γ-Tocotrienol but Not γ-Tocopherol Blocks STAT3 Cell Signaling Pathway through Induction of Protein-tyrosine Phosphatase SHP-1 and Sensitizes Tumor Cells to Chemotherapeutic Agents*

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Although γ-tocotrienol (T3), a vitamin E isolated primarily from palm and rice bran oil, has been linked with anticancer activities, the mechanism of this action is poorly understood. In this study, we investigated whether T3 can modulate the STAT3 cell signaling pathway, closely linked to inflammation and tumorigenesis. We found that γ-T3 but not γ-tocopherol, the most common saturated form of vitamin E, inhibited constitutive activation of STAT3 in a dose- and time-dependent manner, and this inhibition was not cell type-specific. γ-T3 correlated with suppression of proliferation, the accumulation of apoptotic proteins Bcl-2, Mcl-1, and c-IAP2. Both chemically polyamine-depleted cells through the transcription of the anti-apoptotic proteins Bcl-2, Mcl-1, and c-IAP2. Both chemically induced and constitutively active STAT3 protect fibroblasts from ultraviolet-induced apoptosis and antagonize the pro-apoptotic effects of activated STAT1 (16). Thus, STAT3 can contribute to oncogenesis by defending cancer cells from apoptosis (12).

Based on these published results (19, 20), we hypothesized that γ-T3 may modulate the STAT3 cell signaling pathway and sensitize tumor cells to apoptosis. We tested this hypothesis in a series of tumor cell lines. We found that γ-T3 suppressed the apoptotic effects of thalidomide and bortezomib. Overall, our results suggest that activation of STAT3 by various growth factors can suppress apoptosis and promote proliferation, angiogenesis, chemoresistance, and inflammation (15–18). Bhattacharya et al. (18) showed that activated STAT3 precluded apoptosis in polyamine-depleted cells through the transcription of the anti-apoptotic proteins Bcl-2, Mcl-1, and c-IAP2. Both chemically induced and constitutively active STAT3 protect fibroblasts from ultraviolet-induced apoptosis and antagonize the pro-apoptotic effects of activated STAT1 (16). Thus, STAT3 can contribute to oncogenesis by defending cancer cells from apoptosis (12).

This article has been retracted by the publisher. An investigation at MD Anderson determined that the image of nuclei from U266 cells treated with γ-T3 from Fig. 1G had been reused in Fig. 1D of Yadav, V. R., Prasad, S., Kannappan, R., Ravindran, J., Chaturvedi, M. M., Vahtera, L., Parkkinen, J., and Aggarwal, B. B. (2010) Cyclodextrin-complexed curcumin exhibits anti-inflammatory and antiproliferative activities superior to those of curcumin through higher cellular uptake. Biochem. Pharmacol. 80, 1021–1032; the reused image represents different experimental conditions.

Vitamin E includes a group of eight naturally occurring compounds that is further subdivided into two structurally associated subgroups called tocopherols (TP) and tocotrienols (T3), each containing α-, β-, γ-, and δ-forms (1, 2). Although both TPs and T3s demonstrate antioxidant properties (3), only T3s display potent anti-cancer activity at treatment doses that have little or no effect on normal cell proliferation (4–6). T3 displays activity against a variety of chronic diseases, including cardiovascular diseases, neurological diseases, and diabetes, as well as cancer (7–10). Although there is a lot known about TP, as indicated by over 30,000 citations, very little is known about T3. In this review, we summarize the anticancer effects of T3, as well as studies and other chronic diseases is not fully understood. The studies from our laboratory (11) established for the first time that γ-T3 is a potent inhibitor of STAT3 cell signaling pathway both in vitro and in vivo and thus may have potential in prevention and treatment of cancers.

* The abbreviations used are: TP, tocopherol; T3, tocotrienol; STAT3, signal transducer and activator of transcription 3; JAK, Janus-activated kinase; VEGF, anti-vascular endothelial growth factor; IL-6, PARP, poly(ADP-ribose) polymerase; MM, multiple myeloma; PTP, protein-tyrosine phosphatase.

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activation of the STAT3 pathway by activating a protein-tyrosine phosphatase, down-regulated STAT3-regulated proteins, inhibited cell proliferation, and sensitized cancer cells to chemotherapeutic agents.

EXPERIMENTAL PROCEDURES

Reagents—A 50 mM solution of palm oil-derived γ-T3 (from Davos, Singapore) with purity greater than 95% was prepared in dimethyl sulfoxide, stored as small aliquots at −20 °C, and then diluted further in cell culture medium as needed. We purchased Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, Tris, glycine, NaCl, SDS, and bovine serum albumin from Sigma, and we obtained Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic/antimycotic solution. We purchased the following antibodies from Cell Signaling Technology (Beverly, MA), goat anti-mouse HRP from Amersham Biosciences, and goat anti-rabbit Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma) and analyzed under an epifluorescence microscope (Labophot-2, Nikon). Pictures were captured using a Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

Cell Lines—We obtained the human multiple myeloma (MM) cell lines U266, MM.1R, and MM.1S (dexamethasone-sensitive) from Millennium (Cambridge, MA) and thalidomide from Celgene Corporation (Lexington, KY), and goat anti-rabbit HRP from Santa Cruz, CA, and we purchased goat anti-rabbit Alexa 594 from Molecular Probes (Eugene, OR); goat anti-rabbit Alexa 488 (1:100) and goat anti-rabbit Alexa 647 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma) and analyzed under an epifluorescence microscope (Labophot-2, Nikon). Pictures were captured using a Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

Western Blot Analysis—For detection of p-STAT3 (Tyr-705) and STAT3 proteins, γ-T3-treated whole-cell extracts were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 4 mM Na3VO4). Lysates were then centrifuged at 14,000 rpm for 10 min to remove insoluble material. In the in vivo case, pancreatic tumor tissues (75–100 mg/mouse) were minced and incubated on ice for 30 min in 0.5 ml of ice-cold whole-cell lysate buffer (10% Nonidet P-40, 5 mol/liter NaCl, 1 mol/liter HEPEs, 0.1 mol/liter EGTA, 0.5 mol/liter EDTA, 0.1 mol/liter PMSF, 0.2 mol/liter sodium orthovanadate, 1 mol/liter NaF, 2 μg/ml aprotinin, 2 μg/ml leupeptin). The minced tissue was homogenized with a Dounce homogenizer and centrifuged at 16,000 × g at 4 °C for 10 min. The extracted proteins were then resolved on a 7.5% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with anti-p-STAT3 antibodies (1:500) and anti-STAT3 antibodies (1:1,000) overnight at 4 °C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by enhanced chemiluminescence (Amersham Biosciences).

To detect the expression of STAT3-regulated proteins and caspase-3, U266 cells (106 per ml) were treated with 60 μM γ-T3 for 16 h. The cells were then washed and extracted with lysis buffer (10% Nonidet P-40, 500 mM NaCl, 20 mM HEPES, 10 mM EDTA, 10 mM EGTA, 50 mM benzamidine, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin) and centrifuged at 13,000 rpm for 10 min to remove insoluble material. The extracted proteins were then resolved on 10% SDS-PAGE; electrotransferred to nitrocellulose membrane; blotted with antibodies against survivin, Bcl-2, Bcl-xL, cyclin D1, VEGF, and caspase-3; and then detected by enhanced chemiluminescence.

Electrophoretic Mobility Shift Assay—STAT3-DNA binding was analyzed by electrophoretic mobility shift assay using a 32P-labeled high affinity cis-inducible element probe as described previously (22). Briefly, nuclear extracts were prepared from γ-T3-treated cells and incubated with a high affinity cis-inducible element probe (5-CTTCATTTCCCG-3′) and then incubated with rabbit polyclonal anti-human STAT3 antibodies (40 μg/ml) for 2 h at room temperature, and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% nonfat milk, and then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma) and analyzed under an epifluorescence microscope (Labophot-2, Nikon). Pictures were captured using a Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

Immunocytochemistry for STAT3 Localization—γ-T3-treated MM cells were plated on a glass slide by centrifugation using a Cytospin 4 (Thermoshendol), air-dried for 1 h at room temperature, and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human STAT3 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma) and analyzed under an epifluorescence microscope (Labophot-2, Nikon). Pictures were captured using a Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).
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Transfection with SHP-1 siRNA—Human squamous cell carcinoma (SCC4) cells were plated in 6-well plates and allowed to adhere for 24 h. On the day of transfection, 12 μl of HiPerFect transfection reagent (Qiagen) were added to 50 nm SHP-1 siRNA (sense-GCAGGAGUGCCAGGAUCAAtt, antisense-UGUACCUGGACUCUGGctt) (Ambion) in a final volume of 100 μl of culture medium. After 48 h of transfection, cells were treated with γ-T3 for 6 h, and whole-cell extracts were prepared for SHP-1, STAT3, and phospho-STAT3 analysis by Western blot.

Antiproliferative Assay—The antiproliferative effects of γ-T3 against MM cell lines were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide dye uptake method as described earlier (39).

Flow Cytometric Analysis—To determine the effect of γ-T3 on the cell cycle, U266 cells were first synchronized by serum starvation and then exposed to γ-T3 for the indicated time intervals. Thereafter, cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37 °C with 0.1% RNase A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 ng/ml propidium iodide for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a FACS Calibur (Franklin Lakes, NJ).

Immunoblot Analysis of PARP Degradation—γ-T3-induced apoptosis was examined by proteolytic cleavage of PARP. Briefly, cells (2 × 10^6 per ml) were treated with 60 μM γ-T3 for the indicated times at 37 °C. The cells were then extracted by incubation for 30 min on ice in 0.5 ml of buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 ng/ml leupeptin, 2 ng/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 ng/ml benzamidine, 1 mM DTT, and 1 mM sodium orthovanadate. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (40 μg) was resolved on a 10% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with anti-PARP antibody, and then detected by enhanced chemiluminescence.

Live/Dead Assay—Apoptosis was also determined by the Live/Dead assay (Molecular Probes), which measures intracellular esterase activity and plasma membrane integrity, as described previously (23). Briefly, 1 × 10^6 cells were incubated with γ-T3/VELcade/thalidomide alone or in combination for 24 h at 37 °C. Cells were stained with the Live/Dead reagent (5 μM ethidium homodimer, 5 μM calcein) and then incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2).

**RESULTS**

The goal of this study was to determine whether γ-T3 affects the STAT3 activation pathway and, if so, through what mechanism. Among all isoforms of T3s, we used γ-T3 because it exhibits maximum anticancer activity against most types of tumor cells (24). The structure of γ-T3 is shown in Fig. 1A. We evaluated the effect of γ-T3 on both constitutive and IL-6-induced STAT3 activation. For most studies we used MM cells, because STAT3 activation is better understood in these cells. We also investigated the effect of γ-T3 on various mediators of cellular proliferation, cell survival, and apoptosis. Changes in the treatment dose (up to 80 μM) and duration (up to 6 h) of γ-T3 used for STAT3 experiments had no effect on cell viability (data not shown).

**γ-T3 Inhibits Constitutive STAT3 Phosphorylation in a Dose- and Time-dependent Manner**—Whether γ-T3 can modulate constitutive STAT3 activation in MM cells was examined. U266 cells were incubated with different concentrations of γ-T3 for 6 h, and whole-cell extracts were prepared and examined for phosphorylated STAT3 by Western blot analysis using an antibody that recognizes STAT3 phosphorylated at tyrosine 705. As shown in Fig. 1B, γ-T3 inhibited the constitutive activation of STAT3 in U266 cells, with maximum inhibition at 80 μM. Under these conditions, γ-T3 had no effect on the expression levels of STAT3 protein (Fig. 1B, bottom panel). We also determined the incubation time required for γ-T3 to suppress STAT3 activation in U266 cells. As shown in Fig. 1C, γ-T3 inhibited activation of STAT3 in a time-dependent manner, with maximum inhibition occurring at 6 h, again with no effect on the expression of STAT3 protein (Fig. 1C, bottom panel) or on cell viability (data not shown).

**γ-T3 Inhibits Constitutive DNA Binding Activity of STAT3**—Because tyrosine phosphorylation of STAT3 results in its dimerization at the nuclear membrane, thus resulting in gene transcription, we examined whether γ-T3 suppresses DNA binding activity of STAT3. Assay of DNA binding activity as determined by gel retardation analysis indicated that the binding of γ-T3 could inhibit IL-6-induced DNA binding activity of STAT3. No γ-T3 could inhibit DNA binding activity of STAT3.

**γ-T3 Depletes Nuclear Pool of STAT3 in MM Cells**—Because nuclear translocation of STAT3 is central to the function of it (and other transcription factors), we determined whether γ-T3 suppresses nuclear translocation of STAT3. Fig. 1G, as analyzed by Western blot and immunochemistry (Fig. 1H), clearly shows that γ-T3 inhibited the translocation of STAT3 to the nucleus in U266 cells.

**STAT3 Inhibition by γ-T3 Is Not Cell Type-specific**—Next, we determined the effect of γ-T3 on the constitutive activation of STAT3 in DU-145, PC3, and MiaPaCa-2 cell lines. γ-T3 inhibited STAT3 activation in all these cell lines (Fig. 2A). These results clearly indicate that the STAT3 inhibition by γ-T3 is not cell type-specific.

**γ-T3 but Not γ-TP Inhibits STAT3 Activation**—T3s differ from TPs in that T3 is an unsaturated TP that contains three double bonds in the side chain. Whether γ-TP can also inhibit activation of STAT3 in MM cells was examined. As shown in Fig. 2B, γ-T3 inhibited activation of STAT3, but γ-TP did not even at higher doses (Fig. 2C). Thus γ-T3 differs in its biological effects from that of γ-TP.

**γ-T3 Inhibits Inducible STAT3 Phosphorylation in Human Cancer Cells**—Because IL-6 is a growth factor for MM cells and it mediates its effects through induction of STAT3 phosphorylation (25, 26), we determined whether γ-T3 could inhibit IL-6-
induced STAT3 phosphorylation in MMLS cells, which lack constitutively active STAT3. IL-6 induced phosphorylation of STAT3 as early as 5 min, and maximum activation could be seen at 30 min (Fig. 2D). IL-6-induced STAT3 phosphorylation was suppressed by γ-T3 in a time-dependent manner (Fig. 2E). Exposure of cells to γ-T3 for 4 h was sufficient to suppress IL-6-induced STAT3 phosphorylation.

γ-T3 Suppresses Constitutive Activation of c-Src, JAK1, and JAK2—Because STAT3 is also activated by soluble tyrosine kinases of the Src kinase families (27), we determined the effect of γ-T3 on constitutive activation of c-Src kinase in U266 cells. We found that γ-T3 suppressed the constitutive phosphorylation of c-Src kinase (Fig. 3A). The total levels c-Src kinase protein remained unchanged under these conditions (Fig. 3A, bottom panel). STAT3 has been reported to be activated by soluble tyrosine kinases of the JAK family (28); thus, we determined whether γ-T3 affects constitutive activation of JAK1 in U266 cells. We found that γ-T3 suppressed the constitutive phosphorylation of JAK1 (Fig. 3B). The levels of phospho-JAK1 remained unchanged under the same conditions (Fig. 3B, bottom panel).

To determine the effect of γ-T3 on JAK2 phosphorylation, untreated and γ-T3-treated whole-cell lysates were analyzed by Western blot with the anti-phospho-JAK2 antibody. As shown in Fig. 3C, JAK2 was constitutively active in U266 cells, and treatment with γ-T3 inhibited this phosphorylation in a dose-dependent manner.

γ-T3-induced Inhibition of STAT3 Activation Is Reversed by Tyrosine Phosphatase Inhibitor—Because protein-tyrosine phosphatase (PTP) has been implicated in STAT3 inhibition (29), we determined whether γ-T3-induced inhibition of STAT3 tyrosine phosphorylation could be due to activation of a PTP. Treatment of U266 cells with the broad-acting PTP inhibitor sodium pervanadate reversed the γ-T3-induced inhibition of STAT3 activation (Fig. 4A). This suggests that PTPs are involved in γ-T3-induced inhibition of STAT3 activation.

γ-T3 Induces the Expression of
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**FIGURE 2. γ-T3 down-regulates phospho-STAT3 and SHP-1 expression.** A, γ-T3 suppresses phospho-STAT3 levels in a dose-dependent manner. DU145, PC3, and MiaPaCa-2 cells (1 × 10^6/ml) were treated with indicated doses of γ-T3 for 6 h, after which whole-cell extracts were prepared, and phospho-STAT3 level was detected by Western blotting. B, γ-T3 down-regulates constitutively active Src, Jak1, and Jak2. U266 cells (2 × 10^6/ml) were treated with indicated doses of γ-T3 for 6 h, after which whole-cell extracts were prepared, and phospho-JAK1, phospho-JAK2, and phospho-Src levels were detected by Western blotting. C, γ-T3 inhibits STAT3 phosphorylation in pancreatic tumors. γ-T3-treated animals did not express activated STAT3 in tumors, whereas that from vehicle-treated animals express STAT3 phosphorylated protein (VEGF), all reported to be regulated by STAT3 (12). D, γ-T3 inhibits STAT3 phosphorylation in vivo. Whether γ-T3 inhibits STAT3 activation in vivo was examined in human pancreatic tissue from animals treated with γ-T3. The Western blot analysis of tumor tissues showed that tissues from vehicle-treated animals express STAT3 phosphorylated protein, whereas those from γ-T3-treated animals did not (Fig. 4E). These results suggest the critical role of STAT3 in γ-T3-induced cell death.

**FIGURE 3. γ-T3 down-regulates constitutively active Src, Jak1, and Jak2.** A, γ-T3 suppresses phospho-JAK1 levels in a dose-dependent manner. U266 cells (2 × 10^6/ml) were treated with indicated doses of γ-T3 for 6 h, after which whole-cell extracts were prepared, and 40 μg of those extracts were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with phospho-JAK1 antibody. The same blots were stripped and reprobed with respective nonphosphorylated protein antibodies to verify equal protein loading. B, γ-T3 suppresses phospho-JAK2 levels in a dose-dependent manner. U266 cells (2 × 10^6/ml) were treated with indicated doses of γ-T3 for 6 h, after which whole-cell extracts were prepared, and 40 μg of those extracts were resolved on 7.5% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with phospho-JAK2 antibody. The same blots were stripped and reprobed with respective nonphosphorylated protein antibodies to verify equal protein loading.

**FIGURE 4. γ-T3 inhibits STAT3 phosphorylation in pancreatic tumors in vivo.** Whether γ-T3 inhibits STAT3 activation in vivo was examined in human pancreatic tissue from animals treated with γ-T3. The Western blot analysis of tumor tissues showed that tissues from vehicle-treated animals express STAT3 phosphorylation, whereas that from γ-T3-treated animals did not (Fig. 4E). This result clearly indicates that γ-T3 inhibits STAT3 activation not only in vitro but also in vivo.

**γ-T3-induced Inhibition of STAT3 Activation Is Reversed by Gene Silencing of SHP-1—**We determined whether the suppression of SHP-1 expression by siRNA (small interfering RNA) would abrogate the induction of SHP-1 by γ-T3. Western blotting showed that γ-T3-induced SHP-1 expression and that SHP-1 siRNA but not the control scRNA abolished the expression of SHP-1 induction by γ-T3. We also found that γ-T3 failed to suppress STAT3 activation in cells treated with SHP-1 siRNA (Fig. 4C). These results further corroborate our earlier evidence for the critical role of SHP-1 in suppression of STAT3 phosphorylation by γ-T3. SHP-1 gene silencing with siRNA did not suppress constitutive activation of JAK1, JAK2 and Src in SCC4 cells (Fig. 4D).

**SHP-1 siRNA Attenuates Cell Death by γ-T3—**We determined whether the silencing of SHP-1 expression by siRNA would abrogate the γ-T3-induced cell death. We found that silencing of SHP-1 significantly reduced the γ-T3-induced cell death (Fig. 4E). These results suggest the critical role of SHP-1 in γ-T3-induced cell death.

**γ-T3 Inhibits STAT3 Phosphorylation in Pancreatic Tumors in Vivo—**Whether γ-T3 inhibits STAT3 activation in vivo was examined in human pancreatic tissue from animals treated with γ-T3. The Western blot analysis of tumor tissues showed that tissues from vehicle-treated animals express STAT3 phosphorylation, whereas that from γ-T3-treated animals did not (Fig. 4E). This result clearly indicates that γ-T3 inhibits STAT3 activation not only in vitro but also in vivo.

**γ-T3 Down-regulates the Expression of Cell Survival, Proliferative, and Angiogenic Gene Products—**We found that expression of antiapoptotic proteins (Mcl-1, Bcl-xl, Bcl-2, and survivin), cell cycle regulator proteins (cyclin D1), and angiogenic protein (VEGF), all reported to be regulated by STAT3 (12, 31), were modulated by γ-T3 treatment. γ-T3 treatment down-regulated expression of these proteins in a time-dependent manner, with maximum suppression observed at 18 h after the beginning of treatment (Fig. 5A).
Whether equal protein loading. The same blots were stripped and reprobed with SHP-1 antibody to verify equal protein loading. *, p < 0.01.

**FIGURE 4.** γ-T3 regulates STAT3.
A. Pervanadate (γ-T3) induces caspase-3-dependent cleavage of a 118-kDa PARP (Fig. 5C). γ-T3 Inhibits the Proliferation of MM Cells—Because γ-T3 down-regulated the expression of cyclin D1, the gene critical for cell proliferation, we investigated whether γ-T3 inhibits cell proliferation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide method. γ-T3 inhibited proliferation of both MM.1s and MM.1r cells in a dose-dependent manner (Fig. 5D).

**γ-T3 Potentiates the Apoptotic Effect of Bortezomib and Thalidomide in MM Cells—**Recently, the drugs bortezomib, an inhibitor of proteasome, and thalidomide, an inhibitor of TNF receptor expression, have been approved for the treatment of MM (33). We investigated whether γ-T3 can sensitize the cancer cells to these drugs by cotreatment with γ-T3 together with either thalidomide or bortezomib. The cells were then examined by Live/Dead assay. γ-T3 treatment potentiates the apoptotic effects of thalidomide and bortezomib treatment from 18 to 77% (Fig. 5E).

**γ-T3 Causes Caspase-3 Activation and PARP Cleavage—**Whether γ-T3 can activate caspase-3 closely linked to apoptosis was also examined. Treatment of U266 cells with γ-T3 induced caspase-3-dependent cleavage of an 118-kDa PARP protein into an 87-kDa fragment (Fig. 5B).

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**Figure 5.** γ-T3 suppresses STAT3-regulated antiapoptotic gene products, induces apoptosis, and potentiates chemotherapeutic agents. A, γ-T3 suppresses STAT3-regulated antiapoptotic gene products. U266 cells (2 × 10⁶/ml) were treated with 25 μM γ-T3 for the indicated time intervals, after which whole-cell extracts were prepared, and 40 μg portions of those extracts were resolved on 10% SDS-PAGE; the membrane was probed using antibodies against cyclin D1, Bcl-2, Bcl-xL, survivin, and VEGF. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. B, γ-T3 induces caspase-3-dependent PARP cleavage. U266 cells were treated with 60 μM γ-T3 for the indicated times, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to Western blotting against caspase-3 antibody and PARP antibody. The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. C, γ-T3 causes significant accumulation of cells in the sub-G₁ phase. U266 cells (2 × 10⁶/ml) were treated with 25 μM γ-T3 and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. D, effects of γ-T3 on the proliferation of multiple myeloma cells. MM cells were plated in triplicate, treated with the indicated concentrations of γ-T3 for the indicated days, and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. E, γ-T3 potentiates the apoptotic effect of thalidomide (Thal) and Velcade (Val). U266 cells (1 × 10⁶/ml) were treated with 25 μM γ-T3 and 10 ng/ml thalidomide or 20 nm bortezomib alone or in combination for 24 h at 37 °C. Cells were stained with a Live/Dead assay reagent for 30 min, then analyzed under a fluorescence microscope, and 20 random fields were counted. C, Control; T, Thalidomide; V, Velcade; *, p < 0.01; **, p < 0.05 versus control.

Our results demonstrate that γ-T3 can inhibit constitutively active c-Src activity in MM cells. Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks transformation (37, 38). Although γ-T3 alone was inefficient, a combination of γ-T3 plus erlotinib/gefitinib has been reported to inhibit STAT3 activation in murine mammary tumor cells (20). How γ-T3 inhibits activation of STAT3 was not investigated in detail. Another study showed suppression of STAT3 activation in mesothelioma cells by a redox-silent analog of α-tocotrienol (19). Again, the mechanism by which this analog suppresses STAT3 activation was not examined. Thus, our studies are the first to investigate the mechanism by which γ-T3 inhibits STAT3 activation. Previously, α-T3 has been shown to inhibit glutamate-induced pp60 (c-Src) kinase activation in neuronal cells (6), but it had no effect on the activity of recombinant c-Src kinase, suggesting that its mechanism of action may include regulation of SH domains. Besides c-Src, we also found for the first time that γ-T3 can inhibit the activation of JAK1 and JAK2 that has been closely linked with STAT3 activation.

From numerous lines of evidence, we also found that PTP is involved in the down-regulation of STAT3 by γ-T3. First, the broad-acting PTP inhibitor, pervanadate, inhibited the effect of γ-T3 on STAT3 activation. What type of PTP is involved was further investigated. Several PTPs have been reported to regulate STAT3 signaling, including SHP-1 (39), SHP-2 (40), TC-PTP (41), PTEN (42), PTP-1D (43), CD45 (44), and PTP-ε (45). Also, it has been shown that loss of SHP-1 will enhance JAK3/STAT3 signaling in an ALK-positive anaplastic large cell lymphoma (29). In our
We also found that down-regulation of cyclin D1 by γ-T3 in MM cells that express constitutively active STAT3 caused suppression of proliferation and accumulation of cells in sub-G₁. In addition, we found that the apoptotic effects of thalidomide and bortezomib were potentiated by γ-T3.

We also found that γ-T3 down-regulated the expression of VEGF, which is needed for angiogenesis of tumor cells. Previously, we have reported that γ-T3 can inhibit NF-κB activation through inhibition of IkB kinase (IKK) activation (11). Whether inhibition of STAT3 by γ-T3 is connected with suppression of IKK activation is not clear at this point. Although the p65 subunit of NF-κB has been shown to communicate with STAT3 (46), different cytokines and different kinases activate them. Although tumor necrosis factor is the major activator of NF-κB, IL-6 that is regulated by NF-κB, is the most potent inducer of STAT3. Collectively, our results show that γ-T3 inhibits both inducible and constitutive STAT3 activation through the induction of tyrosine kinase phosphatase, which makes it a potentially effective suppressor of tumor cell survival, proliferation, and angiogenesis. Further clinical studies may provide important insights regarding γ-T3 as treatment for cancer and other inflammatory diseases through the suppression of STAT3.

Whether our in vitro results have any relevance to that in vivo was also investigated. We found that γ-T3 inhibited STAT3 activation in tumor tissue from animals treated with the agent in vivo. γ-T3 also induced SHP-1 expression in human tumors from the animals. This indicates that down-modulation of STAT3 activation by γ-T3 may be through up-regulation of SHP-1 in vivo.

Consistent with suppression of STAT3 activation, γ-T3 downregulated the expression of STAT3-regulated genes that are involved in proliferation (cyclin D1) and survival (Bcl-2, Bcl-xL, and Mcl-1) of cancer cells. Although several of these protein have been reported to be down-regulated by γ-T3, the mechanism of this down-regulation is not understood. It is possible that abrogation of NF-κB reported previously (11) and of STAT3 by γ-T3 reported here is responsible for the suppression of proteins involved in survival and proliferation of tumor cells. Suppression of cell survival proteins led to activation of caspases and increased apoptosis.
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