Combined γ-Tocotrienol and Erlotinib/Gefitinib Treatment Suppresses Stat and Akt Signaling in Murine Mammary Tumor Cells

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Abstract. *Background: Heterodimer cooperation between ErbB receptors has limited clinical usefulness of receptor tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib in the treatment of cancer. However, combination treatment of TKIs with γ-tocotrienol targets multiple ErbB receptors and significantly inhibit +SA murine mammary tumor cell growth. Materials and Methods: Cell proliferation was determined by tetrazolium (MTT) assay and immunofluorescent Ki-67 staining. Western blot analysis was used to determine treatment effects on epidermal growth factor (EGF)-dependent mitogenic signaling. Results: Combined treatment of 3 μM γ-tocotrienol with 0.25 μM erlotinib or 0.5 μM gefitinib significantly inhibited +SA cell growth and reduced cyclin D1 and phosphorylated (active) Pdk-1, Akt, Stat3 and Stat5 levels. Conclusion: Combined treatment of γ-tocotrienol with erlotinib or gefitinib prevents ErbB receptor heterodimer cooperation and inhibits EGF-dependent mitogenic signaling in +SA murine mammary tumor cells. These findings strongly suggest that combination treatment may significantly improve therapeutic responsiveness in breast cancer patients.*

Breast cancer is the most common malignancy in women, often characterized by aberrant ErbB receptor signaling (1). The epidermal growth factor (EGF) family of receptors, also known as ErbB receptors in rodents and human epidermal growth factor receptors (HER) in humans, belong to a class of receptor tyrosine kinases that include four members: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 (2). ErbB receptors are activated by binding to appropriate ligands, resulting in receptor dimerization, tyrosine kinase activation, and transphosphorylation at receptor tyrosine kinase residues (3, 4). The tyrosine phosphorylated EGF receptor dimer can interact and activate several important mitogenic signaling pathways as shown in Figure 1, which include the mitogen-activated protein kinase (Mapk); phosphoinositol 3-kinase (PI3K)/PI3K-dependent kinase (Pdk-1)/Akt pathway; and signal transducer and activator of transcription (Stat) pathway (3, 5-7).

Since aberrant ErbB receptor signaling is implicated in various types of cancer, including breast cancer, these receptors are studied as potential targets in the treatment of cancer (7-9). One approach to target ErbB receptors is the use of tyrosine kinase inhibitors (TKIs) which compete with ATP for binding to the kinase domain of the receptor (7). Erlotinib and gefitinib are two such TKIs which reversibly inhibit the phosphorylation and signal transduction events associated with ErbB1 receptor activation (10). Unfortunately, the clinical usefulness of these agents targeting a single ErbB receptor subtype has been limited because of the heterodimer formation between different ErbB family members (8). Heterodimer formation between ErbB family members has been shown to provide greater signal duration, diversification and amplification as compared to their corresponding homodimer receptor complexes, and thereby rescues cancer cells from the inhibitory effect of agents directed against a single ErbB receptor (11, 12). However, heterodimer cooperation among the ErbB family of receptors can be circumvented by targeting multiple ErbB receptors using combination treatment (13).

γ-Tocotrienol, a member of the vitamin E family of compounds, displays potent antiproliferative and apoptotic activity against tumor cells at treatment doses that have little or no effect on normal cell growth and viability (14-16). Recent studies have shown that γ-tocotrienol inhibits EGF-dependent growth of +SA murine mammary epithelial cells by suppressing ErbB3 receptor activity and subsequent reduction in the PI3K/Akt signaling (17, 18). Initial studies have shown that combined treatment with relatively low doses of γ-tocotrienol and erlotinib or gefitinib significantly inhibited +SA mammary cell growth (19). However, the exact mechanism(s) mediating the antiproliferative effects of this combination treatment are presently unknown.

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Key Words: Akt, breast cancer, ErbB receptors, erlotinib, gefitinib, vitamin E, Stat, tyrosine kinase inhibitors, γ-tocotrienol.

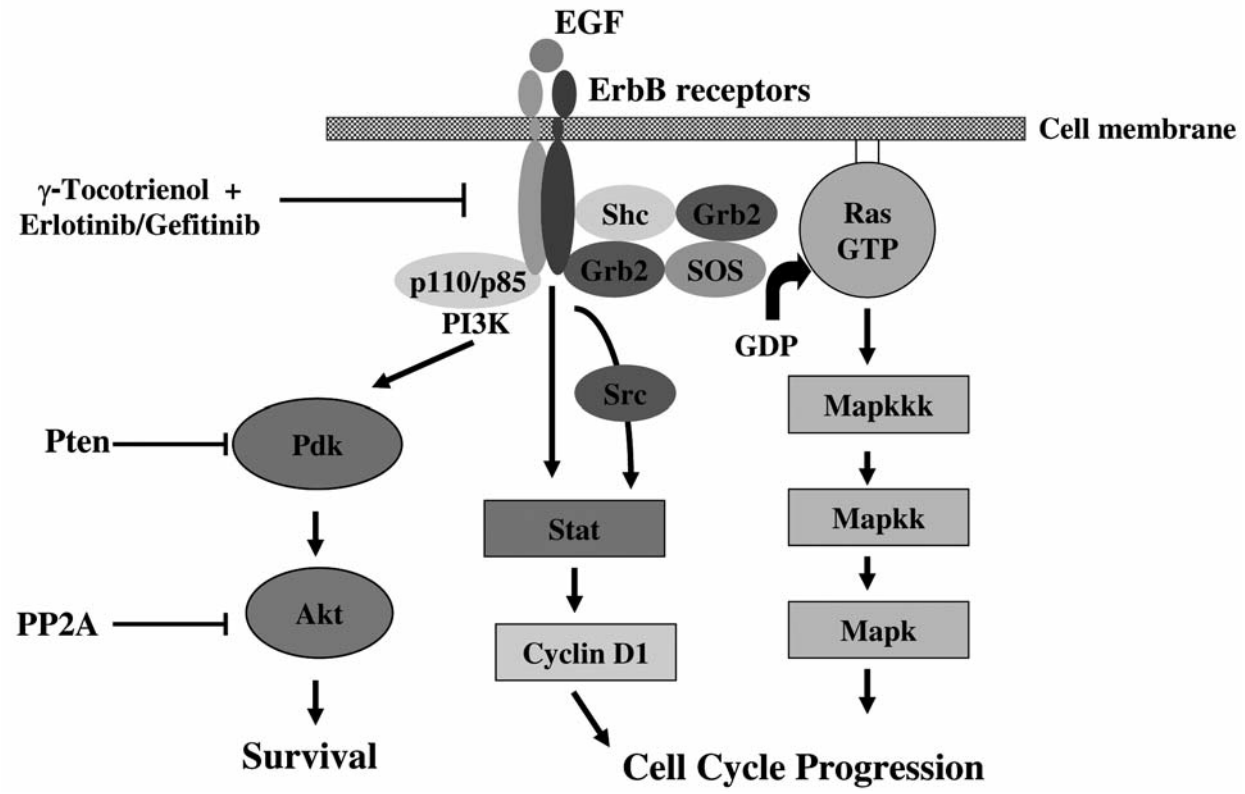

Figure 1. *Signaling pathways activated by ErbB receptors. Activation of ErbB receptors by EGF can lead to the recruitment of PI3K and subsequent activation of Pdk-1 and Akt. Recruitment of adaptor proteins such as Shc, Grb2 and SOS leads to the activation of Ras/Mapk pathway. Tyrosine phosphorylation of ErbB receptors can also activate Stats either directly or through the activation of Src. ErbB receptor activation ultimately leads to the activation of Akt, Mapk and Stat mitogenic signaling.*

In order to elucidate the specific intracellular signaling pathways involved in mediating the growth inhibitory effects of combined γ-tocotrienol and erlotinib or gefitinib treatment in +SA mammary tumor cells, studies were conducted to determine the effects of this combination treatment on the EGF-dependent ErbB receptor activation and downstream signaling of the Stat, PI3K/Pdk-1/Akt and Mapk mitogenic pathways.

Materials and Methods

Reagents and antibodies. All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Purified γ-tocotrienol was provided as a gift by First Tech International Ltd. (Hong Kong, PRC). Erlotinib and gefitinib were generously provided by Genentech (San Francisco, CA, USA) and AstraZeneca (Cheshire, UK), respectively. Antibody for Ki-67 and the respective blocking peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for α-tubulin was purchased from EMD biosciences (La Jolla, CA, USA). Antibodies for total and phosphorylated Stat1, 3, and 5, phospho-Src, phospho-Shc, Mapk, phospho-Mapk, PI3K, phospho-Pdk-1, phospho-Akt, Akt, Pten, phospho-Pten, PP2A, cyclin D1, total and phosphorylated ErbB3 and ErbB4 were purchased from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA). Alexa Fluor 594-conjugated anti-goat antibody was purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, CA, USA).

Cell line and culture conditions. Experiments conducted in the present study represent an extension of the previous findings that have extensively characterized the antiproliferative effects of γ-tocotrienol in the neoplastic +SA mouse mammary epithelial cell line (20). The highly malignant +SA cell line was derived from a mammary adenocarcinoma that developed spontaneously in a female BALB/c mouse (21). The +SA cell line is characterized as being highly malignant, estrogen independent, and displays anchorage-independent growth when cultured in soft agarose gels. When injected back into the mammary gland fat pad of syngeneic female mice, +SA cells form rapidly growing anaplastic adenocarcinoma that are highly invasive and metastasize to lung (21-23). Cell culture conditions have been previously described in detail (14, 16, 24). Briefly, +SA cells were maintained in serum-free defined medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5 mg/ml bovine serum albumin (BSA), 10 μg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml insulin and

10 ng/ml EGF at 37˚C in an environment of 95% air and 5% CO_2 in a humidified incubator. For subculturing, cells were rinsed twice with sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37˚C. The released cells were centrifuged, resuspended in serum-free defined media and counted using hemocytometer.

Experimental treatments. The highly lipophilic γ-tocotrienol was suspended in a solution of sterile 10% BSA as described previously (14, 17). Briefly, an appropriate amount of γ-tocotrienol was dissolved in 100 μl of absolute ethyl alcohol, and then added to a small volume of sterile 10% BSA in water and incubated overnight at 37˚C. This γ-tocotrienol BSA solution was then used to prepare different concentrations of treatment media. Ethanol was added to all treatment media with in a given experiment such that the final concentration of ethanol never exceeded 0.1%. Stock solutions of erlotinib and gefitinib were prepared in DMSO and DMSO was added to all treatment media such that the final concentration was the same in all treatment groups within any given experiment and was always less than 0.1%. For growth studies, cells were plated at a density of 5×10^4 cells/well (6 replicates/group) in 24 well culture plates and treated with control, 3 μM γ-tocotrienol, 0.25 μM erlotinib, 0.5 μM gefitinib alone or in combination for a 4-day culture period. For Western blot analysis of ErbB receptor levels, cells were plated a density of 1×10^6 cells/100 mm culture dish and treated with 3 μM γ-tocotrienol, 0.25 μM erlotinib, or 0.5 μM gefitinib alone or in combination for a 4-day culture period. For immunofluorescent analysis of Ki-67 expression, +SA cells were plated at a density of 2×10^5 cells/well (3 replicates/group) in single well chamber slides and were treated with vehicle containing control media (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone or in combination for a 4-day culture period. In combination studies +SA cells were plated at a density of 1×10^6 cells/100 mm culture dish and exposed to control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone or in combination for a period of 4 days, and then prepared for Western blot analysis of various markers of mitogenic signaling.

Measurement of viable cell number. +SA cell count was determined by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (14, 25). At the end of the treatment, cells were incubated for 4 h with fresh control media containing 0.41 mg/ml MTT at 37˚C. The medium was then removed and MTT crystals were dissolved in 1 ml of isopropanol. The optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount; Packard BioScience Company, Meriden, CT, USA) zeroed against a blank prepared from cell-free medium. The number of cells per well was calculated against a standard curve prepared by plating known cell densities, as determined by hemocytometer, in triplicate at the start of each experiment.

Western blot analysis. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dish and exposed to control or treatment media for a 4-day culture period. Afterwards, cells were washed with PBS and isolated with trypsin and whole cell lysates were prepared and dissolved in Laemmli buffer as described elsewhere (26, 27). The protein concentration in each sample was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein from each sample in a given experiment were loaded onto SDS-polyacrylamide minigels and electrophoresed through 5%-15% resolving gel. Proteins separated on each gel were transblotted at 30 V for 12-16 h at 4˚C onto a polyvinylidene fluoride (PVDF) membrane (PerkinElmer Lifesciences, Wellesley, MA, USA) in a Trans-Blot Cell (Bio-Rad Laboratories) according to the method of Towbin *et al*. (28). The membranes were then blocked with 2% BSA in 10 mM Tris HCl containing 50 mM NaCl and 0.1% Tween 20 pH 7.4 (TBST) and then incubated with specific primary antibodies against ErbB3, phospho-ErbB3, ErbB4, phospho-ErbB4, total and phosphorylated Stat1, 3, and 5, phospho-Src, phospho-Shc, Mapk, phospho-Mapk, PI3K p85 subunit, phospho-Pdk-1, phospho-Akt, Akt, Pten, phospho-Pten, PP2A, cyclin D1, or α-tubulin, diluted 1:5,000 to 1:10,000 in TBST/2% BSA for 2 h. Membranes were washed 5 times with TBST followed by incubation with the respective horseradish peroxide-conjugated secondary antibodies diluted 1:5,000 in TBST/2% BSA for 1 h followed by rinsing with TBST. Protein bound to the antibody was then visualized by chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and images were obtained using a Kodak Gel Logic 1500 Imaging System (Carestream Health Inc, New Haven, CT, USA). The visualization of α-tubulin was used to confirm that there was equal sample loading in each lane. All experiments were repeated at least three times and a representative Western blot image from each experiment is shown in the Figures.

Fluorescent labeling for Ki-67. +SA cells were plated in single-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA) at a density of 2×10^5 cells/well (3 replicates/group) and allowed to attach in serum-free defined media for 24 h. Cells were then incubated with control or treatment media for 4 days in culture and at the end of treatment, cells were washed with 0.05 M Tris buffered saline (TBS) (pH 7.6), fixed with methanol previously cooled to −20˚C, and blocked with 2% donkey serum in TBS for 30 min. Cells were then incubated with specific primary antibody against Ki-67 overnight at 4˚C in 2% donkey serum in TBS. Cells were washed five times with TBS followed by incubation with Alexa Fluor 594-conjugated donkey anti-goat secondary antibody in 2% donkey serum in TBS. The specificity of the Ki-67 antibody used in the immunofluorescent labeling was verified using a blocking peptide that corresponds to the epitope recognized by the primary antibody. Fluorescent images were obtained by using confocal laser scanning microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA). The percentage of +SA cells displaying Ki-67 labeling was determined by counting the number of positive Ki-67 staining cells as a proportion of the total number of cells counted. Cells were counted manually in five photomicrographs taken randomly on each slide in each treatment group in triplicate.

Statistical analysis. Differences among various treatment groups in growth studies were determined by analysis of variance followed by Dunnett's *t*-test. Differences were considered statistically significant at a value of $p<0.05$.

Results

Effects of γ-tocotrienol, erlotinib, or gefitinib treatment alone or in combination on +SA cell growth and ErbB receptor activation. Treatment with subeffective doses of erlotinib (0.25 μM) or γ-tocotrienol (3 μM) alone had no significant effect on +SA cell growth (Figure 2A), nor on intracellular levels of total and phosphorylated ErbB3 and ErbB4 receptors (Figure 2B). In

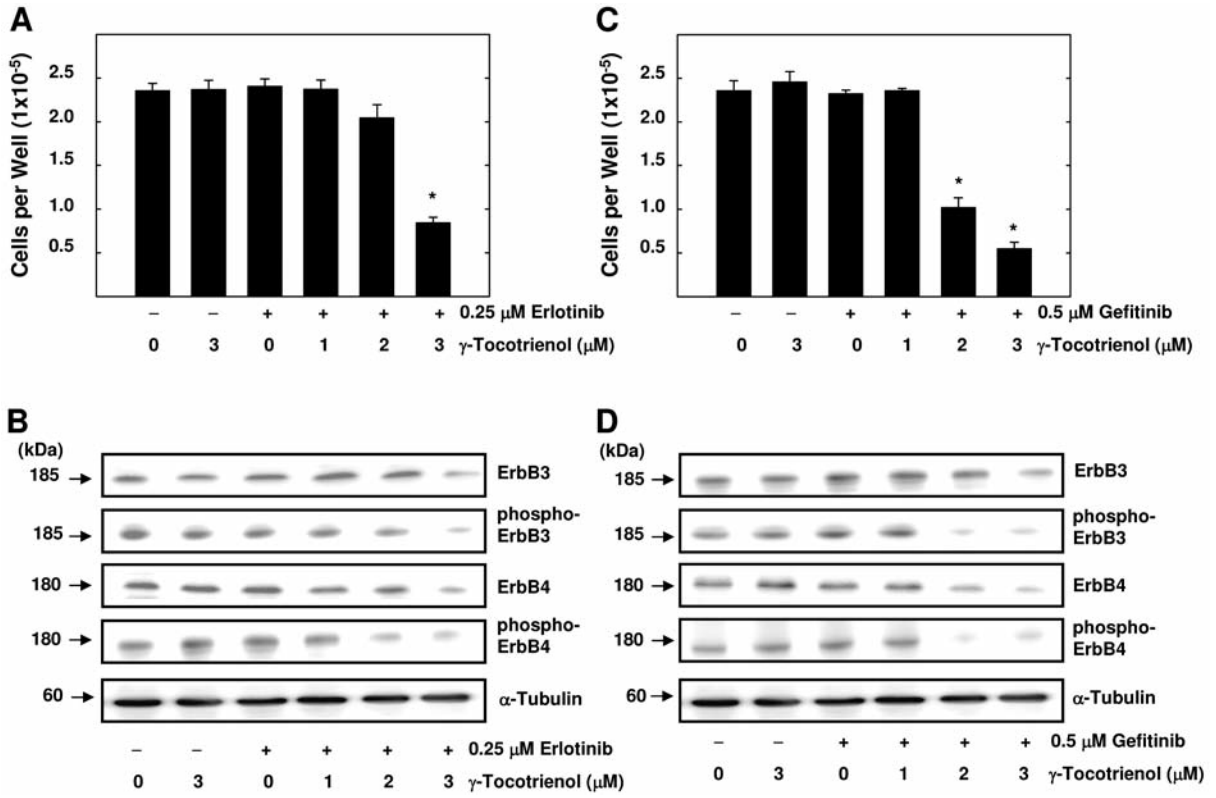

Figure 2. *Effects of combined γ-tocotrienol and erlotinib or gefitinib treatment on +SA cell growth (A, C) and ErbB receptor levels (B, D). Vertical bars indicate the viable cell count±SEM in each treatment group. *p<0.05 as compared to vehicle-treated control group (A and C). Western blot analysis measured total and phosphorylated levels of ErbB3 and ErbB4 receptors and α-tubulin levels (B and D). α-Tubulin was visualized to ensure equal sample loading in each lane. Each Western blot is a representative image of data obtained for experiments that were repeated at least three times.*

contrast, exposure to combined treatment with 0.25 μM erlotinib and 1-3 μM γ-tocotrienol resulted in a dose-dependent decrease in the +SA cell growth (Figure 2A) as compared with vehicle-treated controls. Similarly, treatment with 0.25 μM erlotinib and 3 μM γ-tocotrienol resulted in a relatively large reduction in total and phosphorylated (active) ErbB3 and ErbB4 receptor levels (Figure 2B) as compared to the vehicle-treated control group.

Figure 2C shows that combined treatment with 0.5 μM gefitinib and 1-3 μM γ-tocotrienol resulted in a dose-dependent decrease in +SA cell growth. Similar combination treatment resulted in a large reduction in the total and phosphorylated levels of ErbB3 and ErbB4 receptors at a dose of 0.5 μM gefitinib and 2-3 μM γ-tocotrienol (Figure 2D) as compared to the vehicle-treated control group. However, treatment with 0.5 μM gefitinib or 3 μM γ-tocotrienol alone had no effect on the growth (Figure 2C) nor on ErbB receptor levels and activation (Figure 2D) in +SA cells as compared to the vehicle-treated control group.

Effects of γ-tocotrienol, erlotinib, and gefitinib treatment alone or in combination on Ki-67 labeling. Expression of the Ki-67 antigen is strictly associated with mitogenesis and is therefore used as a marker of actively proliferating cells (29). Positive Ki-67 staining in proliferating +SA cells is characterized by a pink color stain, whereas non-proliferating cells are characterized by the blue color of the DAPI counterstain (Figure 3B). In control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), and 0.5 μM gefitinib (G) treated groups, more than 85% of the +SA cells in culture displayed positive Ki-67 nuclear staining (Figure 3A). However, treatment with similar doses of erlotinib (E, 0.25 μM) or gefitinib (G, 0.5 μM) in combination with a subeffective dose of γ-tocotrienol (T, 3 μM) resulted in fewer than 15% of +SA cells displaying positive staining for Ki-67 (Figure 3A). Confocal images of +SA cells in the various groups are shown in Figure 3B

Effects of γ-tocotrienol, erlotinib, or gefitinib treatment alone or in combination on STAT pathway activation. Figure 4 shows the effect of the combined γ-tocotrienol and erlotinib or gefitinib treatment on the Stat mitogenic signaling after a 4-day treatment exposure of +SA cells. Western blot analyses showed that treatment with 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone did not alter the

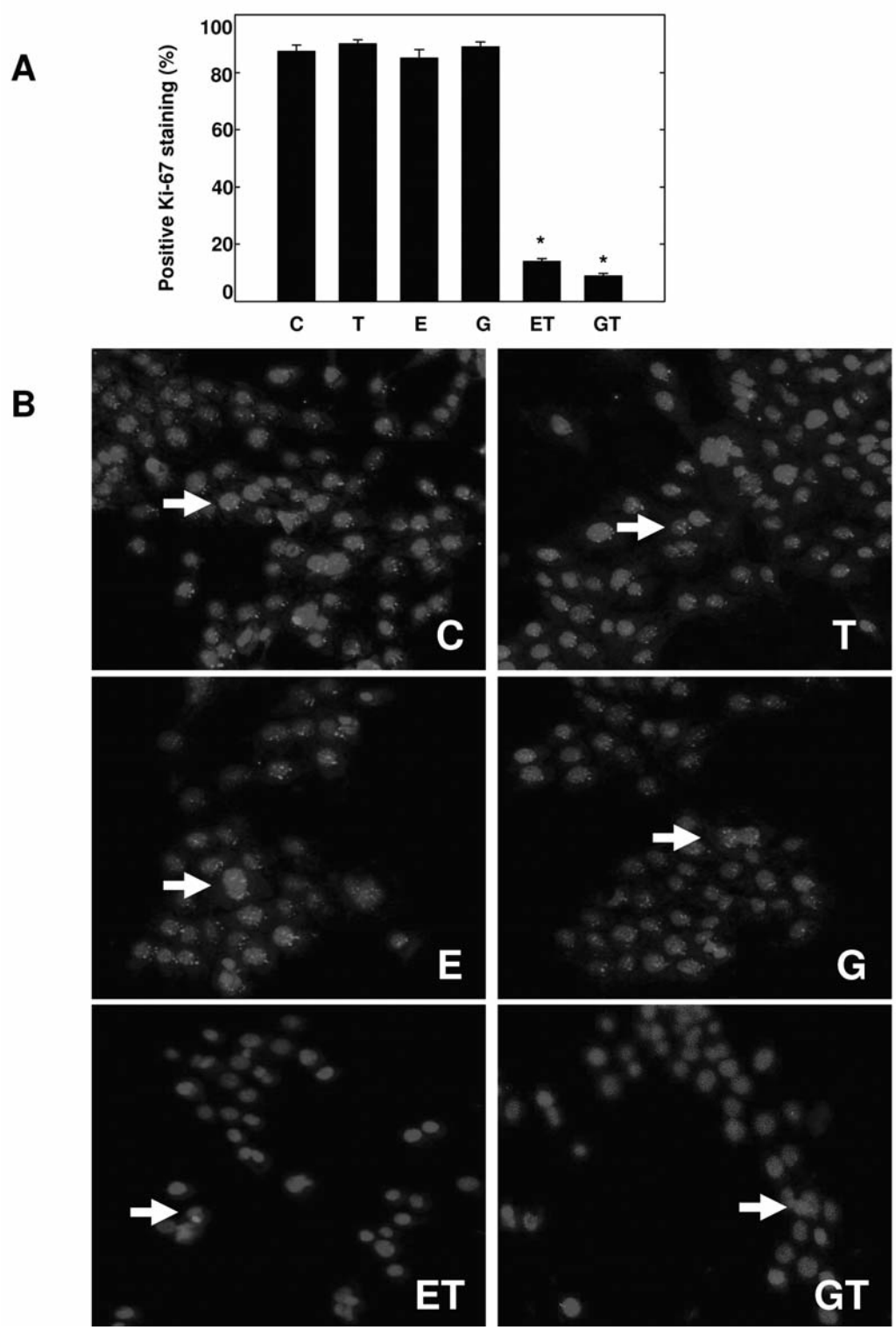

Figure 3. *Effects of combined low-dose treatment of γ-tocotrienol with erlotinib or gefitinib on Ki-67 expression in +SA mammary tumor cells. +SA cells were treated with medium containing vehicle control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone or in combination for a 4-day culture period. Afterwards, cells were fixed with methanol and subjected to immunofluorescent analysis for Ki-67 expression as described in Materials and Methods. A, the percentage of proliferating +SA cells determined by counting cells staining positively for Ki-67 as a proportion of the total number of cells counted in each treatment group. Data are means±SEM. *p<0.05 as compared to vehicle-treated control group (C). B, Fluorescent images of positive Ki-67 staining in +SA cells treated with control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G), alone or in combination following a 4-day culture period. Arrows indicate positive Ki-67 staining in the nucleus counterstained with DAPI. Magnification, ×200.*

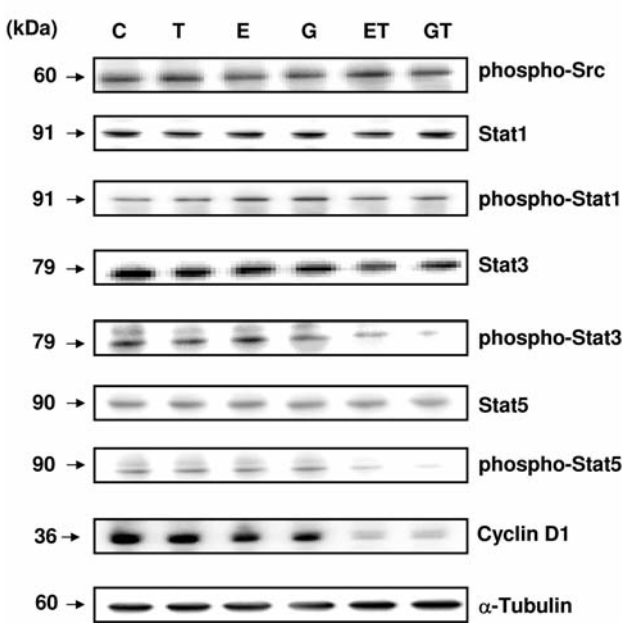

Figure 4. *Effects of combined γ-tocotrienol treatment with erlotinib or gefitinib on the relative intracellular levels of signaling molecules associated with Stat signaling. Western blot analysis of intracellular levels of phospho-Src, total and phosphorylated Stat1, -3, and -5, cyclin D1 and α-tubulin in +SA cells after exposure to control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone and in combination. α-Tubulin was visualized to ensure equal sample loading in each lane. Each Western blot is a representative image of data obtained for experiments that were repeated at least three times.*

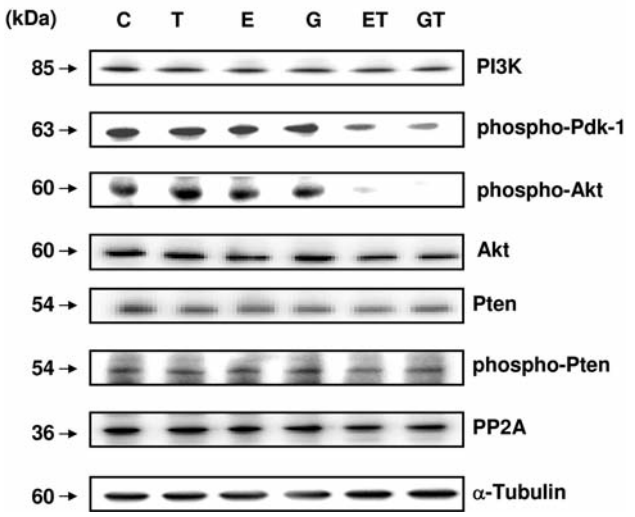

Figure 5. *Effects of combined γ-tocotrienol treatment with erlotinib or gefitinib on the relative intracellular levels of signaling molecules associated with PI3K/Pdk-1/Akt pathway in +SA cells after a 4-day culture period. Western blot analysis of intracellular levels of PI3K p-85 subunit, phospho-Pdk-1, phospho-Akt, Akt, Pten, phospho-Pten, PP2A and α-tubulin in +SA cells after exposure to control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone or in combination for a 4-day culture period. α-Tubulin was visualized to ensure equal sample loading in each lane. Each Western blot is a representative image of data obtained for experiments that were repeated at least three times.*

relative intracellular levels of phospho-Stat3, phospho-Stat5 and cyclin D1, whereas combination treatment with 0.25 μM erlotinib and 3 μM γ-tocotrienol (ET) or 0.5 μM gefitinib and 3μM γ-tocotrienol (GT) resulted in marked reduction in the levels of phospho-Stat3, phospho-Stat5 and cyclin D1 as compared to the vehicle-treated control group (C) (Figure 4). Furthermore, intracellular levels of phospho-Src, phospho-Stat1, Stat1, Stat3, and Stat5 were not found to differ greatly among any of the treatment groups (Figure 4).

Effects of γ-tocotrienol, erlotinib, or gefitinib treatment alone or in combination on Akt pathway activation. The effects of combined erlotinib or gefitinib treatment with γ-tocotrienol on the intracellular levels of PI3K p85 subunit, phospho-Pdk-1, phospho-Akt, Akt, Pten, phospho-Pten and PP2A in +SA mammary tumor cells after a 4-day treatment period are shown in Figure 5. Western blot analyses showed that treatment with 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone had no effect on the relative intracellular levels of phospho-Pdk-1 and phospho-Akt, but combined treatment of 0.25 μM erlotinib with 3 μM γ-tocotrienol (ET) or 0.5 μM gefitinib with 3μM γ-tocotrienol (GT) caused a relatively large decrease in phospho-Pdk-1 and phospho-Akt levels as compared to the vehicle-treated controls (C). Total intracellular levels of PI3K p85 subunit, Akt, Pten, phospho-Pten, and PP2A catalytic subunit (active) showed no differences among any of the treatment groups (Figure 5).

Effects of γ-tocotrienol, erlotinib, or gefitinib treatment alone or in combination on Mapk pathway activation. Effects of the combination treatment on the Mapk mitogenic signaling pathway are shown in Figure 6. Western blot analysis showed that intracellular levels of phospho-Shc, phospho-Mapk and Mapk were similar in all treatment groups (Figure 6).

Discussion

The results in this study demonstrate that the enhanced antiproliferative effects of combined γ-tocotrienol and erlotinib or gefitinib treatment are associated with a reduction in EGF-dependent Stat and PI3K/Pdk-1/Akt mitogenic signaling. Combined low-dose treatment of γ-tocotrienol (3 μM) with erlotinib (0.25 μM) or gefitinib (0.5 μM) caused a decrease in EGF-dependent cell proliferation, as indicated by a nearly 70%

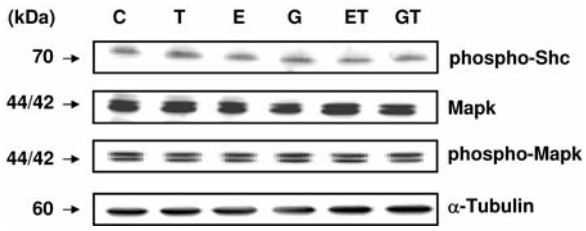

Figure 6. *Effects of combined γ-tocotrienol treatment with erlotinib or gefitinib on the relative intracellular levels of signaling molecules associated with Mapk pathway in +SA cells after a 4-day culture period. Western blot analysis of intracellular levels of phospho-Shc, total and phosphorylated Mapk, and α-tubulin in +SA cells after exposure to control (C), 3 μM γ-tocotrienol (T), 0.25 μM erlotinib (E), or 0.5 μM gefitinib (G) alone and in combination for a 4-day culture period. α-Tubulin was visualized to ensure equal sample loading in each lane. Each Western blot is a representative image of data obtained for experiments that were repeated at least three times.*

decrease in positive Ki-67 staining. These same combination treatments also caused a corresponding large decrease in the intracellular total and phosphorylated (active) levels of ErbB3 and ErbB4 receptors associated with suppression in Stat and PI3K/Pdk-1/Akt mitogenic signaling. However, this same combination treatment did not affect Mapk signaling.

Elevation in ErbB3 receptor expression is associated with poor prognosis in various types of cancer including breast cancer (30, 31). In addition, recent studies have shown that cancer cells escape the antiproliferative effects of specific ErbB-targeted agents such as ErbB1 receptor TKIs (erlotinib and gefitinib) through kinase inactive ErbB3 receptor (12). γ-Tocotrienol has previously been shown to inhibit EGF-dependent growth and ErbB3 signaling in highly malignant +SA mammary epithelial cells (14, 17, 18). The results from the present study demonstrate that subeffective doses of γ-tocotrienol significantly enhanced the inhibitory effects of erlotinib or gefitinib on the ErbB3 receptor activity and growth of neoplastic +SA mammary epithelial cells. Recent advances in understanding breast carcinogenesis suggest that ErbB4 receptors selectively regulate the growth of breast cancer cells and are often coexpressed with ErbB3 receptors (30, 32). The present study demonstrates that combined treatment with subeffective doses of γ-tocotrienol and erlotinib or gefitinib markedly reduced the total and phosphorylated levels of ErbB4 receptor.

Enhanced mitogenic signaling of Stat, PI3K/Pdk-1/Akt and Mapk pathways has been correlated with advanced tumor progression and poor prognosis in breast cancer patients (9). Stats comprise a family of transcription factors that participate in regulating numerous cellular processes such as proliferation, differentiation, cell survival, apoptosis and angiogenesis (33). Stats are activated through tyrosine phosphorylation by members of the Janus kinase (JAK) family of intracellular non-receptor tyrosine kinases that transduce mitogenic signals from activated hormone, growth factor and cytokine receptors (6). Phosphorylated Stats form homo- or heterodimers, translocate to the nucleus and function to regulate transcription of genes associated with cell cycle progression (33). Growth factor-dependent phosphorylation of Stats is a transient process, but Stat proteins were found to be constitutively active in tumor cells (33). Although seven different Stat proteins have been identified, Stat1, Stat3, and Stat5 are often found to be constitutively active in various types of tumors (6, 33).

The effect of combined γ-tocotrienol and erlotinib and gefitinib treatment on the EGF-dependent Stat signaling had not previously been investigated. Results in this study demonstrated that combined low-dose treatment with γ-tocotrienol and erlotinib or gefitinib inhibited the activation of Stat3 and Stat5, but had no effect on the relative intracellular levels of phospho-Stat1 in +SA mammary tumor cells. Stat3 and Stat5 function to promote cell cycle progression and inhibit apoptosis, whereas Stat1 is considered to act as a tumor suppressor and its role in tumor progression and oncogenesis is not well understood and remains controversial (6). Additional studies showed that EGF-dependent activation of Stat3 and Stat5 up-regulates cyclin D1 expression leading to enhanced cell cycle progression (33). Results in the present study demonstrate that combined low-dose treatment with γ-tocotrienol and erlotinib or gefitinib downregulated the cyclin D1 expression, contributing to growth inhibition of neoplastic +SA mammary tumor cells. Other studies show that Stats (particularly Stat3) can also be activated by Src, a non-receptor protein tyrosine kinase (34, 35). However, results in the present study showed that combined γ-tocotrienol and erlotinib or gefitinib treatment had no effect on the Src activation, and suggests that combined treatment-induced reduction in Stat3 phosphorylation is independent of Src activity.

ErbB receptor dimerization and activation can also lead to the recruitment of PI3K. PI3Ks represent a family of enzymes which are ubiquitously expressed and activated by various membrane receptors (5). One class of PI3Ks is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. ErbB receptors activate PI3K by recruiting its p85 regulatory subunit, resulting in allosteric activation of p110 catalytic subunit. ErbB3 and ErbB4 receptors have six and one p85-binding sites respectively and hence are potent stimulators of PI3K, as compared to ErbB1 and ErbB2 receptors which bind to PI3K through adaptor proteins (36). Recruitment of the PI3K by activated ErbB receptors leads to the phosphorylation of membrane phospholipids which in turn recruit effectors such as Pdk-1 which act to phosphorylate and activate Akt. Activation of Akt subsequently leads to the phosphorylation of an array of proteins in the cytoplasm and nucleus that regulate cell growth and apoptosis.

The present study shows that combined γ-tocotrienol and erlotinib or gefitinib treatment-induced growth inhibition is associated with a decrease in the phosphorylation (activation) of Pdk-1 and Akt. Furthermore, combined treatment-induced reduction in Pdk-1 and Akt phosphorylation does not result from an increase in Pten and PP2A phosphatase activity. These findings support previous studies that demonstrated γ-tocotrienol-induced reductions in Akt phosphorylation and activation were not associated with increase phosphatase activity (17). Together, these observations suggest that combination treatment inhibits ErbB3 and ErbB4 receptor activity, and subsequently blocks the recruitment of PI3K and further activation of Pdk-1 and Akt. Interestingly, combination treatment had no effect on the Mapk signaling. It is possible that in this cell line, EGF-dependent activation of Mapk signaling might primarily involve ErbB1 receptor activation. Previous studies have shown that γ-tocotrienol had no effect on ErbB1 receptor activation (18) and hence, may not significantly affect Mapk signaling in these cells. Additional studies are required to investigate this possibility. Nevertheless, combined treatment of γ-tocotrienol with erlotinib or gefitinib induces sufficient suppression in Stat and Akt mitogenic signaling to cause a significant inhibition of neoplastic +SA mammary tumor cell growth.

In summary, the combination of γ-tocotrienol and erlotinib or gefitinib treatment inhibits the growth of mammary tumor cells specifically by suppressing EGF-dependent Stat and Akt mitogenic signaling. These findings strongly suggest that tocotrienols used in combination with other agents that target ErbB receptors may enhance therapeutic responsiveness in breast cancer patients with aberrant EGF receptor signaling.

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