

γ -Tocotrienol-induced autophagy in malignant mammary cancer cells

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Abstract

γ -Tocotrienol, a member of the vitamin E family of compounds, displays potent antiproliferative and cytotoxic effects in a variety of cancer cell types at treatment doses that have little or no effect on normal cell viability or growth. Autophagy is a tightly regulated lysosomal self-digested process that can either promote cell survival or programmed cell death, but the role of autophagy in mediating γ -tocotrienol-induced cytotoxicity in breast cancer is not presently completely understood. Mouse (+SA) and human (MCF-7 and MDA-MD-231) mammary tumor cells lines were exposed to 0–40 μ mol/L γ -tocotrienol for a 24 h treatment period. γ -Tocotrienol treatment caused a relatively large increase in the accumulation of monodansylcadaverine (MDC)-labeled vacuoles, a marker of autophagosome formation, in all tumor cell lines. Results also showed that γ -tocotrienol treatment induced an increased conversion of microtubule-associated protein, 1A/1B-light chain 3, from its cytosolic form (LC3B-I) to its lipidated form (LC3B-II), increased Beclin-1 levels, and increased acridine orange staining as determined by flow cytometry analysis, providing further evidence of γ -tocotrienol-induced autophagy in these mammary cancer cell lines. In contrast, similar treatment with γ -tocotrienol was not found to increase autophagy marker expression in immortalized mouse (CL-S1) and human (MCF-10A) normal mammary epithelial cell lines. Treatment with γ -tocotrienol also caused a reduction in PI3K/Akt/mTOR signaling and a corresponding increase in the Bax/Bcl-2 ratio, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) levels in these cancer cell lines, suggesting that γ -tocotrienol-induced autophagy may be involved in the initiation of apoptosis. In summary, these findings demonstrate that the cytotoxic effects of γ -tocotrienol are associated with the induction of autophagy in a mouse and human mammary cancer cells.

Keywords: γ -Tocotrienol, autophagy, breast cancer, Protein kinase B (Akt), mammalian target of rapamycin

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Introduction

Autophagy is a tightly regulated cellular self-catabolic process in which cytoplasmic components are sequestered in double membrane vesicles called autophagosomes, which then fuse with lysosomes to form autophagolysosomes.^{1–4} Autophagy provides an efficient means for the degradation and recycling of excessive or damaged cellular organelles and proteins, and can be involved in either promoting cell survival or initiating programmed cell death.^{1–4} The execution and regulation of autophagy is dependent on autophagy-related gene (ATG) products that exist in all organisms and act to initiate the self-digestion of intracellular cytoplasm, proteins, and organelles.^{5,6} Microtubule-associated proteins called light chain 3 (LC3) or yeast Atg8 exist in two forms. During basal conditions this protein exists in its cytosolic form called LC3B-I. However, during autophagy LC3B-I is converted to its lipidated form called LC3B-II, which is associated with the autophagosome and leads to

the formation of autophagic vacuoles.⁷ Beclin-1 is a mammalian ortholog of yeast Atg6, and forms a complex with class III phosphatidylinositol 3-kinase (PI3K) that mediates the localization of autophagy targeted proteins into the autophagic vesicles.⁸ Studies have shown that enhanced or overexpression of Beclin-1 is associated with the suppression of tumorigenesis and plays an important role in autophagic mediated programmed cell death.⁹

Vitamin E represents a family of compounds that is further divided into two subgroups called tocopherols and tocotrienols. Tocopherols and tocotrienols are very similar in their chemical structure, but only tocotrienols display potent anticancer activity at treatment doses that have little or no effect on normal cell growth or viability.^{10–12} Apoptosis requires activation of initiator caspases that subsequently activate effector caspases, which then trigger downstream processes associated with cellular self-destruction.¹³ Previous studies have shown that γ -tocotrienol

treatment induced programmed cell death both a time- and dose-dependent manner, characterized by an increase in caspase activation and poly (ADP-ribose) polymerase (PARP) cleavage.^{13,14} Numerous studies have shown that tocotrienol-induced apoptosis can be mediated by the intrinsic, extrinsic, and/or endoplasmic reticulum stress apoptotic pathways, depending on the particular cell type being investigated.¹⁵ In addition, tocotrienol-induced apoptosis was found to be associated with a significant decrease in PI3K/protein kinase B (Akt)/mTOR signaling.^{16,17}

Previous investigations have shown tocotrienol treatment induces autophagy in various cell types.^{18–21} However, the exact role of autophagy in mediating γ -tocotrienol-induced cytotoxicity against breast cancer cells is not presently understood. Treatment with γ -tocotrienol was found to induce autophagy and apoptosis in rat pancreatic stellate¹⁸ and prostate cancer cells,²¹ whereas other studies have shown that γ -tocotrienol treatment was cardioprotective and prevented apoptosis in ischemic cardiomyocytes.²⁰ In this present study, experiments were conducted to characterize γ -tocotrienol-induced autophagy in highly malignant mouse (+SA), and human estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) malignant mammary cancer cell lines to determine its role in tocotrienol-induced cytotoxicity. In addition, the effects of γ -tocotrienol in promoting the conversion of LC3B-I to LC3B-II, Beclin-1 expression, and mitogenic and apoptotic signaling were also examined.

Materials and methods

Reagents and antibodies

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Purified γ -tocotrienol (>98% purity) was generously provided by First Tech International Ltd. (Hong Kong). Antibodies for LC3B, Beclin-1, Akt, phosphorylated-Akt, Bcl-2, Bax, cleaved caspase-3, cleaved PARP, and phosphorylated-mammalian target of rapamycin (phosphorylated-mTOR) were purchased from Cell Signaling Technology (Beverly, MA). Antibody for α -tubulin was purchased from Calbiochem (San Diego, CA). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA).

Cell lines and culture conditions

The mouse +SA mammary epithelial cell line was originally isolated from a mammary adenocarcinoma that developed spontaneously in a BALB/c female mouse.^{22–24} The +SA cell line was characterized as being highly malignant, estrogen independent, and displayed anchorage-independent growth when cultured in soft agarose gels. CL-S1 cells are preneoplastic “normal” mouse mammary epithelial cells that were derived from the hyperplastic D1 cell line that spontaneously arose in BALB/c mice. The CL-S1 cell line was immortal in culture and formed only hyperplastic nodules, not solid tumors, upon transplantation into the mouse mammary gland fat pad.^{10,23,25} Cell culture and experimental procedures have been previously described in detail (McIntyre, 2000 #753; Akl, 2012 #1494).

Briefly, +SA and CL-S1 cells were maintained in serum-free defined control media consisting of Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 5 mg/mL bovine serum albumin (BSA), 10 μ g/mL transferrin, 100 U/mL soybean trypsin inhibitor, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL EGF, and 10 μ g/mL insulin. The estrogen-receptor negative MDA-MB-231 and estrogen-receptor positive MCF-7 breast carcinoma cell lines and the MCF-10A, an immortalized “normal” human mammary epithelial cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 and MCF-7 breast cancer cells were cultured in modified Dulbecco's modified Eagle Medium (DMEM)/F12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 μ g/mL insulin. MCF-10A cells were maintained in DMEM/F12 supplemented with 5% horse serum, 0.5 μ g/mL hydrocortisone, 20 ng/mL EGF, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10 μ g/mL insulin. All cells were maintained at 37°C in an environment of 95% air and 5% CO₂ in humidified incubator. For subculturing, cells were rinsed twice with sterile Ca²⁺ and Mg²⁺-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 containing media, and counted using a hemocytometer.

Experimental treatment

To dissolve the highly lipophilic γ -tocotrienol in aqueous culture media, a stock solution of γ -tocotrienol was prepared by suspending it in a solution of sterile 10% BSA as described previously.^{10,11,26} Briefly, an appropriate amount of γ -tocotrienol was dissolved in 100 μ L of absolute ethyl alcohol and then added to a small volume of sterile 10% BSA in water and incubated overnight at 37°C. This stock solution was used to prepare various concentrations of the treatment media. Ethanol was added to all treatment groups in a given experiment such that the final concentration of ethanol never exceeds 0.1%.

Measurement of viable cell number

For growth studies, cells (+SA, CL-S1, MCF-7, MDA-MB-231 and MCF-10A) were initially plated at a seeded density of 5×10^3 cells/well in 96-well culture plates (6 wells/group) and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given fresh media containing various doses of γ -tocotrienol. Cells in all groups were fed fresh treatment media every other day for a 96 h treatment period. For cytotoxicity studies, all cells were initially plated at an initial density of 1×10^4 cells/well in 96-well culture plates and maintained on control media. After a three-day culture period (approximately 70% confluent), cells were then divided into different treatment groups (6 wells/group) and treated with 0–40 μ mol/L γ -tocotrienol for a 24-h culture period. Afterwards, viable cell number was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, as described

previously.^{27,28} Briefly, media was replaced in all treatment groups with fresh control media containing 0.41 mg/mL MTT. After a 3-h incubation period, medium was removed, MTT crystals were dissolved in dimethyl sulfoxide (DMSO; 100 μ L/well), and optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company, Meriden, CT) zeroed against a blank prepared from cell-free medium. Numbers of cells/well were calculated against a standard curve prepared by plating known cell densities, as determined by hemocytometer, in triplicate at the beginning of each experiment.

Detection of autophagic vacuoles with monodansylcadaverine (MDC)

All cells (+SA, CL-S1, MCF-7, MDA-MB-231 and MCF-10A) were initially plated at a density of 7×10^5 cells/well in six-well culture plates and maintained on control media for three days (approximately 70% confluent). Afterwards, cells were then divided into different treatment groups and treated with 0–40 μ mol/L γ -tocotrienol for a 24-h culture period. The next day, all cells were treated with 0.05 mmol/L MDC and 0.2 μ mol/L ethidium bromide at 37°C for 15 min.²⁹ Afterwards, cells were washed with PBS and then lysed in 500 μ L of 10 mmol/L Tris-HCl, pH 8, 0.1% triton X-100.³⁰ Intracellular MDC accumulation in each well was measured by fluorescence plate reader (FLx800, BioTek, Winooski, VT) with excitation wavelength 360 nm and emission filter 525 nm. Total DNA content per well was determined by with excitation wavelength 530 nm and emission filter 590 nm. Normalization of MDC fluorescence intensity was performed by dividing MDC fluorescence by total DNA for each well. The MDC fluorescence/DNA was expressed as specific activity (arbitrary units)/well.

Visualization of MDC-labeled vacuoles

Malignant cells (+SA, MCF-7 and MDA-MB-231) were initially plated at a density of 5×10^4 cells/chamber in eight-chamber glass culture slides and maintained on control media for a 48-h culture period (approximately 70% confluent). Afterwards, cells were divided into different groups and treated with 0–40 μ mol/L γ -tocotrienol for 24 h. The next day, all cells were treated with 0.05 mmol/L MDC in PBS at 37°C for 15 min. Cells were then washed with PBS and then the slides were mounted with Vectashield medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories IN., Burlingame, CA). The fluorescence images were captured using LSM Pascal confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY).

Quantification of acidic vesicular organelles (AVOs) with acridine orange staining

Malignant cells (+SA, MCF-7 and MDA-MB-231) were planted at the density of 1×10^6 cells/100 mm culture dish and maintained in control media for a three-day culture period (approximately 70% confluent). Cells were then divided into different groups and treated with 0–40 μ mol/L γ -tocotrienol for a 24-h culture period.

The next day, cells were then stained with acridine orange (1 μ g/mL) for 15 min in culture.³¹ Afterwards, the cells were isolated with trypsin and washed in PBS. The cell pellets were resuspended in 1.0 mL of PBS and analyzed on a BD FACSCalibur flow cytometer with BD CellQuest Pro program (BD Bioscience, San Jose, CA).

Western blot analysis

All cells (+SA, CL-S1, MCF-7, MDA-MB-231 and MCF-10A) were planted at the density of 1×10^6 cells/100 mm culture dish and maintained in control media for three days (approximately 70% confluent). Cell were then divided into different groups and treated with 0–40 μ mol/L γ -tocotrienol for a 24-h culture period. Afterwards, the cells were isolated with trypsin, washed in PBS, then whole cell lysates were prepared as described previously, in Laemmli buffer.^{32,33} Protein concentration in each sample was determined using Bio-Rad protein assay kit (Bio Rad, Hercules, CA). Equal amounts of protein (30 μ g/lane) from each sample were then subjected to electrophoresis through 7.5–20% sodium dodecyl sulfate-polyacrylamide minigels. Proteins from minigels were transplotted at 30 V for 12–16 h at 4°C onto a single 8" \times 6.5" polyvinylidene fluoride (PVDF) membrane (PerkinElmer Life Sciences, Wellesley, MA) in a Trans-Blot Cell (Bio-Rad) according to the methods of Towbin et al.³⁴ These PVDF membranes were then blocked with 2% BSA in 10 mmol/L Tris-HCl containing 50 mmol/L NaCl and 0.1% Tween 20, pH 7.4 (TBST) then incubated with specific primary antibodies against LC3B, Beclin-1, Bcl-2, Bax, Akt, phosphorylated-Akt, cleaved caspase-3, cleaved PARP and phosphorylated-mTOR diluted 1:1000 to 1:5000 in 2% BSA in TBST overnight at 4°C. Membranes were incubated in primary antibodies against α -tubulin, diluted 1:5000 in 2% BSA in TBST for 2 h at room temperature. Membranes were then washed five times in TBST and incubated with respective horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in 2% BSA in TBST for 1 h at room temperature followed by washing in TBST five times. Blots were then visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL). Images of protein bands from all treatment groups within a given experiment were acquired using Kodak Gel Logic 1500 Imaging System (Carestream Health Inc., New Haven, CT). Visualization of α -tubulin was used to ensure equal sample loading in each lane. Image of protein bands were acquired and scanning densitometric analysis was performed with Kodak molecular imaging software version 4.5 (Carestream Health Inc). All experiments were repeated at least three times and a representative Western blot image from each experiment was shown in the figures.

Statistical analysis

Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A difference of $P < 0.05$ was considered statistically significant as compared with the vehicle-treated control group or as defined in the figure legends.

Results

Antiproliferative and cytotoxic effects of γ -tocotrienol

The effect of various doses of γ -tocotrienol on mouse murine malignant (+SA) and normal (CL-S1) mammary epithelial cells, and human malignant (MCF-7, MDA-MB-231) and normal (MCF-10A) mammary epithelial cells after a 96-h culture period are shown in Figure 1. Treatment with 3.5–4 $\mu\text{mol/L}$ γ -tocotrienol significantly inhibited +SA cell growth, whereas treatment with 4–8 $\mu\text{mol/L}$ and 4–10 $\mu\text{mol/L}$ γ -tocotrienol significantly inhibited MCF-7 and MDA-MB-231 cell growth, respectively, in a dose-response manner as compared with the cells in their corresponding vehicle-treated control group (Figure 1). However, treatment with similar doses of γ -tocotrienol was found to have little or no effect on the growth of normal mouse CL-S1 and human MCF-10A mammary epithelial cells (Figure 1).

The cytotoxic effects following an acute 24 h treatment exposure to various doses of γ -tocotrienol on +SA, CL-S1, MCF-7, MDA-MB-231, and MCF-10A cell viability are shown in Figure 2. Treatment with 8–24 $\mu\text{mol/L}$, 8–40 $\mu\text{mol/L}$, and 16–44 $\mu\text{mol/L}$ γ -tocotrienol significantly reduced +SA, MCF-7, and MDA-MB-231 viable cell number, respectively, as compared with their corresponding vehicle-treated control group. In contrast, treatment with similar doses of γ -tocotrienol was found to have little or no effect on the viability of normal mouse CL-S1 and human MCF-10A mammary epithelial cells (Figure 2).

γ -Tocotrienol effects on fluorescent autophagy marker intensity

Accumulation of autofluorescent MDC in mature autophagic vacuoles (autophagolysosomes) is a specific marker for autophagy. Following a 24-h treatment period with cytotoxic doses of γ -tocotrienol, MDC autofluorescent intensity was significantly increased in a dose-responsive manner in +SA, MCF-7, and MDA-MB-231 mammary cancer cells, as compared with cells in their respective vehicle-treated control groups (Figure 3). In contrast, treatment with the same doses of γ -tocotrienol had no effect on MDC autofluorescent intensity in normal mouse CL-S1 and normal human MCF-10A mammary epithelial cells (Figure 3). Representative photomicrographs of treatment effects on positive MDC autofluorescent staining in +SA (Figure 4a), MCF-7 (Figure 4b), and MDA-MB-231 (Figure 4c) breast cancer cells after a 24-h treatment period is shown in Figure 4. Treatment with γ -tocotrienol was found to increase autophagic vacuole MDC labeling within the cytoplasm and perinuclear area in a dose-dependent manner in each of the different mammary cancer cell lines (Figure 4).

Flow cytometry analysis of γ -tocotrienol effects on AVO levels

Flow cytometric analysis was performed to quantify γ -tocotrienol-induced autophagy in +SA (Figure 5a), MCF-7 (Figure 5b), and MDA-MB-231 (Figure 5c) breast cancer cells as indicated by positive acridine orange staining, a direct indicator of cellular AVO levels. As shown in

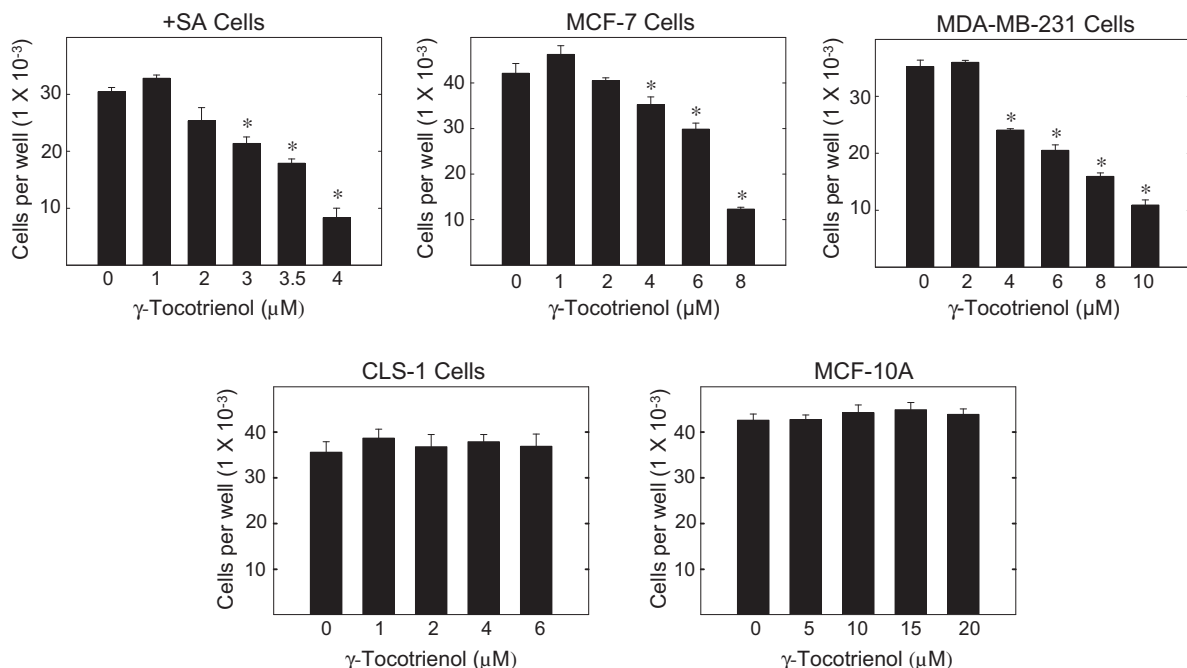


Figure 1 Antiproliferative effects of γ -tocotrienol on mouse +SA mammary tumor and human MCF-7 and MDA-MB-231 breast cancer cells, and mouse CL-S1 and human MCF-10A immortalized normal mammary epithelial cells. All cells were seeded at density of 5×10^3 cells/well (6 replicates/group) in 96-well plates and exposed to treatment media for a four-day culture period. Afterwards, viable cell count was determined using the MTT colorimetric assay. Vertical bars indicate mean cell number \pm SEM in each treatment group. * $P < 0.05$ as compared with their respective vehicle-treated control group. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

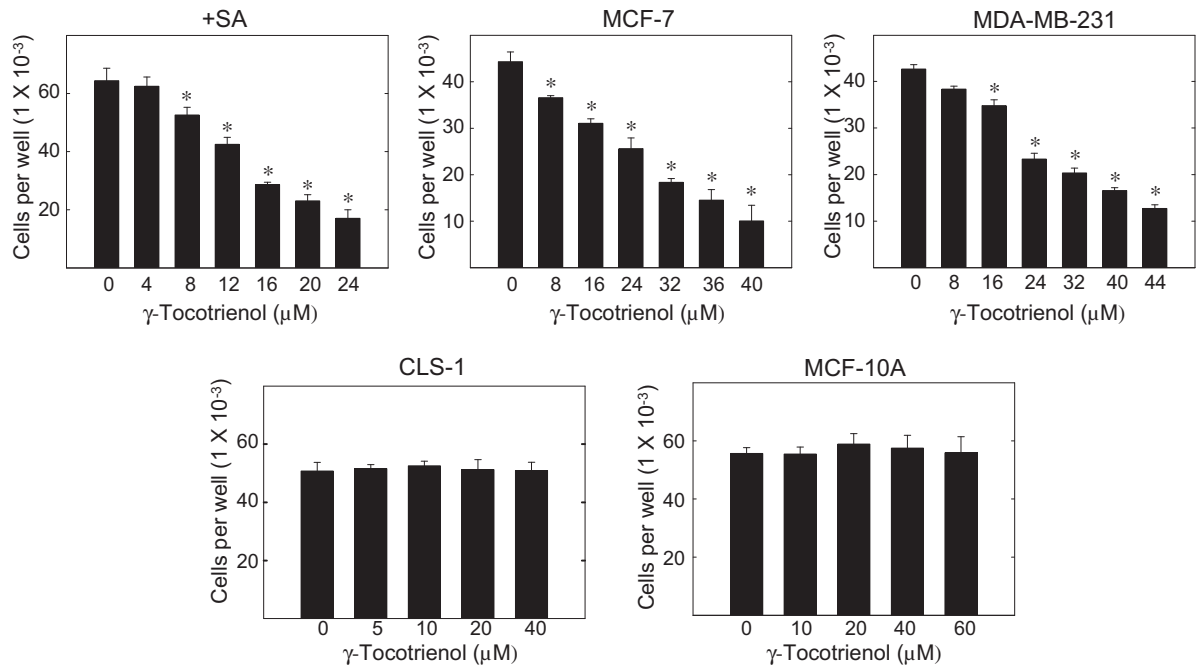


Figure 2 Cytotoxic effect of γ -tocotrienol on mouse + SA mammary tumor and human MCF-7 and MDA-MB-231 breast cancer cells, and mouse CLS-1 and human MCF-10A immortalized normal mammary epithelial cells. All cells were seeded at density of 1×10^4 cells/well (6 replicates/group) in 96-well plates and maintained on media for a three-day culture period (approximately 70% confluent). Afterwards, cells were divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. The next day, viable cell count was determined using the MTT colorimetric assay. Vertical bars indicate mean cell number \pm SEM in each treatment group. * $P < 0.05$ as compared with their respective vehicle-treated control group. MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide

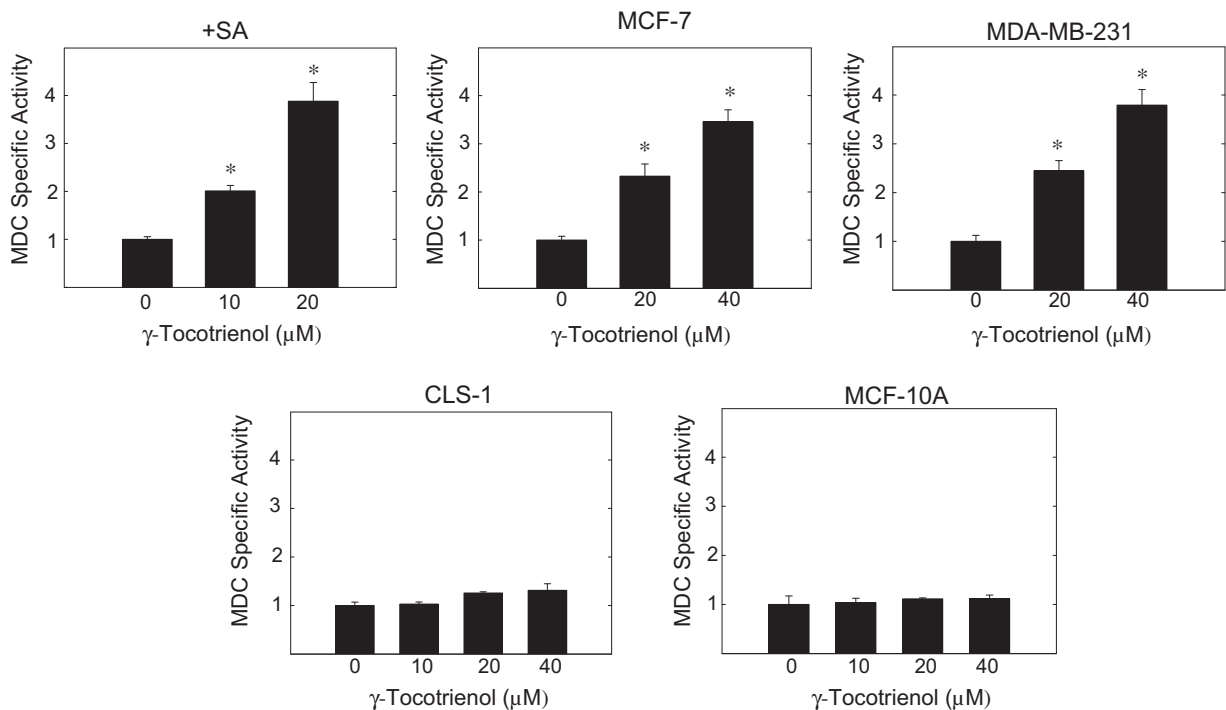


Figure 3 Effects of γ -tocotrienol on autofluorescent autophagy marker intensity in mouse + SA mammary tumor and human MCF-7 and MDA-MB-231 breast cancer cells, and mouse CLS-1 and human MCF-10A immortalized normal mammary epithelial cells. All cells were plated in six-well culture plates and maintained on control media for three days (approximately 70% confluent). Afterwards, cells were divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. The next day, cells in all treatment groups were treated with 0.05 mmol/L MDC and 0.2 μ mol/L ethidium bromide for 15 min at 37°C. Cells were then washed, lysed, and MDC (wavelength 360 nm and emission filter 525 nm) and ethidium bromide (excitation wavelength 530 nm and emission filter 590) fluorescence was measure in each well. Normalization of treatment effects in each well was determined by dividing MDC fluorescence by ethidium bromide fluorescence (total DNA). Vertical bars indicate normalized mean MDC fluorescence \pm SEM expressed as specific activity (arbitrary units) for each treatment group. * $P < 0.05$ as compared with their respective vehicle-treated control group. MDC: monodansylcadaverine

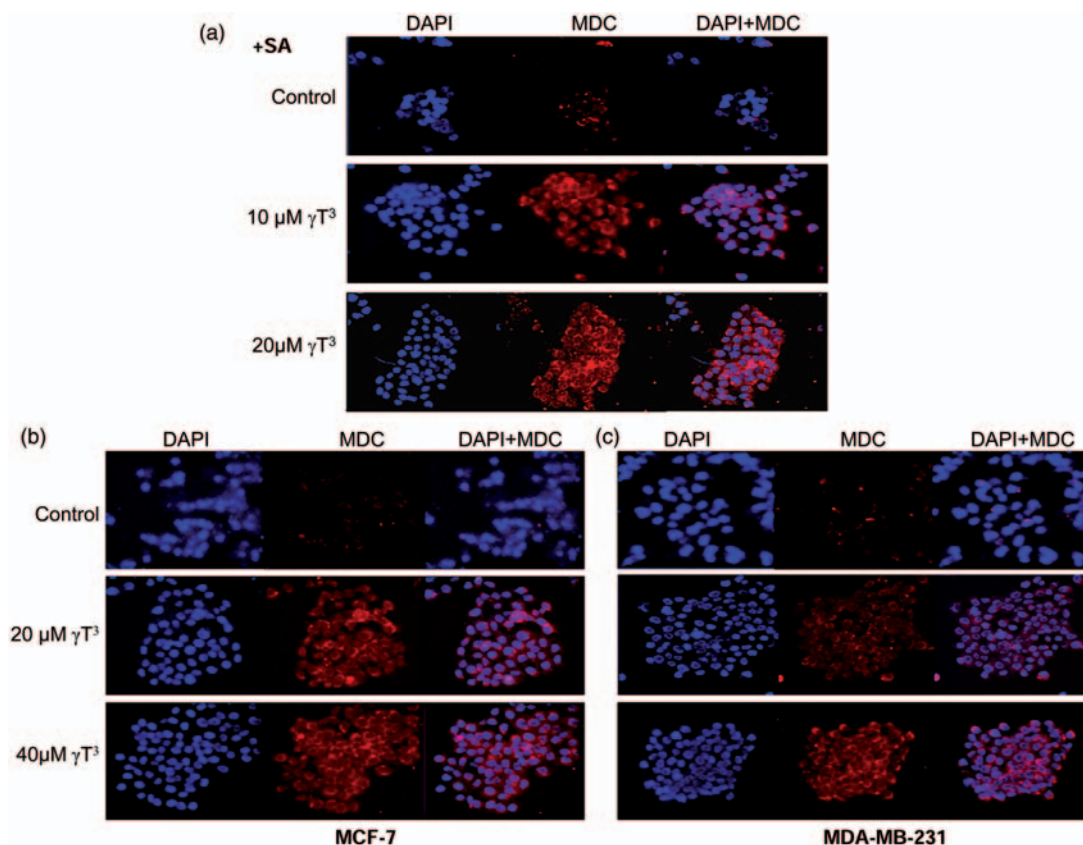


Figure 4 Effects of γ -tocotrienol treatment on autophagic vacuoles MDC fluorescence intensity in mouse (a) +SA, human (b) MCF-7, and (c) MDA-MB-231 mammary tumor cells. All cells were plated in six-well culture plates and maintained on control media three days (approximately 70% confluent). Afterwards, cells were divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. The next day, cells in all treatment groups were treated with 0.05 mmol/L MDC for 15 min at 37°C. Afterwards, all cells were washed, mounted, and subjected to fluorescent analysis by confocal microscopy. Red staining indicates MDC labeled vesicles and blue staining indicates cell nuclei counter-stained with DAPI. Magnification of each photomicrograph is 200 \times . DAPI: 4',6-diamidino-2-phenylindole; MDC: monodansylcadaverine

Figure 5, green (x-axis, 510–530 nm, FL1-H channel) and red (y-axis, >650 nm, FL3-H channel) fluorescence emission with blue (488 nm) excitation from 1×10^4 cells/group was measured in dot plots with a FACSCalibur from BD Biosciences using CellQuest Software. An increase in intensity the bright red fluorescence (top right quadrant) is reflective of a corresponding increase in AVO and autophagy. Treatment with γ -tocotrienol induced a dose-responsive increase in AVO levels in +SA (from 4.6% to 28.66%), MCF-7 (from 2.6% to 17.33%), and MDA-MB-231 (from 4.16% to 25.16%) breast cancer cells as compared with their respective vehicle-treated control groups (Figure 5).

γ -Tocotrienol effects on LC3B-I, LC3B-II, and Beclin-1 levels

Western blot analysis shows that following a 24 h treatment with 0–40 μ mol/L γ -tocotrienol treatment had little or no effect on the conversion of LC3B-I to LC3B-II in immortalized normal mouse (CL-S1) and human (MCF-10A) mammary epithelial cells, an indication that breast cancer cells are more sensitive to γ -tocotrienol-induced autophagy than normal cells (Figure 6a). In contrast, similar treatment with γ -tocotrienol resulted in a dose-dependent conversion of LC3B-I (cytosolic form) to LC3B-II

(the phosphatidylethanolamine-conjugated form associated with autophagosomes), and a corresponding increase in relative Beclin-1 levels (the protein product of the autophagy-related gene) (ATG6) in +SA, MCF-7, and MDA-MB-231 cells, as compared with their respective vehicle-treated control groups (Figure 6b). γ -Tocotrienol treatment also significantly increased the LC3B-II/LC3B-I ratio in all breast cancer cell lines, a positive indicator of autophagy, as compared with their respective vehicle-treated control groups (Figure 6c).

γ -Tocotrienol effects on Bcl-2, Bax, cleaved caspases-3, and cleaved PARP levels

Western blot analysis showed that following a 24-h treatment period, 0–40 μ mol/L γ -tocotrienol caused a dose-responsive decrease in Bcl-2 (anti-apoptotic) and corresponding increase in Bax (pro-apoptotic) protein levels in +SA, MCF-7, and MDA-MB-231 breast cancer cells as compared with their respective vehicle-treated control group (Figure 7a). Figure 7b shows that γ -tocotrienol treatment significantly increased the ratio of Bax/Bcl-2 in each of the tumor cell lines. In addition, γ -tocotrienol treatment was found to increase cleaved PARP and cleaved caspase-3 (activated) levels in each of these breast cancer cell

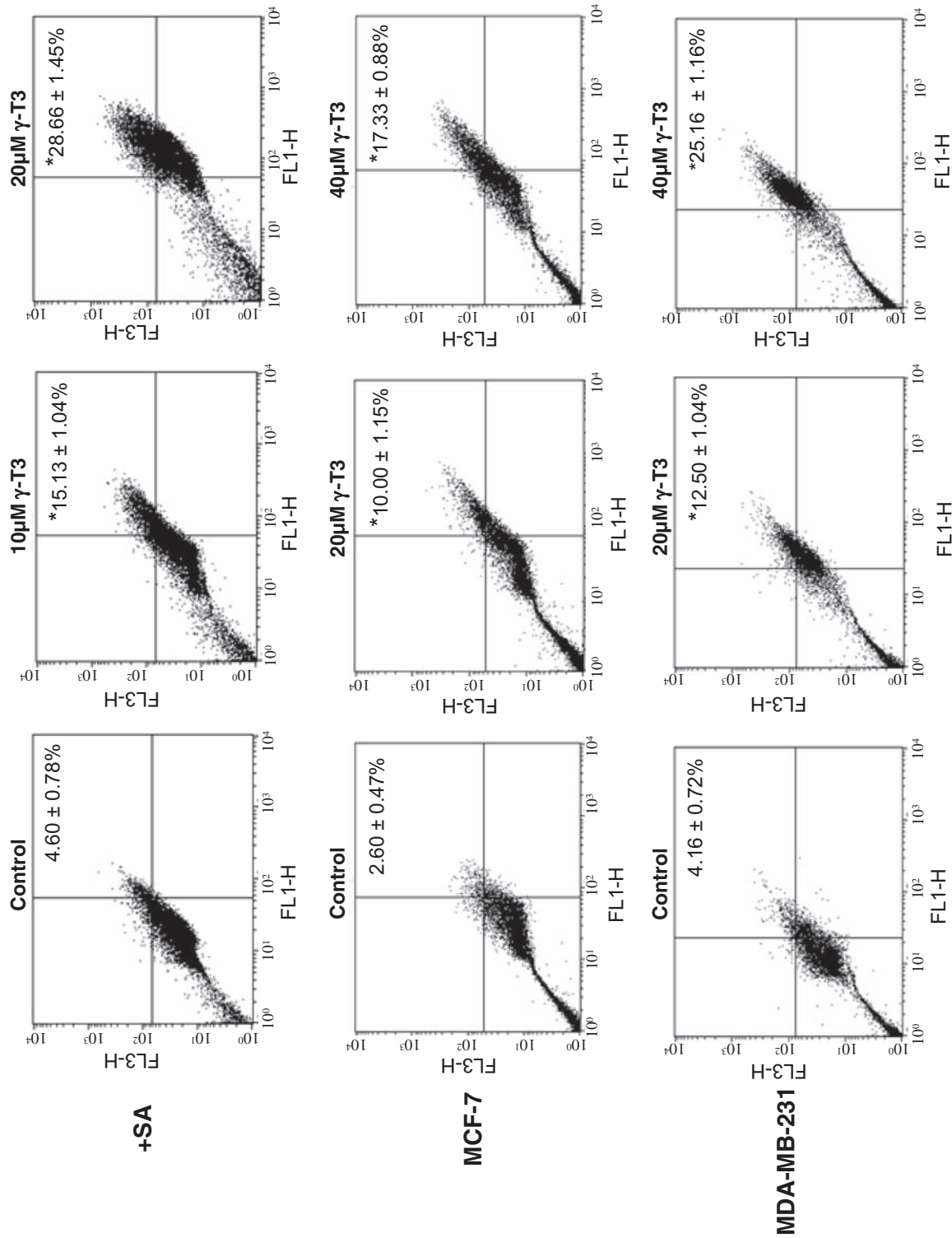


Figure 5 Effects of γ -tocotrienol on acidic vesicular organelle (AVO) levels as determined by flow cytometry in mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cells. All cells were plated in six-well culture plates and maintained on control media for three days (approximately 70% confluent). Cells were then divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. Afterwards, cells were incubated with 1 μ g/mL acridine orange for 15 min at 37 °C and then subjected to flow cytometry analysis. Dot plots were generated using CellQuest Software. Cells displaying positive acridine orange staining indicate elevations in AVO, a cellular marker for autophagy, appear in the upper right quadrant of each dot plot. The numbers appearing in the upper right quadrant of each dot plot represent the mean percentage \pm SEM of cells with AVO in their respective treatment group. * $P < 0.05$ as compared with cells in their respective vehicle-treated control group.

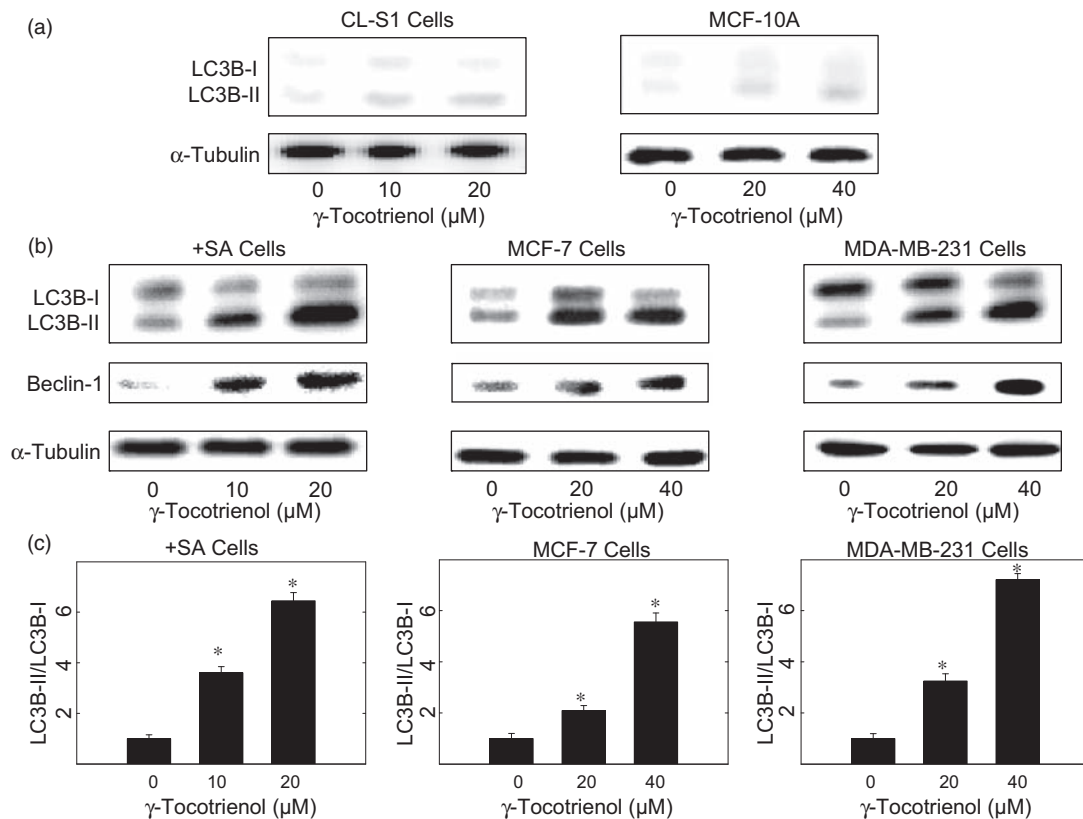


Figure 6 Western blot analysis of γ -tocotrienol effects on the relative protein levels that serve as markers for autophagy in (a) immortalized normal mouse (CL-S1) and human (MCF-10A) mammary epithelial cells, and (b) mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cells. All cells were initially plated at 1×10^6 cells/100 mm culture plate and maintained on control media for three days (approximately 70% confluent). Cells were then divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. Afterwards, whole cell lysates were prepared from cells in each treatment group for subsequent separation by polyacrylamide gel electrophoresis (50 μ g/lane) followed by Western blot analysis for LC3B and Beclin-1. (c) Scanning densitometric analysis was performed on all blots done in triplicate and the integrated optical density of each band was normalized with corresponding α -tubulin. Vertical bars indicate the LC3B-II/LC3B-I ratio in optical density in each treatment group \pm SEM. * $P < 0.05$ as compared with their respective vehicle-treated control group

lines (Figure 7). These findings indicate that γ -tocotrienol-induced autophagy is associated with the initiation of caspase-dependent apoptosis in mouse (+SA) and human (MCF-7 and MDA-MB-231) breast cancer cells.

Effects of γ -tocotrienol on the PI3K/Akt/mTOR signaling

Western blot analysis showed that 24 h treatment with 0–40 μ mol/L γ -tocotrienol resulted in a relatively large dose-dependent decrease in PI3K, phosphorylated-Akt (activated), and phosphorylated-mTOR (activated) levels in mouse (+SA) and human (MCF-7 and MDA-MB-231) breast cancer cells, as compared with cells in their respective vehicle-treated control groups (Figure 8). These findings suggest that γ -tocotrienol-induced autophagy was associated with corresponding attenuation in PI3K/Akt/mTOR signaling.

Discussion

These results demonstrate that γ -tocotrienol-induced cytotoxicity is directly associated with the induction of autophagy in the highly malignant mouse (+SA), and human

estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) mammary tumor cells. Treatment with cytotoxic doses of γ -tocotrienol induced a dose-dependent increase in positive MDC fluorescence, a direct indicator of autophagosome levels, and a corresponding increase in positive acridine orange staining, a direct marker for AVO (AVO or autophagolysosome) levels in these cells. Furthermore, treatment with similar doses of γ -tocotrienol was found to have little or no effect in stimulating autophagic activity in immortalized normal mouse (CL-S1) and human (MCF-10A) mammary epithelial cells, suggesting that breast cancer cells are more sensitive to γ -tocotrienol-induced autophagy than normal cells. These same treatments were also found to stimulate the conversion of LC3B-I to LC3B-II and increase in Beclin-1 expression, demonstrating elevations in cellular markers for autophagosome and autophagic vacuoles formation, respectively, in each of these breast cancer cell lines. γ -Tocotrienol-induced autophagy was also associated with a reduction in intracellular levels of phosphorylated-mTOR (active) and Bcl-2. Since mTOR activity is directly associated with a suppression of autophagy and Bcl-2 acts to suppress Beclin-1 levels, these findings indicate possible intracellular signaling

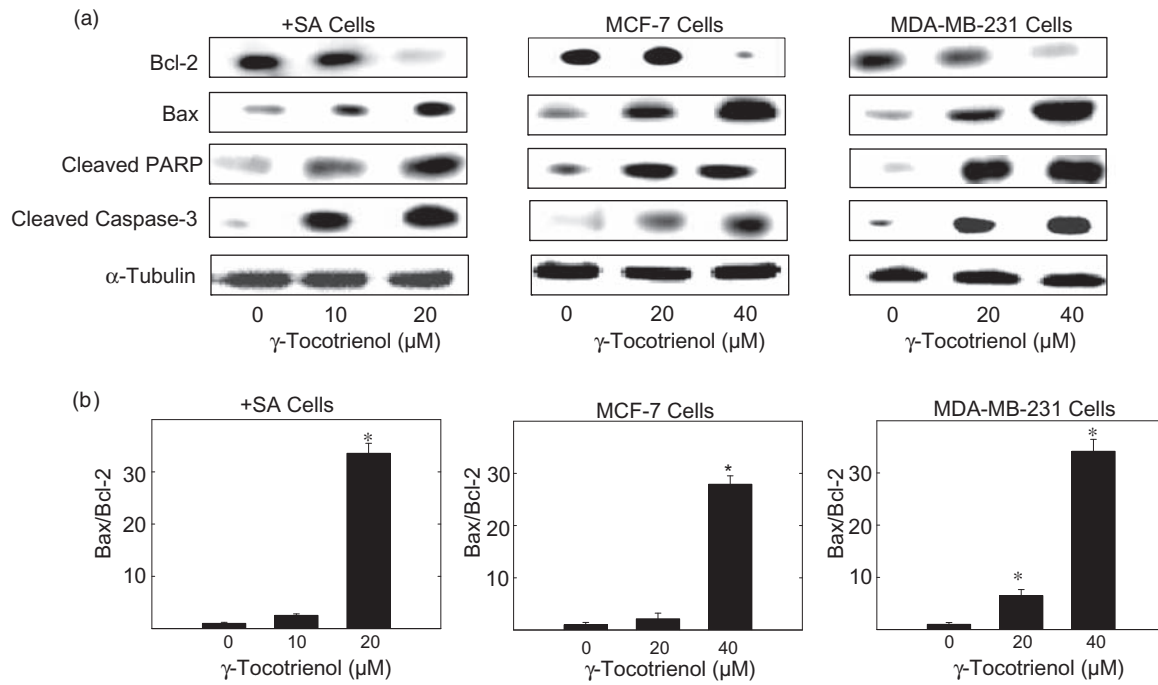


Figure 7 (a) Western blot analysis of γ -tocotrienol effects on the relative levels of apoptotic protein markers in mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cells. All cells were initially plated at 1×10^6 cells/100 mm culture plate and maintained on control media for three days (approximately 70% confluent). Cells were then divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. Afterwards, whole cell lysates were prepared from cells in each treatment group for subsequent separation by polyacrylamide gel electrophoresis (30 μ g/lane) followed by Western blot analysis for Bcl-2, Bax, cleaved PARP, and cleaved caspase-3. (b) Scanning densitometric analysis was performed on all blots done in triplicate and the integrated optical density of each band was normalized with corresponding α -tubulin. Vertical bars indicate the Bax/Bcl-2 ratio in optical density in each treatment group \pm SEM. * $P < 0.05$ as compared with their respective vehicle-treated control group. PARP: poly (ADP-ribose) polymerase

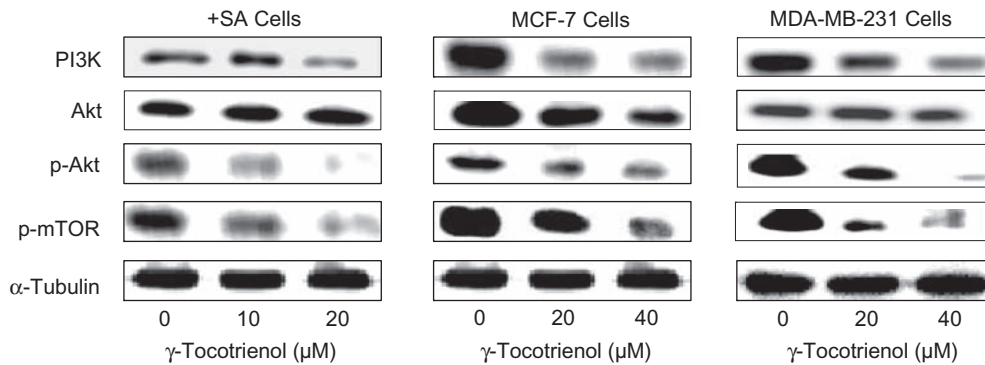


Figure 8 Western blot analysis of γ -tocotrienol effects on the relative levels of PI3K/Akt/mTOR signaling proteins in mouse (+SA) and human (MCF-7 and MDA-MB-231) mammary cancer cells. All cells were initially plated at 1×10^6 cells/100 mm culture plate and maintained on control media for three days (approximately 70% confluent). Cells were then divided into the various treatment groups, media was removed, and cells were exposed to their respective treatment media for a 24-h culture period. Afterwards, whole cell lysates were prepared from cells in each treatment group for subsequent separation by polyacrylamide gel electrophoresis (30 μ g/lane) followed by Western blot analysis for PI3K, Akt, phosphorylated-Akt (p-Akt), and phosphorylated-mTOR (p-mTOR). The visualization of α -tubulin was used to ensure equal sample loading in each lane. All experiments were repeated at least three times

mechanisms that may be involved in mediating tocotrienol-induced autophagy in estrogen-dependent and independent breast cancer cells.

Autophagy is a catabolic process that provides a means by which cells can dispose of unnecessary or dysfunctional intracellular organelles, membranes and proteins, and recycle useful cellular components.^{2,3,5,6,35} During induction of autophagy, cytoplasmic and intracellular organelles are sequestered within double-membrane vesicles called

the autophagosomes. These vesicles then fuse with lysosomes to form autophagolysosomes, and the sequestered material is then degraded by lysosomal enzymes.² However, the role of autophagy in cancer is complex and not completely understood. Experimental evidence indicates that autophagy may have a dual function in cancer that can either promote or suppress tumor cell survival.^{2,3,5,6,35} However, it still remain to be clarified whether autophagic activity in dying cancer cells is reflective of a

compensatory mechanism that is trying to prevent death or is directly involved in promoting cellular destruction.

Results in the present study demonstrate for the first time that γ -tocotrienol-induced autophagy in mouse and human breast cancer cell lines is associated with tumor suppression and cancer cell death. Treatment with γ -tocotrienol induced a dose-dependent increase in autophagosome and autophagolysosome formation in +SA, MCF-7, and MDA-MB-231 mammary tumor cells. MDC is an autofluorescent substance that accumulates in autophagosomes and an increase in positive MDC staining is an established indicator of increased autophagic activity in cells.^{29,30} Results showed that treatment with cytotoxic doses of γ -tocotrienol resulted in a corresponding increase in positive MDC staining in all breast cancer cell types examined. Similarly, acridine orange is a lysosomotropic dye that accumulates in AVOs or autophagolysosomes and emits a bright red fluorescence. Positive acridine orange staining is another established cellular marker for autophagic activity.^{31,36} Results in the present study also demonstrated that γ -tocotrienol treatment induced a dose-dependent increase in positive acridine orange AVO staining and the induction of autophagy in all mammary cancer cell lines.

Another cellular marker for autophagy is the conversion of LC3B-I to LC3B-II.⁷ LC3B-I is a soluble microtubule-associated protein that is widely distributed in the cytoplasm of normal and cancerous mammalian cells.⁷ During autophagy, LC3B-I undergo a process of lipidation and is conjugated to phosphatidylethanolamine to form LC3B-II, which readily accumulates and attaches to the autophagosome membrane. Treatment with γ -tocotrienol resulted in a dose-dependent decrease in LC3B-I and corresponding increase in LC3B-II levels in +SA, MCF-7, and MDA-MB-231 breast cancer cells. These findings confirm and extend previous findings which showed that tocotrienol treatment promotes the conversion of LC3B-I to LC3B-II in other cell types^{18,20,21} and supports the hypothesis that tocotrienol-induced autophagy promotes cytotoxicity and death in breast cancer cells.

A key regulatory protein in autophagy is Beclin-1. Beclin-1 localizes within the trans-Golgi network, where it participates in the formation of autophagosomes.³⁷ In addition, Bcl-2 is an anti-apoptotic protein that can bind to and inhibit the activity of Beclin-1 and subsequently lead to a suppression autophagy.³⁸ Experimental evidence shows that an acute 24 h exposure to γ -tocotrienol was found to enhance expression of Beclin-1 levels in mouse (+SA) and human (MCF-7 and MDA-MB-231) breast cancer cell lines. These same treatments were also found to cause a decrease in Bcl-2 and corresponding increase in Bax levels in these same tumor cell lines. Previous studies have suggested that autophagy in cancer cells can lead to type II programmed cell death, as well as caspase-dependent and -independent apoptosis.^{2,4,39,40} It has been suggested that excessive autophagy destroys large quantities of cellular proteins and organelles that can ultimately lead to cellular self-destruction.³⁵ Previous studies have shown that an increase in the Bax/Bcl-2 ratio is directly related to an increase in cancer cell susceptibility to apoptosis and self-destruction.⁴¹⁻⁴³ Bax is a pro-apoptotic protein that will

form heterodimers with Bcl-2 and inhibit its function.⁴⁴⁻⁴⁶ Results showed that treatment with γ -tocotrienol increased the Bax/Bcl-2 ratio in all breast cancer cell lines and is associated with increased apoptotic activity in these cancer cell lines. This suggestion is further evidence by the finding that γ -tocotrienol treatment was also found to increase intracellular levels of cleaved (activated) caspases-3 and cleaved PARP, a downstream target of the activated caspases-3. These findings are consistent with previous finding that shown high dose treatment with γ -tocotrienol-induced caspase activation.^{47,48} In these studies, γ -tocotrienol-dependent apoptosis was mediated by endoplasmic reticulum stress-induced caspase activation.²⁸ Taken together, these finding strongly suggest that γ -tocotrienol-induced autophagy is associated with a large increase in Beclin-1 levels and a corresponding increase in the Bax/Bcl-2 ratio that ultimately leads to the initiation in caspase-mediated apoptosis in breast cancer cells (Figure 9).

Excessive and/or unregulated PI3K/Akt/mTOR signaling is often important in mediating mitogen-dependent growth and survival in various types of cancer cells, and inhibition of this pathway results in tumor cell growth arrest and apoptosis. Enhanced PI3K/Akt/mTOR activity is also associated with increased cancer progression to a highly metastatic and invasive phenotype and the development of resistance to traditional chemotherapeutic agents.⁴⁹ The PI3K/Akt/mTOR signaling also plays an important role in regulation of autophagy.^{50,51} Recent studies have shown that inhibition of the PI3K/Akt/mTOR pathway was associated with triggering autophagy in cancer cells.^{4,52} γ -Tocotrienol has been shown to be a potent inhibitor of PI3K/Akt/mTOR activity, and cancer cells are significantly more sensitive to this effect than normal cells.¹⁰ Since apoptosis and autophagy share many common signaling elements (Akt, mTOR, Bcl-2, Bax, etc.), it is possible that the magnitude and nature of the stimuli may determine which form of cell death predominates. Autophagy has shown to mediate cell death in case of apoptosis resistance cancer cells in response to anticancer agent that would otherwise lead to apoptosis.^{53,54} Nevertheless, crosstalk between apoptosis and autophagy signaling is complex and additional studies are required to clarify the exact mechanisms that are involved in determining whether the anticancer effects of γ -tocotrienol are mediated by autophagy and/or apoptosis in a particular cancer cell type. Although the exact intracellular target site for γ -tocotrienol remains unknown, results in the present study provides additional evidence to suggest that γ -tocotrienol-induced autophagy is mediated by a reduction in PI3K/Akt/mTOR signaling (Figure 9). Previous studies have shown that γ -tocotrienol inhibits HER receptor member activation and signaling,^{16,32} suggesting that its site of action may be at the level of the plasma membrane. However, since γ -tocotrienol treatment also acts to suppress Bcl-2 levels and stimulate the conversion of LC3-I to LC3-II, these finding also suggest that γ -tocotrienol may also be acting on cytoplasmic targets to induce autophagy (Figure 9). Further studies are required to specifically identify the exact intracellular sites targeted by γ -tocotrienol that are involved in the induction of autophagy.

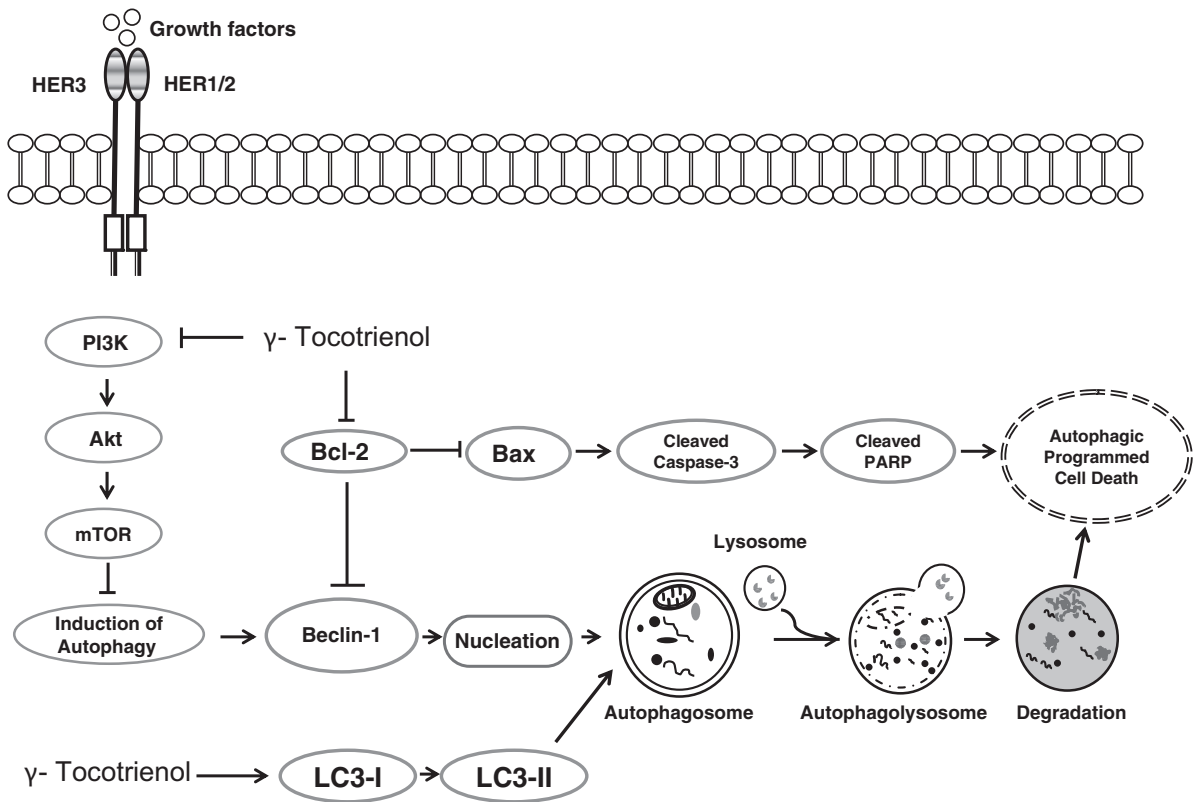


Figure 9 Schematic representation of γ -tocotrienol-induced autophagy in malignant mammary tumor cells

In summary, experimental evidence is provided which shows that γ -tocotrienol-induced autophagy in breast cancer cells appears to be mediated by a decrease in PI3K/Akt/mTOR signaling and Bcl-2 expression, and a corresponding increase in Beclin-1 levels and the conversion of LC3B-I to LC3B-II in mouse and human breast cancer cell lines. In addition, these findings also suggest that γ -tocotrienol-induced autophagy may also be involved in the initiation of apoptosis, as evidenced by the finding that these same treatments were found to increase the intracellular Bax/Bcl-2 ratio, as well as increased levels of cleaved caspase-3 and PARP in each of the mammary cancer cell lines were investigated.

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