Tocotrienol combination therapy results in synergistic anticancer response

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1. ABSTRACT

Vitamin E represents a family of compounds that is divided into two subgroups called tocopherols and tocotrienols, which act as important antioxidants that regulate peroxidation reactions and control free-radical production within the body. However, many of the biological effects of vitamin E are mediated independently of its antioxidant activity. Although tocopherols and tocotrienols have the same basic chemical structure characterized by a long phytyl chain attached to a chromane ring, only tocotrienols display potent anticancer activity, by modulating multiple intracellular signaling pathways associated with tumor cell proliferation and survival, and combination therapy with other chemotherapeutic agents result in a synergistic anticancer response. Combination therapy is most effective when tocotrienols are combined with agents that have complementary anticancer mechanisms of action. These findings strongly suggest that the synergistic antiproliferative and apoptotic effects demonstrated by combined low dose treatment of γ-tocotrienol with other chemotherapeutic agents may provide significant health benefits in the prevention and/or treatment of breast cancer in women, while at the same time avoiding tumor resistance and toxic side effects associated with high dose monotherapy.

2. INTRODUCTION

Cancer is an inclusive term that represents a group of more than 100 different diseases characterized by uncontrolled cellular growth, local tissue invasion, and distant metastases. Collectively, cancer is second only to cardiovascular disease as the most common cause of death in the United States (1). Breast cancer is the most prevalent malignancy in women in the world (2). However, because of major advances in early detection and treatment, breast cancer mortality has decreased during the past decade (1). While mutations in specific tumor repressor genes, such as BRCA1 and BRCA2, have been identified to predispose women to breast cancer, these gene mutations are quite rare and are only directly responsible for an estimated 10-15% of all breast cancers (3). The cause for the remaining 85-90% of breast cancers in women remains less clear and unpredictable. Breast carcinogenesis is a multistage process that is initiated by a single genomic mutation, and subsequent mutations leads to a progression in malignant phenotypic characteristics, including increased anaplastic histological morphology, resistance to anticancer or endocrine therapy, and enhanced invasive and metastatic potential (4-13). Current strategies for treating cancer have focused on developing drugs directed against specific molecular targets associated with tumor cell growth and progression. Experimental evidence suggests that the
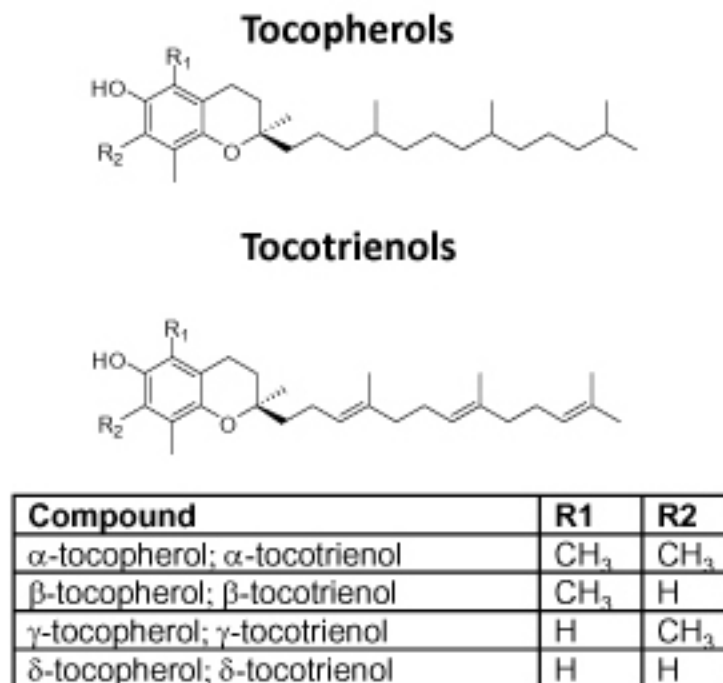
Compound	R1	R2
α-tocopherol; α-tocotrienol	CH₃	CH₃
β-tocopherol; β-tocotrienol	CH₃	H
γ-tocopherol; γ-tocotrienol	H	CH₃
δ-tocopherol; δ-tocotrienol	H	H

Figure 1. General structure of vitamin E compounds.

dietary supplementation with specific forms of vitamin E can provide significant protection at multiple stages of mammary carcinogenesis (14-19), and strongly suggests that tocotrienols may have great potential in the prevention and treatment of cancer because of multiple mechanisms of action directed against tumor cell proliferation and viability. Furthermore, it is now firmly established that anticancer chemotherapy is most effective when multiple drugs with complimentary mechanisms of action are given in combination. Combination chemotherapy results in each drug being used at its optimal dose to provide an enhanced and even synergistic therapeutic response, while at the same time reducing or eliminating the likelihood of adverse and toxic side effects. Depending on the particular type of cancer being treated, studies have shown that combined low dose tocotrienol treatment with other specific chemotherapeutic agents, results in significantly increased anticancer effectiveness as compared to high dose monotherapy (20-25).

3. VITAMIN E COMPOUNDS

Vitamin E is a general term representing a family of compounds that is further divided into two subgroups called tocopherols and tocotrienols. Tocopherols are commonly found in high concentrations in a wide variety of foods, whereas tocotrienols are relatively rare and found in appreciable levels only in a few specific vegetable fats, such as palm oil (19, 26). Although chemically very similar, tocopherols have a saturated, whereas tocotrienols have an unsaturated phytyl chain attached to a chromane ring structure (Figure 1). Each subgroup of vitamin E contains several isoforms and individual tocopherols and

tocotrienols isoforms differ from each other based on the number of methyl groups bound to their chromane ring. These subtle differences in their chemical structure have shown that individual tocotrienol isoforms display significantly greater anticancer activity than their corresponding tocopherol isoforms (27-29). These findings are particularly interesting because they are observed using treatment doses that have little or no effect on cell growth or viability (27). Furthermore, dose-response studies have also shown that IC_{50} treatment doses for individual tocotrienol isoforms were 5-6 times lower than their corresponding LD_{50} cytotoxic doses (27), suggesting that the antiproliferative and apoptotic effects of tocotrienols are mediated through different mechanisms (27, 30). All vitamin E compounds are potent natural antioxidants that act to prevent the spoilage, maintain flavor and enhance the nutritional value of dietary fat. However, natural antioxidants also provide significant health benefits by preventing the damaging effects of peroxidation reactions and free radical production within the body (26, 31, 32). Uncontrolled production of free radicals is associated with damage to cell structures, reduced cellular function, and implicated as a cause of various diseases, such as arteriosclerosis and cancer (26, 31, 32). However, studies have shown that the anticancer effects of tocotrienols are not dependent on antioxidant potency, but rather the action of tocotrienols to modulate specific intracellular signaling pathways (28, 29, 33, 34). In summary, numerous studies investigating the intracellular mechanisms responsible for mediating antiproliferative and cytotoxic effects of tocotrienols and have provided strong experimental evidence to suggest that tocotrienols may provide

significant health benefits in preventing or reducing the risk of breast cancer in women.

4. TOCOTRIENOLS AS ANTICANCER AGENTS

Initially, the anticancer effects of tocotrienols were discovered in studies investigating the effects of high dietary fat intake on mammary tumorigenesis in laboratory animals. Initial studies showed that high dietary intake of palm oil inhibited carcinogen-induced mammary carcinogenesis in rats (17, 35), while palm oil diets stripped of tocotrienols had no protective effect (36). Furthermore, the anticancer effects of tocotrienols *in vitro* are firmly established (27-29, 33, 34, 37-39). Although these studies strongly suggest that it is the tocotrienols in palm oil diets that are responsible for inhibiting mammary tumorigenesis, this hypothesis has been difficult to prove *in vivo*. Dietary supplementation with isolated tocotrienols or the tocotrienol-rich-fraction of palm oil (TRF) has produced conflicting results (40). Other studies showed that dietary supplementation with TRF inhibited growth of +SA mammary tumors transplanted in female syngeneic BALB/c mice (41). However, these antitumor effects of dietary TRF supplementation did not produce a typical dose-responsive effect. These findings indicate that the anticancer effectiveness of oral administration of tocotrienols may be limited due to inefficient or saturated uptake/transport mechanisms within the gut and circulation. Nevertheless, experimental evidence strongly suggested that tocotrienols may provide potential health benefits in the treatment of breast cancer in women if a method could be developed to further optimize tocotrienol delivery to the mammary tumors.

5. MECHANISMS MEDIATING THE ANTIPROLIFERATIVE EFFECTS OF TOCOTRIENOLS

Experimental studies have shown that tocotrienols significantly inhibit the growth of breast cancer cells (33, 42). Specifically, tocotrienols have been found to inhibit EGF-dependent mitogenic actions in normal and neoplastic mammary epithelial cells (43). The EGF receptor is a member of the ErbB family of receptors (44-46). This receptor family is comprised of four related receptors defined as the EGF receptor (ErbB1/HER1), ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 (44-46). Activation of EGF receptor tyrosine kinase activity results in the recruitment and phosphorylation of several intracellular substrates (44-46). A major downstream signaling pathway activated by EGF receptor is the Ras/Raf/MEK/ERK pathway, also known as the MAPK pathway (47). Activation of Ras initiates a multistep phosphorylation cascade that leads to the activation of ERK1 and ERK2 (47). Both ERK1 and ERK2 are known to regulate transcription of molecules that are linked to cell proliferation, survival, and transformation (47). Another important target in EGF receptor signaling is phosphatidylinositol 3-kinase (PI3K) and the downstream protein-serine/threonine kinase, Akt. Akt activation then triggers a cascade of responses from cell growth, survival and motility (47).

Cell culture experiments have shown that tocotrienol treatment causes a reduction in MAPK mitogenic signaling in preneoplastic and neoplastic mammary epithelial cells (34, 43, 48). Further findings suggest that tocotrienols indirectly attenuate EGF-dependent MAPK mitogenic signaling by inhibiting early events involved in EGF receptor stimulation of cAMP production (34). Additionally, growth inhibitory concentrations of tocotrienols were found to inhibit EGF-dependent PKCα translocation from the cytosolic to membrane fraction (43). Such finding suggests that the antiproliferative effects of tocotrienols are mediated, at least in part, by a reduction in PKCα activation (43).

Studies have also demonstrated that tocotrienols are potent inhibitors of PI3K/Akt activation (49). Highly malignant +SA mouse mammary epithelial cells exposed to various concentrations of γ-tocotrienol displayed a dose-dependent suppression in +SA mammary tumor cell growth and a corresponding decrease in the phosphorylated Akt (activated) levels (50). Other studies showed that γ-tocotrienol treatment had no direct inhibitory effect on Akt or PI3K activity, indicating that the inhibitory effects of tocotrienol occurred upstream of these enzymes (50). Furthermore, γ-tocotrienol was found to have little effect on ErbB1 receptor tyrosine phosphorylation, but did cause a significant decrease in the ErbB3 tyrosine phosphorylation. Such findings strongly suggest that the antiproliferative effects of γ-tocotrienol in +SA cells are mediated by suppression in ErbB3 activation and subsequent reduction in PI3K/Akt mitogenic signaling (50). Subsequent studies showed that treatment with growth inhibitory doses of γ-tocotrienol cause a large decrease in ErbB3, ErbB4, and to a lesser extent ErbB2 receptor levels, and EGF-dependent ErbB2-4 tyrosine phosphorylation (activation), but had no effect on ErbB1 receptor levels or activation (20, 21). This same treatment 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/Akt, but not MAPK mitogenic signaling (20, 21).

Additional studies showed that the antiproliferative effects of tocotrienols also include the suppression of the synthesis of isoperenoid intermediates from the mevalonate biosynthesis pathway (23). These intermediates are thought to be involved in the prenylation of several signal transduction proteins including Ras proteins, which are essential for mitogenic signaling (23, 42). The rate limiting enzyme in the mevalonate pathway is 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase. This pathway provides essential intermediates required for the anchorage of signaling proteins to the membrane in close contact with various membrane bound receptors (51).

Additional studies showed that tocotrienol treatment decreased NFκB transcriptional activity. NFκB proteins constitute an inducible family of transcription factors that have been implicated in the regulation of cell proliferation, cell survival, tumor development as well as malignant transformation (52-55). EGF-induced activation
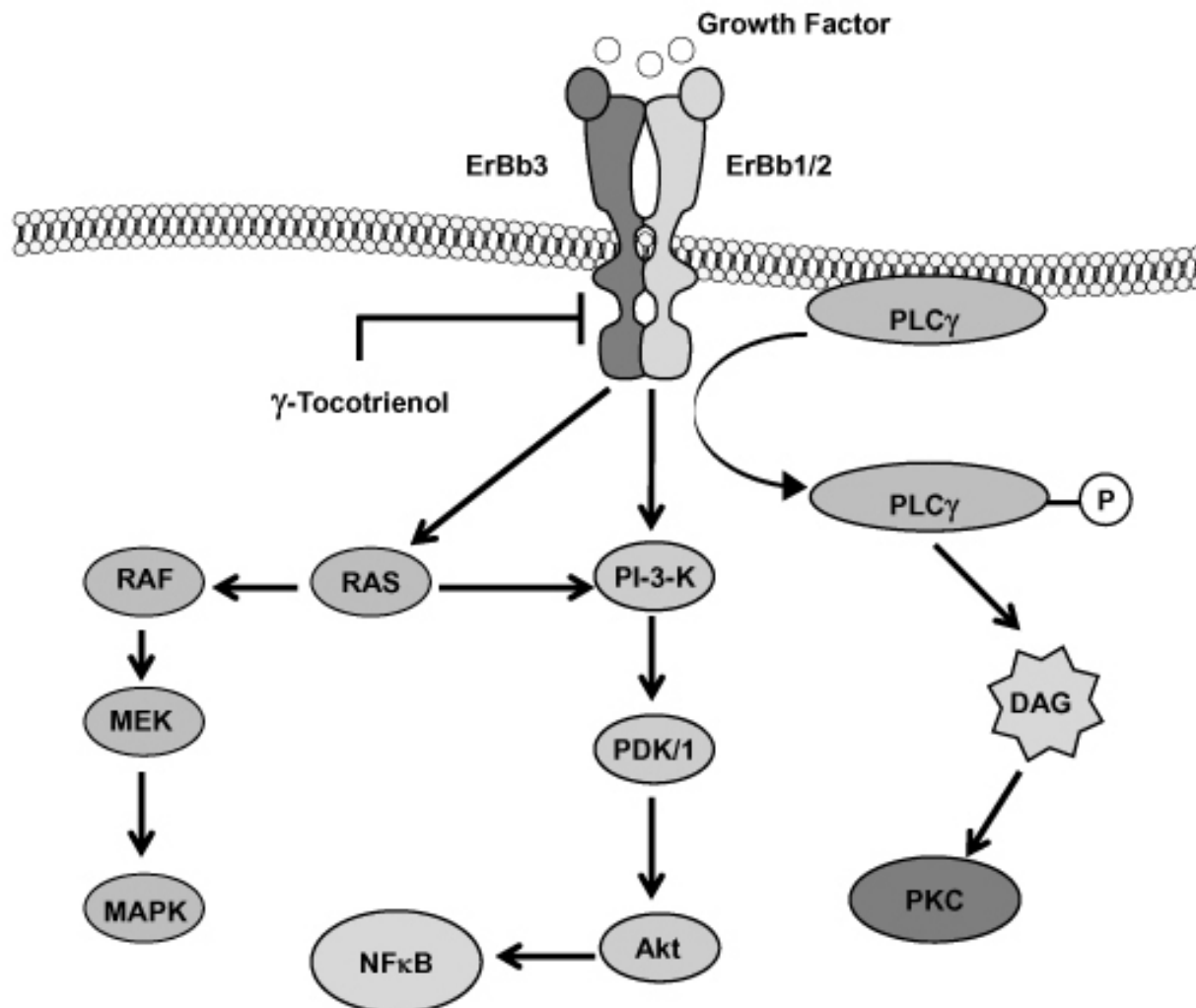
Figure 2. Summary of major mitogen-induced mitogenic signaling pathways modulated by γ-tocotrienol.

of the PI3K/Akt mitogenic pathway has been shown to enhance NFκB transcriptional activity (53, 54). NFκB exists as a heterodimer composed of the p65 and p50 subunits, and it is the p65 subunit that contains the transcriptional activation domain required for initiating gene transcription (52). The IκB inhibitory proteins bind to NFκB and suppress transcriptional activity by preventing its translocation for the cytoplasm to the nucleus (52). EGF-dependent activation of PI3K and Akt has been shown to stimulate the activation of IκB kinase (IKK), an enzyme that phosphorylates IκB, which promotes IκB degradation and subsequently NFκB activation (52, 54, 56). Experimental evidence suggests that the inhibitory effects of tocotrienol on NFκB activation, appear to result from a reduction in IKK-α/β and enhanced IκB binding and suppression of NFκB transcriptional activity. Since Akt and NFκB play a critical role in development and progression of mammary tumorigenesis, and overexpression of activated Akt and NFκB is associated with the development of resistance to chemotherapy in a majority of breast cancer cases (52, 54-56), these findings

suggest that tocotrienols may have potential value as chemotherapeutic agents against multidrug resistant forms of breast cancer. The intracellular targets of γ-tocotrienol in suppressing mitogenic signaling in +SA mammary tumor cells are shown in Figure 2.

6. MECHANISMS MEDIATING THE APOPTOTIC EFFECTS OF TOCOTRIENOLS

Apoptosis or programmed cell death is an essential process in embryogenesis, tissue growth, differentiation, and homeostasis as a protective mechanism to remove superfluous or malfunctioning cells from the organism (57). Excessive cell death can result in degenerative diseases like Alzheimer and Parkinson, whereas defects in apoptosis can result in excessive cell growth in diseases like cancer (57). Apoptosis can be induced by several signal transduction pathways that are tightly regulated and linked to other cellular events such as inflammatory responses and proliferation (57). Since apoptosis is well characterized and highly regulated, the
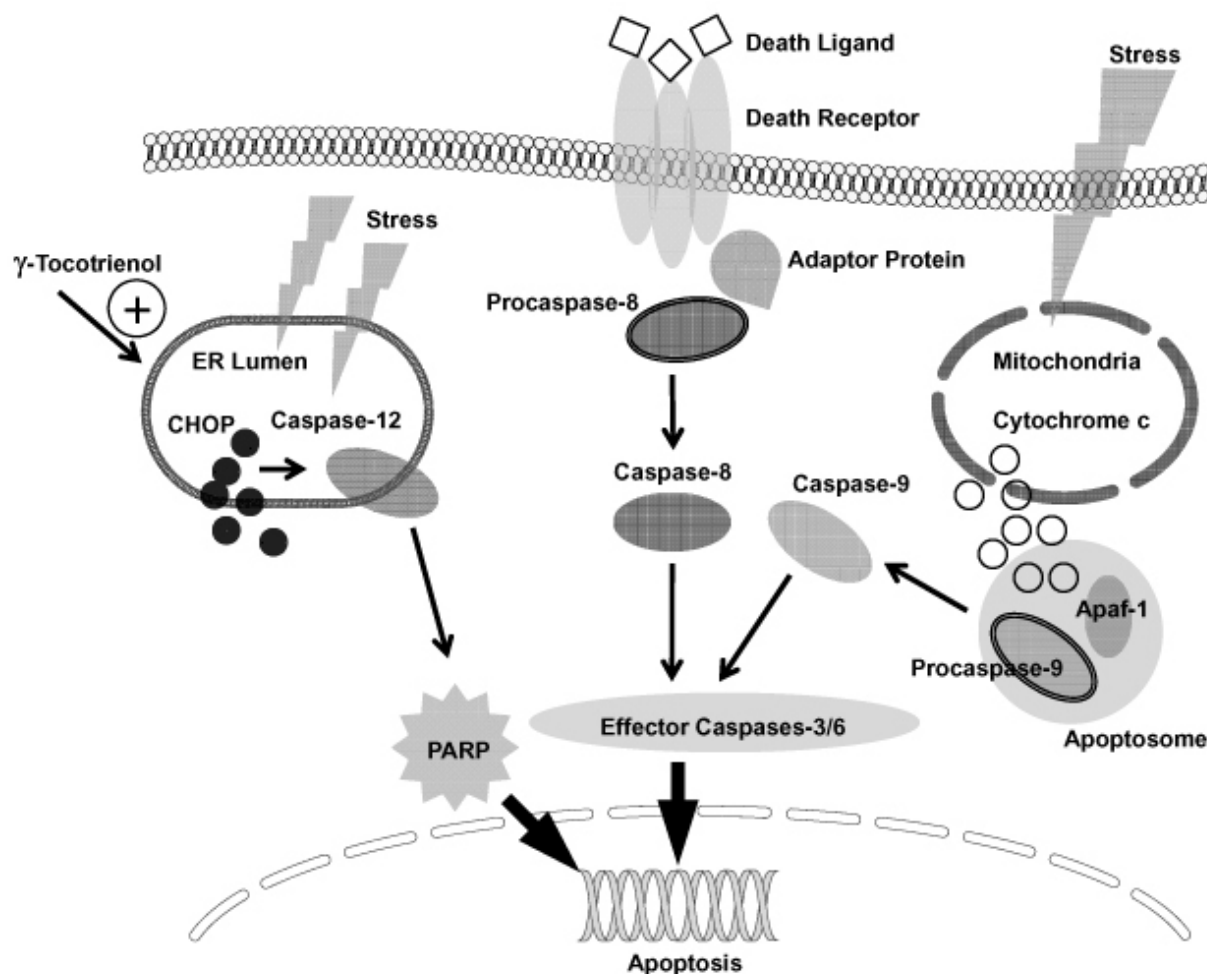
Figure 3. Summary of major apoptotic signaling pathways modulated by γ-tocotrienol.

various signaling components involved in mediating apoptosis makes attractive targets for therapeutic intervention (58). Various types of cellular stress stimuli have been shown to trigger apoptosis, including chemotherapeutic agents, irradiation, oxidative stress, and endoplasmic reticulum (ER) stress (58).

Caspases, a family of cysteine proteases, act as common death effector molecules in apoptosis (58). Caspases exist as inactive proenzymes and upon activation, cleave various substrates within the cytoplasm or nucleus (58). This leads to many of the morphologic features of apoptotic cell death including polynucleosomal DNA fragmentation, loss of overall cell shape, and nuclear shrinking (58). During apoptosis caspases are activated by different mechanisms. There are two basic types of caspases (34). Initiator caspases exclusively cleave and activate other caspases, and effector caspases that cleave other proteins and are responsible for most features of apoptosis (34). Various mechanisms can initiate caspase activation. One such mechanism is mediated by death receptor activation. Stimulation of death receptors within the tumor necrosis factor (TNF) receptor superfamily, such

as Fas or TRAIL receptors by their respective ligands results in receptor aggregation and recruitment of the adaptor molecule FADD and procaspase-8 to form the death inducing signaling complex (DISC) (58). Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases (58). A second mechanism for initiating apoptosis is mitochondrial stress. Caspase activation in this pathway is initiated by the release from the mitochondrial intermembrane space of pro-apoptogenic factors such as cytochrome c and apoptosis inducing factor (AIF) (58). The release of cytochrome c into the cytosol results in caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex (58). A third mechanism for initiating apoptosis is endoplasmic reticulum (ER) stress. The ER has been primarily recognized as a compartment for protein folding and assembly, a pool of free calcium, and a site for lipid and sterol biosynthesis (59). The ER ensures that only correctly folded proteins can be transported out of the ER while unfolded or misfolded proteins are retained in the ER and eventually degraded (59). ER stress activates a set of signaling pathways termed the unfolded protein response

(UPR) (60). If the stressed cells fail to adapt to and recover from ER stress through the UPR-mediated survival programs, the UPR will initiate apoptotic pathways to remove the stressed cells (59). The intracellular targets of γ-tocotrienol in activating apoptosis in +SA mammary tumor cells are shown in Figure 3.

Numerous reports related to the role of tocotrienols in mediating cancer cell apoptosis have been published. Earlier studies in +SA mammary tumor cells had demonstrated involvement of caspase-8 and -3 activation (61). In these studies, treatment with 20 μM γ-tocotrienol increased the relative levels of cleaved (active) p20 subunit of caspase-8 and cleaved (active) p18 subunit of caspase-3 throughout 24 hours treatment duration (61). In addition, tocotrienol treatment caused a corresponding decrease in intracellular FLIP levels, an antiapoptotic protein that inhibits caspase-8 activation (49, 62). Studies also showed that tocotrienol-induced reductions in FLIP levels was directly associated with a rapid decrease in PI3K/PDK-1/Akt pathway signaling and Akt activity (49, 62). Subsequent studies were conducted to determine the mechanism mediating tocotrienol-induced caspase-8 activation (62). Interestingly, treatment of +SA cells with 100 ng/mL TNFα, 100 ng/mL Fas ligand (FasL), 100 ng/mL TRAIL, or 1 μg/mL apoptosis-inducing Fas antibody had all failed to induce death in examined cancer cells (62). These data indicated that this mammary tumor cell line is resistant to death receptor-induced apoptosis (62). Additionally, cytotoxic treatment with 20 μM γ-tocotrienol had no effect on total membrane or cytosolic levels of Fas, FasL, or FADD and did not also induce translocation of Fas, FasL, or FADD from the cytosolic to the membrane fraction (62). Such results provided the evidence that γ-tocotrienol-induced caspase-8 activation is unlikely to be associated with death receptor apoptotic signaling in +SA mammary tumor cells (62, 63).

Other studies showed that tocotrienol-induced apoptosis in these +SA mammary tumor cells was not associated with a disruption or loss of mitochondrial membrane potential, or the release of cytochrome c into the cytoplasm (64). Interestingly, apoptotic +SA cells showed a paradoxical decrease in mitochondrial levels of anti-apoptotic proteins Bcl-2 and Bcl-x$_L$, suggesting that mitochondrial membrane stability and integrity might be enhanced for a limited period of time following acute tocotrienol treatment (64). In contrast, characterization of tocotrienol-induced apoptosis in MDA-MB-231 breast cancer cells demonstrated the activation of mitochondrial-mediated death pathway (65). In this study, γ-tocotrienol induced a collapse in mitochondrial membrane potential and release of cytochrome c from the mitochondria, but the expression of Bax and Bcl-2 (mRNA and protein) did not change, and poly- (ADP-ribose)-polymerase (PARP) cleavage was not detected, suggesting that caspases were not involved in the γ-tocotrienol-induced apoptosis (65).

Recently, the involvement of ER stress in mediating tocotrienol apoptotic actions has been established. Studies conducted in +SA mammary tumor cells showed that treatment with 15–40 μM γ-tocotrienol induced +SA cell death in a dose-responsive manner, and these effects were associated with a corresponding increase in PARP-cleavage and activation of protein kinase-like endoplasmic reticulum kinase/eukaryotic translational initiation factor/activating transcription factor 4 (PERK/eIF2a/ATF-4) pathway, a marker of ER stress response (66). In addition, these treatments also caused a large increase in C/EBP homologous protein (CHOP) levels, a key component of ER stress mediated apoptosis that increases expression of tribbles 3 (TRB3) (66). Knockdown of CHOP attenuated γ-tocotrienol-induced PARP-cleavage, CHOP, and TRB3 expression (66). In addition, γ-tocotrienol treatment decreased levels of full-length caspase-12 levels, an indication of caspase-12 cleavage and activation. Additional studies showed that treatment of human MDA-MB-231 and MCF-7 cells with γ-tocotrienol induced cleavages of PARP as well as caspase-8, -9, and -3 (67). Additional analyses showed that γ-tocotrienol activated c-Jun NH (2)-terminal kinase (JNK) and p38 MAPK, and upregulated death receptor 5 (DR5) and CHOP (67). Silencing either JNK or p38 MAPK reduced the increase in DR5 and CHOP and partially blocked γ-tocotrienol-induced apoptosis (67). Both DR5 and CHOP upregulation were required for γ-tocotrienol-induced apoptosis, and DR5 was transcriptionally regulated by CHOP after γ-tocotrienol treatment (67). Moreover, γ-tocotrienol increased the level of other ER-stress markers (67). Taken together, these results suggest that upregulation of DR5 by γ-tocotrienol treatment is dependent on JNK and p38 MAPK activation which is mediated by ER stress (67).

7. BENEFITS OF COMBINATION THERAPY

Ideally, chemotherapy would destroy cancer cells without harming normal cells, but most traditional cancer chemotherapeutic agents are not very selective and as such, damage normal cells and cause significant adverse and/or toxic side effects. Common side effects resulting from traditional cancer chemotherapy include nausea, vomiting, loss of appetite, weight loss, fatigue, and low blood cell counts that lead to anemia and increased risk of infections, hair loss, and infertility, while other side effects are associated with specific types of anticancer drugs. Some drugs may cause organ damage such as in the lungs, heart, or liver, while other drugs may increase the risk of developing other forms of cancer such as leukemia several years after treatment. Recently, a new approach for limiting side effects and enhancing therapeutic outcome utilizes drugs that target specific pathways and processes vital to the cancer cell growth and survival. Furthermore, cancer chemotherapy has been found to be more effective when drugs that work by different mechanisms given in combination at optimal doses, while at the same time reducing or eliminating the adverse or toxic side effects associated with high dose monotherapy. Furthermore, combination chemotherapy is used sometimes not as a cure, but as a means to reduce symptoms and prolong life in patients with advanced

forms of cancer that cannot be treated with radiation therapy or surgery.

8. COMBINATION TREATMENT OF TOCOTRIENOLS WITH TRADITIONAL CANCER CHEMOTHERAPY

The rationale for using tocotrienols in combination therapy is based on the principle that resistance to any single agent could be overcome by using multiple agents with different or complimentary mechanisms of action. Preliminary studies in our laboratory were conducted using subeffective doses γ-tocotrienol in combination with different doses of traditional chemotherapeutic agents including: doxorubicin and cisplatin (cell cycle non-specific); paclitaxel and vinblastine (microtubule inhibitors, G_2/M phases); irinotecan (topoisomerase I inhibitors, S-phase); methotrexate and 5-fluorouracil (antimetabolites, S-phase), in order to assess if combination therapy induced an additive, synergistic or antagonistic anticancer response. Results from these studies indicated that combined treatment of γ-tocotrienol with any of these chemotherapeutic agents, for the most part, resulted in an additive, but not synergistic anticancer response (data not published).

Other experiments demonstrated the synergistic anticancer effects of vitamin E in combination with other anticancer agents. Investigators showed the inhibitory effects of the TRF and tamoxifen on growth of the estrogen receptor positive MCF-7 and the estrogen receptor negative MDA-MB-435 cells (68). In addition to considering the individual effects of these compounds, investigators considered the growth inhibitory effects of equal mixtures (w/w) of tamoxifen with TRF or the three individual tocotrienols within TRF. In MCF-7 cells, concentrations of tamoxifen required for 50% inhibition of cell proliferation were greatly reduced when combined with equal mixtures of γ- or δ-tocotrienol, respectively. However, combination of α-tocotrienol with tamoxifen slightly increased the concentration of tamoxifen needed for 50% inhibition of cell proliferation in this same tumor cell line (68). Tamoxifen inhibited growth of estrogen receptor negative MDA-MB-435 cells at a much higher concentration than that required for MCF-7 cells and combined treatment with TRF, α-, γ-, or δ-tocotrienol greatly reduced the dose of tamoxifen required to inhibit cell growth by 50%. Other reports showed similar effects of combining tocotrienols to tamoxifen therapy (39). Treatment with TRF and individual α-,γ-, or δ-tocotrienols can also inhibit the growth of the human breast cancer cell line ZR-75–1, in the presence as well as in the absence of estradiol (39). However, in the same cell line, α-tocopherol had no effect on the growth of the ZR-75–1 cells in either absence or presence of estradiol. Further experiments found that TRF inhibited the growth of ZR-75–1 cells in the presence of tamoxifen (10^{-7}M and 10^{-8}M). Individual tocotrienol fractions (α-, γ-, and δ-tocotrienols) also inhibited the growth of ZR-75–1 cells in the presence of 10^{-8}M estradiol and 10^{-8}M of the pure antiestrogen ICI 164 384 (39). Additional studies showed that combined treatment with γ-

tocotrienol, resveratrol, and epigallocatechin gallate (EGCG) significantly inhibited growth of estrogen receptor positive MCF-7 breast cancer cells (69). This growth suppression was mediated by G1 cell cycle arrest and reduced expression of cyclin D1 (69).

These findings indicate that use of γ-tocotrienol in combination with other therapies does not necessarily result in a synergistic anticancer response. The known anticancer mechanisms of action of γ-tocotrienol involves modulating specific intracellular pathways associated with receptor tyrosine kinase mitogenic signaling and apoptosis (28, 29, 33, 34, 41). However, these specific actions of γ-tocotrienol may not be complementary with the mechanism of action of every chemotherapeutic agent. These findings have made it clearly evident that γ-tocotrienol used in combination therapy, may not always produce a synergistic therapeutic response. Nevertheless, experimental evidence has also shown that when γ-tocotrienol is combined with the proper type of drug, profound synergistic anticancer activity can result (20-25). These findings are summarized below.

9. COMBINATION TREATMENT OF γ-TOCOTRIENOL WITH STATINS

Statins represent a class of drugs that are widely used to lower high blood cholesterol levels. However, evidence shows that statins also act as chemoprotective agents against various types of cancers (70-72), particularly breast cancer (73). Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, an enzyme catalyzing the conversion of HMGCoA to mevalonate, the rate limiting step in cholesterol biosynthesis. Statin-induced inhibition of mevalonate synthesis also reduces the synthesis of downstream non-sterol products such as farnesyl pyrophosphate. These non-sterol products are essential for the isoprenylation of intracellular second messenger mitogenic signaling proteins like Ras. Early investigations showed that HMGCoA inhibitor blockade of mevalonate synthesis induced cell cycle arrest *in vitro* (74-77) and inhibited tumor growth *in vivo* (78). Nevertheless, the use of statins in cancer trials has been greatly limited by their high-dose toxicity that is characterized by severe myopathy liver toxicity, gastrointestinal dysfunction, and even death (79, 80). In many cancer cells, HMG-CoA reductase activity has been found to be over expressed or unregulated (51). In this respect, statins (competitive inhibitors of HMG-CoA reductase) also display antitumor activity. However, clinical use of statins as anticancer agents has been limited because of high dose toxicity (51). The synergistic antiproliferative effects of γ-tocotrienol and statins is now well established (23-25). +SA mammary tumor cells were subjected to treatment with individual statins, γ-tocotrienol, or a combination of selected statins with γ-tocotrienol (23). Results showed that treatment with 3-4 μM γ-tocotrienol or 2-8 μM of individual statins, simvastatin, lovastatin, and mevastatin resulted in a significant decrease of +SA cell growth, whereas treatment with 10-100 μM pravastatin had no effect on cancer cell
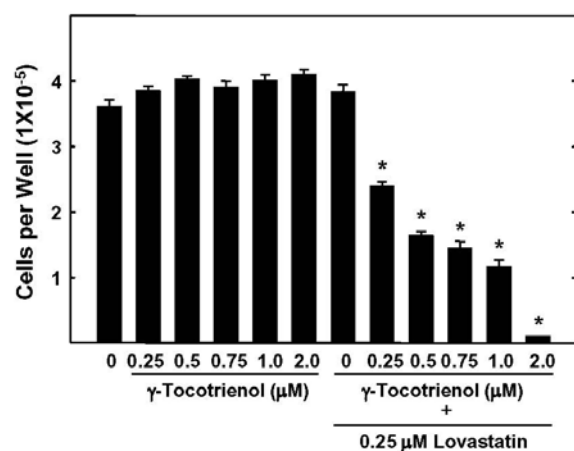
Figure 4. Effects of combined γ-tocotrienol and statin (lovastatin) treatment alone on neoplastic +SA mammary epithelial cells growth. Cells were plated at a density of 5×10^4 cells/well (6 wells/group) in 24-well culture plates and treated for 4-days. Afterwards, viable cell number was determined by MTT assay. Vertical bars indicate the mean cell count ±SEM in each treatment group. *$P < 0.05$, as compared to the vehicle-treated control group.
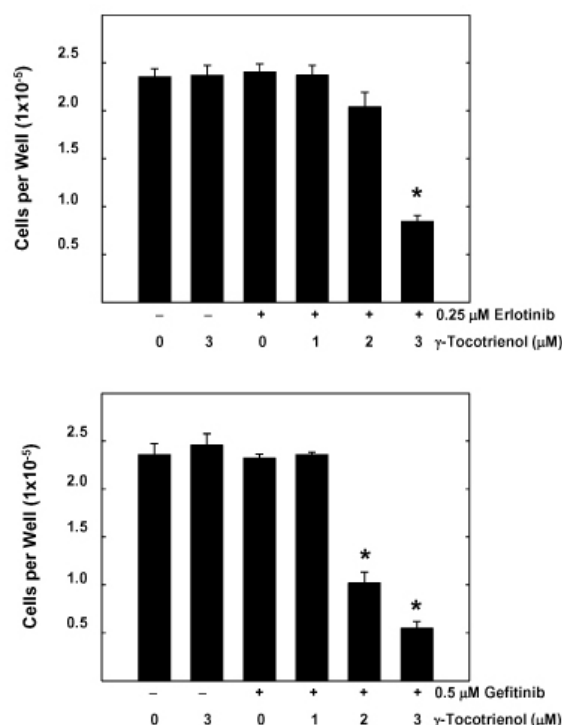

Figure 5. Effects of combined γ-tocotrienol and erlotinib or gefitinib treatment on neoplastic +SA mammary epithelial cell growth. Vertical bars indicate the viable cell count ± SEM in each treatment group. *$P < 0.05$ as compared to vehicle-treated control group.

growth (23). Combined treatment of subeffective doses (0.25 or 10 μM) of individual statins with subeffective

doses (0.25-2.0 μM) of γ-tocotrienol resulted in a synergistic inhibition in +SA cell proliferation (23). Additional studies showed that combined treatment of γ-tocotrienol and statins resulted in a relatively large decrease in intracellular levels of activated MAPK, JNK, p38, and Akt (23). The synergistic antiproliferative effects of combined γ-tocotrienol and lovastatin on +SA mammary tumor cell growth is shown in Figure 4.

10. COMBINATION TREATMENT OF TOCOTRIENOLS WITH RECEPTOR TYROSINE KINASE INHIBITORS

Recently, agents have been developed for the treatment of cancer that inhibit ErbB/HER receptors, including monoclonal antibodies, tyrosine kinase inhibitors, immunotoxin conjugates, antisense oligonucleotides, and bispecific antibodies (81). Among these agents, monoclonal antibodies and tyrosine kinase inhibitors have advanced to clinical trials (81). However, the clinical usefulness of such agents as monotherapy has been found to be limited due to the ability of cancer cells to circumvent the actions of these drugs through the cooperation of different ErbB/HER receptors (33, 34). As a result, recent research has focused on the effect of combinational treatments directed against multiple ErbB/HER receptors. Initial studies showed improved therapeutic responsiveness with combined treatment of the tyrosine kinase inhibitor, gefitinib, with the monoclonal antibody, cetuximab, as compared to monotherapy targeting only a single receptor subtype in a panel of human cancer cell lines (47). In addition, recent experimental evidence has demonstrated that γ-tocotrienol treatment potentiates the anticancer effects of the tyrosine kinase inhibitors erlotinib and gefitinib (20). In these studies, treatment of malignant +SA mammary epithelial cells with 3.5 μM γ-tocotrienol, 0.5 μM erlotinib or 1.0 μM gefitinib alone, significantly inhibited +SA tumor cell growth (20, 21). Interestingly, combined treatment with subeffective doses of erlotinib (0.25 μM) or gefitinib (0.5 μM) with subeffective doses of γ-tocotrienol (0.5-3.0 μM) synergistically inhibited growth and induced apoptosis in mammary tumor cancer cells (20, 21). This same treatment 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 the MAPK signaling (20, 21). The synergistic antiproliferative effects of combined γ-tocotrienol and erlotinib or gefitinib on +SA mammary tumor cell growth is shown in Figure 5.

11. COMBINATION TREATMENT OF TOCOTRIENOLS WITH CYCLOOXYGENASE-2 (COX-2) INHIBITORS.

Similarly, synergistic antiproliferative effects were observed in mammary tumor cells following treatment with combined low dose celecoxib (cyclooxygenase-2 inhibitor) and γ-tocotrienol treatment (22). These effects were found to be associated with a reduction in prostaglandin E_2 synthesis, and decrease in
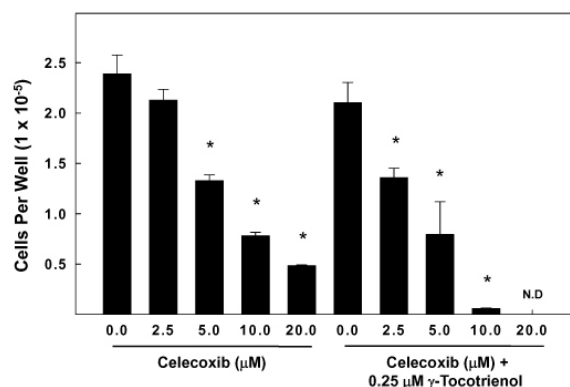
Figure 6. Antiproliferative effect of γ-tocotrienol and celecoxib alone and in combination on neoplastic +SA mouse mammary epithelial cell growth. Cells were initially plated at a density of 5×10^4 cells/well (6 wells/group) in 24-well plates and exposed to treatments for a 4-day culture period. Vertical bars indicate the mean cell count ±SEM in each treatment group. *$P < 0.05$ as compared to the vehicle-treated control group.

COX-2, phospho-Akt (active), and phospho-NFκB (active) levels (22). Treatment with high doses of γ-tocotrienol or celecoxib alone inhibited Akt activation and downstream signaling and NFκB activation. Similar treatment with γ-tocotrienol also decreased concentration and activation of ErbB2-4 receptors, whereas celecoxib only inhibited ErbB2-4 receptor activation. In contrast, combined treatment with subeffective doses of γ-tocotrienol and celecoxib resulted in a large decrease ErbB2-4 receptor levels and activation, a decrease in PGE_2 levels, and a corresponding increase in prostaglandin EP2 and EP4 receptor levels. Combined treatment also induced an increase in the prostaglandin catabolizing enzyme, PGDH. The synergistic anticancer effects of combined low dose γ-tocotrienol and celecoxib treatment in +SA mammary tumor cells are mediated by COX-2-dependent mechanisms associated with a suppression in PGE2 levels, as well as, COX-2-independent mechanisms associated with a reduction in ErbB2-4 receptor levels, activation, and subsequent reduction in downstream Akt and NFκB mitogenic signaling. The synergistic antiproliferative effects of combined γ-tocotrienol and celecoxib on +SA mammary tumor cell growth are shown in Figure 6.

12. CONCLUSIONS

Recent studies demonstrate the synergistic anticancer effects of combined γ-tocotrienol treatment with some, but not all chemotherapeutic agents. Combination therapy is most effective when the anticancer mechanism of action of γ-tocotrienol compliments the mechanism of action of the other drug. Specifically, the synergistic effects of γ-tocotrienol and statins was found to be due to the cooperative action of γ-tocotrienol to cause a down-regulation in HMGCoA reductase levels and statins to directly inhibit HMGCoA activity. Since this synergistic anticancer effect was observed using very low doses of

each agent, these finding suggest that combined low dose treatment with γ-tocotrienol and statins may have potential value in the treatment of breast cancer without causing myotoxicity that is associated with high dose statin monotherapy. Likewise, the combination of γ-tocotrienol and receptor tyrosine kinase inhibitors synergistically inhibits the growth of mammary tumor cells by suppressing inhibiting multiple members of the ErbB receptor family and subsequent EGF-dependent Stat and Akt mitogenic signaling. Since the clinical usefulness of receptor tyrosine kinase inhibitors, such as erlotinib and gefitinib, which target a single ErbB receptor subtype, has been limited because of the heterodimer formation between different ErbB family members, these findings strongly suggest that tocotrienols used in combination with these agents targets multiple ErbB receptors and overcomes heterodimer cooperation. These findings strongly suggest the potential use of combination therapy of γ-tocotrienol with receptor tyrosine kinase inhibitors in order to enhance therapeutic responsiveness in breast cancer patients with aberrant EGF receptor signaling. Similarly, the synergistic antiproliferative effects of combined low dose γ-tocotrienol and celecoxib against mammary tumor cells have been shown to be mediated by both COX-2-dependent and COX-2-independent mechanisms. The COX-2 dependent anticancer effects of both γ-tocotrienol and celecoxib result from suppression in COX-2 activity and PGE2 production, while the COX-2-independent mechanisms result from the attenuation of EGF-dependent activation ErbB receptors and second messenger mitogenic signaling. Taken together these findings demonstrate the synergistic antiproliferative effects of combined low dose treatment of γ-tocotrienol with other chemotherapeutic agents, and strongly suggest that combination therapy may provide significant health benefits in the prevention and/or treatment of breast cancer in women, while at the same time avoiding tumor resistance or toxic effects associated with high dose monotherapy.

13. ACKNOWLEDGEMENTS

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