Anticancer Effects of Combined γ-Tocotrienol and PPARγ Antagonist Treatment are Associated with a Suppression in Adipogenic Factor Expression

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Abstract: Cancer cells reprogram their metabolism to meet the demands of accelerated growth. Glucose is the primary source of energy for cancer cells, but under conditions of high-energy demand lipids and free fatty acids become increasingly important. PPARγ is a member of the nuclear receptor superfamily and acts to regulate adipocyte differentiation and lipid metabolism. However, in many types of cancer, PPARγ activity is elevated in order to increase production of adipogenic factors [1, 2]. γ-Tocotrienol is an isoform of vitamin E that displays potent anticancer activity [3]. Previous studies have shown that the antiproliferative effects of combined treatment of γ-tocotrienol with PPARγ antagonists was associated with a reduction in PPARγ activity, expression of PPARγ and RXR, and suppression in Akt activation in MCF-7 and MBA-MB-231 human breast cancer cells [4]. The present study was conducted to determine the effects of combination treatment with these agents on adipogenic factor levels in rapidly proliferating human breast cancer cells. Western blot and qRT-PCR studies showed that combined treatment of γ-tocotrienol with PPARγ antagonists not only suppressed the adipogenic proteins, C/EBPβ and SREBP-1c, but also decreased their target lipogenic enzymes, ap2, FAS, and HMGCoR. However, treatment effects were also observed in PPARγ silenced breast cancer cells, indicating that these effects are mediated through PPARγ-independent mechanism. These findings suggest the combined treatment of γ-tocotrienol with PPARγ antagonist may have potential as a therapeutic strategy in the treatment of breast cancer.

Keywords: γ-Tocotrienol, PPARγ, Breast Cancer, C/EBPβ, SREBP-1c, FASN.

1. INTRODUCTION

Cancer cells characteristically display an elevated metabolism and consume vast amounts of energy to support their growth and survival. Although, glucose is the primary energy source for cancer cells, during conditions of high-energy demand, lipids and free fatty acids can replace glucose as an important energy source. Adipocytes modulate lipid metabolism and are also involved in activating signaling pathways associated with promoting chronic inflammation [5]. Although it has not yet been firmly established that lipids derived from insulin-resistant adipocytes have a direct impact on cancer cell metabolism, evidence does suggest that activation of adipocytes appears to be involved in supporting growth and proliferation in malignant cells [5]. Peroxisome proliferator activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, is expressed predominantly in adipose tissue, where it functions to regulates adipocyte differentiation and lipid metabolism [6]. Studies have shown that PPARγ levels are elevated in many types of cancer tissues [1, 7, 8]. PPARγ may function in cancer cells to increase production of adipogenic factors and lipogenic proteins in support of the high metabolic demands of cancer cells [2, 9]. Therefore, strategies targeting the inhibition of PPARγ may be beneficial in the treatment of cancer by acting to decrease adipogenic proteins and genes in rapidly proliferating cancer cells.

PPARγ regulates adipogenesis by activating families of transcription factors such as CCAAT-enhancer-binding proteins (C/EBP) and sterol regulatory element binding protein (SREBP; also called ADD1) [10, 11]. Studies have shown that of the three isoforms of C/EBP (C/EBPα, C/EBPβ, and C/EBPδ), PPARγ directly regulates C/EBPα and C/EBPβ to maintain a terminally differentiated state of adipocytes that is absolutely required for the function of fat-selective enhancers such as adipocyte fatty acid binding protein (also called ap2) [12, 13]. In addition, PPARγ regulates function of SREBP-1c and SREBP2, which are important regulators of fatty acid uptake and enhance fatty acid (FA) synthesis by modulating activity of key lipogenic enzymes such as FA synthase (FASN) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoR) [14-16]. Investigations have also shown that specific C/EBP and SREBP isoforms are over expressed in many types of cancers in order to compensate for the elevated energy demand displayed by these cells [17, 18].

γ-Tocotrienol is a naturally occurring isoform within the vitamin E family of compounds that displays potent

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anticancer activity [19, 20] by suppression of growth-factor-dependent mitogenic signaling [21, 22]. *In vitro* studies have previously shown that mammary tumor cells exposed to γ-tocotrienol treatment display a significant reduction in 3-hydroxy-3-methylglutaryl-CoA reductase activity through posttranscriptional down-regulation of this enzyme [23]. Additional studies showed that γ-tocotrienol inhibits adipogenesis in 3T3-L1 preadipocytes by suppressing insulin-induced ap2 protein and Akt phosphorylation [24], and reduce SREBP expression in human fibroblasts [25].

The antiproliferative effects of γ-tocotrienol may be due to changes in PPARγ activity [26], Studies conducted in human breast cancer cells showed that the antiproliferative effects resulting from combined treatment of γ-tocotrienol with PPARγ antagonists (GW9662 or T0070907) was associated with a reduction in PPARγ expression and activity [4]. However, the effects of these combination treatments on lipogenic enzymes have not yet been determined. Therefore, studies were conducted to determine the effects of combination treatment of γ-tocotrienol with PPARγ antagonists on C/EBP and SREBP isoforms and their target lipogenic genes. In addition, experiments were conducted to understand whether these effects are mediated by PPARγ-dependent versus PPARγ-independent mechanisms.

2. MATERIALS AND METHODS

2.1. Reagents and Antibodies

Unless stated otherwise, all reagents were purchased from Sigma Chemical Company (St. Louis, MO). Isolated γ-tocotrienol (>98% purity) used in experimentation was acquired from First Tech International Ltd (Hong Kong). The PPARγ antagonists, GW9662 and T0070907, were purchased from Cayman Chemicals (San Diego, CA). Cells grown in culture were maintained in media that contain fetal bovine serum (FBS) that was purchased from American Type Culture Collection (Manassas, VA). Primary antibodies used in Western blot analysis for β-actin, PPARγ, C/EBPα, C/EBPβ, lipin 1, FASN, ap2, and HMGCoR were purchased from Cell Signaling Technology (Beverly, MA), while the primary antibody directed against SREBP-1c was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody used in Western blot analysis was goat anti-rabbit and was purchased from PerkinElmer Biosciences (Boston, MA).

2.2. Cell Lines and Culture Conditions

The human breast cancer cell lines, MCF-7 and MDA-MB-231, used in experimentation were purchased from American Type Culture Collection (Manassas, VA). MCF-7 is an estrogen-receptor-positive, whereas MDA-MB-231 is an estrogen-receptor negative, ductal epithelial cancer breast cell. Both breast cancer cell lines were maintained in a 1:1 mixture of modified Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 10% fetal bovine serum, 10μg/ml insulin, 100 U/ml penicillin, 0.1 mg/ml streptomycin. Cells were maintained in a humidified incubator at 37°C in a 95% air and 5% CO_2 environment. Prior to experimentation, cells were harvested for subculturing, media was removed and cells were twice washed with sterile Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline (PBS). Cells were then incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37°C. Afterward, the now released cells were collected by centrifugation, and then resuspended in serum containing media. The number of harvested cells was then calculated using a hemocytometer as previously described in detail [19].

2.3. Experimental Treatments

All isoforms of vitamin E are highly lipophilic. Therefore, in order to dissolve γ-tocotrienol into the aqueous culture media, it must first be suspended in a sterile 10% bovine serum albumin (BSA) solution [19, 20]. This is accomplished by first taking a known amount of purified γ-tocotrienol and dissolving it in 100 μL of 100% ethanol. Once dissolved, this γ-tocotrienol/ethanol solution is then added to a small volume of sterile 10% BSA in water to produce a final 10 mM γ-tocotrienol/BSA stock solution. This stock solution is then incubated overnight at 37°C with continuous shaking. From this stock solution, different concentration of γ-tocotrienol containing treatment media is prepared. Likewise, stock solutions of the highly lipophilic PPARγ antagonist, GW9662 and T0070907, were prepared by dissolving these agents in sterile dimethyl sulfoxide (DMSO), and then the appropriate amount of this stock solution was then added directly to treatment media. Ethanol and/or DMSO can induce toxic effects to cells in culture if concentrations reach levels greater than 1%. Therefore, as a treatment control, the levels ethanol and/or DMSO were adjusted in all control and treatment media so that exposure to these agents was the same for all cells in a particular experiment. The

final concentration of ethanol and/or DMSO within any given experiment was always less than 0.1%.

2.4. Growth Studies

In dose-response studies examining the antiproliferative effects of the various treatments, MCF-7 and MDA-MB-231 breast cancer cells were isolated, resuspended and counted as described above in section 2.2. Cells were then seeded at a density of 1×10^4 cells suspended in 100 µL control media into each well of a 96-well culture plate, returned to the incubator and allowed to adhere to the bottom of the plate. The next day, cells were divided into different treatment groups and the original media was removed. Cells were then washed with 100 µL of sterile PBS and then all wells received 100 µL of their respective treatment media containing either 0-12 µM GW9662, T0070907 or 0-6 µM γ-tocotrienol alone or combination and then plates were returned to the incubator. Treatment media was replaced with fresh media every other day throughout the 4-day experimental period.

2.5. Measurement of Viable Cell Number

MCF-7 and MDA-MB-231 breast cancer cell number was determined in 96-well culture plates (8 wells/group) by the 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay using a modification of the method previously described in detail [19, 20]. On the day of assay, treatment medium was replaced with fresh control medium containing 0.5 mg/mL MTT, and cells were returned to the incubator for 3 h. At the end of the incubation period, treatment media was removed, and the MTT crystals were dissolved in 100 µL of DMSO. The optical density of each sample was read at 570 nm on a microplate reader (Spectracount; Packard Bioscience Company, Meriden, CT) that was zeroed against a blank prepared from cell-free cultures. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometer, in triplicate at the start of each experiment. Previous studies showed that γ-tocotrienol treatment does not affect the specific activity of the MTT assay [19, 20].

2.6. Western Blot Analysis

In studies measuring treatment effects on PPARγ signaling protein or lipogenic factor expression by Western blot analysis, 1×10^6 MCF-7 or MDA-MB-231 breast cancer cells were resuspended in 10 mL of control medium and plated in 100 mm culture plates. Plates were then returned to the incubator to allowed

cells to attach to the bottom of the plate. The next day, media was removed and the cells were washed with 10 mL sterile PBS, divided into the various treatment groups and fed 10 mL of their respective treatment media. Cells were then returned to the incubator. Fresh media was replaced every other day for the remainder of the 4-day experimental period. Afterwards, cells were twice washed with sterile PBS, and isolated with trypsin as described above. Whole cell lysates were prepared by resuspending cells in lysis buffer, then boiled for 5 min, sonicated, microcentrifuged at $4^{\circ}C$ and the supernatant was collected [22, 27, 28]. The protein concentration in each sample was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein from each sample in a given experiment was then loaded onto SDS-polyacrylamide minigels and electrophoresed through a 15% resolving gel. Separated proteins on each gel were then transferred to a polyvinylidene fluoride (PVDF) membrane at 30 V for 12-16 h at $4^{\circ}C$ (PerkinElmer Lifesciences, Wellesley, MA) in a Trans-Blot Cell (Bio-Rad, Hercules, CA) according to the method of Towbin *et al.* [29]. Nonspecific antibody binding sites were blocked on each transblotted membranes by incubating membranes in 2% BSA in 10 mM Tris HCl containing 50 mM NaCl and 0.1% Tween 20 pH 7.4 (TBST) for 2 h, followed by an incubation with specific primary antibodies against PPARγ, C/EBPα, C/EBPβ, SREBP-1c, lipin 1, FASN, ap2, HMGCoR or β-actin, diluted 1:500 to 1:5000 in TBST/2% BSA for 2 h. Afterwards, membranes are washed 5 times with TBST followed by incubation with their respective horseradish peroxide-conjugated secondary antibodies diluted 1:3000 to 1:5000 in TBST/2% BSA for 1 h, and then washed again with TBST. Specific target protein bands on each membrane were then visualized by chemiluminescence (Pierce, Rockford, IL) according to the manufacturer's instructions. Images were obtained using a Syngene Imaging System (Frederick, MD). The visualization of β-actin in each sample was used as a control standard to verify the equal sample loading occurred in each lane. Protein bands images were then acquired and densitometric analysis was performed with the Kodak molecular imaging software version 4.5 (Carestream Health Inc, Rochester, NY). All experiments were repeated at least three times. Representative Western blot images from each experiment are shown in the Figures.

2.7. Transient Transfection and Luciferase Reporter Assay

In transfection and reporter assays, 2×10^4 MCF-7 or MDA-MB-231 breast cancer cells were resuspended in

100 µL of control medium and plated in each well of a 96-well culture plate. Plates were then returned to the incubator to allow cells to attach to the bottom of the plate. The next day, media was replaced with fresh control media. Cells in each well were then transfected with 32 ng of PPRE X3-TK-luc (Addgene plasmid #1015) (Kim *et al.*, 1998) and 3.2 ng of renilla luciferase plasmid per well (Promega, Madison, WI) using 0.8 µL of lipofectamine^{TM} 2000 transfection reagent (Invitrogen, Grand Island, NY) and returned to the incubator. Following a 6 h incubation period, the transfection media was removed, cells were washed in 100 µL sterile PBS, and then exposed to 100 µL of control or treatment media and returned to the incubator, and fresh media was provided every other day. After a 4-day experimental period, cells were lysed with 75 µL of passive lysis buffer and treated according to manufacturer's instructions using dual-glo luciferase assay system (Promega, Madison, WI). Renilla activity in each sample was used to normalize luciferase activity. Experimental results are represented as the mean-fold change in activity observed in treated cells, as compared to the activity observed in vehicle-treated control cells.

2.8. Quantitative Real-Time PCR

In qPCR studies, 2×10^5 MCF-7 or MDA-MB-231 breast cancer cells were resuspended in 2 mL of control medium and plated in each well of a 6-well culture plate and then returned to the incubator to allowed cells to attach to the bottom of the plate. The next day, cells were divided into different treatment groups (3 wells/group) culture media was removed, washed with sterile PBS, then fed fresh media containing their respective treatments that included 0-6.4 µM GW9662, 0-6.4 µM T0070907 or 2-3µM γ-tocotrienol alone or in combination and then return to the incubator. Cells were fed fresh media every other day during the 4-day culture period. Total RNA was extracted using Applied Biosystems (Carlsbad, CA) RNA kit according to the manufacturer's instructions. First-strand cDNA was generated from total RNA for each sample using the cDNA kit from Applied Biosystems (Carlsbad, CA) according to the manufacturer's instructions. Taqman PCR probes and gene-specific primer pairs were generated for PPARγ, RXR, C/EBPβ, SREBP-1c, and GAPDH using Integrated DNA technologies (Coralville, IA). qRT-PCR was performed on an Applied Biosystems (Carlsbad, CA) Prism 7900 Sequence Detection System. Reactions were prepared in triplicate for each gene using Taqman gene expression assays. During thermal

cycling, the threshold cycle (C_t) is defined as the cycle number when amplification of a specific PCR product is detected. The average C_t value of GAPDH was subtracted from average C_t value of target genes (PPARγ, RXR, C/EBPβ, SREBP-1c) to normalize the amount of sample RNA added to the reaction. Relative quantification describes the fold change in expression of a gene of interest in a test sample relative to a calibrator sample. With the comparative C_t ($\Delta\Delta C_t$) method, the level of the target gene mRNA in treatment samples relative to control samples was determined.

2.9. Statistical Analysis

Differences among the various treatment groups in growth studies and Western blot studies were determined by analysis of variance followed by Dunnett's multiple range test. Differences were considered statistically significant at a value of $P < 0.05$.

3. RESULTS

3.1. Antiproliferative Effects of γ-Tocotrienol and PPARγ Antagonists (GW9662 or T0070907) when Given Alone and in Combination

Treatment with 3-4 µM γ-tocotrienol, or 3.2-6.4 µM GW9662 or T0070907 was found to significantly inhibit growth of MCF-7 cells in a dose-responsive manner as compared to cells in the vehicle treated control group (Figure **1A** and **B**). However, when combined with a subeffective dose (3.2 µM) of the PPARγ antagonist, GW9662 and T0070907, the growth inhibitory effects of 1-4 µM γ-tocotrienol was significantly enhanced in MCF-7 breast cancer cells (Figure **1C** and **D**). Similarly, treatment with 4-6 µM γ-tocotrienol, or 6.4-12 µM GW9662 or T0070907 was found to significantly inhibit growth of MDA-MB-231 cells in a dose-responsive manner as compared to cells in the vehicle treated control group (Figure **1E** and **F**). However, the growth inhibitory effects of 3-6 µM γ-tocotrienol was significantly enhanced when given in combination with a subeffective dose (6.4 µM) of the PPARγ antagonists, GW9662 and T0070907, in MDA-MB-231 breast cancer cells (Figure **1G** and **H**).

3.2. Effects of γ-Tocotrienol and PPARγ Antagonists (GW9662 or T0070907) Given Alone or in Combination on PPARγ and RXR mRNA Levels and PPRE Mediated Reporter Activity

qRT-PCR analysis shows that treatment with 2 µM (MCF-7 cells) or 3 µM (MDA-MB-231 cells) γ-tocotrienol alone did not affect PPARγ and RXR mRNA
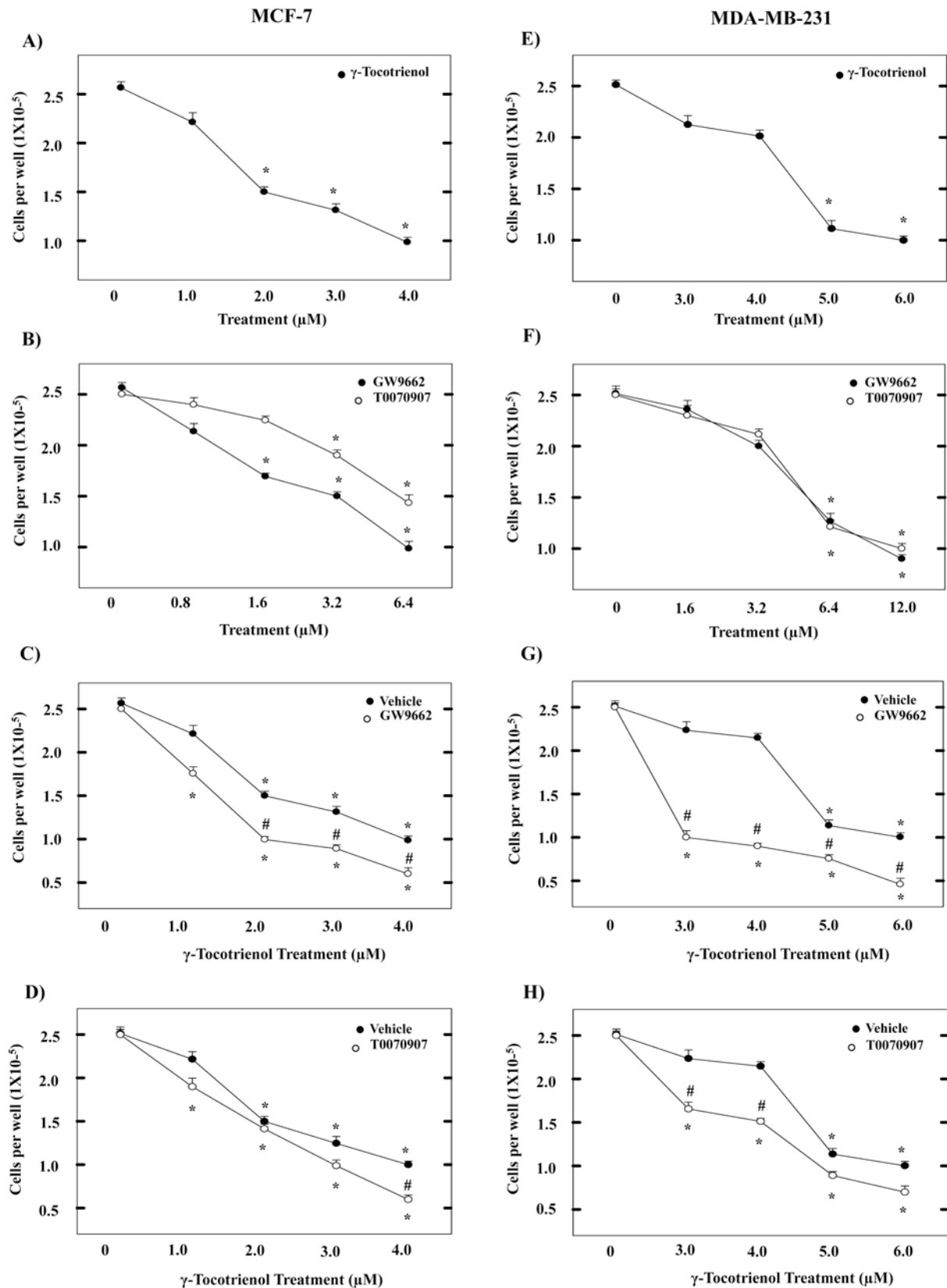
Figure 1: Antiproliferative effects of γ-tocotrienol and PPARγ antagonists (GW9662 and T0070907) on (**A**, **B**, **C**, **D**) MCF-7 and (**E**, **F**, **G**, **H**) MDA-MB-231 breast cancer cells. Cells were plated at a density of 1x10^{4} cells/well in 96-well culture plate (3 replicates per group) and exposed to treatment media for a 4-day period. Afterwards viable cell number was determined using MTT colorimetric assay. Vertical bars indicate mean cell count ± SEM in each treatment group. *$P< 0.05$ as compared with vehicle-treated controls.
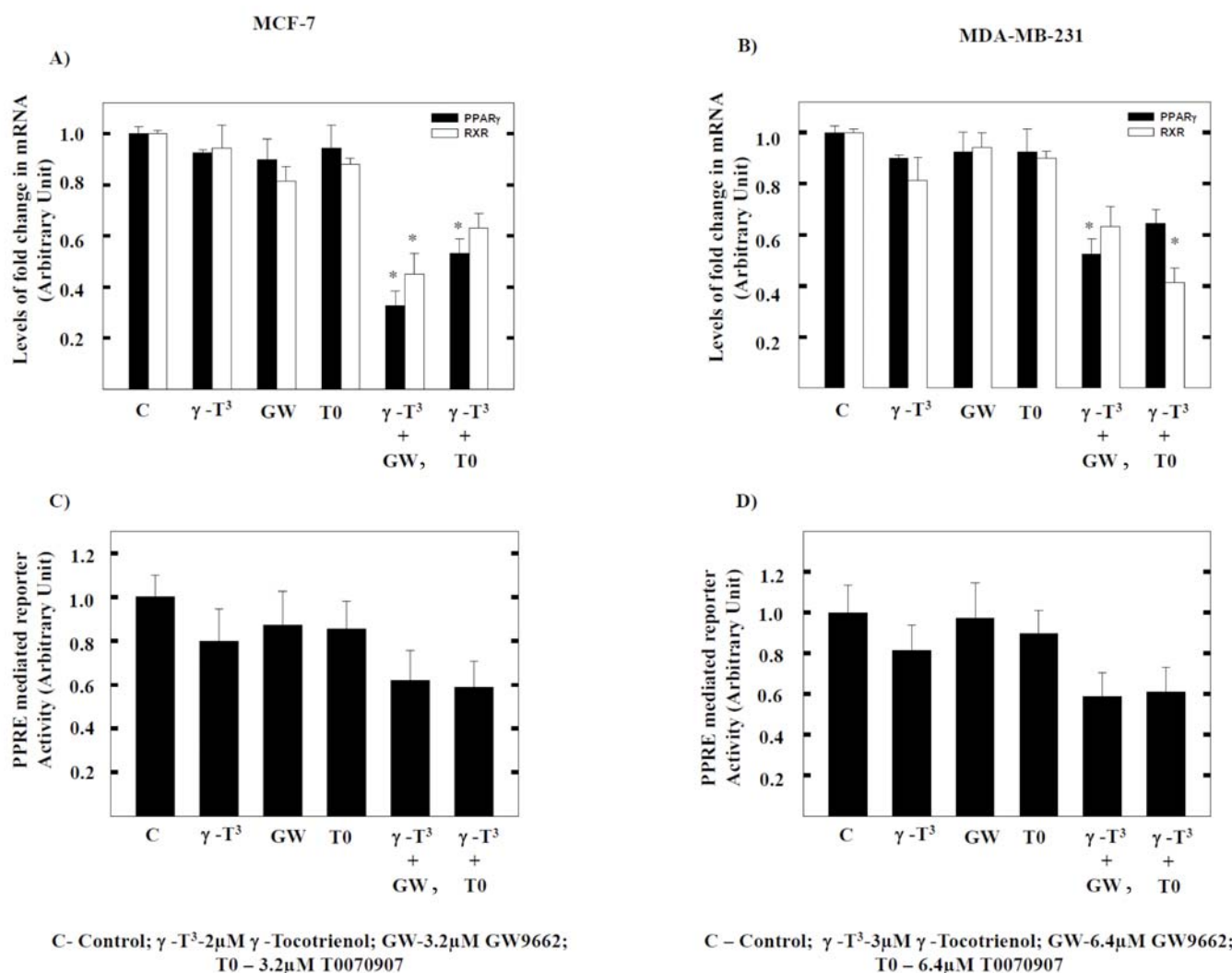
Figure 2: qRT-PCR analysis of treatment of γ-tocotrienol, GW9662, and T0070907 given alone or in combination on the levels of PPARγ and RXR in (**A**) MCF-7 and (**B**) MDA-MB-231 breast cancer cells after a 4-day incubation period. Cells were plated at a density of 2×10^5 cells/well (3 replicates per group) in 6-well plate and treated with control or treatment media for a 4-day culture period. Changes in mRNA levels of PPARγ and RXR were normalized to mRNA level of GAPDH and represented as bar graph. Vertical bars indicate the normalized C_t value ± SEM (Arbitrary Unit) in each treatment group. Luciferase assay to determine PPRE mediated reporter activity in (**C**) MCF-7 and (**D**) MDA-MB-231 breast cancer cells after a 4-day incubation period. Cells were plated at a density of 2×10^4 cells/well (3 replicates per group) in 96-well plate. Transfections were performed as given in the protocol. Results were calculated as raw luciferase units divided by raw renilla units. Vertical bars indicate PPRE mediated reporter activity ± SEM (Arbitrary Unit). *$P < 0.05$ as compared with vehicle-treated controls.

levels (Figure **2A** and **B**) and PPRE mediated reporter activity (Figure **2C** and **D**) as compared to the vehicle treated controls. Treatment with 3.2 μM GW9662 or T0070907 alone in MCF-7 cells or 6.4 μM GW9662 or T0070907 alone in MDA-MB-231 cells had little or no effect on PPARγ or RXR mRNA levels (Figure **2A** and **B**) and PPRE mediated reporter activity (Figure **2C** and **D**). However, combined treatment with similar doses of γ-tocotrienol and GW9662 or T0070907 resulted in a relatively significant decrease in PPARγ and RXR mRNA levels (Figure **2A** and **B**) and PPRE mediated reporter activity in MCF-7 and MDA-MB-231 breast cancer cell lines as compared to vehicle treated-control cells (Figure **2C** and **D**).

3.3. Effects of γ-Tocotrienol and PPARγ Antagonists (GW9662 and T0070907) Given Alone or in Combination on C/EBPα and C/EBPβ Levels

Western blot analysis shows that treatment with 2 μM (MCF-7 cells) or 3 μM (MDA-MB-231 cells) γ-tocotrienol and 3.2 μM (MCF-7 cells) or 6.4μM (MDA-MB-231 cells) of the PPARγ antagonists, GW9662 or T0070907 alone did not induce change in expression of C/EBPα and C/EBPβ as compared to the vehicle treated controls (Figure **3A**, **B**, **C**, and **D**). However, combined treatment with these same doses of γ-tocotrienol and GW9662 or T0070907 caused a significant reduction in levels of C/EBPβ without any
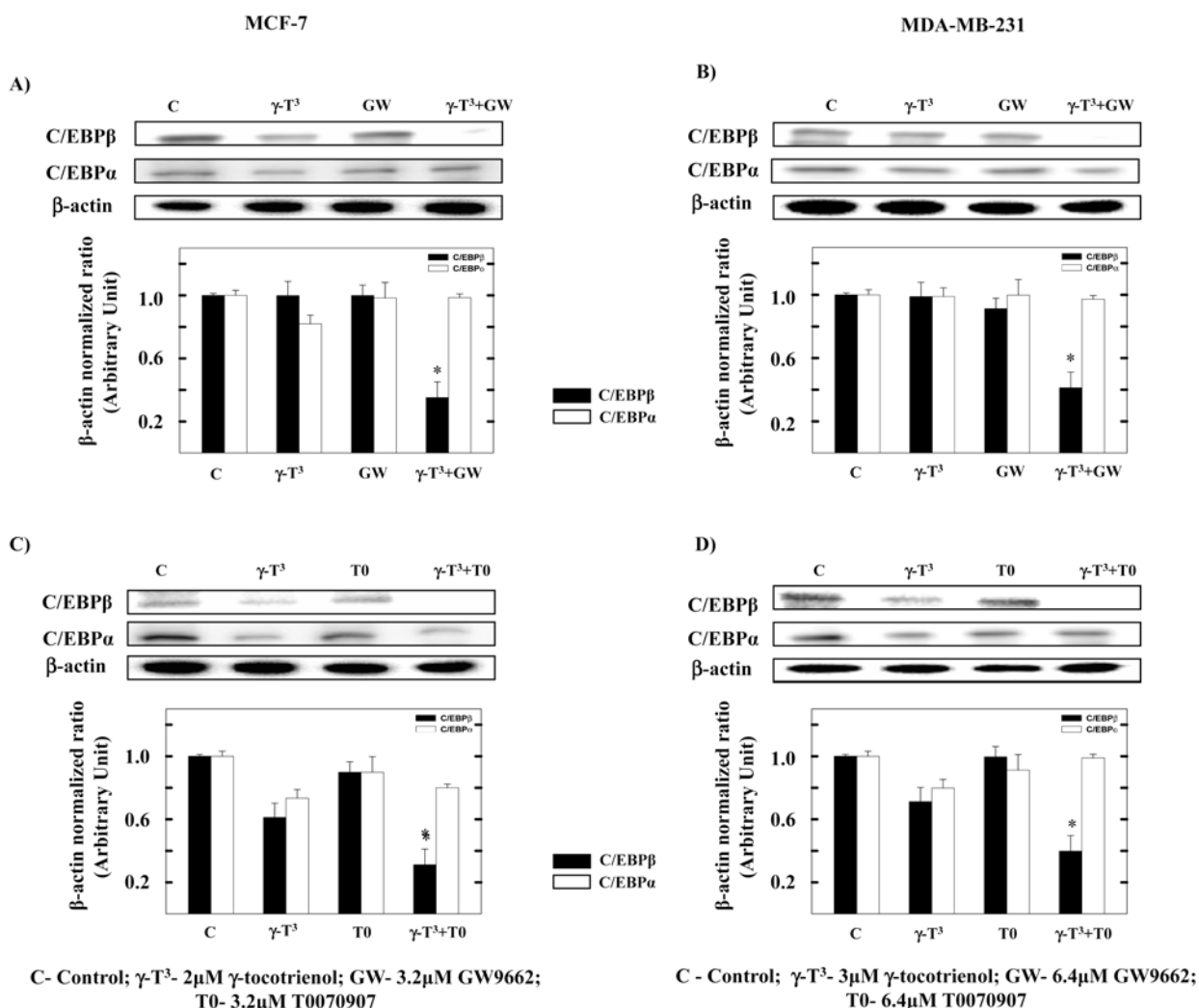
Figure 3: Western blot analysis of treatment of γ-tocotrienol, GW9662, and T0070907 given alone or in combination on the levels of C/EBPα and C/EBPβ after a 4-day incubation period in (**A** and **C**) MCF-7 and (**B** and **D**) MDA-MB-231 breast cancer cells. Cells were plated at 1X10⁶ cells/100 mm culture dish and treated with control or treatment media for 4-day incubation period. Afterwards, whole cell lysates were prepared from each treatment group for subsequent separation by polyacrylamide gel electrophoresis (50µg/lane) followed by Western blot analysis. Scanning densitometric analysis was performed on all the blots done in triplicate and the integrated optical density of each bond was normalized with corresponding β-actin, as shown in bar graphs below their respective Western blot images. Vertical bars in the graphs indicate the normalized integrated optical density of bands visualized in each lane ± SEM (Arbitrary Unit). *P< 0.05 as compared with vehicle-treated controls.

change in the expression of C/EBPα in both MCF-7 and MDA-MB-231 cells as compared to vehicle-treated controls (Figure **3A**, **B**, **C**, and **D**).

3.4. Effects of γ-Tocotrienol and PPARγ Antagonist GW9662 and T0070907 Given Alone or in Combination on SREBP-1c, SREBP 2, and Lipin 1 Levels

Western blot analysis shows that treatment with 2 µM (MCF-7 cells) or 3 µM (MDA-MB-231 cells) γ-tocotrienol and 3.2 µM (MCF-7 cells) or 6.4 µM (MDA-MB-231 cells) of the PPARγ antagonists, GW9662 or T0070907 alone did not induce change in the expression of SREBP-1c, SREBP 2 and lipin 1 as compared to the vehicle treated controls (Figure **4A**, **B**,

C, and **D**). However, combined treatment with these same doses of γ-tocotrienol and GW9662 or T0070907 caused a significant reduction in levels of SREBP-1c and lipin 1 without change in the expression of SREBP 2 in both MCF-7 and MDA-MB-231 cells as compared to vehicle-treated controls (Figure **4A**, **B**, **C**, and **D**).

3.5. Effects of γ-Tocotrienol and PPARγ Antagonist GW9662 and T0070907 Given Alone or in Combination on mRNA Levels of C/EBPβ and SREBP-1c

qRT-PCR analysis shows that treatment with 2 µM (MCF-7 cells) or 3 µM (MDA-MB-231 cells) γ-tocotrienol and 3.2 µM (MCF-7 cells) or 6.4 µM (MDA-
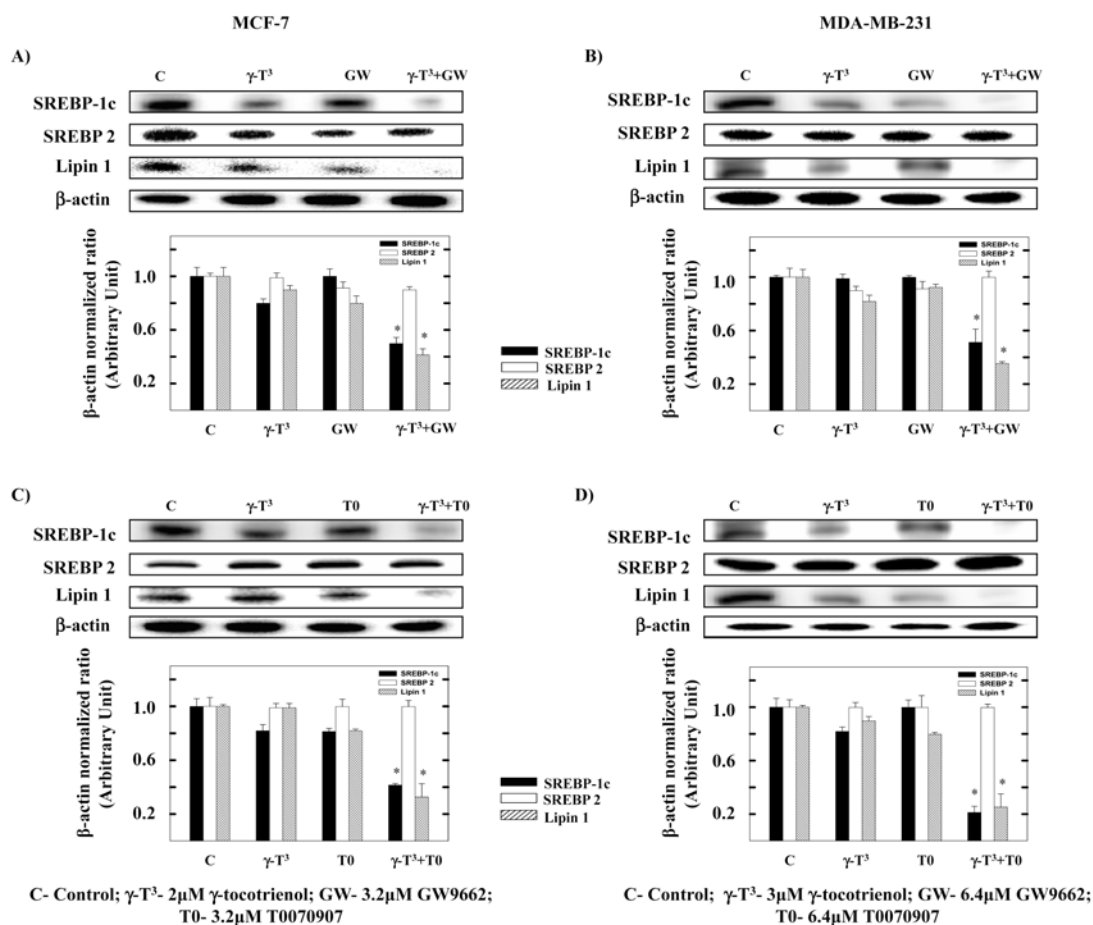
Figure 4: Western blot analysis of treatment of γ-tocotrienol, GW9662, and T0070907 given alone or in combination on the levels of SREBP-1c, SREBP 2 and Lipin 1 after a 4-day incubation period in (**A** and **C**) MCF-7 and (**B** and **D**) MDA-MB-231 breast cancer cells. Cells were plated at 1X10^6 cells/100 mm culture dish and treated with control or treatment media for 4-day incubation period. Afterwards, whole cell lysates were prepared from each treatment group for subsequent separation by polyacrylamide gel electrophoresis (50μg/lane) followed by Western blot analysis. Scanning densitometric analysis was performed on all the blots done in triplicate and the integrated optical density of each bond was normalized with corresponding β-actin, as shown in bar graphs below their respective Western blot images. Vertical bars in the graphs indicate the normalized integrated optical density of bands visualized in each lane ± SEM (Arbitrary Unit). *P< 0.05 as compared with vehicle-treated controls.
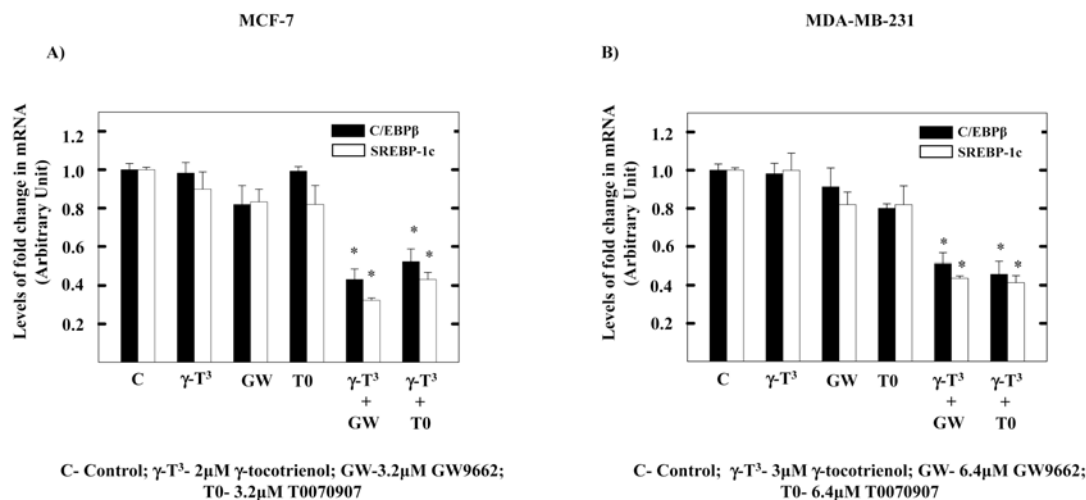

Figure 5: qRT-PCR analysis of treatment of γ-tocotrienol, GW9662, and T0070907 given alone or in combination on the levels of C/EBPβ and SREBP-1c in (**A**) MCF-7 and (**B**) MDA-MB-231 breast cancer cells after a 4-day incubation period. Changes in mRNA levels of C/EBPβ and SREBP-1c were normalized to mRNA level of GAPDH and represented as bar graph. Vertical bars indicate the normalized C$_t$ value ± SEM (Arbitrary Unit) in each treatment group. *P< 0.05 as compared with vehicle-treated controls.

MB-231 cells) of the PPARγ antagonists, GW9662 or T0070907 alone did not induce any change in the mRNA levels of C/EBPβ and SREBP-1c (Figure **5A** and **B**). However, combined treatment with these same doses of γ-tocotrienol and GW9662 or T0070907 caused a significant reduction in mRNA levels of C/EBPβ and SREBP-1c in both MCF-7 and MDA-MB-231 cells as compared to vehicle-treated controls (Figure **5A** and **B**).

3.6. Effects of γ-Tocotrienol and PPARγ Antagonist GW9662 and T0070907 Given Alone or in Combination on FAS, ap2, and HMGCoR Levels

Western blot analysis shows that treatment with 2 μM (MCF-7 cells) or 3 μM (MDA-MB-231 cells) γ-tocotrienol and 3.2 μM (MCF-7 cells) or 6.4 μM (MDA-MB-231 cells) of the PPARγ antagonists, GW9662 or

T0070907 alone did not induce any change in expression of FAS, ap2 and HMGCoR as compared to the vehicle-treated controls (Figure **6A**, **B**, **C**, and **D**). However, combined treatment with these same doses of γ-tocotrienol and GW9662 or T0070907 caused a significant reduction in levels of FAS, ap2, and HMGCoR in both MCF-7 and MDA-MB-231 cells as compared to vehicle-treated controls (Figure **6A**, **B**, **C**, and **D**).

3.7. Effects of γ-Tocotrienol and PPARγ Antagonist GW9662 and T0070907 Given Alone or in Combination on Levels of C/EBPβ and SREBP-1c in PPARγ siRNA Transfected PPARγ Positive MCF-7 and MDA-MB-231 Breast Cancer Cells

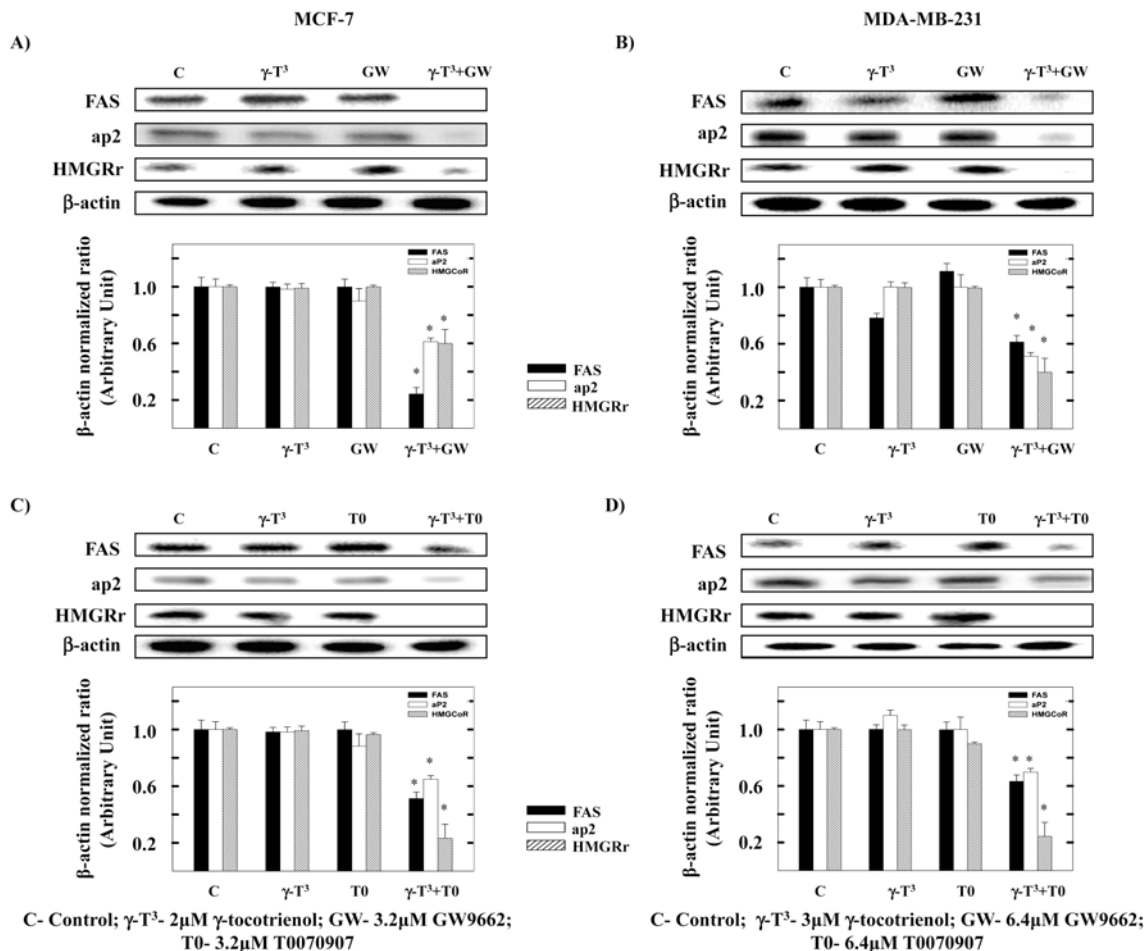PPARγ positive MCF-7 and MDA-MB-231 breast cancer cells were transfected with PPARγ siRNA.

Figure 6: Western blot analysis of treatment of γ-tocotrienol, GW9662, and T0070907 given alone or in combination on the levels of FAS, ap2 and HMGCoR after a 4-day incubation period in (**A** and **C**) MCF-7 and (**B** and **D**) MDA-MB-231 breast cancer cells. Cells were plated at 1X10^6 cells/100 mm culture dish and treated with control or treatment media for 4-day incubation period. Afterwards, whole 6 cell lysates were prepared from each treatment group for subsequent separation by polyacrylamide gel electrophoresis (50μg/lane) followed by Western blot analysis. Scanning densitometric analysis was performed on all the blots done in triplicate and the integrated optical density of each bond was normalized with corresponding β-actin, as shown in bar graphs below their respective Western blot images. Vertical bars in the graphs indicate the normalized integrated optical density of bands visualized in each lane ± SEM (Arbitrary Unit). *P< 0.05 as compared with vehicle-treated controls.
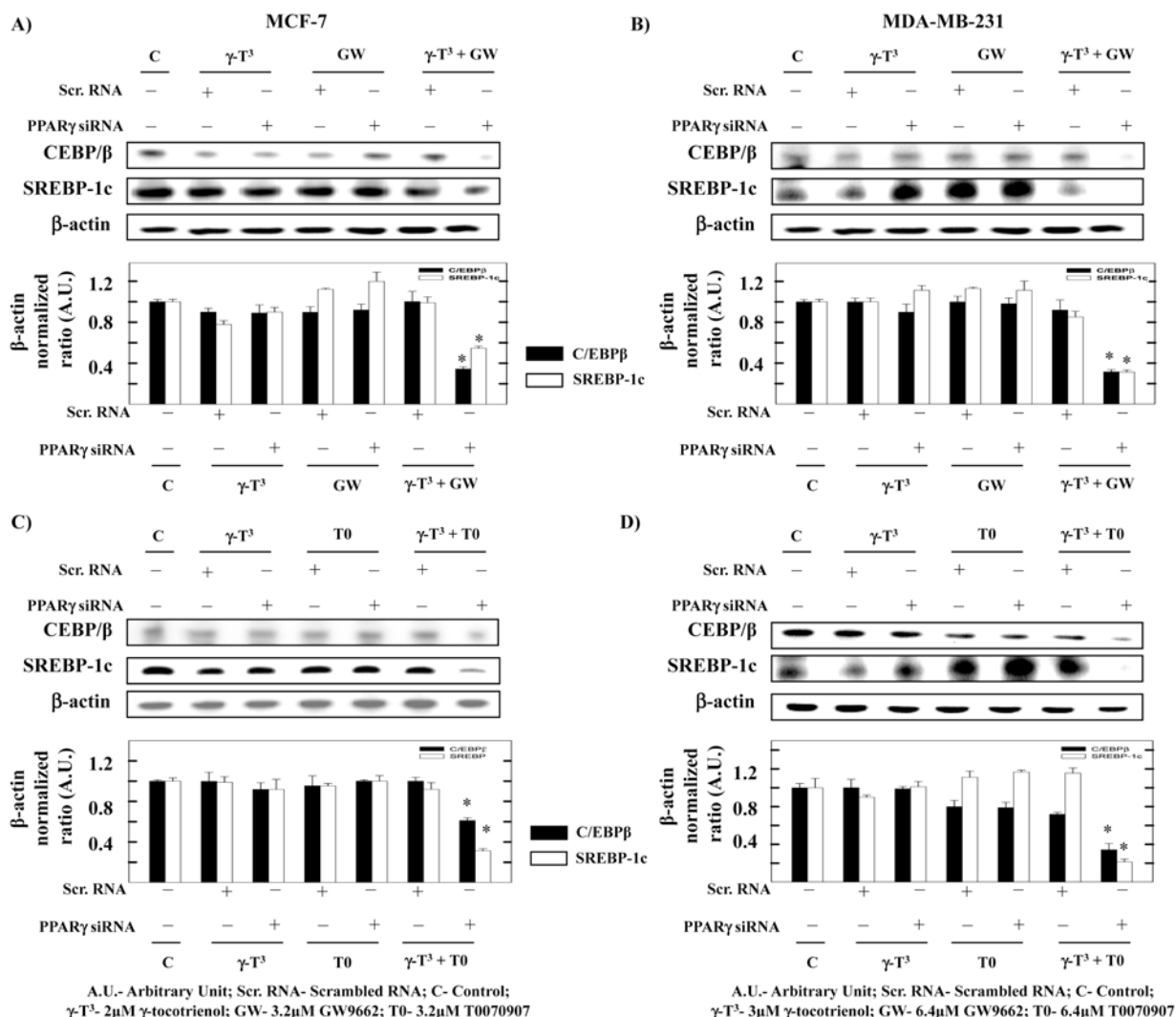
Figure 7: Western blot analysis of treatment effects on levels of C/EBPβ and SREBP-1c in PPARγ siRNA transfected (**A** and **C**) MCF-7 and (**B** and **D**) MDA-MB-231 cells. Cells were plated at a density of 2×10^5 cells/well (3 replicates per group) in 6-well plate in 2ml antibiotic free media. Transfections were performed using 5μL lipofectamine^TM 2000 according to the manufacturer's protocol. Cells were then exposed to control or treatment media for a 4-day culture period. Afterwards whole cell lysates were prepared from each treatment group for subsequent separation by polyacrylamide gel electrophoresis (50μg/lane) followed by Western blot analysis. Scanning densitometric analysis was performed on all the blots done in triplicate and the integrated optical density of each bond was normalized with corresponding β-actin, as shown in bar graphs below their respective Western blot images. Vertical bars in the graphs indicate the normalized integrated optical density of bands visualized in each lane ± SEM (Arbitrary Unit). *$P < 0.05$ as compared with vehicle-treated controls.

Western blot analysis showed that treatment with 2 μM γ-tocotrienol (MCF-7) or 3 μM (MDA-MB-231 cells) γ-tocotrienol and 3.2 μM (MCF-7 cells) or 6.4 μM (MDA-MB-231 cells) of the PPARγ antagonists, GW9662 or T0070907 alone did not induce any change in the expression of C/EBPβ and SREBP-1c in comparison to vehicle treated controls or cells transfected with scrambled RNA (Figure **7A**, **B**, **C**, and **D**). However, combined treatment with these same doses of γ-tocotrienol and GW9662 or T0070907 caused a reduction in levels of effects in the expression of C/EBPβ and SREBP-1c as compared to vehicle treated controls or cells transfected with scrambled RNA in

PPARγ positive MCF-7 and MDA-MB-231 breast cancer cells (Figure **7A**, **B**, **C**, and **D**).

4. DISCUSSION

Previous studies showed that combined treatment of γ-tocotrienol with PPARγ antagonists (GW9662 and T0070907) resulted in a synergistic inhibition of MCF-7 and MDA-MB-231 breast cancer cell growth that was mediated by a direct decrease in PPARγ activity and expression [4]. Results in the present study extend these previous findings by demonstrating that combination treatment effects induced by γ-tocotrienol and PPARγ antagonists is associated with a

corresponding reduction in adipogenic transcription factors in these human breast cancer cells. Western blot and qRT-PCR studies show that combination treatment resulted in a large reduction in adipogenic protein expression, particularly C/EBPβ, SREBP-1c, ap2, FAS, and HMGCoR. Additional studies showed that combination treatment induced inhibition of C/EBPβ and SREBP-1c was still observed in MCF-7 and MDA-MB-231 cells transfected with PPARγ siRNA, indicating that treatment effects occur independently of PPARγ expression and activity. These results demonstrate that combined treatment of γ-tocotrienol with PPARγ antagonists decreased levels of C/EBPβ, SREBP-1c and their target lipogenic enzymes (ap2, FAS, and HMGCoR) in highly proliferating breast cancer cells. These findings suggest that similar combination therapies may provide an important strategy in the treatment of breast cancer.

There is compelling evidence indicating the important role of PPAR family members in lipid sensing and utilization and to aid in cellular protection against lipid excess. Particularly, PPARγ plays a role in fatty acid uptake and transport (e.g. by adipocytes), and acts to control inflammation that can arise from increased adipocyte differentiation and proliferation [30, 31]. However, cancer cells characteristically display high levels of lipids and lipid precursors together with increased levels of PPARγ [32]. It has been hypothesized that PPARγ may act to promote the growth and survival of these highly proliferating malignant cell populations [32]. Numerous investigations have established that γ-tocotrienol is a potent anticancer agent that inhibits the growth of mouse [3, 33] and human [34, 35] breast cancer cells. In addition, γ-tocotrienol has been shown to inhibit adipogenesis in 3T3-L1 preadipocytes by suppressing insulin-induced Akt phosphorylation [24]. Studies have also shown that combined treatment of γ-tocotrienol with other traditional chemotherapies results in synergistic or additive antiproliferative effects against various types of cancer cells [3, 33]. Results in the present study further emphasize the importance of down-regulation of PPARγ in cancer cells as a potential therapeutic strategy [1, 8, 36].

PPARγ regulates adipogenesis with the help of C/EBP family of proteins [37]. Studies conducted on mammary tumor samples show that increased expression of PPARγ results in a corresponding increase in C/EBPβ, and subsequent increase in cancer cell proliferation [38]. In contrast, other studies showed that C/EBPα was not involved in cancer

progression [17]. Additional reports have shown that PPARγ regulates the SREBP family of transcription factors that are associated with adipocyte differentiation, cholesterol synthesis, and lipid metabolism [39, 40]. PPARγ induces ectopic expression of AAD/SREBP-1c and a related transcription factor SREBP 2 in 3T3L1 cells and HepG2 cells [41]. SREBP-1c and SREBP 2 in turn regulate lipid metabolism by activating FASN [42]. Studies show that there is an increased expression of SREBP-1c and FASN mRNA levels, while SREBP-2 showed less variation in MCF-7 breast cancer cells [18]. Furthermore, targeting SREBP-1c and FASN has recently been recommended as a treatment against breast cancer [43]. PPARγ regulates nuclear translocation of SREBP-1c by controlling lipin 1, a phosphatidic acid phosphatase in 3T3-L1 adipocytes [44]. Lipin 1 is also associated with insulin sensitivity and glucose metabolism in human samples [45]. The decreased level of lipin 1 in the present study suggests that combination treatment may also have a potential role in decreasing glucose metabolism in breast cancer cells. Additional experiments are needed to confirm this hypothesis.

A study conducted by Sim *et al.*, showed that PPARγ antagonist, GW9662 decreased levels of SREBP and HMGCoR to inhibit cholesterol synthesis in human glial progenitor cells. This study suggests the potential role of PPARγ antagonists in inhibiting cholesterol synthesis [46]. HMGCoR is the rate-limiting enzyme in mevalonate pathway for cholesterol synthesis [47]. Interestingly, the mevalonate pathway is constitutively active in malignant cells due to elevated and unregulated HMGCoR activity [23]. Tocotrienols appear to reduce HMGCoR activity by triggering both the retention of SREBP-1c on the endoplasmic reticulum and the degradation of HMGCoR, thereby inducing the reduction in both mRNA and protein HMGCoR levels [25, 48, 49]. Furthermore, combined treatment with subeffective doses of γ-tocotrienol and various statins (HMGCoR inhibitors) was found to significantly inhibit +SA mammary tumor cell growth associated with a large reduction in HMGCoR expression [23].

PPARγ induced expression of C/EBPβ is associated with an increase in adipogenesis and synthesis of adipocyte-selective ap2 (fatty acid binding protein) [50]. This adipocyte-selective protein strikes a balance between availability of metabolic resources and the control of inflammatory responses. Studies have shown that these proteins are heavily and aberrantly

expressed in tumors where they appear to regulate growth and survival responses. Therefore, ap2 offers targeting opportunities for the development of therapeutic treatment for not only cancer, but also other metabolic diseases [51]. Apart from its role as an adipogenic protein, some studies suggest that ap2 may also be involved in breast cancer cell invasion and metastasis, and suggest that ap2 might be used as a diagnostic marker for breast cancer [52]. Other studies have shown that anticancer effects induced by PPARγ antagonists are mediated, in part, by PPARγ-independent mechanisms [53, 54]. These findings are further supported by results in the present study demonstrating that PPARγ ligands mediate adipogenesis through PPARγ-independent mechanisms.

In summary, results in the present study show that combined treatment of γ-tocotrienol with PPARγ antagonists suppress adipogenic proteins and their target genes C/EBPβ, SREBP-1c, ap2, FAS, and HMGCoR. Furthermore, these combination treatment induced effects were found to occur independently of PPARγ expression and activity. Taken together, these findings demonstrate the potential importance of combined treatment of γ-tocotrienol with PPARγ antagonists as a therapeutic strategy in treatment of breast cancer.

CONFLICT OF INTEREST

ACKNOWLEDGEMENTS

ABBREVIATIONS

Akt	=	Protein kinase B
BSA	=	Bovine serum albumin
CEBP	=	CCAAT enhancer binding protein
DMEM	=	Dulbecco's modified eagle medium
DMSO	=	Dimethyl sulfoxide
EDTA	=	Ethylene diamino tetra acetic acid
FA	=	Fatty acid
FASN	=	Fatty acid synthase
FBS	=	Fetal bovine serum
FABP	=	Fatty acid binding protein
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	=	Phosphate buffer solution
PEPCK	=	Phophoenolpyruvate carboxykinase
PPARγ	=	Peroxisome proliferator activated receptor gamma
PPRE	=	Peroxisome proliferator activated receptor
PVDF	=	Polyvinylidene fluoride
RNA	=	Ribonucleic acid
ScrRNA	=	Scrambled RNA
siRNA	=	Silence RNA
SDS	=	Sodium dodecyl sulfate
SREBP	=	Sterol regulatory binding protein
TBST	=	Tris buffered saline with tween 20
UV	=	Ultraviolet

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