

Research Article

Driven to Death: Inhibition of Farnesylation Increases Ras Activity and Promotes Growth Arrest and Cell Death

Mandy Geryk-Hall, Yanwen Yang, and Dennis P.M. Hughes

Abstract

To improve cancer outcomes, investigators are turning increasingly to small molecule medicines that disrupt vital signaling cascades, inhibit malignant growth, or induce apoptosis. One vital signaling molecule is Ras, and a key step in Ras activation is membrane anchoring of Ras through prenylation, the C-terminal addition of a lipid anchor. Small molecule inhibitors of farnesyltransferase (FTI), the enzyme most often responsible for prenylating Ras, showed clinical promise, but development of FTIs such as tipifarnib has been stalled by uncertainty about their mechanism of action, because Ras seemed unimpeded in tipifarnib-treated samples. Interpretation was further complicated by the numerous proteins that may be farnesylated, as well as availability of an alternate prenylation pathway, geranylgeranylation. Our initial observations of varied response by cancer cell lines to tipifarnib led us to evaluate the role of FTI in Ras signal alteration using various tumor models. We describe our novel counterintuitive finding that endogenous Ras activity increases in cancer cell lines with low endogenous Ras activity when farnesyltransferase is inhibited by either tipifarnib or short hairpin RNA. In response to tipifarnib, variable growth arrest and/or cell death correlated with levels of activated extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Sensitivity to tipifarnib treatment was shown by growth inhibition and by an increase in subdiploid cell numbers; cells with such sensitivity had increased activation of ERK and p38 MAPK. Because Ras must be prenylated to be active, our findings suggest that geranylgeranylated N-Ras or K-Ras B interacts differently with downstream effector proteins in sensitive cancer cells responding to tipifarnib, switching the balance from cell proliferation to growth inhibition. *Mol Cancer Ther*; 9(5); 1111–9. ©2010 AACR.

Introduction

There is great interest in disrupting cell signaling as a means of combating cancer, and one method is by blocking protein prenylation. Prenylation is a posttranslational process in which a lipid anchor is added to the C-terminus of proteins to allow membrane association (1–3). This lipid modification increases the hydrophobicity of the proteins and enhances interaction with membrane lipids and with other proteins (2, 3).

There are two forms of prenylation, farnesylation and geranylgeranylation, mediated by the enzymes farnesyltransferase and geranylgeranyltransferase. Prenylated proteins contain a CAAX motif at their C-terminus: a cysteine, two aliphatic amino acids (leucine, isoleucine,

or valine), and a C-terminal serine, methionine, leucine, or glutamine (4, 5). This CAAX motif, along with the linker region at the C-terminus of proteins, determines which prenyl group is added to the protein (6).

Farnesyltransferase inhibitors (FTI) are small molecules that inhibit prenylation of multiple proteins, including Ras, RhoB, lamin A/C, and the centromere proteins (CENP-E and CENP-F; refs. 1, 7, 8). Tipifarnib is a FTI that blocks farnesylation by competitively binding to the CAAX motif in proteins (4, 9, 10). In clinical trials, tipifarnib, when used alone or in combination with chemotherapy, has produced therapeutic responses in many cancers, including acute myeloid leukemia (AML), breast cancer, head and neck squamous cell carcinoma, myelodysplastic syndrome, and myeloproliferative disorders (1). The intent of using FTIs therapeutically was to block activation of Ras, which is oncogenic in many cancers or is overactivated as a result of being downstream of oncogenic receptor tyrosine kinases as is often the case in breast carcinomas.

Ras proteins are small guanosine triphosphatases (GTPase) that regulate growth, differentiation, survival, and cell death (11–13). After Ras is prenylated, it associates with the cell membrane, functioning in signal transduction (5, 14, 15) and cycling between active (Ras-GTP)

Authors' Affiliation: Department of Pediatrics Research, Children's Cancer Hospital, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Corresponding Author: Dennis P.M. Hughes, Department of Pediatrics Research, Children's Cancer Hospital, The University of Texas M.D. Anderson Cancer Center, Unit 853, P.O. Box 301402, Houston, TX 77030. Phone: 713-563-9270; Fax: 713-563-5406. E-mail: DPHughes@mdanderson.org

doi: 10.1158/1535-7163.MCT-09-0833

©2010 American Association for Cancer Research.

and inactive (Ras-GDP) states (11, 12, 16). There are four highly homologous Ras proteins: K-Ras A, K-Ras B, N-Ras, and H-Ras (9, 12, 13, 16). All of which contain a CAAX motif in their C-terminus and can be farnesylated; K-Ras B and N-Ras can also be geranylgeranylated (17). Oncogenic Ras mutations are commonly found in cancer, and the specific mutated Ras species (if any) in a particular tumor is likely to determine whether FTI therapy will be useful (7). We chose to look at FTI effects in osteosarcoma because no oncogenic Ras mutations have been identified in osteosarcoma (18) perhaps because of the constitutive activity of receptor tyrosine kinases providing a similar effect on cell signaling (19).

Ras activates multiple pathways, and the best characterized are the mitogen-activated protein kinase (MAPK) cascades. The MAPK family includes extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH₂-terminal kinase, and p38. There are three evolutionarily conserved sequential kinases: MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK (20, 21), which are activated through phosphorylation by a small GTP-binding proteins such as Ras. There is overlap and cross talk in these signaling cascades that regulate the balance of cell survival. Ras was originally thought to contribute only to proliferation and cell survival. However, Ras also has been shown to have roles in apoptosis, growth arrest, and senescence through activation of these same signaling molecules, depending on cellular context, the presence or absence of other regulatory molecules, and the persistence of the signal (12, 16, 22–24).

We wanted to determine what effect FTI had on a disease that has identified no known mutations in Ras. This study is aimed to expand the knowledge of how FTI's exert their antineoplastic effects. The purpose of this study was to identify if FTI is effective in decreasing proliferation in cancer cells with wild-type Ras. Increased Ras activity observed with tipifarnib treatment led us to determine the activation status of downstream effector molecules of Ras. ERK and p38 pathways were increased in osteosarcoma cells that underwent growth arrest in response to tipifarnib treatment. We therefore treated osteosarcoma cells with tipifarnib alone, with the geranylgeranyltransferase inhibitor (GGTI) GGTI-298 alone, or with combined tipifarnib and GGTI-298 to determine the prenylation status of N-Ras and K-Ras B. The results suggested that when farnesylation is blocked with tipifarnib, N-Ras and K-Ras B are geranylgeranylated and their downstream signaling is altered, resulting in decreased cell survival and proliferation.

Materials and Methods

Cell culture and experimental reagents

Human osteosarcoma cell lines were maintained as follows: OS187, COL, CCH-OS-M, and CCH-OS-D cells were grown in DMEM 1X media (Invitrogen) with 10% fetal bovine serum (HyClone), 1% penicillin-streptomycin

solution (Gemini Bio-Products), and 1% L-glutamine (Lonza). COL, CCH-OS-M, and CCH-OS-D were given additional 1% insulin transferrin selenium-A solution (Invitrogen). SaOS2, LM7, and HOS were grown in MEM with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, 1% MEM essential vitamins (Lonza), 1% sodium pyruvate (MP Biomedicals), and 1% nonessential amino acid mixture (Cambrex Biosciences). AML cell lines THP-1 and U937 (a kind gift from Dr. Patrick Zweidler-McKay, M.D. Anderson, Houston, TX) were maintained in RPMI 1640 high-glucose 1X media (Invitrogen), with 10% fetal bovine serum and 1% penicillin-streptomycin. All cells were incubated in a humidified atmosphere at 37°C with 5% CO₂.

OS187, COL, SaOS2, LM7, and HOS have been described (19, 25, 26). CCH-OS-M and CCH-OS-D are primary osteosarcoma cell lines derived from patients at the Children's Cancer Hospital at The University of Texas M.D. Anderson Cancer Center. CCH-OS-D was obtained from a core needle biopsy of a proximal femur lesion in an 18-year-old man who also presented with pulmonary metastases. CCH-OS-M was obtained from the malignant pleural effusion of a 15-year-old boy with recurrent pulmonary and pleural-based osteosarcoma 13 months after initial diagnosis of right proximal humerus osteosarcoma. Experiments were conducted between passages 8 and 13 for both cell lines.

Downstream Ras targets. OS187, COL, and SaOS2 were exposed to tipifarnib at the following concentrations for 24 hours: 0, 0.01, 0.1, and 1 $\mu\text{mol/L}$.

N-Ras and K-Ras alternate prenylation. OS187, COL, and SaOS2 cells were treated with 1 $\mu\text{mol/L}$ tipifarnib, 4 $\mu\text{mol/L}$ GGTI-298, no drug, or both drugs for 24 hours.

Chemicals. Tipifarnib (Johnson & Johnson) and GGTI-298 (Calbiochem) were dissolved in DMSO as 10 mmol/L stock solutions.

Cell viability assay

Osteosarcoma cells were plated in six-well tissue culture plates at 100,000 per well. The following day, tipifarnib was added to achieve the following concentrations: 0, 0.01, 0.1, and 1 $\mu\text{mol/L}$, in triplicate. Cell nuclei were counted at 24, 48, 72, and 96 hours as follows: The medium was removed from the plates and the cells were washed with 2 mL PBS. HEPES (0.5 mL of 0.01 mol/L)/MgCl₂ (0.015 mol/L) buffer was added, and cells were rocked for 5 minutes at room temperature to equilibrate. After equilibration, 50 μL of 0.132 mol/L Bretol with 0.525 mol/L glacial acetic acid were added, and the cells were rocked for 10 minutes at room temperature. A solution consisting of 3.5 mL of 0.9% NaCl and 0.5% formalin was added to fix the nuclear membranes, and 2 mL of the solution containing the nuclei were placed into an auto-sampler cup. Nuclei (5–25 μm) were counted by using an automated Vi-Cell Analyzer (Beckman Coulter).

Invasion assay

Invasiveness was measured in a BD Matrigel invasion chamber, as described previously (27). Cells were treated

with tipifarnib for 48 hours before plating, and drug was included in upper and lower chambers throughout the assay. Invasive cells were counted after 48 hours.

Cell cycle analysis

Cells were cultured with tipifarnib for 72 hours, and then dead and live cells were collected and incubated overnight at 4°C with 0.005% propidium iodide and 0.1% Triton X-100 diluted in PBS. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Western blot analysis

Lysates were collected as follows: The medium was removed, plates were washed with cold PBS, and 1 mL of lysis buffer, as described previously (28), was added to each plate, and plates were incubated on ice for 35 minutes. Lysates were collected and centrifuged at 10,000 rpm for 10 minutes. Protein concentrations were determined by bicinchoninic acid analysis (Thermo Scientific). Whole-cell lysates were separated by SDS-PAGE on a 12% or 15% polyacrylamide gel and transferred to nitrocellulose membrane according to standard techniques. Membranes were probed with use of the following antibodies: phospho-44/42 MAPK, MAPK/ERK kinase (MEK) 1/2, phospho-MEK 1/2, phospho-p38 MAPK, p38 MAPK, MKK3, phospho-MKK3/6, phospho-MSK1, phospho-ATF-2, and pan-Ras (1:1,000; all from Cell Signaling); MAPK/ERK (1:1,000; Promega); Rap-1 (Millipore); and actin (1:1,000; Sigma). Horseradish peroxidase-conjugated anti-rabbit IgG (1:4,000; GE Healthcare) was used as secondary antibody for all antibodies listed above. Membranes were probed with use of HDJ-2 (Thermo Scientific), K-Ras, and N-Ras (Santa Cruz Biotechnology). Horseradish peroxidase-labeled polyclonal anti-mouse Ig (1:5,000; BD Biosciences) was used as secondary antibody for antibodies listed above. Chemiluminescent signal was detected by the Immobilon Western blotting detection system (Millipore). Densitometry values are relative to actin and are representative of three experiments.

Ras activity

OS187, COL, and SaOS2 osteosarcoma cells were exposed to 0.01, 0.1, or 1 $\mu\text{mol/L}$ tipifarnib or no drug. LM7, HOS, CCH-OS-M, and CCH-OS-D were treated with 0 and 1 $\mu\text{mol/L}$. Lysates were made, and 1.5 mg/mL protein was used for the Ras activity assay (Cell Biolabs, Inc.). Immunoprecipitation and immunoblotting were conducted according to the manufacturer's directions.

Short hairpin RNA

Designing oligonucleotides. Two hairpin structures predicted to anneal to RNA of the β subunit of farnesyltransferase (FT β ; see Results for discussion of FT β) were designed with use of software provided by Invitrogen (29) and purchased from Sigma. Lowercase letters indicate sequence used for ligation only: 5'-untranslated region,

1011 (top strand, 5'-gatctGCGATTGAAGGAGGATT-CACGAATGAAATCCTCCTTCAAATCGCtttttC-3'; bottom strand, 5'-tcgagAAAAAAGCGATTGAAGGAGGATTTCATTTCGTGAAATCCTCCTTCAAATCGCa-3'); open reading frame, 1927 (top strand, 5'-gatctGGTCCACCAAGATGAGTTCTCGAAAGAACTCATCTTGGTGGGACCTtttttC-3'; bottom strand, 5'-tcgagAAAAAAGGTCCCACCAAGATGAGTTCTTTCGAGAACTCATCTTGGTGGGACCa-3').

Annealing oligonucleotides. The annealing buffer was 100 mmol/L NaCl and 50 mmol/L HEPES (pH 7.4). One microliter of 3 mg/mL stocks of top and bottom strand oligonucleotides were incubated with 48 μL of annealing buffer at the following temperatures for 4 minutes each: 94°C, 80°C, 75°C, 70°C, 65°C, 60°C, and 55°C. The mixture was incubated at room temperature for 1 hour and at 4°C for 20 minutes. To generate a retroviral expression system for short hairpin RNA (shRNA) expression, the MigR1 retroviral expression vector, coexpressing green fluorescent protein as a selection marker, was manipulated to contain a U6 promoter upstream of the hairpin sequence; this vector is now termed MigU6. Hairpins were ligated into retroviral vector MigU6 by using a standard T4 ligation reaction.

Retroviral transduction. MigU6 empty vector, scrambled control, 1011, or 1922 were used to make replication-incompetent retrovirus, which was then used to infect osteosarcoma cells. To generate virus, 293T cells were seeded at 140,000 per well in a six-well dish. After 24 hours, the following were incubated for 5 minutes: tube A, 2 μg of MigU6 vectors, 2 μg of VSVG, 2 μg of PCGP (kind gifts from Dr. Patrick Zweidler-McKay), and 250 μL Opti-MEM (Invitrogen); tube B, 12 μL of Lipofectamine (Invitrogen) and 250 μL Opti-MEM. A and B were combined and incubated at room temperature for 30 minutes; 500 μL of each complex were added to one well of 293T cells. After 8 hours, the complex was removed, fresh medium was added, and the plate was incubated at 32°C. Supernatant was collected at 24 hours and centrifuged at 2,500 rpm for 2 minutes. Next, 2.5 mL of viral supernatant and 8 $\mu\text{g/mL}$ polybrene (Sigma) were added to OS187 and SaOS2 cells. These plates were centrifuged at 2,500 rpm for 1 hour and then incubated at 32°C for 24 hours. Viral medium was removed and fresh medium was added. Cells were sorted for green fluorescent protein twice to generate a polyclonal population of transduced cells.

Lysates were generated and Ras activity was assayed as described above. Parallel samples of lysate were tested by Western blotting for FT β , and knockdown was confirmed by densitometric analysis (ImageJ processing software, NIH).

Statistics

Significance was assessed by Student's *t* test (Statistica Software) with an α error threshold of 0.05. All experiments were conducted at least three times.

Results

Tipifarnib decreases cell viability and causes cell cycle arrest in sensitive cell lines and decreases invasiveness in all cells. To determine the response of osteosarcoma cells to farnesyltransferase inhibition, three osteosarcoma cell lines were treated with increasing concentrations of tipifarnib. Cell viability was assayed by counting nuclei after chemical lysis of the plasma membrane to avoid counting dead or dying cells. There was a range of growth inhibition to tipifarnib among the various osteosarcoma cells. OS187 cells were the most sensitive, yielding reduced counts with as little as 0.001 $\mu\text{mol/L}$ tipifarnib (data not shown). COL also showed a dose-dependent cell

count reduction with tipifarnib. The reduced cell yields for OS187 and COL by 72 hours were significant at 0.01 and 0.1 $\mu\text{mol/L}$, with P values of 0.001 and 0.003, respectively, whereas SaOS2 did not show a significant reduction in cell yield in response to tipifarnib at 1 $\mu\text{mol/L}$ concentration after 96 hours of exposure (Fig. 1A). Variable responsiveness to tipifarnib has also been reported in numerous other cancer cell types, including AML and pancreatic cancer (30–32).

Phase-contrast light microscopic examination of OS187 cells after farnesyltransferase inhibition revealed enlarged, markedly “swollen” cells compared with untreated control cells after 24 hours (Fig. 1B, inset). We wanted to know if the reduced cell yields were due to

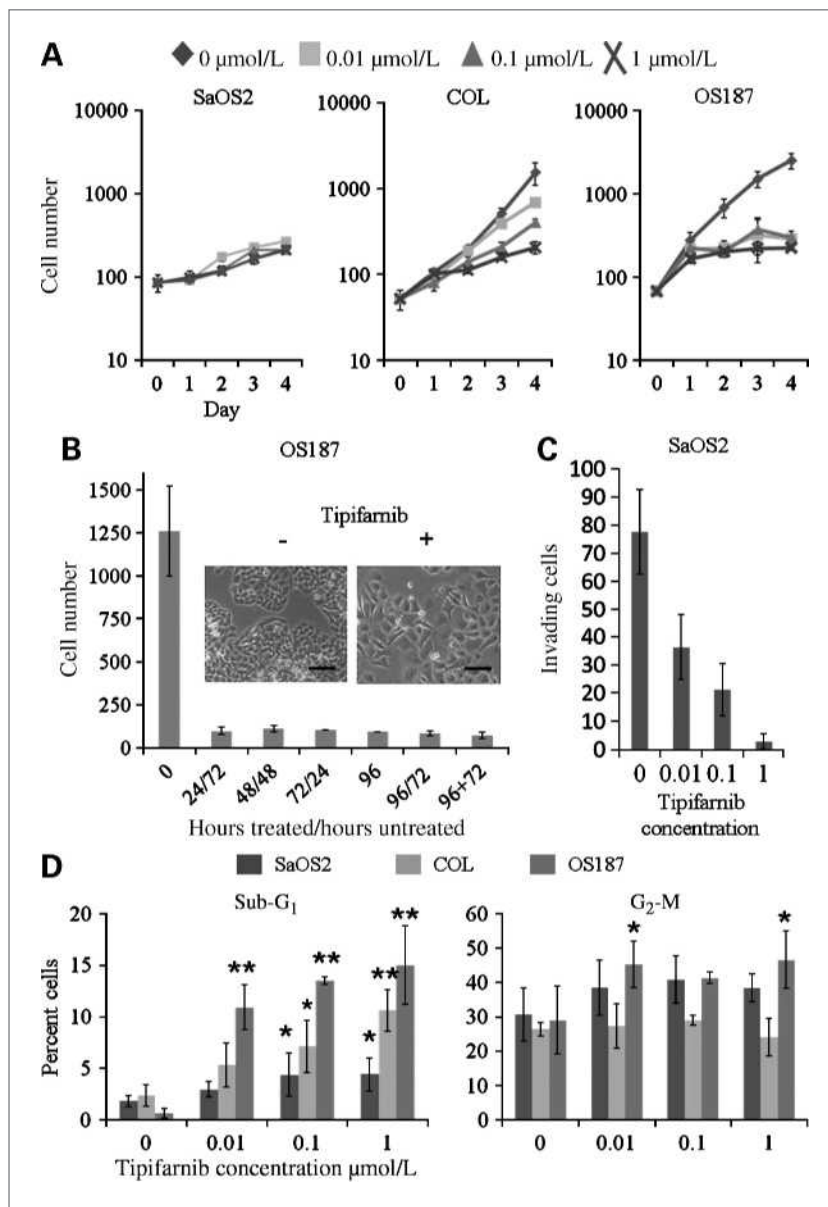


Figure 1. Differential susceptibility to tipifarnib treatment. A, cell viability (yield) analysis of sensitive, intermediate, and resistant osteosarcoma cells with increasing tipifarnib concentrations by Coulter counter. B, OS187 cells were treated with tipifarnib for 24, 48, or 72 hours. The medium was removed and replaced with fresh medium containing no drug. Cell counts at 96 hours were compared with those of untreated cells and with those of cells treated for 96 hours. Inset, phase-contrast light micrographs of OS187 cells cultured either in standard conditions or in 1 $\mu\text{mol/L}$ tipifarnib. Scale bar, 0.1 mm. C, SaOS2 cells were treated with tipifarnib for 48 hours and then assayed for invasive ability. D, cell cycle analysis conducted by flow cytometry: cells were incubated with tipifarnib at the indicated concentrations for 72 hours. *, $P = 0.05$; **, $P \leq 0.01$. Data are representative of three or more experiments.

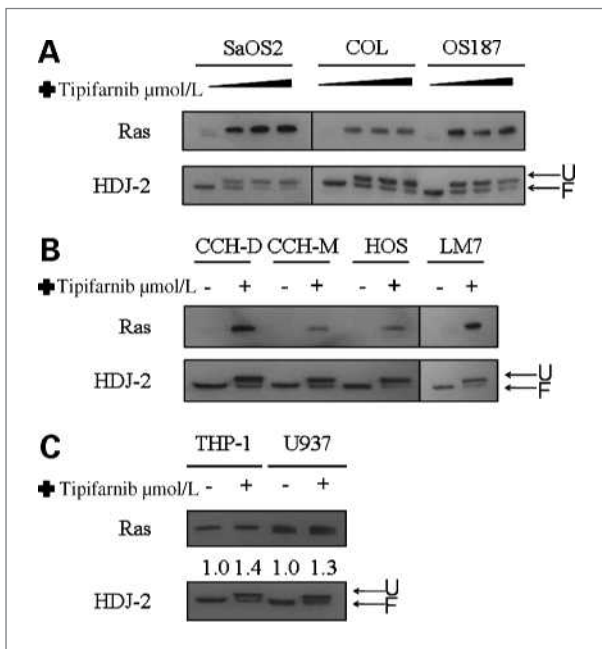


Figure 2. Ras activity increases with tipifarnib treatment. A, Ras activity assay in OS187, COL, and SaOS2. Cells were treated with 0, 0.01, 0.1, or 1 $\mu\text{mol/L}$ tipifarnib for 24 hours, lysates were collected, activated Ras was precipitated with Raf-1 Ras-binding domain, and activated Ras was measured by immunoblot. Immunoblot of HDJ-2 is a control assessing farnesyltransferase inhibition. U, unfarnesylated; F, farnesylated. B, cells from four additional osteosarcoma cell lines treated with 1 $\mu\text{mol/L}$ tipifarnib and compared with untreated cells. Lysates were assayed as in A. C, two AML cell lines were subjected to the same parameters as in B. Densitometric analysis shows relative increases of activated Ras with tipifarnib treatment. Data are representative of three or more experiments.

growth arrest of viable cells and, if so, whether the cells could recover or whether the reduced yields were due to cell loss. To assess the duration of response to farnesyltransferase inhibition, OS187 cells were exposed to tipifarnib for 24, 48, or 72 hours and then washed and grown in fresh medium. After a total of 96 hours, cells were counted as above (Fig. 1B). No difference in cell yield was seen between cells treated with tipifarnib for only 24 hours and cells treated longer, regardless of washout duration.

We wished to know whether inhibition of farnesyltransferase would affect other hallmarks of cancer, especially invasion, a necessary step in metastasis. We therefore assessed the effect of FTI on invasion with use of Matrigel invasion assays. OS187 and COL cells showed decreased invasiveness that correlated with loss of cell viability data in Fig. 1A (data not shown). Of interest, SaOS2 cells, which had shown little growth arrest with farnesyltransferase inhibition, showed decreased invasiveness even at drug concentrations so low they had no effect on tumor cell growth (Fig. 1C).

Previous reports have shown that cells with wild-type Ras undergo growth arrest in the G_2 -M phase in response to FTI (33). To determine whether increased growth arrest occurs in osteosarcoma cell lines when farnesyla-

tion is inhibited, cells were treated with tipifarnib for 72 hours. Cell cycle analysis was conducted on detached and attached cells. OS187 cell numbers in G_2 -M increased by 72 hours, but no significant effect on cells in the G_2 -M cycle was seen in COL or SaOS2 cells (Fig. 1D). However, by 72 hours, dose-responsive increases in the number of sub- G_1 cells were seen in OS187, COL, and SaOS2 cells, with the greatest increase in OS187, a moderate increase in COL, and a relatively small increase in SaOS2. There was no increase in G_0 - G_1 -phase or S-phase cells at any treatment or time point. This indicates a correlation between sub- G_1 cells and growth arrest in cells that show reduced yield after FTI.

Blocking farnesyltransferase increases Ras activity. Several studies have examined the effects of FTIs on downstream effector molecules of Ras, with varied results: some investigators found decreased activation, whereas others detected no change in activation of effector proteins in response to inhibition of farnesyltransferase (23, 32, 34) and therefore have been quick to disregard Ras as a protein that confers sensitivity to FTI.

To determine the effect of FTI on Ras activity in osteosarcoma cells, we used an assay that precipitates activated Ras through binding to the Ras-binding domain of Raf-1 conjugated to agarose beads. All osteosarcoma cells examined had low baseline levels of Ras activity when grown in standard conditions. We were surprised to find, however, that for all osteosarcoma cells, FTI caused upregulation of Ras activity (Fig. 2A and B). Two AML cell lines, THP-1 and U937, which have shown growth inhibition when treated with tipifarnib, were tested for activation of Ras in response to FTI (30, 31). THP-1 has an activating mutation at the 12 codon of N-Ras and contains no known mutations in K-Ras or H-Ras. U937 has no known mutations in any Ras proteins. Although U937 has no known mutations in Ras, U937 and THP-1 both have high endogenous expression of activated Ras,

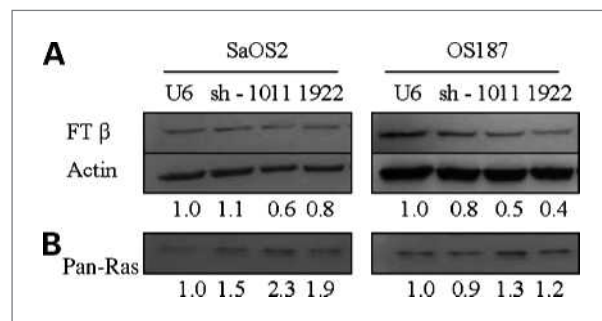


Figure 3. Ras activity is increased with inhibition of FT β in OS187 and SaOS2 cells. Retrovirally encoded shRNA specific for FT β was transduced into OS187 and SaOS2. A, Western blot shows knockdown of FT β . B, Ras activity assay shows increased Ras activity with knockdown of FT β . Densitometric analysis shows relative decrease in FT β expression (A) and relative increase in Ras activity (B). U6, empty vector; sh, scrambled control hairpin; 1011 and 1922, two distinct shRNAs specific for FT β . Analysis was done as in Fig. 2.

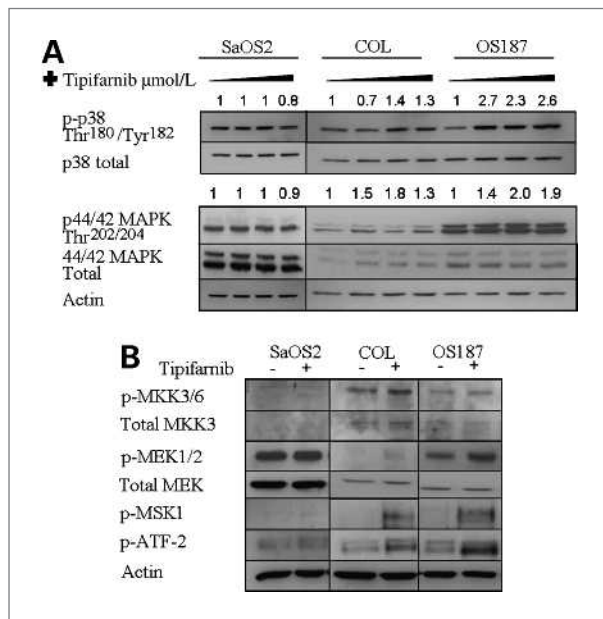


Figure 4. Downstream effectors of Ras are increased in sensitive osteosarcoma cell lines. **A**, phosphorylated and/or total levels of Ras downstream effectors in response to tipifarnib treatment. Cells were treated with 0, 0.01, 0.1, or 1 $\mu\text{mol/L}$ tipifarnib for 24 hours. Numbers above each blot show the proportionate change in densitometry relative to actin compared with the untreated cell sample. **B**, signaling molecules upstream and downstream of p38 and ERK are increased with farnesyltransferase inhibition. Cells were treated with 1 $\mu\text{mol/L}$ tipifarnib for 24 hours, and lysates were assayed by Western blotting. Analysis was done as in Fig. 2.

which is further increased when treated with 1 $\mu\text{mol/L}$ tipifarnib for 24 hours (Fig. 2C).

Farnesyltransferase and geranylgeranyltransferase share a common α subunit but have separate β subunits. To verify that blocking farnesylation increases Ras, we used shRNA targeted to FT β . We created two shRNAs: one targeted to the open reading frame and one to the 5'-untranslated region of FT β . Both shRNAs were transduced into OS187 and SaOS2. Knockdown was quantified by Western blotting (Fig. 3A). Ras activation was measured as above. Increased Ras activity was observed in the FT β shRNA-transduced cells. This is the first report, to our knowledge, that shows increased activity of Ras in response to blocking farnesylation.

Tipifarnib treatment results in increased activation of ERK and p38 MAPK in sensitive cell lines. Inhibition of farnesyltransferase led to increased Ras activity, measured by Raf binding assays, but it was unclear whether this effect reflected an actual increase in Ras signaling. We assessed both ERK and p38. Activation of both pathways remained unchanged or decreased in SaOS2 cells (Fig. 4A). The endogenous level of activated ERK was low in COL, but it was increasingly activated in response to FTI. ERK and p38 were increasingly activated in OS187 and COL when farnesylation was blocked. Because p38 and ERK activation seemed to be associated

with growth arrest after FTI, we examined the activation levels of proteins upstream and downstream of p38 and ERK in response to tipifarnib treatment. MKK3/6 and MEK1/2 are kinases upstream of p38 and ERK, respectively. Phosphorylated levels of these kinases were increased in OS187 and COL when treated with tipifarnib (Fig. 4B; Table 1). We also measured the response of ATF-2, a downstream effector protein of p38, and MSK1, a downstream effector protein of ERK and p38. Both were increasingly phosphorylated in response to FTI in OS187 and COL. Phosphorylated levels of MKK3/6, MSK1, and ATF-2 were low or undetectable in SaOS2, whereas phosphorylated MEK1/2 levels remained unchanged. We concluded that osteosarcoma cells that undergo growth arrest and/or cell death in response to FTI have increased ERK and p38 activation as a part of that response.

N-Ras and K-Ras B are alternately prenylated with tipifarnib treatment. On the basis of the above work, we hypothesized that N-Ras and K-Ras B were geranylgeranylated when farnesyltransferase was inhibited. We therefore treated cells with a FTI (tipifarnib) alone, with a GGTI (GGTI-298) alone, or with a combination of both. Anti-Rap-1 staining verified that geranylgeranylation was blocked (35), and anti-HDJ-2 showed that farnesylation was blocked (36). A shift to a higher mobility band represents unprenylated forms of proteins. After validation that the targets were blocked, the prenylation status of the Ras isoforms was measured. For both pan-Ras and N-Ras, two bands were seen when farnesyltransferase only was blocked, but almost all of the protein was in the unprenylated form when both farnesylation and geranylgeranylation were blocked. The persistence of prenylated Ras when farnesylation was blocked (as shown by the HDJ-2 mobility) suggests that some N-Ras could be geranylgeranylated when farnesylation was prevented (Fig. 5A). Although endogenous levels of K-Ras are difficult to detect in osteosarcoma cells using available antibodies, it is clear that there was no shift in mobility of K-Ras when only farnesyltransferase or geranylgeranyltransferase was inhibited (Fig. 5A). Blocking both enzymes did alter mobility, suggesting that either form

Table 1. Densitometry from Fig. 4B Western blot

	SaOS2	COL	OS187
Phospho-MKK3/6	0.52	1.46	1.15
Total MKK3/6	0.92	1.49	0.91
Phospho-MEK1/2	1.05	2.77	1.40
Total MEK	1.05	1.60	0.85
Phospho-MSK1	0.78	37.12	11.09
Phospho-ATF-2	1.76	2.30	2.76

NOTE: Analysis was done as stated in Fig. 4.

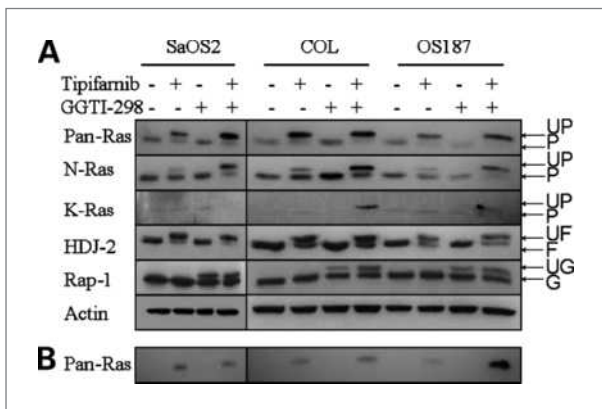


Figure 5. K-Ras and N-Ras are alternately prenylated with tipifarnib treatment. Cells were treated with 1 $\mu\text{mol/L}$ tipifarnib or 4 $\mu\text{mol/L}$ GGTI-298, alone or in combination, for 24 hours. A, cell lysates were assayed by Western blotting. P, prenylated; UP, unprenylated; G, geranylgeranylated; UG, ungeranylgeranylated. B, Ras activity assay was measured on the same samples; assay was done as in Fig. 2. Analysis was done as in Fig. 2.

of prenylation was readily available for K-Ras. These results indicated that N-Ras and K-Ras are both alternately prenylated when farnesylation is blocked.

To assess the effect on Ras activity of the inhibition of geranylgeranylation, parallel lysates were tested for Ras activation by Raf binding, as described above. Increased Ras activity was found only in cells in which farnesyltransferase was inhibited (Fig. 5B). There was no difference in Ras activity observed between untreated cells and cells in which geranylgeranyltransferase was inhibited, showing that the increased Ras activation was a result of FTI specifically and not a general effect of prenylation inhibition.

Discussion

We showed that farnesyltransferase inhibition by either tipifarnib or shRNA to FTI increases Ras activity. Although the nature of the relationship between increased Ras activation and cancer cell response remains to be determined, one intriguing possibility is that when farnesyltransferase is inhibited, the resulting increase in Ras activity contributes to growth arrest and/or cell death.

An increase of Ras activity was confirmed by the upregulation of key downstream targets of Ras signaling in response to tipifarnib treatment. Specifically, we showed the effect of FTI on endogenous levels of Ras and MAPK proteins by using pharmacologic means to influence the prenylation status of Ras. We showed that decreasing farnesylated Ras increases the activation of ERK and p38 MAPK. The upregulation of MAPK family members found in growth-arrested OS187 and COL cells, compared with the downregulation and/or unchanged levels in cells without growth arrest (SaOS2), suggests that the functional increase in Ras signaling may be linked to the response seen in cells that undergo growth arrest.

Decreased proliferation, apoptosis, and senescence have all been reported responses to FTI (30, 34, 37, 38). Although Ras activity normally is associated with growth factor responses, proliferation, survival, and an increase in many of the hallmark behaviors of cancer, there are circumstances in which Ras signaling can be associated with reduced survival (23, 24, 39–42). The outcome of Ras signaling depends on the cellular context, the presence or absence of other regulatory molecules, and the persistence of the signal (16, 24). Fibroblasts with wild-type p53 become susceptible to apoptosis when Ras is transiently expressed (16). Likewise, oncogenic activation of Ras in fibroblasts or primary human cells converts p53 into an inducer of senescence (41, 42). SaOS2 is p53 null, which may render this cell line “resistant” to Ras-mediated cell death signals induced by FTI, due to lack of a key regulator of senescence and cell death. It may be that resistance to tipifarnib in many cancers may be due to a lack of key downstream proteins, such as p53 in SaOS2, which would account for senescence or cell death.

Many other proteins are farnesylated. Inhibition of farnesylation of these other proteins (such as RhoB, CENP-E, and CENP-F) may contribute to the decreased proliferation and growth arrest seen in OS187 and COL. However, increased Ras activity, in combination with increased phosphorylation of ERK and p38 MAPK, is a key finding in responsive cells and likely contributes to the decreased proliferation and growth arrest. An increase in Ras activity in response to inhibition of farnesylation suggests that growth arrest and/or cell death may rely on a shift from farnesylated to geranylgeranylated Ras. The relationship between G₂-M arrest and Ras signaling remains to be confirmed with experiments that are outside the scope of this report. The relationship between decreased invasiveness and increased Ras activity when farnesylation is inhibited is also unclear and may be due to the functional inhibition of H-Ras. H-Ras has been reported to increase invasiveness of various carcinomas (43, 44). Raponi et al. (45) found that expression of *RASGRP1*, a guanine nucleotide exchange factor that activates Ras, predicted sensitivity to tipifarnib treatment in AML. They report that *RASGRP1* normally activates N-Ras and H-Ras, but blockage of H-Ras by FTI may be responsible for some antitumor effects. The loss of H-Ras function due to FTI could account for the decreased invasiveness found in all three cell lines tested, especially SaOS2, which was otherwise not responsive to FTI after 96 hours of treatment with tipifarnib. Antisense to *AKT2* caused a loss of invasiveness in pancreatic carcinoma cells (46), and phosphorylated AKT was decreased in SaOS2 and OS187 in response to tipifarnib treatment.¹ Negligible expression of H-Ras mRNA or protein was seen in any osteosarcoma cell line tested.¹ However, loss of H-Ras or loss of farnesylated N-Ras or K-Ras B might result in decreased

¹ Unpublished data.

phosphorylated AKT and therefore decreased invasiveness, although we recognize that other prenylated proteins also may contribute to this effect.

Our findings were consistent with previous studies that have reported that both FTI and GGTI are required for inhibition of K-Ras processing (47). Tipifarnib treatment alone partially inhibited N-Ras prenylation, but the combination treatment inhibited prenylation of N-Ras to a greater extent. It is well accepted that Ras must be localized to a membrane to cause activation of downstream signaling proteins. Therefore, increased Ras activation resulting from inhibiting farnesylation suggests that Ras must be alternately prenylated because unprenylated Ras would not remain membrane associated and would not cause activation of ERK, p38, etc. These findings, along with the continued presence of prenylated Ras (as assessed by SDS-PAGE mobility) after either FTI or GGTI treatments alone, strongly suggest that N-Ras and K-Ras are alternately geranylgeranylated when farnesylation is inhibited.

The lack of mutations in Ras combined with the expression of proteins important in cell death or senescence suggests that some osteosarcoma patients might achieve clinical benefit from FTI. Because growth arrest was the predominant effect seen *in vitro*, we would not expect a clear loss of tumor volume by Response Evaluation Criteria in Solid Tumors criteria from such treatment but might see stable disease. Indeed, tumor shrinkage is uncommon with osteosarcoma therapy because tumors

often calcify with treatment. *In vivo* studies are necessary to determine whether tipifarnib treatment induces stable disease in orthotopic xenograft models, which would lend support to pursuing clinical trials for this disease.

In conclusion, Ras activity is increased when farnesylation is inhibited. The combination of Ras activation and increased activation of MAPK family members in cells with decreased cell viability and growth arrest suggests that Ras is functionally important for response to tipifarnib treatment. Understanding how signaling pathways are altered in response to geranylgeranylated N-Ras and K-Ras B is of increasing importance and may lead to novel findings of how Ras signaling is able to switch from proliferative to antineoplastic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Jori Zemel Children's Bone Cancer Foundation, Rosalie B. Hite Fellowship, The University of Texas Graduate School of Biomedical Sciences, and National Cancer Institute grant 5 K08 CA118730.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/09/2009; revised 02/16/2010; accepted 03/01/2010; published OnlineFirst 04/20/2010.

References

- Brunner TB, Hahn SM, Gupta AK, Muschel RJ, McKenna WG, Bernhard EJ. Farnesyltransferase inhibitors: an overview of the results of preclinical and clinical investigations. *Cancer Res* 2003;63:5656-68.
- Casey PJ. Biochemistry of protein prenylation. *J Lipid Res* 1992;33:1731-40.
- Winter-Vann AM, Casey PJ. Post-prenylation-processing enzymes as new targets in oncogenesis. *Nat Rev Cancer* 2005;5:405-12.
- Adjei AA. Blocking oncogenic Ras signaling for cancer therapy. *J Natl Cancer Inst* 2001;93:1062-74.
- Zujewski J, Horak ID, Bol CJ, et al. Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. *J Clin Oncol* 2000;18:927.
- Maurer-Stroh S, Eisenhaber F. Refinement and prediction of protein prenylation motifs. *Genome Biol* 2005;6:R55.
- Lancet JE, Karp JE. Farnesyltransferase inhibitors in hematologic malignancies: new horizons in therapy. *Blood* 2003;102:3880-9.
- Pan J, Yeung S-CJ. Recent advances in understanding the anti-neoplastic mechanisms of farnesyltransferase inhibitors. *Cancer Res* 2005;65:9109-12.
- Sousa SF, Fernandes PA, Ramos MJ. Unraveling the mechanism of the farnesyltransferase enzyme. *J Biol Inorg Chem* 2005;10:3-10.
- Reid TS, Beese LS. Crystal structures of the anticancer clinical candidates R115777 (tipifarnib) and BMS-214662 complexed with protein farnesyltransferase suggest a mechanism of FTI selectivity. *Biochemistry* 2004;43:6877-84.
- Khawaja A, Dockrell ME, Hendry BM, Sharpe CC. Prenylation is not necessary for endogenous Ras activation in non-malignant cells. *J Cell Biochem* 2006;97:412-22.
- Ashery U, Yizhar O, Rotblat B, Kloog Y. Nonconventional trafficking of Ras associated with Ras signal organization. *Traffic* 2006;7:119-26.
- Sherman LS, Ratner N. Immunocytochemical assay for Ras activity. *Methods Enzymol* 2001;348-55.
- Basso AD, Kirschmeier P, Bishop WR. Thematic review series: lipid posttranslational modifications. Farnesyl transferase inhibitors. *J Lipid Res* 2006;47:15-31.
- Shaw NJ, Bishop NJ. Bisphosphonate treatment of bone disease. *Arch Dis Child* 2005;90:494-9.
- Berthiaume LG. Insider information: how palmitoylation of Ras makes it a signaling double agent. *Sci STKE* 2002;2002:pe41.
- Whyte DB, Kirschmeier P, Hockenberry TN, et al. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J Biol Chem* 1997;272:14459-64.
- Antillon-Klussman F, Garcia-Delgado M, Villa-Elizaga I, Sierrasesumaga L. Mutational activation of ras genes is absent in pediatric osteosarcoma. *Cancer Genet Cytogenet* 1995;79:49-53.
- Hughes DP, Thomas DG, Giordano TJ, McDonagh KT, Baker LH. Essential erbB family phosphorylation in osteosarcoma as a target for CI-1033 inhibition. *Pediatr Blood Cancer* 2006;46:614-23.
- Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004;68:320-44.
- Junttila MR, Li S-P, Westermarck J. Phosphatase-mediated cross-talk between MAPK signaling pathways in the regulation of cell survival. *FASEB J* 2008;22:954-65.
- Bivona TG, Quatela SE, Bodemann BO, et al. PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. *Mol Cell* 2006;21:481-93.
- Ninomiya Y, Kato K, Takahashi A, et al. K-Ras and H-Ras activation

- promote distinct consequences on endometrial cell survival. *Cancer Res* 2004;64:2759–65.
24. Yang R, Piperdi S, Gorlick R. Activation of the RAF/mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase pathway mediates apoptosis induced by chelerythrine in osteosarcoma. *Clin Cancer Res* 2008;14:6396–404.
 25. Lisle JW, Choi JY, Horton JA, Allen MJ, Damron TA. Metastatic osteosarcoma gene expression differs *in vitro* and *in vivo*. *Clin Orthop Relat Res* 2008;466:2071–80.
 26. Santala P, Larjava H, Nissinen L, Riikonen T, Maatta A, Heino J. Suppressed collagen gene expression and induction of $\alpha 2\beta 1$ integrin-type collagen receptor in tumorigenic derivatives of human osteogenic sarcoma (HOS) cell line. *J Biol Chem* 1994;269:1276–83.
 27. Zhang P, Yang Y, Zweidler-McKay PA, Hughes DPM. Critical role of notch signaling in osteosarcoma invasion and metastasis. *Clin Cancer Res* 2008;14:2962–9.
 28. Tibes R, Qiu Y, Lu Y, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. *Mol Cancer Ther* 2006;5:2512–21.
 29. <http://www.invitrogen.com/site/us/en/home.html>.
 30. Raponi M, Belly RT, Karp JE, Lancet JE, Atkins D, Wang Y. Microarray analysis reveals genetic pathways modulated by tipifarnib in acute myeloid leukemia. *BMC Cancer* 2004;4:56.
 31. Yanamandra N, Colaco NM, Parquet NA, et al. Tipifarnib and bortezomib are synergistic and overcome cell adhesion-mediated drug resistance in multiple myeloma and acute myeloid leukemia. *Clin Cancer Res* 2006;12:591–9.
 32. Venkatasubbarao K, Choudary A, Freeman JW. Farnesyl transferase inhibitor (R115777)-induced inhibition of STAT3(Tyr705) phosphorylation in human pancreatic cancer cell lines require extracellular signal-regulated kinases. *Cancer Res* 2005;65:2861–71.
 33. Ashar HR, James L, Gray K, et al. Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with the microtubules. *J Biol Chem* 2000;275:30451–7.
 34. Caraglia M, Marra M, Leonetti C, et al. R115777 (Zarnestra)/zoledronic acid (Zometa) cooperation on inhibition of prostate cancer proliferation is paralleled by Erk/Akt inactivation and reduced Bcl-2 and bad phosphorylation. *J Cell Physiol* 2007;211:533–43.
 35. Efuet ET, Keyomarsi K. Farnesyl and geranylgeranyl transferase inhibitors induce G₁ arrest by targeting the proteasome. *Cancer Res* 2006;66:1040–51.
 36. Davis AR, Alevy YG, Chellaiah A, Quinn MT, Mohanakumar T. Characterization of HDJ-2, a human 40 kD heat shock protein. *Int J Biochem Cell Biol* 1998;30:1203–21.
 37. Brassard DL, English JM, Malkowski M, Kirschmeier P, Nagabhushan TL, Bishop WR. Inhibitors of farnesyl protein transferase and MEK1,2 induce apoptosis in fibroblasts transformed with farnesylated but not geranylgeranylated H-Ras. *Exp Cell Res* 2002;273:138–46.
 38. Wang C-C, Liao Y-P, Mischel PS, Iwamoto KS, Cacalano NA, McBride WH. HDJ-2 as a target for radiosensitization of glioblastoma multiforme cells by the farnesyltransferase inhibitor R115777 and the role of the p53/p21 pathway. *Cancer Res* 2006;66:6756–62.
 39. Shao J, Sheng H, DuBois RN, Beauchamp RD. Oncogenic Ras-mediated cell growth arrest and apoptosis are associated with increased ubiquitin-dependent cyclin D1 degradation. *J Biol Chem* 2000;275:22916–24.
 40. Vos MD, Ellis CA, Elam C, Ulku AS, Taylor BJ, Clark GJ. RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. *J Biol Chem* 2003;278:28045–51.
 41. Castro ME, del Valle Guijarr M, Moneo V, Carnero A. Cellular senescence induced by p53-ras cooperation is independent of p21waf1 in murine embryo fibroblasts. *J Cell Biochem* 2004;92:514–24.
 42. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 1997;88:593–602.
 43. Gotzmann J, Huber H, Thallinger C, et al. Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF- $\beta 1$ and Ha-Ras: steps towards invasiveness. *J Cell Sci* 2002;115:1189–202.
 44. Zhang Z, Yao R, Li J, et al. Induction of invasive mouse skin carcinomas in transgenic mice with mutations in both H-ras and p53. *Mol Cancer Res* 2005;3:563–74.
 45. Raponi M, Lancet JE, Fan H, et al. A 2-gene classifier for predicting response to the farnesyltransferase inhibitor tipifarnib in acute myeloid leukemia. *Blood* 2008;111:2589–96.
 46. Cheng JQ, Ruggeri B, Klein WM, et al. Amplification of AKT2 in human pancreatic cancer cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci U S A* 1996;93:3636–41.
 47. Sun J, Qian Y, Hamilton AD, Sebti SM. Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. *Oncogene* 1998;16:1467–73.

Correction: Driven to Death: Inhibition of Farnesylation Increases Ras Activity in Osteosarcoma and Promotes Growth Arrest and Cell Death

Following publication of this article (Mol Cancer Ther 2010;9:1111–9), which was featured in the May 2010 issue of *Molecular Cancer Therapeutics* (1), new data, including DNA fingerprint analysis, *in vivo* experiments, and protein biochemistry studies, were obtained showing that two cell lines used for this study, OS 187 and COL, are not osteosarcoma cell lines. The authors state that they are the first group to report and characterize these cell lines (2), though OS 187 was not generated by the group. The authors are correcting their publications that have used OS 187 and COL. DNA fingerprint analysis for OS 187 is identical to HCT 15, a common colon cancer cell line included in the NCI60 panel. COL has a unique DNA fingerprint, but several *in vitro* and *in vivo* analyses recently described (3) demonstrate that it is, in fact, a neuroblastoma.

Although the report was written with the assumption that all lines used were osteosarcoma cell lines, it is now clear that these results cannot be interpreted to represent osteosarcoma biology specifically, and that any references specific to osteosarcoma in the body of the article should be disregarded. The title and abstract now are revised to reflect this change. The biology of Ras signaling and response to farnesyltransferase inhibitors, especially in cell lines with low endogenous Ras activity, is likely to be general and not specific to one particular disease type. Indeed, the observation of similar effects in cell lines of diverse origins speaks to the generalizability of these results. Thus, although the identity of the cell lines requires correction, the authors stand by the observations reported.

The corrected title is "Driven to Death: Inhibition of Farnesylation Increases Ras Activity and Promotes Growth Arrest and Cell Death."

The corrected abstract is as follows:

To improve cancer outcomes, investigators are turning increasingly to small molecule medicines that disrupt vital signaling cascades, inhibit malignant growth, or induce apoptosis. One vital signaling molecule is Ras, and a key step in Ras activation is membrane anchoring of Ras through prenylation, the C-terminal addition of a lipid anchor. Small molecule inhibitors of farnesyltransferase (FTI), the enzyme most often responsible for prenylating Ras, showed clinical promise, but development of FTIs such as tipifarnib has been stalled by uncertainty about their mechanism of action, because Ras seemed unimpeded in tipifarnib-treated samples. Interpretation was further complicated by the numerous proteins that may be farnesylated, as well as availability of an alternate prenylation pathway, geranylgeranylation. Our initial observations of varied response by cancer cell lines to tipifarnib led us to evaluate the role of FTI in Ras signal alteration using various tumor models. We describe our novel counterintuitive finding that endogenous Ras activity increases in cancer cell lines with low endogenous Ras activity when farnesyltransferase is inhibited by either tipifarnib or short hairpin RNA. In response to tipifarnib, variable growth arrest and/or cell death correlated with levels of activated extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Sensitivity to tipifarnib treatment was shown by growth inhibition and by an increase in subdiploid cell numbers; cells with such sensitivity had increased activation of ERK and p38 MAPK. Because Ras must be prenylated to be active, our findings suggest that geranylgeranylated N-Ras or K-Ras B interacts differently with downstream effector proteins in sensitive cancer cells responding to tipifarnib, switching the balance from cell proliferation to growth inhibition.

Please note that the article posted online has been corrected as described and no longer matches the May 2010 print version.

References

1. Geryk-Hall M, Yang Y, Hughes DPM. Driven to death: inhibition of farnesylation increases Ras activity in osteosarcoma and promotes growth arrest and cell death. *Mol Cancer Ther* 2010;9:1111-9.
2. Hughes DPM, Thomas DG, Giordano TJ, Baker LH, McDonagh KT. Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. *Cancer Res* 2004;64:2047-53.
3. Hua Y, Gorshkov K, Yang Y, Wang W, Zhang N, Hughes DP. Slow down to stay alive: HER4 protects against cellular stress and confers chemoresistance in neuroblastoma. *Cancer* 2012;118:5140-54.

Published OnlineFirst August 5, 2014.

doi: 10.1158/1535-7163.MCT-14-0537

©2014 American Association for Cancer Research.

Molecular Cancer Therapeutics

Driven to Death: Inhibition of Farnesylation Increases Ras Activity and Promotes Growth Arrest and Cell Death

Mandy Geryk-Hall, Yanwen Yang and Dennis P.M. Hughes

Mol Cancer Ther 2010;9:1111-1119. Published OnlineFirst April 20, 2010.

Updated version Access the most recent version of this article at:
doi:[10.1158/1535-7163.MCT-09-0833](https://doi.org/10.1158/1535-7163.MCT-09-0833)

Cited articles This article cites 45 articles, 26 of which you can access for free at:
<http://mct.aacrjournals.org/content/9/5/1111.full#ref-list-1>

Citing articles This article has been cited by 4 HighWire-hosted articles. Access the articles at:
<http://mct.aacrjournals.org/content/9/5/1111.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://mct.aacrjournals.org/content/9/5/1111>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.