The Ras Inhibitor Farnesylthiosalicylic Acid as a Potential Therapy for Neurofibromatosis Type 1

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Abstract

Purpose: Farnesylthiosalicylic acid (FTS) is a Ras inhibitor that dislodges all active Ras isoforms from the membrane. We assessed the ability of FTS to reverse the transformed phenotype of neurofibromatosis type 1 (NF1)–associated tumor cell lines of malignant peripheral nerve sheath tumor (MPNST).

Experimental Design: nf1 mutations were genotyped, allelic losses were analyzed, and neurofibromin expression levels were determined in MPNST cell lines ST88-14, S265P, and 90-8. The effects of FTS on GTP-bound Ras (Ras-GTP) and its prominent downstream targets, as well as on cell morphology, anchorage-dependent and anchorage-independent growth, and tumor growth in mice, were assessed.

Results: The MPNST cell lines were biallelic, NF1 inactive, and neurofibromin deficient. We show that FTS treatment shortened the relatively long duration of Ras activation and signaling to extracellular signal-regulated kinase, Akt, and RalA in all NF1-deficient MPNST cell lines (NF1 cells) to that observed in a non-NF1, normally expressing neurofibromin MPNST cell line. These effects of FTS led to lower steady-state levels of Ras-GTP and its activated targets. Both anchorage-dependent and anchorage-independent growth of NF1 cells were dose dependently inhibited by FTS, and the inhibition correlated positively with Ras-GTP levels. NF1 cells were found to possess strong actin stress fibers, and this phenotype was also corrected by FTS. NF1 tumor growth in a nude mouse model was inhibited by oral FTS.

Conclusions: FTS treatment of NF1 cells normalized Ras-GTP levels, resulting in reversal of the transformed phenotype and inhibition of tumor growth. FTS may therefore be considered as a potential drug for the treatment of NF1.

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with pleiotropic manifestations, including cafe-au-lait spots, increased risk of benign and malignant tumor formation, and a variety of neurologic syndromes (1–3). Patients with NF1 are predisposed to development of a variety of tumors; malignant peripheral nerve sheath tumors (MPNST), astrocytic brain tumors (glioblastomas), pilocytic astrocytomas in the visual pathway, leukemias, and pheochromocytomas (1–3). The disease gene localizes to 17q11.2, encompasses multiple exons that span 350-kb genomic DNA, and encodes the protein neurofibromin (4, 5). The exact functions of most of the domains of this protein are unknown, but a region encoded by exons 23 to 29 of the gene was shown to act as a Ras–GTPase-activating protein (Ras-GAP), which facilitates the hydrolysis and inactivation of the active GTP-bound Ras (Ras-GTP) to the inactive GDP-bound form (6, 7). In line with its Ras-GAP activity, deficiency of neurofibromin in NF1-derived cells is associated with an increase in the active Ras-GTP (7–10), a biochemical feature also observed in tumor cell lines containing Ras activated by point mutations (11–13).

Importantly, many studies have shown that NF1 tumors and cell lines exhibit increased amounts of Ras-GTP even when the amounts of p120 Ras-GAP and Ras proteins are normal (7–10). In addition to the predicted effect of NF1 deficiency on activated Ras-GTP levels, aberrant activation of downstream Ras effectors has been documented in several NF1-deficient tumors (14–17). Experiments in vitro have shown that microinjection of either Ras antibody or the Ras-GAP catalytic domain (GAP-related domain) into NF1 cells inhibited their growth (8, 9). Furthermore, point mutations affecting the Ras-GAP activities of neurofibromin have been detected in NF1 patients, reinforcing the notion that inactivation of GAP activity contributes to NF1 symptoms (18). A host of animal models (19, 20) provide additional support for the significance of aberrant Ras-GTP and its signaling pathways in determining the phenotypic expression of mutant NF1; mouse NF1+−/− fibroblasts, astrocytes, melanocytes, and mast cells exhibit increased proliferation rates and constitutively activated Ras signaling (19, 20); mice or flies with heterozygous inactivating mutations...
in both nf1 and p120 Ras-GAP genes exhibit a more severe phenotype (19, 20).

Taken together, these observations strongly suggest that the defective ability of neurofibromin to down-regulate active Ras-GTP plays an important role in the phenotypic expression of NF1 and NF1-associated tumors. They also support the notion that Ras inhibitors might alleviate some of the NF1 symptoms. This notion was tested in experiments that showed the ability of farnesyltransferase inhibitors (FTI) to inhibit the growth of NF1-associated MPNST cell lines (21), neurofibromin-deficient hematopoietic cells (14), and mouse Schwann cells (22). FTIs inhibit Ras activity by blocking Ras farnesylation, a critical and essential step required for anchoring the Ras protein to the inner cell membrane and ensuring its biological activity (23). A major problem with the use of FTIs is that they can block the functions of H-Ras only, not of the highly abundant K-Ras and N-Ras isoforms (23).

The present study was aimed at the development of a novel rational therapeutic strategy for NF1 by using the Ras inhibitor farnesylthiosalicylic acid (FTS; refs. 24, 25). FTS is a potent Ras inhibitor that acts in a rather specific manner on the active, GTP-bound forms of H-Ras, N-Ras, and K-Ras proteins (24, 25). FTS competes with Ras-GTP for binding to specific saturable binding sites (26) in the plasma membrane, resulting in mislocalization of active Ras and facilitating Ras degradation (26). This competition prevents active Ras from interacting with its prominent downstream effectors and results in reversal of the transformed phenotype in transformed cells that harbor activated Ras (25). As a consequence, Ras-dependent cell growth and transforming activities, both in vitro and in vivo, are strongly inhibited (24, 25). Here, we examined the effects of FTS on the Ras-signaling cascade and on biochemical and phenotypic characteristics in neurofibromin-deficient MPNST cell lines.

Materials and Methods

Materials

FTS was a gift from Thyreos (Newark, NJ). The enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL); mouse anti-pan-Ras antibody (Ab-3) was from Calbiochem (La Jolla, CA); mouse anti-phosphorylated extracellular signal-regulated kinase (ERK) and mouse anti-tubulin (AK-15) antibodies were from Sigma-Aldrich (St. Louis, MO); rabbit anti-ERK and rabbit anti-neurofibromin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-Akt and rabbit anti-phosphorylated Akt (Ser473; 4E2) antibodies were from Cell Signaling Technology (Beverly, MA); mouse anti-p120 Ras-GAP antibody was from Upstate Biotechnology (Lake Placid, NY); and mouse anti-RalA antibody was from Transduction Laboratories (Lexington, KY). Peroxidase goat anti-mouse IgG and peroxidase goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Genetic analysis of the nf1 gene

Using high molecular weight DNA from ST88-14, 90-8, and T265P21 cell lines as a template, exon-specific PCR amplification was applied to the 60 exons of the nf1 gene in each cell line. The resulting PCR products were analyzed by denaturing high-performance liquid chromatography to target abnormal migration patterns heralding possible sequence alterations. For optimal detection of heterozygous mutations, PCR amplification products (amplicons) of the cell lines (putatively containing only the mutated copy of the nf1 gene) were mixed with amplicons of non-NF1 cells. Following denaturing high-performance liquid chromatography analysis, all abnormally migrating fragments were sequenced using the ABI Prism BigDye and a semiautomated sequencing kit (PE Biosystems, Foster City, CA).

Alleotyping of cell lines using 17q markers DNA from all cell lines was used as a template for amplification using four 17q markers (D17S33, D17S1166, D17S250, and an intronic polymorphic sequence within intron 39). PCR primers and protocols were done as described previously (27) and analysis of PCR products was carried out on the ABI Prism 310 apparatus or using an RsrI restriction enzyme digest (for D17S33).

Cell culture procedures. The human NF1 MPNST cell lines ST88-14, T265P21, and 90-8 and the non-NF1 human schwannoma cell line STS26T were obtained from Dr. Nancy Ratner (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). T265P21, 90-8, and STS88-14 cells were maintained in RPMI 1640/15% FCS medium and STS26T cells were maintained in DMEM/10% FCS as described elsewhere (24). Cells were plated at a density of 1 × 10^4 per 10-cm plate for biochemical and immunoblotting assays, 2.5 × 10^4 per well in 24-well plates for cell growth assays, or on glass coverslips (5 × 10^4 per 35-mm dish) for labeling of actin cytoskeletal elements. The cells were incubated for 24 hours, treated with the indicated concentration of FTS or with the vehicle (0.1% DMSO; control) for the times specified in each of the experiments, and then subjected to the various assays as described below. The effect of FTS on cell growth was estimated by direct counting of cells collected from each well as described earlier (24).

Western immunoblotting, Ras-GTP and RalA-GTP assays, and confocal microscopy

Unless otherwise indicated, cells were lysed 48 hours after being treated with lysis buffer as described previously (26) Lysates containing 50 to 100 μg protein were subjected to SDS-PAGE followed by Western immunoblotting, as described (26, 28, 29), with one of the following antibodies: pan-Ras antibody (Ab-3; 1:2,000), anti-phosphorylated ERK antibody (1:10,000), anti-ERK antibody (1:2,000), anti-Akt antibody (1:1,000), anti-phosphorylated Akt antibody (1:2,000), anti-tubulin antibody (1:5,000), anti-RalA antibody (1:5,000), anti-p120 Ras-GAP antibody (1:1,000), or anti-neurofibromin antibody (1:200). The immunoblots were then exposed to either peroxidase goat anti-mouse IgG (1:7,500) or peroxidase goat anti-rabbit IgG (1:2,000). Protein bands were visualized by enhanced chemiluminescence and quantified by densitometry with Image Master VDS-CL (Amersham) using TINA 2.0 software (Ray Tests). To obtain reliable comparisons between replicated experiments for statistical analysis, we used a standardization procedure, in which we normalized the density of each given protein band as recorded with its specific antibody. Normalization was achieved by collecting and averaging all the data obtained for a given band (e.g., for the Ras-GTP band) in a set of experiments, irrespective of cell type or treatment. The standard average density was defined as 1.0, and each of the individually determined bands was related to this value. Accordingly, the normalized value of a single band would be 1.0 if it was equal to the standard value or would range between values above and below 1.0 (respectively representing values higher and lower than the standard). The normalized values thus obtained were used to calculate mean ± SD and to determine, using Student’s t test, the statistical significance of differences between populations. Lysates containing 500 μg protein were used to determine Ras-GTP by the glutathione S-transferase (GST)–Ras-binding domain pull-down assay, and this was followed by Western immunoblotting with pan anti-Ras antibody as described earlier (28). Lysates containing 500 μg protein were used to determine RalA-GTP by the GST-RalBD pull-down assay followed by Western immunoblotting with anti-RalA antibody (30). Actin cytoskeletal elements were labeled with rhodamine-phalloidin,
and digital fluorescence images were collected on a Zeiss LSM 510 confocal microscope (Göttingen, Germany) fitted with fluorescein and rhodamine filters as described (25).

**Soft agar assays and animal experiments**

Noble agars (2% and 0.6%; Difco, Sparks, MD) were prepared in double-distilled water and autoclaved. The 2% agar was melted and mixed with medium (DMEM X2 with 20% FCS, 100 µg/mL penicillin, and 0.1 µg/mL streptomycin) and the mixture (50 µL) was placed in 96-well plates to provide the base agar (at a final concentration of 1%). ST88-14 (5,000 per well) and STS26T (15,000 per well) cells were suspended in medium (DMEM X2 mixed with 0.6% agar), and 50 µL of the mixture were plated on the base agar. FTS or vehicle containing DMEM/10% FCS (100 µL) was added to the wells. The plates were incubated for 14 to 21 days at 37°C and colonies were then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL) and photomicrographed. The number of colonies per well was determined using the ImagePro software.

**Results**

**Pathogenic nf1 gene mutations and neurofibromin deficiency in NF1-associated MPNST cell lines.** The NF1-associated cell lines used here were originally derived from human MPNST and were therefore assumed to possess an nf1/0/0 genotype. As a preliminary step, we considered it important to genotype the nf1 gene mutation in these cell lines in order define the specific pathogenic inactivating nf1 mutation. We detected a

![Fig. 1](#)
heterozygous nonsense mutation (C910T) in codon 304 (R304X) of exon 7 of both 90-8 (Fig. 1) and ST88-14 (data not shown) cell lines. This is a well-known pathogenic mutation that leads to exon 7 skipping (31). Another mutation detected in the 90-8 cell line was a 7-bp deletion in exon 23a of the nf1 gene (GATCCTT). This mutation is predicted to lead to inactivation of the nf1 gene and is apparently the “second hit,” which, in concert with the nonsense mutation, inactivates the wild-type allele. These results predict that 90-8 cells are deficient in neurofibromin, confirming the earlier

![Fig. 2. Steady-state levels of Ras-GTP correlate positively with neurofibromin deficiency and growth inhibition by FTS. A, typical photomicrographs of control and FTS-treated NFI and non-NFI cells. The NFI cell lines ST88-14, 90-8, and T265P21, as well as non-NFI STS26T cells, were grown for 2 days in the absence and presence of 50 \( \mu \text{mol/L} \) FTS and then photomicrographed. Typical photomicrographs of vehicle-treated (control) and FTS-treated cells. B, FTS inhibits growth of NFI and non-NFI cells in a dose-dependent manner. ST88-14, 90-8, T265P21, and STS26T cells were grown for 3 days in the absence and presence of the indicated concentrations of FTS. They were then detached, collected, and counted as described in Materials and Methods. The numbers of cells in the FTS-treated cultures (expressed as percentages of the cell numbers in control cultures) as a function of FTS concentration for each cell line. Points, means (\( n = 4 \)); bars, SD. Similar results were obtained in three additional experiments. C, correlation curve relating the amounts of Ras-GTP to the IC50 values for FTS-induced growth inhibition in NFI and non-NFI cells. Correlations between the IC50 values [points, mean (\( n = 4 \)); bars, SD] evaluated from the FTS dose-dependent inhibition curves shown in (B) and the normalized Ras-GTP levels (arbitrary units; see Materials and Methods) evaluated from experiments as shown in Fig. 1D [points, mean (\( n = 9 \)); bars, SD]. \( R^2 \) is the correlation coefficient.
The amount of neurofibromin detected in 50 analysis showed that the non-NF1-associated STS26T cell lines and a specific anti-neurofibromin antibody. The Western immunoblot analysis using cell lysates from all four fibromin deficient, neurofibromin expression was assessed by translocation has been characterized previously in a MPNST cell line, as evident from the minimal signal of clonality, there seems to be a residual DNA contribution from allelic loss. These allelotyping results show that the cell lines the same pattern in all four markers, a pattern consistent with all NF1 cell lines consistently showed a pattern of a single dominant allele and another one that seemed to be a lesser intensity allele, with the three intragenic markers (see Fig. 1B). Using the D17S250 marker cell line 90-8 showed clearly intensity allele, with the three intragenic markers (see Fig.1B). The other cell lines showed the same pattern in all four markers, a pattern consistent with allelic loss. These allelotyping results show that the cell lines originate from monoclonal tumors, but despite their monoclonality, there seems to be a residual DNA contribution from a non tumorous tissue, as evident from the minimal signal of the “lost allele.” In this regard, it is worth noting that a 17q11.2 translocation has been characterized previously in a MPNST cell line (33) and that recombination hotspot in NF1 microdeletion patients has been characterized as well (35).

To establish conclusively that these cell lines are neurofibromin deficient, neurofibromin expression was assessed by Western immunoblot analysis using cell lysates from all four cell lines and a specific anti-neurofibromin antibody. The analysis showed that the non-NF1-associated STS26T cells expressed significant amounts of neurofibromin, equivalent to the amount of neurofibromin detected in 50 μg protein from total brain homogenate, which served as a standard (Fig. 1C). In contrast, only small amounts of neurofibromin were detectable in ST88-14, 90-8, and T265P21 cells (Fig. 1C). Immunoblot analysis with anti-p120 Ras-GAP antibody indicated, nonetheless, that the amounts of p120 Ras-GAP in ST88-14, 90-8, and T265P21 cells were indistinguishable from the amounts in STS26T cells (Fig. 1C).

Steady-state levels of Ras-GTP correlate positively with neurofibromin deficiency and growth inhibition by FTS. Next, we examined whether the steady-state levels of active Ras in the MPNST cell lines correlated with neurofibromin deficiency. Total Ras and Ras-GTP in the NF1 cell lines ST88-14, 90-8, and T265P21, as well as in the non-NF1 STS26T cells, were determined by using the GST–Ras-binding domain of Raf-1 pull-down assay and Western immunoblotting with anti-Ras antibody. The amounts of total Ras in all cell lines were clearly comparable, but the amounts of Ras-GTP varied (Fig. 1D). Ras-GTP (mean ± SD; n = 6) in the non-NF1 STS26T cells was very low (0.17 ± 0.14% of the total Ras protein) but was far higher in the STS26T, 90-8, and ST88-14 cells (3 ± 2.5%, 7.5 ± 1.8%, and 7.7 ± 1.8% of the total Ras protein), respectively (Fig. 1D). Thus, the relatively large amounts of Ras-GTP detected in the NF1 MPNST cell lines and the relatively small amounts in the non-NF1 STS26T MPNST cells (Fig. 1D) correlate well with the observed neurofibromin deficiency (Fig. 1C), consistent with a recent report on Ras-GTP levels in these cell lines (36).

Next, we examined the effects of the Ras inhibitor FTS on the growth rates of the NF1 cell lines ST88-14, 90-8, and T265P21, as well as of the non-NF1 STS26T cells. Typical photomicrographs of control and FTS-treated (50 μmol/L STS26T, T265P21, ST88-14, and 90-8 cells (Fig. 2A) show a drug-induced reduction in cell number. Under the conditions used, there was no significant cell death evident by Hoechst dye-exclusion assay (data not shown). Treatment of the cells with different concentrations of FTS (12.5–100 μmol/L) induced a dose-dependent decrease in cell number, with estimated IC50 values of 35 ± 7, 42 ± 13, 42 ± 9, and 53 ± 10 μmol/L (mean ± SD; n = 4) in T265P21, ST88-14, 90-8, and STS26T cells, respectively (Fig. 2B). Interestingly, sensitivity to FTS as judged by these values showed good inverse correlation with apparent amounts of Ras-GTP in the cell lines studied: the larger the amount of Ras-GTP, the lower the IC50 value (Fig. 2C).

FTS down-regulates Ras-GTP and inhibits Ras signaling in NF1 cell lines. One plausible explanation for the above results is that inhibition of the cellular growth of NF1 MPNST cells by FTS might be attributable to FTS-induced inhibition of active Ras and its downstream signals. To examine this possibility, we assessed the effects of FTS on steady-state levels of Ras and on Ras-GTP levels in each of the studied cell lines. All four cell lines were treated with the vehicle (control) or with 75 μmol/L FTS for 48 hours. FTS treatment induced relatively small (15-30%) but significant (P < 0.05) reductions in Ras levels in all cell lines (Fig. 3A). Even greater reductions in the steady-state levels of Ras-GTP (25-55%; P < 0.03) were observed in TS265P21, 90-8, and ST88-14 cells (Fig. 3A). The low basal levels of Ras-GTP in STS26T cells precluded any attempt at accurate analysis of Ras-GTP in the presence of FTS, although total Ras was clearly decreased in these cells, as it was in the NF1 cell lines (Fig. 3A). These experiments showed that FTS acts as a Ras inhibitor, which down-regulates active Ras in NF1 cells. FTS (75 μmol/L; 48 hours) had no effect on the amounts of p120 Ras-GAP in the NF1 and non-NF1 cells (data not shown), indicating that the decrease in active Ras-GTP resulted from a direct effect of the Ras inhibitor on active Ras and was not achieved indirectly by increasing p120 Ras-GAP.

Next, we examined whether the observed down-regulation of active Ras by FTS was accompanied by inhibition of Ras signaling. We assessed the effect of FTS on steady-state levels of phosphorylated ERK, phosphorylated Akt, and RalA-GTP as read-outs of the three prominent Ras pathways, Ras/Raf/MEK/ERK, Ras/phosphatidylinositol 3-kinase/Akt, and Ras/RalGEF/RalA, respectively (37, 38). The results of these experiments showed that FTS caused a significant reduction in phosphorylated ERK, phosphorylated Akt, and RalA-GTP in all NF1 cell lines (Fig. 3B) but did not affect the amounts of total ERK, total Akt, or total RalA. FTS also caused a significant reduction in RalA-GTP in the non-NF1 STS26T cells (Fig. 3B) and a small (10-20%), unexplained increase in phosphorylated ERK and phosphorylated Akt. Thus, down-regulation of active Ras in NF1 cells was accompanied by a reduction in activation of three prominent Ras downstream pathways.
FTS restores an attenuated Ras signal termination in NF1 cells. We postulated that the Ras signaling in NF1 cells would be relatively prolonged owing to the deficiency of neurofibromin and that the Ras inhibitor FTS would shorten that signal.

To test these notions, we did a detailed kinetic study, in which we determined the time courses of serum-stimulated GTP loading of Ras and serum-stimulated activation of signals downstream of Ras. The cells were treated with FTS and serum starved (0.5% serum) for 24 hours before being stimulated with 10% serum for the indicated times. Results of a typical experiment with the non-NF1-derived cells, STS26T, are shown in Fig. 4A. As shown, stimulation with 10% serum induced a strong and rapid increase (within 2 minutes) in Ras-GTP, lasting for at least 10 minutes. The increase was transient, however, and by 30 or 60 minutes after serum stimulation, the amounts of Ras-GTP were substantially reduced (Fig. 4A). This serum-stimulated increase in Ras-GTP was strongly inhibited by 20 μmol/L FTS (Fig. 4A). Notably, unlike in STS26T cells grown in the presence of serum (steady-state conditions), where Ras-GTP was undetectable (Fig. 1D), we observed a robust increase in Ras-GTP in the serum-starved cells on stimulation with serum.
This increase might be due to the reported rapid degradation of NF1 in response to serum, which would have prevented inactivation of Ras-GTP (39). It is also worth noting that at the low serum concentration used for serum starvation, the FTS concentration needed to inhibit Ras was relatively low (Fig. 4A) compared with that needed in cells grown with serum (Fig. 3).

Phosphorylated ERK and phosphorylated Akt in STS26T cells also showed a serum-stimulated transient increase, which was slightly delayed (by 3-5 and 5-10 minutes, respectively) compared with that observed in Ras-GTP. This delay might be accounted for by the fact that phosphorylated ERK and phosphorylated Akt are downstream targets of Ras (37, 38). Nonetheless, as for serum activation of Ras, the increases in phosphorylated ERK and phosphorylated Akt levels were transient, and after 60 minutes, were substantially reduced (Fig. 4A). FTS also inhibited the serum-stimulated increase in phosphorylated ERK and in phosphorylated Akt (Fig. 4A). Under these conditions, FTS had no effect on the total amounts of Ras, ERK, or Akt. Thus, serum stimulation of STS26T cells resulted in transient activation of Ras and its downstream signals, and this, as expected, was inhibited by the Ras inhibitor.

Next, we conducted a similar set of experiments using NF1 T265P21 (Fig. 4B), ST88-14 (Fig. 4C), and 90-8 (Fig. 4D) cells. In each of these cell lines, we observed a strong and rapid (within 2 minutes) serum-stimulated increase in Ras-GTP, similar to that observed in the non-NF1 cells. However, in marked contrast to the observation in the latter cells, the serum-stimulated increase in Ras-GTP levels in the NF1 cells was maintained for at least 30 minutes, by which time Ras-GTP levels in the non-NF1 cells had already declined. Similarly, the signals to ERK and Akt in the NF1 cells were more prolonged (Fig. 4B-D) than in the non-NF1 cells (Fig. 4A), and phosphorylated ERK and phosphorylated Akt levels were still relatively high even 60 minutes after serum stimulation. Importantly, in all the NF1 cell lines, FTS not only reduced the serum-stimulated increases in Ras-GTP, phosphorylated ERK, and phosphorylated Akt levels but also shortened the duration of the signals (Fig. 4B-D). Thus, in the presence of FTS, the duration of the Ras signal in the NF1 cells (5-10 minutes) was similar to that observed in the non-NF1 cells.

**FTS affects cytoskeleton reorganization in NF1 cells.** A recent study showed that loss of neurofibromin induces excessive formation of actin stress fibers in HT1080 and HeLa cells (40). We therefore examined whether stress fiber formation related to neurofibromin deficiency is detectable in the NF1 MPNST cells and, if so, whether this phenotype can be reversed by FTS. We carried out a comparative analysis of cytoskeleton reorganization in the NF1 MPNST cells and in non-NF1 STS26T MPNST cells. Control or FTS-treated cells (75 μmol/L) were stained with rhodamine-labeled phalloidin and subjected to confocal fluorescence microscopy. A, typical image collected from controls and FTS-treated cells. B, statistical analysis of the results was done. The number of cells with stress fibers, expressed as a percentage of the total cell number, was determined by counting the cells containing stress fibers and the total number of cells in a field. Cells in three to four fields were counted in each of three experiments. Columns, mean (n = 3); bars, SD. *, P < 0.01; **P < 0.001.
proportion of cells with stress fibers was 86 ± 6.7%, 82 ± 6.1%, and 87 ± 6.4% in T265P21, ST88-14, and 90-8 cells, respectively, and significantly lower in the non-NF1 STS26T cells (50 ± 11.8%; P < 0.05; Fig. 5B). Consistently with earlier observations in non-NF1 Ras-transformed cells, we found that FTS induced a significant increase of 27 ± 11.9% (mean ± SD; P < 0.05, n = 3) in stress fiber formation in the non-NF1 STS26T cells (Fig. 5). In marked contrast, FTS induced a significant decrease in the numbers of cells with stress fibers (from 82-87% to 11-20%) in the NF1 cell lines T265P21, ST88-14, and 90-8 (Fig. 5B). Thus, nearly all of the NF1 cells lost their stress fibers. These results showed clearly reversal of the transformed phenotype of the NF1 cells by the Ras inhibitor FTS.

**FTS inhibits the anchorage-independent growth of ST88-14 and STS26T NF1 cells.** Next, we examined whether FTS can inhibit the transforming activity associated with NF1 deficiency. To this end, we did soft agar assays and determined the effect of FTS on the anchorage-independent growth. ST88-14 and STS26T cells were plated in soft agar and then treated with 0.1% DMSO as a control or with FTS (25, 50, or 75 μmol/L) and grown for 3 or 2 weeks, respectively. Results of a typical experiment (Fig. 6A) show that control ST88-14 and STS26T cells developed approximately 130 and 670 colonies per plate, respectively, within this time period. FTS inhibited colony formation of both cell lines; in the presence of 50 μmol/L FTS, for example, colony formation was inhibited by 60% in STS26T cells and by 57% in ST88-14 cells. In the presence of 75 μmol/L FTS, colony formation was inhibited by 84% and 87% in ST88-14 and STS26T cells, respectively. Unlike ST88-14 and STS26T, the other two MPNST cell lines, T265P21 and 90-8, did not form colonies in soft agar.

**Tumor growth in ST88-14 cells is inhibited by FTS in nude mice.** We also assessed the ability of FTS to inhibit tumor growth in nude mice implanted with ST88-14 cells. These cells develop tumors in nude mice as shown previously (42). We used two types of treatment. The first was i.p. administration of 1, 5, or 10 mg/kg FTS as described earlier (24) The second was oral administration, which required higher FTS doses of 20, 40, 60, and 80 mg/kg. Six weeks after the treatment, mice were killed and tumors were removed and weighed. As shown in Fig. 6B, both the i.p. and the oral FTS treatments caused dose-dependent inhibition of tumor growth: the i.p. protocol yielded a significant inhibition of 49% (P < 0.05) at a dosage of 10 mg/kg FTS, whereas the oral protocol yielded a significant inhibition of 38% (P < 0.01) at a dosage of 80 mg/kg.

**Discussion**

Several lines of evidence strongly support the notion that active Ras, in the context of neurofibromin deficiency, plays an important role in the etiology and clinical manifestations of NF1 (43–46). These publications lend credence to the concept of using Ras inhibitors to treat some of the symptoms of this disease. The data presented here further strengthen this concept and show that the Ras inhibitor FTS can be considered at least as a candidate for NF1 therapy. First, we confirmed that the
MPNST ST88-14, T265P21, and 90-8 cells chosen for this study are genetically biallelic, NF1 inactive, and are deficient in neurofibromin protein. Second, in agreement with earlier studies (9), we observed relatively large amounts of Ras-GTP in these cells. We observed that the high levels of Ras-GTP found in these cells were associated with neurofibromin deficiency. Third, we showed that FTS reduced the steady-state levels of Ras-GTP in the NF1 cells, resulting in the inhibition of three major downstream targets of Ras: ERK, Akt, and RalA. Fourth, we showed that FTS inhibited the anchorage-dependent growth of the NF1 cells, attenuated their anchorage-independent growth in soft agar, and inhibited NF1 tumor growth in a nude mouse model.

Earlier studies used FTIs in attempts to inhibit Ras and NF1 cell growth (14, 21, 22). The published effects of FTIs on NF1 cell growth, similar to that of FTS, are only partial, perhaps both pharmacologic agents fail to induce NF1 cell death. It is however worth noting that, despite the fact that FTIs were originally developed to block the processing and function of oncogenic Ras proteins, it is now widely accepted that inhibiting Ras processing is not the most important downstream consequence of FTI action (23). FTIs do not block the highly abundant K-Ras and N-Ras isoforms (23). Indeed, it was shown that the FTI L-744,832 inhibited H-Ras but not N-Ras processing in nfi1−/− hematopoietic cells (14). This and the demonstration that glialoma formation in NF1 reflects preferential activation of K-Ras in astrocytes (47) combined with the observed role of N-Ras in ST-88 and 90-8 NF1 cells (48) may indicate the importance of Ras inhibitors that affect the K-Ras and N-Ras isoforms. FTS, which is a non-FTI Ras inhibitor, interferes with Ras membrane anchorage and inhibits the functions of all activated Ras isoforms (19, 20).

Sensitivity of the NF1 cells to FTS, as judged by growth-inhibition curves, showed good inverse correlation with the apparent amounts of Ras-GTP in the cells: the greater the amount of Ras-GTP, the lower the IC_{50} value. This finding is in line with results obtained with other FTS-treated cell lines, in which cells with a high Ras-signal output were shown to be more sensitive to FTS than cells, in which Ras activity is relatively low (24). Our detailed kinetic analysis of serum-stimulated non-NF1 MPNST cells (STS26T) and NF1 cells disclosed that Ras signals in NF1 cells take longer to fade leading to prolonged activation of Ras, as well as of ERK, Akt, and RalA. Thus, in line with earlier reports (10), neurofibromin deficiency seems to result in prolongation of Ras signaling. Our results strongly suggest that the relatively high steady-state levels of active Ras exhibited by the NF1 cells are mainly attributable to the lack of neurofibromin GTPase activity. Thus, once these neurofibromin-deficient cells receive growth factor signals, the receptor-mediated GTP loading is terminated at a relatively slow pace, weakening the signal termination. Signal termination in the non-NF1 STS26T cells is likely to proceed at a higher rate owing to the mechanisms that allow rapid recovery of NF1 (39). Recent studies showed that neurofibromin is rapidly degraded by the ubiquitin-proteasome pathway in response to growth factors and that this response is followed by its restoration shortly after growth factor treatment. Interestingly and in accordance with the results described above, nfi1-deficient mouse embryonic fibroblasts exhibit enhanced activation of Ras as well as prolonged Ras and ERK activities and proliferate in response to subthreshold levels of growth factors (39). These experiments suggested that the dynamic proteosomal regulation of neurofibromin represents an important mechanism of controlling both the amplitude and the duration of Ras-mediated signaling (39).

An important finding of the current study was that FTS not only reduced the serum-stimulated increases in Ras-GTP, phosphorylated ERK, and phosphorylated Akt in all NF1 cell lines but also forced a shorter signal. Thus, in the presence of FTS, the duration of the Ras signal in the NF1 cells (5-10 minutes) was similar to that observed in the non-NF1 cells. We thus concluded that, with respect to Ras activation, neurofibromin deficiency can apparently be corrected by FTS.

Transformation by Ras is often accompanied by the disappearance of actin stress fibers, which is attributable to the effects of active Ras on Ras-dependent regulation of the Rho family of GTPases (49, 50). Other groups as well as ours have shown previously that Ras transformation of rat-1 cells is associated with loss of actin stress fibers (25, 51), which reappear on inhibition of the active Ras in these cells by FTS (25) or FTIs (51). A novel and different observation of the present study was that nearly all NF1 cells possessed strong actin stress fibers, in sharp contrast to the non-NF1 cell line. This phenomenon seemed to be associated with increased amounts of Ras-GTP in the NF1 cells because FTS induced the complete disappearance of actin stress fibers in these cells. Consistently with these observations, recent studies showed that loss of neurofibromin induced excessive formation of actin stress fibers in HT1080 and HeLa cells (40). In those cell lines, which are derived from tumors not associated with the NF1 phenotype, this effect required Ras activation and was reversed by transfection with the NF1 GAP-related domain. It thus seems that inhibition of active Ras-GTP, either by the NF1 GAP-related domain (40) or by FTS, induces disappearance of stress fibers.

In conclusion, it seems that Ras inhibition by FTS induces reversal of the aberrant Ras-associated transformed phenotype in NF1 cells and that this can explain the observed attenuation of NF1 tumor growth in FTS-treated animals. These findings pave the way to attempts to develop a treatment with FTS to inhibit Ras-dependent NF1 tumor growth.

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