., Scolnick, E. M., Oliff, A. & Gibbs, J. B. (1993) *Science* **260,** 1937–1937.
21. Lebowitz, P. F., Sakamuro, D. & Prendergast, G. C. (1997) *Cancer Res.* **57** (4)**,** 708–713.
22. Nagasu, Y., Yoshimatsu, K., Rowell, C., Lewis, M. D. & Garcia, A. M. (1995) *Cancer Res.* **55** (22), 3510–3514.
23. Kohl, N. E., Wilson, F. R., Mosser, S. D., Giuliani, E., DeSolms, S. J., Conner, M. W., Anthony, N. J., Holtz, W. J., Gomez, R. P. & Lee, T. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9141–9145.
24. Manne, V., Yan, N., Carboni, J. M., Tuomari, A. V., Ricca, C. S., Brown, J. G., Andahazy, M. L., Schmidt, R. J., Patel, D. & Zahler, R. (1995) *Oncogene* **10,** 1763–1779.
25. Sun, J., Qian, Y., Hamilton , A. D. & Sebti, S. M. (1995) *Cancer Res.* **55,** 4243–4247.
26. Gibbs, J. B. & Oliff, A. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 146–166.
27. Rowell, C.A., Kowalczyk , J. J., Lewis, M. D. & Garcia, A. M. (1997) *J. Biol. Chem.* **272,** 14093–14097.
28. Whyte, D. B., Kirschmeier, P., Hockenberry, T. N., Nunez-Olivia, I., James, L., Catino, J. J., Bishop, W. R. & Pai, J. K. (1997) *J. Biol. Chem.* **272,** 14459–14464.
29. Cox, A. D. & Der, C. J. (1992) *Curr. Opin. Cell Biol.* **4,** 1008–1016.
30. Cox, A. D. & Der, C. J. (1997) *Biochim. Biophys. Acta.* **1333,** F51–F71.
31. Marciano, D., Ben-Baruch, G., Marom, M., Egozi, Y., Haklai, R. & Kloog, Y. (1995) *J. Med. Chem.* **38** (8), 1267–1272.
32. Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y. & Kloog, Y. (1995) *J. Biol. Chem.* **270,** 22263–22270.
33. Marciano, D., Aharonson, Z., Varsano, T., Haklai, R. & Kloog, Y. (1997) *Bioorg. Med. Chem. Lett.* **7,** 1709–1714.
34. Aharonson, Z., Gana-Weisz, M., Varsano, T., Haklai, H., Marciano, D. & Kloog, Y. (1998) *Biochim. Biophys. Acta* **1406,** 40–50.
35. Haklai, R., Gana-Weisz, M., Elad, G., Marciano, D., Egozi, Y., Ben-Baruch, G. & Kloog, Y. (1998) *Biochemistry* **37,** 1306–1314.
36. Niv, H., Gutman, O., Henis, Y. & Kloog, Y. (1999) *J. Biol. Chem.* **274,** 1606–1613.
37. Gana-Weisz, M., Haklai, R., Marciano, D., Egozi, Y., Ben-Baruch, G. & Kloog, Y. (1997) *Biochem. Biophys. Res. Commun*. **239,** 900–904.
38. van Elsas, A., Zerp, S., van der Flier, S., Kruse-Wolters, M., Vacca, A., Ruiter, D. G. & Schrier, P. I. (1995) *Recent Res. Cancer Res.* **139,** 57–67.
39. Van Elsas, A., VanDeursen, E., Wilders, R., Van Den BergBakker, C. A. M. & Schrier, P. I. (1994) *J. Invest. Dermatol.* **103,** Suppl., 117s–121s.
40. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D., Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlien, R. L., Cobb, M. H. & Krebs, E. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6142–6146.
41. Cobb, M. H. & Goldsmith, E. J. (1995) *J. Biol. Chem.* **270,** 14843–14846.
42. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56,** 779–827.
43. Weisz, B., Giehl, K., Gana-Weisz, M., Egozi ,Y., Ben-Baruch, G., Marciano, D., Gierschicken, P. & Kloog, Y. (1999) *Oncogene* **18,** 2579–2588.
44. Klint, P., Kanda, S., Kloog, Y. & Claesson-Welsh, L. (1999) *Oncogene* **18,** 3354–3364.
45. Stadelmann, W. K., Rapaport, D. P., Soong, J.-S., Reintgen, D. S., Buzaid, A. C. & Balch, C. M. (1998)in *Cutaneous Melanoma*, eds. Balch, C. M., Houghton, A. N., Sober, A. J. & Soong, S.-J. (QMP, St. Louis), pp. 11–35.
46. Gutierrez, L., Magee, A. L., Marshal, C. J. & Hancock, J. F. (1989) *EMBO J.* **8,** 1093–1098.
47. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M. & Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 9690–9697.
48. Mineo, C., James, G. L., Smart, E. J. & Anderson, G. W. (1996) *J. Biol. Chem.* **271,** 11930–11935.
49. Simons, K. & Ikonen, E. (1997) *Nature (London)* **387,** 569–572.
50. Symons, M. (1996) *Trends Biochem. Sci.* **21,** 178–181.
51. Joneson ,T., White, M. A., Wigler, M. H. & Bar-Sagi, D. (1996) *Science* **271,** 810–812.
52. Egozi, Y., Weisz, B., Gana-Weisz, M., Ben-Baruch, G. & Kloog, Y. (1999) *Int. J. Cancer* **80,** 911–918.