RRR-α-Tocopherol succinate down-regulates oncogenic Ras signaling

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Abstract

α-Tocopherol succinate (TS), an analogue of vitamin E, has growth-inhibitory activity in a wide spectrum of in vitro and in vivo cancer models. Here, we report that modulation of oncogenic Ras is associated with TS activity. TS inhibits the proliferation and induces apoptosis of NIH3T3 cells stably transfected with oncogenic K-Ras and H-Ras, but not NIH3T3 cells expressing empty vector. TS treatment resulted in decreased Ras protein levels in oncogenic Ras expressing NIH3T3 cells but not in parental NIH3T3 cells. Treatment with TS suppressed the levels of phospho-Akt and phospho-Erk1/2 in oncogenic Ras expressing NIH3T3 cells. Overexpression of constitutively active phosphoinositide-3-kinase, Akt, and Mek1/2 significantly attenuated TS growth inhibition of oncogenic Ras-transformed NIH3T3 mouse fibroblast cell lines. In addition, transcriptional targets of oncogenic Ras such as c-Myc, cyclin D1, and E2F1 were down-regulated by TS in oncogenic Ras-expressing cells. The above TS effects on oncogenic Ras signaling were also observed in endogenous oncogenic K-Ras expressing HCT 116 (human colon cancer) and MDA-MB-231 (human breast cancer) cells. Taken together, these data show that TS down-regulation of the Ras signaling pathways that are mediated by Mek/Erk and phosphoinositide-3-kinase/Akt plays, at least in a critical role in TS inhibition of proliferation and survival of transformed cells. This data supports further investigation of the chemopreventive and therapeutic potential of TS in tumors that are dependent on activated Ras signaling and identifies phosphor-Erk and phosphor-Akt as potential biomarkers of TS activity. [Mol Cancer Ther 2006;5(2):309–16]

Introduction

RRR-α-Tocopherol succinate (TS), a member of the vitamin E family, is semi-synthesized from an α-tocopherol precursor, prepared from vegetable oil distillate. TS has been shown to have preclinical activity in a wide spectrum of solid tumors including melanoma, breast, prostate, gastric, mesothelioma, and colorectal cancers (1–6). Preclinical data indicate that TS is one of the most effective analogues of vitamin E in terms of inhibitory activity against cancer cells in vitro and in vivo (3, 7). The cancer-inhibitory activity of TS has been shown to involve inhibition of proliferation (1, 3, 8, 9), cell cycle progression (10, 11), invasion (12), angiogenesis (7, 13), and survival of cancer cells (1, 9, 11, 14–18). TS is also known to inhibit the growth of transformed cells, but not normal cells, in culture (14, 19–22).

The antiproliferative activity of TS has been shown to involve the induction of transforming growth factor-β responsiveness (10, 23, 24), increased expression of the cyclin-dependent kinase inhibitor p21 Waf1/Cip1 (25–27), and inhibition of the activity of the cell cycle progression transcription factor E2F (28–30). The pro-apoptotic activity of TS has been associated with the induction of Ras response (31–33), activation of c-Jun (22, 34–36), and induction of the apoptotic cascade by mitochondria destabilization (1, 37, 38). Recently, we showed that TS antangiogenic activity was associated with inhibition of vascular endothelial growth factor expression (7, 13). Although several potential mechanisms have been associated with TS activity, the oncogenic targets of TS are poorly understood. The multiple effects of TS on the phenotype of cancer cells led us to postulate that TS affects one or more major oncogenic pathway(s) that modulate multiple characteristics of the cancer cell.

Ras proteins are key regulators of signaling pathways that modulate many cellular characteristics of transformation, including cell division and survival. Mutation of the Ras gene, which renders the Ras protein in a persistent activated state, leads to malignant transformation. Although the Ras oncogene alone may not be sufficient to transform cells, the premise that Ras oncogene mutations play a pivotal role in cellular transformation and tumorigenesis is based on the high frequency of Ras mutations in human cancer and the transforming capability of mutant Ras in transforming immortal cell lines (39–41). Oncogenic Ras overexpressing
NIH3T3 cells form tumors in mice (42). Thirty percent of all human cancers express oncogenically activated Ras proteins due to activating mutations in the Ras gene (43). Ras can also be activated in cancer cells by alterations of upstream or downstream signaling components. For example, epidermal growth factor receptor is overexpressed or mutationally activated in many human cancers, and hyperactivation of epidermal growth factor receptor tyrosine kinase activity, in turn causes persistent activation of Ras and Ras-mediated signaling upstream of Ras. Mutations of BRAF and AKT2 or deletion of the tumor suppressor gene PTEN increases the expression of Ras downstream effectors (44–49). Thus, many human cancers without mutations in the Ras gene have activated Ras signaling.

In this study, we show that cancer cell lines and NIH3T3 cells that express oncogenic Ras are sensitive to TS inhibition of growth, survival, and colony formation. In contrast, NIH3T3 cells that do not express Ras were resistant to TS. Furthermore, we present data which showed that expression of constitutively active AKT and MEK attenuated the antiproliferative activity of TS.

Materials and Methods

Chemicals

TS and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were made at a concentration of 100 mmol/L in ethanol and stored at −20°C.

Cell Culture and Drug Treatment

The sources of the cell lines used in this study are as follows: human breast cancer cell line (MDA-MB-231), human colon cancer cell line (HCT-116), immortalized nontumorigenic human breast cell line (MCF-10A), immortalized nontumorigenic mouse fibroblast cell line (NIH3T3), and normal hepatocytes (AM1-12), were purchased from American Type Culture Collection (Manassas, VA). MDA-MB-231 (50) and HCT-116 (51) cells overexpress mutated Ras whereas MCF-10A, NIH3T3, and AM1-12 do not express oncogenic Ras. NIH3T3 cells stably transfected with constitutively active H-Ras61L (H-Ras/NIH3T3), K-RasV12 (K-Ras/NIH3T3), and empty vector pcDNA3 (pcDNA3/NIH3T3), which were already available in our laboratory, were maintained in DMEM with 10% fetal bovine serum and containing 400 μg of geneticin per mL. MDA-MB-231 cells were cultured in DMEM with 10% fetal bovine serum whereas HCT-116 cells were cultured in McCoy’s 5a medium with 1.5 mmol/L L-glutamine and 10% fetal bovine serum. H-Ras/NIH3T3, K-Ras/NIH3T3, and pcDNA3/NIH3T3 cell lines were grown in DMEM with 10% fetal bovine serum containing 400 μg/mL of geneticin (G418, Mediatech Inc., Herndon, VA). All the cell lines were grown and treated in an incubator with 37°C and 5% CO2. Viability assay, cell cycle analysis assay, and apoptosis assay were done in treated polystyrene 96-well plates, 100 mm plates, and six-well plates, respectively (Costar, Corning NY).

The indicated number of cells were allowed to attach overnight and fresh medium was added before the treatment. A designated concentration of α-tocopherol and TS were added to the cells with medium. The cells were collected using 0.05% trypsin EDTA (Mediatech) following the completion of a specified incubation period before cell cycle and apoptosis analyses.

Viability Assay

Drug-induced changes in the number of live cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (2 × 105 cells in 100 μL/well) were grown overnight in 96-well plates. Fresh medium (100 μL) with or without drug was added and incubated at 37°C at 5% CO2 for 96 hours. Three hours before the end of the incubation period, 20 μL of PBS containing MTT (5 mg/mL) was added to each well. Following this, the plates were centrifuged at 200 × g for 5 minutes and the medium was removed. The precipitate was then resuspended in 100 μL of DMSO. The absorbance was measured on a Bio-Rad (Hercules, CA) plate reader at 540 nm. All the experiments were independently done thrice in triplicate.

Apoptosis Assay

Trypsinized cells were counted and 5 × 105 cells per well were grown overnight in a six-well plate and the indicated concentration of α-tocopherol, TS, or ethanol was added to the cells in fresh medium. After 24 hours, the cells were collected and resuspended in a 100 μL staining solution containing Annexin V fluorescein and propidium iodide in Annexin V binding buffer (Annexin V-FITC; BD PharMingen, San Diego, CA). Following incubation at room temperature for 15 minutes, the cells were analyzed by flow cytometry following the manufacturer’s instructions. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide). Results obtained from three independent experiments were analyzed.

Anchorage-Independent Assay

Polystyrene six-well plates were precoated with a 3 mm layer of 0.7% soft agar in DMEM containing 10% fetal bovine serum. Then cells were trypsinized, and 2.5 × 105 cells were suspended in triplicate in 0.35% soft agar with or without the indicated concentration of α-tocopherol, TS, or ethanol. The cells were fed every 5 days for 2 to 5 weeks and images of anchorage-independent colonies were taken in bright-field using an inverted microscope. Images from three independent experiments in triplicate were analyzed and representative images are presented.

Western Blot Analysis

Immunoblot analysis of H-Ras, pan-Ras (Santa Cruz Biotechnology, Santa Cruz, CA), vascular endothelial growth factor (Abcam, Cambridge, MA), phospho-ERK1/2, ERK1/2, phospho-cRaf (Cell Signaling, Beverly, MA), c-Myc, E2F1 (Zymed, San Francisco, CA), Retinoblastoma
(Delta Biolabs, Gilroy, CA) cyclin D1 (Cell Signaling), and β-actin (Sigma Chemical) were done using specific antisera or monoclonal antibodies as below. Briefly, 2 × 10⁶ cells were grown overnight in 100 mm plates and the indicated concentration of drugs were added in fresh medium. After 24 hours of treatment, whole cell lysates were prepared in a lysis buffer containing 20 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1.5 μg each of aprotinin and leupeptin per milliliter, 10 mmol/L NaF, 10 mmol/L Na3P04, 3 mmol/L sodium vanadate, and 15 mmol/L glycerol phosphate. Nuclear fractions from the treated and untreated cells were prepared as previously described. Briefly, nuclear extracts were prepared from cultured cells in hypertonic buffer (20 mmol/L HEPES, pH 7.9; 420 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 20% glycerol, 20 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L Na2P04, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L aprotinin, 1 mmol/L leupeptin, and 1 mmol/L antipain). Lysates were incubated for 30 minutes on ice and centrifuged at 15,000 rpm for 15 minutes at 4°C prior to Western blotting. Fifty micrograms of lysates were loaded for each sample onto SDS-PAGE gel. Horizontal scanning densitometry was done on Western blots by using acquisition into Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and analysis by the Image Quant Program. β-Actin was used as an internal loading control.

**Transient Transfection of Constitutively Active Phosphoinositide-3-Kinase, Mek1 and Mek2, and AKT1 and AKT2**

The plasmids containing constitutively active phosphoinositide-3-kinase (PI3K), Mek1 and Mek2, and AKT were kindly provided by Dr. Julie Y. Djeu (H. Lee Moffitt Cancer and Research Institute). Mek1 and Mek2 plasmids were a kind gift from Dr. Said Sebti, Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute (Tampa, FL). DNA sequencing facilities at the H. Lee Moffitt Cancer Center & Research Institute confirmed the orientations and sequences of the genes. DNA transfection was done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were trypsinized after 48 hours of transfection and MTT viability assay was done with or without the indicated drug treatments. All the transient transfection experiments were done thrice in triplicate. Expression of the constitutively active plasmids was confirmed by Western blotting of expressed proteins.

**Reporter Assays**

**Constructs.** To produce a pGL3CCD1 construct, the coding sequence of cyclin D1 promoter (a kind gift from Dr. Pledger, H. Lee Moffitt Cancer Center & Research Institute) was subcloned into the KpnI and SalI sites of pGL3-basic luciferase vector (Promega, Madison, WI).

**Dual Luciferase Assay.** DNA transfection was done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) reagents following the manufacturer’s instructions. Briefly, 50% to 70% confluent cells in six-well plates (two wells per condition) were transfected with 2.5 μg of promoter reporter plasmid DNA (pGL3CCD1) and 0.25 μg of internal control pRL-CMV (Promega). Starting 6 hours after transfection, cells were left untreated or treated with either vehicle or TS for a period of 48 to 72 hours. Cells were then washed twice with PBS and lysed using passive lysis buffer (Promega). Aliquots of the lysates were then used to measure the Firefly luciferase and Renilla luciferase activities. Luciferase activity was normalized by the internal control readout. Assays were done in triplicate, with duplicate sets in each experiment.

**Immunofluorescence Microscopy**

Cells (1 × 10⁵) were grown overnight on a treated microscope cover glass (Fisher Scientific, Pittsburgh, PA) and treated with the indicated compounds(s) in fresh medium. After 24 hours of treatment, cells were washed twice in PBS, fixed in 4% paraformaldehyde for 10 minutes, and permeabilized in 0.5% Triton X-100/PBS for 10 minutes. After blocking with 3% bovine serum albumin/PBS, cells were incubated with primary antibodies at room temperature in a humid chamber for 1 hour, followed by three washes in PBST and incubation with either Alexa Fluor 488 chicken anti-rabbit IgG (H+L) or Alexa Fluor 594 donkey anti-mouse IgG (H+L) 2 μg/mL (from Molecular Probes, Carlsbad, CA) for 30 minutes at room temperature. Cells were washed thrice in PBST before nuclear staining with 0.1 μg/mL of 4',6-diamino-2-phenylindole and analysis by fluorescence microscopy (QIMaging Camera and IPLab 3.6 software using ×100 magnification objective). Negative controls for immunohistochemistry experiments with TS included use of isotype- and subclass-matched monoclonal antibodies when employing monoclonal antibodies, and preimmune serum when employing polyclonal antibodies.

**Transfactor Profiling Assay for c-Myc**

Using c-Myc Transfactor kit (BD Biosciences, Franklin Lakes, NJ), we measured changes in c-Myc transcription factor DNA–binding in response to TS treatment following the manufacturer’s instructions. Briefly, cell lysates were incubated with DNA-binding sequences precoated in 96-well plates and the bound protein was quantitated using horseradish peroxidase–labeled polyclonal c-Myc antibody and horseradish peroxidase substrate. Color intensity was measured on a plate reader.

**Statistical Analyses**

Results were expressed as averages ± SE. Analyses of modifications in treated cells used Student’s t test. P < 0.05 was considered to be significant (Sigma Plot). Comparison of viability percentages in transfected cells was done using GraphPad Prism version 4 software.

**Results**

**Oncogenic K-Ras and H-Ras – Transformed NIH3T3 Cells Are More Sensitive than Parental NIH3T3 Cells to TS’s Ability to Inhibit Cell Proliferation and Induce Apoptosis**

To determine the role of oncogenic Ras in the sensitivity of neoplastic cells to TS, we examined the effects of TS on
mouse fibroblasts, stably transfected with either oncogenic K-Ras (K-Ras/NIH3T3) or oncogenic (H-Ras/NIH3T3). The effect of TS treatment on these cells was compared with control parental mouse fibroblast cells (NIH3T3) and vector-only transfected NIH3T3 cells (PcDNA3/NIH3T3) that do not express activated oncogenic Ras. We also examined the effect of TS treatment on human breast cancer cell line (MDA-MB-231) and human colon cancer cell line (HCT116) expressing oncogenic K-Ras. The effects of TS treatment were also evaluated in immortalized but nontransformed human breast epithelial cell line (MCF10A) and mouse hepatocyte cell line (AML-12). TS significantly inhibited the growth of oncogenic Ras-expressing cells (K-Ras/3T3, H-Ras/3T3, MDA-MB-231, and HCT-116; Fig. 1A) with an IC₅₀ of ~20 μmol/L. In contrast, up to 100 μmol/L of TS had no effect on the nontransformed cells in culture (NIH3T3, MCF-10A, and AML-12). Furthermore, α-tocopherol, the chemical species that is usually implied by the generic term—vitamin E—without the succinate moiety, had no significant inhibitory effect on oncogenic Ras-expressing cells or nontransformed cells.

The ability of TS to induce apoptosis in the oncogenic Ras-expressing human cancer cells and NIH3T3 oncogenic Ras-transformed murine fibroblasts was investigated by Annexin V staining. For these experiments, cells were incubated with medium, vehicle, or increasing concentrations of TS (0–100 μmol/L) for 24 hours. The α-tocopherol levels at the maximum TS concentration of 100 μmol/L were also evaluated. As shown in Fig. 1B, TS induced apoptosis in the four cell lines with activated oncogenic Ras signaling (H-Ras/NIH3T3, K-Ras/NIH3T3, MDA-MB-231, and HCT116). The three nontransformed cells (AML-12, MCF-10A, and NIH3T3) were not affected by TS with concentrations as high as 100 μmol/L. In contrast, α-tocopherol had no apoptotic effect on either the normal cells or the malignant cells.

We next investigated the effect of TS on the anchorage-independent growth of transformed cells. The clonogenicity of the cells was measured in semisolid agarose plates. TS treatment resulted in a significant reduction in the size and number of colonies at concentrations of 5 to 40 μmol/L (Fig. 1C). α-Tocopherol without the succinate moiety did not significantly alter the size or number of colonies compared with vehicle control (Fig. 1C). TS Decreases Cellular Ras Protein Levels and Its Immediate Downstream Targets

To investigate whether the antineoplastic activity of TS involves the modulation of Ras, we did Western analysis of TS-treated and TS-untreated HCT116, MDA-MB-231, H-Ras/NIH3T3, and NIH3T3 cells. As shown in Fig. 2, TS induced significantly decreased levels of oncogenic Ras protein. In contrast, TS treatment increased Ras protein levels in NIH3T3 parental cells.

One of the important steps in the activation of Ras-dependent signal transduction pathway is the phosphorylation of AKT and Erk1/2. To determine whether TS suppression of oncogenic Ras expression affects the activation of Ras downstream effectors AKT and Erk1/2 in HCT-116 (Fig. 3A), MDA-MB-231 (Fig. 3B), and H-Ras/NIH3T3 (Fig. 3C), we did Western analysis. Cells were treated for 24 hours with vehicle or TS and resultant cell lysates were immunoblotted, using antibodies against phospho-Erk1/2 (pErk1/2), Erk1/2 and phospho-AKT (pAKT), and AKT (Fig. 3). TS treatment caused a significant inhibition of pErk1/2 and pAKT at 40 μmol/L. Specific inhibitors of Mek (PD98059) and PI3K (wortmannin) were used as positive controls.

Overexpression of Constitutively Active PI3K, AKT1/2, and MEK1/2 Attenuates the Effects of TS

To determine if the TS-mediated decrease in p-Erk1/2 and p-Akt levels play a significant role in the ability of TS to
inhibit anchorage-dependent proliferation, we ectopically expressed constitutively active Mek1/2, PI3K, and AKT in NIH3T3 cells stably expressing oncogenic H-Ras and measured the effect of TS treatment. The expression of the constitutively active plasmids was confirmed by Western analysis (Fig. 4A) and the effect of TS on viability/ proliferation was determined by MTT assay (Fig. 4B). At 10 μmol/L TS, empty vector–transfected cells were inhibited by 50%, whereas constitutively active MEK–and constitutively active AKT–transfected cells were not affected. Constitutively active PI3K also significantly attenuated the inhibitory effect of TS at 18 μmol/L. Constitutively active MEK1/2 significantly attenuated the antiproliferative activity of TS in H-Ras/NIH3T3 as shown by an increase in the IC50 of TS from 18 μmol/L in the mock/empty vector–transfected cells to an IC50 of 35 μmol/L of TS in MEK1/2–transfected cells (P = 0.001). These results suggest that both the Ras-Mek-Erk and Ras-PI3K-AKT limbs of the Ras signaling pathways are required for TS antiproliferative activity in oncogenic Ras-transformed cells.

TS Inhibits Ras Transcriptional Targets E2F, Cyclin D1, and c-Myc

Because E2F, cyclin D1, and c-Myc are known oncogenic Ras transcriptional targets that mediate cell proliferation, we determined the TS-induced changes in these protein levels. As shown in Fig. 5A, the protein expression of known transcriptional targets of Ras-E2F1, cyclin D1, and c-Myc were effectively repressed by TS in the nuclear fractions of MDA-MB-231 and H-Ras/NIH3T3 cells. To further confirm the effect of TS on E2F1, we did fluorescent immunohistochemistry of TS-treated MDA-MB-231. We observed a significant decrease of E2F1 expression in the nucleus and retinoblastoma endocytosis in cytoplasm with TS treatment (Fig. 5B). Because activated Ras is known to regulate cyclin D1 expression at the level of transcription, we determined whether TS down-regulates cyclin D1 transcriptional activity using cyclin D1 promoter in a luciferase reporter construct (Fig. 5C). TS caused significant repression of cyclin D1 transcriptional activity. Further evidence of TS inhibition of Ras transcriptional targets was obtained by measuring the effect of TS on c-Myc transcriptional activity in MDA-MB-231 cells (Fig. 5D). TS caused a significant inhibition of c-Myc transcriptional activity as measured by c-Myc transfactor profiling assay.
Discussion

We have shown that the active anticancer analogue of vitamin E, TS, can block oncogenic Ras signaling in NIH3T3 cells stably expressing oncogenic Ras, and that oncogenic Ras signaling is, at least in part, responsible for the antiproliferative effects of TS.

The observation of TS modulation of oncogenic Ras signal transduction pathway brings together many previous reports of the potential mechanisms of TS anticancer effects. TS modulation of oncogenic Ras signaling could explain TS induction of transforming growth factor-β responsiveness (10, 23, 24), increased expression of the cyclin-dependent kinase inhibitor p21 Waf1/Cip1 (25–27), and inhibition of the activity of the cell cycle progression transcription factor E2F (28–30) because activated Ras signaling is known to modulate these factors. TS modulation of oncogenic Ras could also play a role in its proapoptotic activity associated with mitochondrial destabilization via AKT inactivation (1, 37, 38). Recently, we showed that TS antiangiogenic activity was associated with the inhibition of vascular endothelial growth factor expression (7, 13). Oncogenic Ras is known to modulate vascular endothelial growth factor via RAS-mitogen-activated protein kinase signaling.

Our findings are consistent with previous work, which showed a correlation of TS activity with decreased H-Ras mRNA in the B16F10 mouse melanoma cell line (52) and in the NBP2 mouse neuroblastoma cell line (53). In contrast to these correlative studies, our experiments provide direct evidence that TS inhibition of growth and survival of NIH3T3 cells was dependent on the expression of oncogenic Ras. The mechanism by which TS modulates oncogenic Ras is unknown. Recently, 6-O-carboxypropyl-α-tocotrienol, a redox-silent derivative of α-tocotrienol was reported to inhibit Ras activation by altering posttranslational processing of Ras protein due to inhibition of Ras farnesylation (54). We did not observe modulation of Ras farnesylation by TS in our system (data not shown). Tocopherols and tocotrienols are lipid-soluble antioxidants collectively known as vitamin E. Although TS is an ester derivative of tocopherol, 6-O-carboxypropyl-α-tocotrienol is an ether derivative of tocotrienol. The differences in chemical structures could explain the difference in Ras farnesylation. Because hydrophobic TS binds to the cellular membrane, it is possible that TS perturbs oncogenic signaling by modulating the homeostasis of the Ras activation complex at the plasma membrane rather than during farnesylation. Further investigation of this hypothesis with more biochemical analysis of the interaction of TS and membrane-bound activated Ras complex is warranted.

In this article, we showed that the inhibition of p-Akt and p-Erk1/2 levels is critical to TS antiproliferative activity. Indeed, constitutively active Mek, PI3K, and AKT attenuated the growth-inhibitory activity of TS in H-Ras/NIH3T3 cells. This suggests that TS functions upstream of MEK and PI3K. TS modulation of key proteins of two different but connected Ras mitogenic pathways provides an explanation for the potent antiproliferative and proapoptotic effects of TS on Ras-transformed cells. Further support that TS interferes with oncogenic Ras-mediated signal transduction came from promoter-reporter (cyclin D1) and DNA-binding (c-Myc) assays that measured cyclin D1 and c-Myc transcriptional activity. The complex crosstalk within oncogenic Ras pathways, and between Ras and other oncogenic pathways, makes it likely that the mechanism of action of TS involves other signaling events that interact with oncogenic Ras signaling.

The specificity of the inhibitory activity of TS in oncogenic Ras NIH3T3 cells underscores the potential of TS as a lead compound for the development of new anticancer drugs that target oncogenic Ras-dependent tumors. Our data does not indicate that TS functions exclusively through oncogenic Ras inhibition. TS is known to inhibit transformed cells that may not have activated Ras signaling. However, we provide strong evidence that oncogenic Ras signaling is targeted by TS. Our data supports further investigation of the chemopreventive
and therapeutic potential of TS in tumors that are dependent on activated Ras signaling. Furthermore, this work identifies phospho-Akt and phospho-Erk as potential biomarkers of TS antitumor activity.

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