Growth Inhibition of Human MDA-MB-231 Breast Cancer Cells by δ-Tocotrienol Is Associated with Loss of Cyclin D1/CDK4 Expression and Accompanying Changes in the State of Phosphorylation of the Retinoblastoma Tumor Suppressor Gene Product

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Abstract. *Tocotrienols, a subgroup within the vitamin E family of compounds, have shown antiproliferative and anticancer properties, however, the molecular basis of these effects remains to be elucidated. In this study, the effect of δ-tocotrienol on cell cycle arrest was assessed by studying the retinoblastoma protein (Rb) levels and phosphorylation status, levels of E2F (a transcription factor critically involved in the G1/S-phase transition of the mammalian cell cycle; originally identified as a DNA-binding protein essential for early region 1A-dependent activation of the adenovirus promoter designated E2), and other cell cycle controlling proteins in estrogen receptor-negative MDA-MB-231 breast cancer cells. The cell growth assay demonstrated that exposure of the MDA-MB-231 cells to δ-tocotrienol (1-20 μM) resulted in a dose- and time-dependent inhibition of cell growth as compared with vehicle treated cells and the magnitude of growth inhibition was higher at 10 and 20 μM treatment for 48 and 72 h. The phosphorylation status of Rb plays a central role in the control of the cell cycle at the G0/G1-phase. δ-Tocotrienol treatment reduced the total Rb and its phosphorylation at the Ser780, Ser795, Ser 807/811 and Thr826 positions in a dose- and time-dependent fashion. The site-specific inhibition of the phosphorylation of Rb by δ-tocotrienol was tightly associated with a marked reduction in the expression of cyclin D1 and its regulatory partner cyclin-dependant kinase 4 (CDK4), which is responsible for the phosphorylation of Rb at Ser780, Ser795, Ser 807/811 and Thr826. In addition, δ-tocotrienol also reduced the expression of E2F that occurred simultaneously with the loss of Rb phosphorylation and inhibition of cell cycle progression. Interestingly, δ-tocotrienol also caused a marked reduction in the expression of G2/M regulatory proteins including cyclin B1 and CDK1. To the best of our knowledge, this study was the first to reveal that the target of cell proliferative inhibitory action of δ-tocotrienol in a model estrogen receptor-negative human breast cancer cell line MDA-MB-231 is mediated by the loss of cyclin D1 and associated suppression of site-specific Rb phosphorylation, suggesting its future development and use as an anticancer agent.*

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Key Words: Breast cancer, MDA-MB-231 cells, δ-tocotrienol, cell cycle, retinoblastoma, cyclin D1.

Among women worldwide, breast cancer is the most common cause of cancer death. The latest statistics indicated that about 1.3 million women are diagnosed with breast cancer annually worldwide and about 465,000 die from the disease. Women in the U.S. have a 1 in 8 lifetime chance of developing invasive breast cancer and a 1 in 33 chance of breast cancer causing their death (1). Thus, there is a great need for new alternative agents for the prevention and treatment of breast cancer. The use of naturally occurring compounds for chemoprevention, ones that are present in dietary sources, is considered to be a practical approach for the prevention of breast cancer. It has been suggested that one-third of all cancer deaths could be avoided through this approach (2).

During the past decade, much evidence has accumulated demonstrating the anticancer activity of specific forms of vitamin E. Vitamin E occurs in nature as at least eight different isoforms that include α-, β-, γ-, and δ-isomers of both tocopherol and tocotrienol. Structurally, these compounds are similar, except that tocotrienols have an unsaturated side-chain with three double bonds, whereas tocopherols have a fully saturated side-chain (3). Although both tocopherols and tocotrienols are potent antioxidants, only tocotrienols display potent antiproliferative and apoptotic activity against breast cancer cells that can be characterized as

δ-tocotrienol > γ-tocotrienol > α-tocotrienol > δ-tocopherol > γ- and α-tocopherol (4, 5). The accumulation of tocotrienols in cells has been shown to be much greater than that of tocopherols and this might be one of the reasons that tocotrienols have a more significant effect than tocopherols (6).

Tocotrienols and tocotrienol-rich fractions of palm oil led to the induction of apoptosis in malignant carcinoma cells, such as breast (3, 7), colon (8), prostate (9) and liver (10) cancer cells, by various inhibitory mechanisms. Antioxidant effects (6), the suppression of HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) activity (11), pro-apoptotic effects (9, 12), the regulation of mitogenesis (13), and anti-angiogenic potential (14) have been proposed as mechanisms of the antiproliferative effects of tocotrienols. These findings are of particular interest because these antiproliferative effects were observed using treatment doses that had little or no effect on normal mammary epithelial cell growth (4, 5). The relative selectivity displayed by tocotrienols against mammary tumor cells suggests that these compounds may provide significant therapeutic benefit in the prevention or treatment (or both) of breast cancer in women.

To date, there have been several publications regarding the use of α- and γ-tocotrienol to inhibit the growth of human breast cancer cells and other tumor cells (3). However, while previous studies have reported that δ-tocotrienol induced growth inhibition in human breast cancer cells (4, 15-17), its growth suppressive mechanism remains to be elucidated. The purposes of the present study were to investigate the effects of δ-tocotrienol on cell growth inhibition in MDA-MB-231 cells; to determine the level of retinoblastoma (Rb) phosphorylation in MDA-MB-231 cells and to explore the possible mechanism of regulating signals in MDA-MB-231 cells.

Materials and Methods

Reagents. δ-Tocotrienol was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The primary antibodies anti-Rb, anti-p-Rb Ser780, anti-p-Rb Ser795, anti-p-Rb Ser807/811, anti-p-Rb Thr826, anti-E2F1, anti-cyclin D1, anti-cyclin B1, anti-CDK4, anti-CDK1 and anti-actin, and the secondary antibodies were purchased from Santa Cruz Biotechnology. (Santa Cruz, CA, USA). Fetal calf serum, L-15 medium, penicillin and streptomycin were purchased from Cellgro Inc. (Herndon, VA, USA). All the other chemicals and solvents used were of analytical grade.

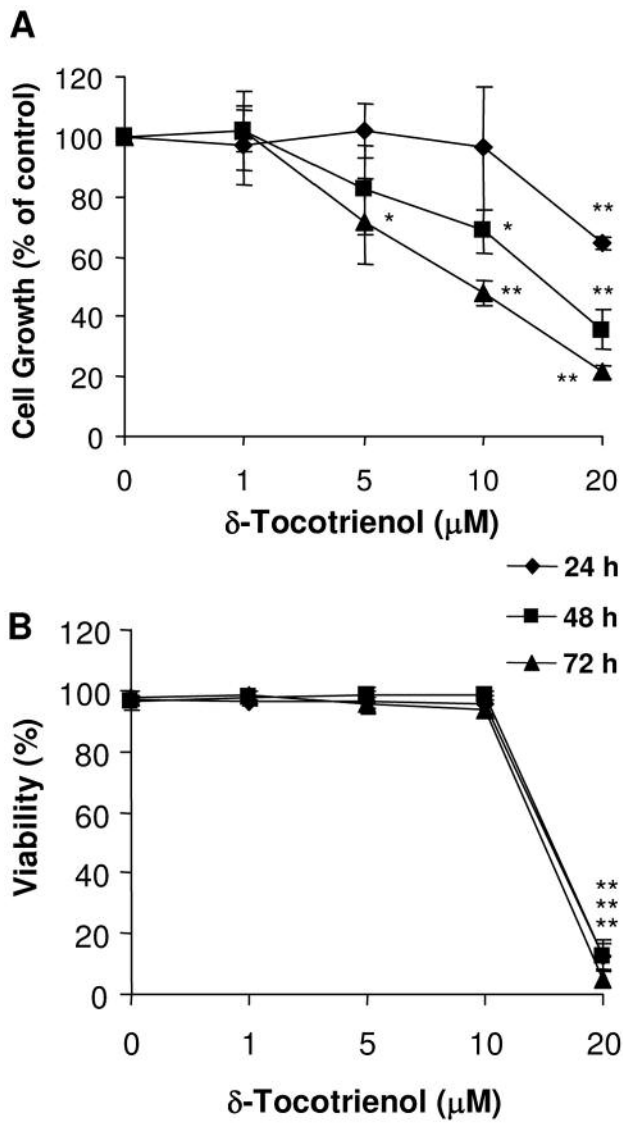
Cell culture and treatment. Human MDA-MB-231 breast cancer cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA) and maintained in L-15 medium supplemented with penicillin, streptomycin and 10% heat inactivated fetal calf serum in a humidified atmosphere at 37˚C, without CO_2 as previously described (18). δ-Tocotrienol, dissolved in ethanol, was added to the culture media to the final concentration specified in the text. The final concentration of ethanol in the culture medium during δ-tocotrienol treatment did not exceed 0.1% (v/v), and the same concentration of ethanol was added to the control dishes.

Cell growth assay. The MDA-MB-231 cells were plated at 5×10^4 cells/ml in 6-well plates under standard culture conditions. After 24 h, the cells were treated with either ethanol alone or 1-20 μM doses of δ-tocotrienol. After 24, 48 or 72 h of treatment, the cells were trypsinized, collected and counted using a hemocytometer. Trypan blue dye exclusion was used to determine cell viability.

Colony formation assay. The colony formation assay was performed as described previously with some modifications (18). The MDA-MB-231 cells were plated in 6-well tissue culture plates at a density of 800 cells/ml, 2 ml/well. After 24 h, the cells were treated with either ethanol alone or 1-20 μM doses of δ-tocotrienol, followed by an additional 8-day incubation to allow colonies to form. The colonies were fixed and stained with 1.25% crystal violet, washed to remove excessive dye and imaged by a scanner. Quantitative changes in clonogenicity were determined by extracting the colonies with 10% acetic acid and measuring the absorbance of the extracted dye at 595 nm.

Western blotting. For Western blotting, the MDA-MB-231 cells were cultured as detailed above and treated with either vehicle alone or with 1, 5 or 10 μM δ-tocotrienol and harvested by trypsinization after 24 or 72 h. The harvested cells were collected by centrifugation, washed with PBS and lysed in ice-cold RIPA buffer (50 mM Tris (pH 7.4) 150 mM NaCl, 1 mM EDTA, 1% Triton® X-100, 1% deoxycholate, 0.1 % SDS, 1 mM dithiothreitol and 10 μl/ml protease inhibitor cocktail). After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 12,000 g for 15 min. The protein content of the cell lysates were determined by a Coomassie protein assay kit (Pierce, IL, USA) with bovine serum albumen (BSA) as standard. An aliquot from each sample (20 μg of protein) was boiled with sample buffer for 5 min, and then resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred to a nitrocellulose membrane (Whatman, Middlesex, UK) and then blocked in TBST buffer (10 mM Tris (pH 7.5) 100 mM NaCl and 0.05% Tween 20) containing 3% nonfat dried milk overnight at 4˚C. The blots were probed with the primary antibodies (anti-Rb, anti-p-Rb Ser780, anti-p-Rb Ser795, anti-p-Rb Ser807/811, anti-p-Rb Thr826, anti-E2F1, anti-cyclin D1, anti-cyclin B1, anti-CDK4, anti-CDK1, anti-actin) overnight and then washed three times in TBST, followed by incubation for 1 h with horseradish peroxidase-coupled anti-mouse IgG or anti-rabbit IgG. The blots were then washed in TBST and visualized by an enhanced chemiluminescent (ECL) detection system (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). In some instances, the blots were stripped by incubation in stripping buffer (62.5 mM Tris (pH 6.7) 100 mM β-mercaptoethanol and 2% SDS) for 5 min at 50˚C and then reprobed with other antibodies. The intensity of the specific immunoreactive bands was quantified by densitometry and expressed as a ratio compared to the internal control.

Data analysis. The results are expressed as mean±standard deviation (SD). Differences between groups were assessed by one-way analysis of variance using the SPSS software package for Windows (SPSS Inc., Chicago, IL, USA). *Post hoc* testing was performed for inter-group comparisons using the least significance difference (LSD) test; statistical significance at *p*-values <0.001, <0.01 has been given respective symbols in the figures.



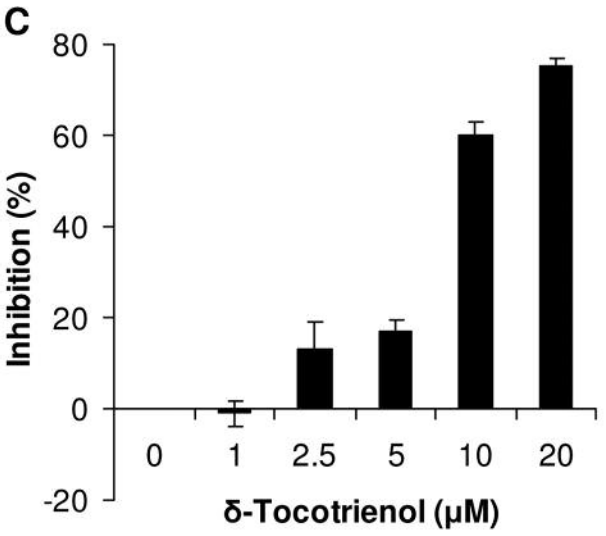


Figure 1. *Effect of δ-tocotrienol on cell growth and viability in MDA-MB-231 cells. (A) Cells were treated with increasing doses (1, 5, 10, 20 μM) of δ-tocotrienol and the cell numbers were counted at 24, 48 or 72 h by hemocytometer. The cell growth was expressed relative to the control. These results are mean±SD for three experiments; * and ** indicate statistical significance of p<0.01 and p<0.001, respectively, when compared to vehicle-treated controls. (B) Cell viability was measured at the end of each time-point using the trypan blue dye exclusion assay. The percentage of viable cells was calculated and expressed as mean±SD for three experiments; * and ** indicate statistical significance of p<0.01 and p<0.001, respectively, when compared to vehicle-treated controls. (C) Clonogenicity of MDA-MB-231 cells. Cells were plated, in triplicate, in 6-well tissue culture plates at a density of 800 cells/ml in the absence or presence of the indicated concentration of δ-tocotrienol. After 1 week, colonies were stained with 1.25% crystal violet, and quantified by extraction with 10% acetic acid and measuring the absorbance at 595 nm. Values are expressed as mean±SD for three experiments.*

Results

Effect of δ-tocotrienol on MDA-MB-231 cell growth. The δ-tocotrienol treatment resulted in dose- and time-dependent inhibition of cell growth, compared with the controls, and the magnitude of cell growth suppression was higher at 72 h exposure to 10 and 20 μM δ-tocotrienol (52% and 78% respectively; Figure 1A). δ-Tocotrienol-induced cell growth arrest was accompanied by a decrease in cell viability at the 20 μM dose (Figure 1B). However, treatment with 1-10 μM δ-tocotrienol had minimal effect on the viability of the MDA-MB-231 cells, which suggested that extensive death of the MDA-MB-231 cells was occurring only at the higher dose. In the clonogenic assay, colony formation reflects cell survival. The results in Figure 1C showed that the inhibition of colony formation occurred in a dose-dependent fashion with the δ-tocotrienol treatment.

Effect of δ-tocotrienol on Rb phosphorylation at specific sites. To elucidate the role of Rb in δ-tocotrienol-induced MDA-MB-231 cell growth inhibition, the total Rb expression and the phosphorylated state of Rb were assessed by Western blot using phospho-specific antibodies (Figure 2). Treatment of the MDA-MB-231 cells with δ-tocotrienol caused a significant reduction of Rb phosphorylation at specific serine sites. The densitometric analysis of the blots showed that phosphorylation at Ser780, Ser795 and Ser 807/811 decreased by 40, 57 and 59% , respectively, at 24 h, and by 60, 47 and 67% at 72 h after 10 μM δ-tocotrienol treatment. Similarly, δ-tocotrienol exposure also resulted in reduced phosphorylation at Thr826:

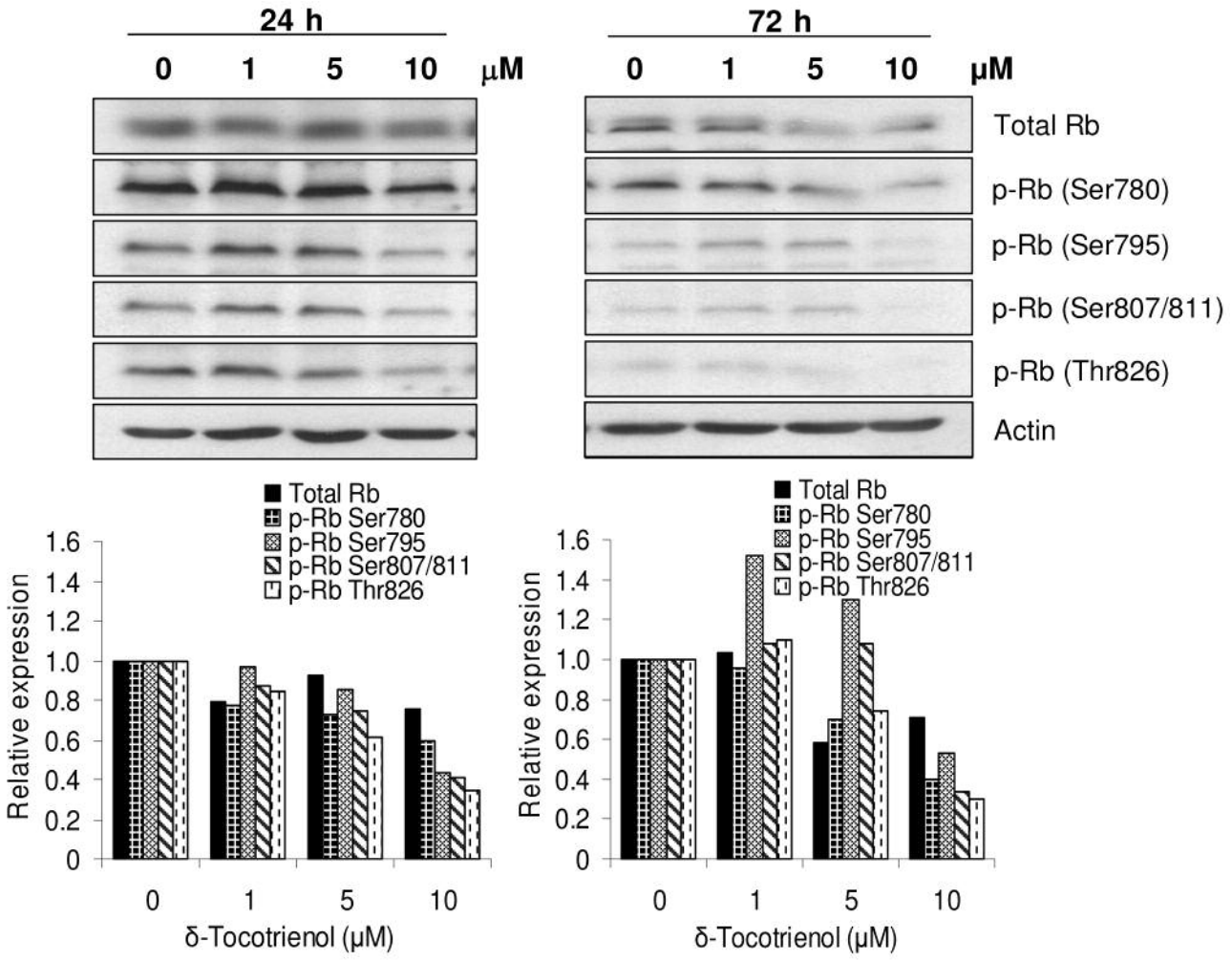


Figure 2. *Effect of δ-tocotrienol on Rb phosphorylation in MDA-MB-231 cells. Cells were exposed to1, 5 or 10 μM concentrations of δ-tocotrienol for 24 or 72 h. Cell lysates were prepared, fractionated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed for total Rb and phosphorylated Rb at Ser780, Ser795, Ser807/811 and Thr826 positions. The blots were stripped and reprobed with actin which served as loading control. Densitometric data provided under the immunoblots are normalized to a level of 1.0 present in control cells in relation to the expression of actin in each sample.*

65% decrease at 24 h and 70% decrease at 72 h at the 10 μM dose. No change in Rb phosphorylation was found at the Thr821 site (data not shown). δ-Tocotrienol was not effective in modulating the total Rb protein levels, except after 72 h treatment at the 5 and 10 μM doses, which showed a 41 and 29% decrease, respectively, compared with the control.

Effect of δ-tocotrienol on cyclin D1 and E2F expression. Because cyclin D1 and its regulatory partner CDK4 are responsible for the phosphorylation of Rb at Ser780, Ser795, Ser 807/811 and Thr826 (19), the effect of δ-tocotrienol on cyclin D1 and CDK4 levels was investigated. The δ-tocotrienol treatment resulted in a marked decrease in the protein levels of cyclin D1 at 24 and 72 h. Densitometric analysis showed a decrease of 21, 30 and 38 % at 24 h after treatment with 1, 5 and 10 μM δ-tocotrienol, respectively (Figure 3). The reduced cyclin D1 expression was also accompanied by reduced expression of its regulatory partner, CDK4. The most profound loss of cyclin D1 was observed as early as 24 h.

E2F transcription factors are known to exist in a bound form with Rb, inhibiting their transcriptional activity, however, after a growth signal that phosphorylates Rb, E2Fs are released from Rb and thus allow specific gene transcription (20). As shown in Figure 3, δ-tocotrienol treatment of the MDA-MB-231 cells resulted in a strong decrease in E2F1 protein expression in a dose-dependent manner. Up to 64 and 73 % decreases were observed in the E2F1 protein levels after 10 μM δ-tocotrienol exposure of MDA-MB-231 cells for 24 and 72 h, respectively.

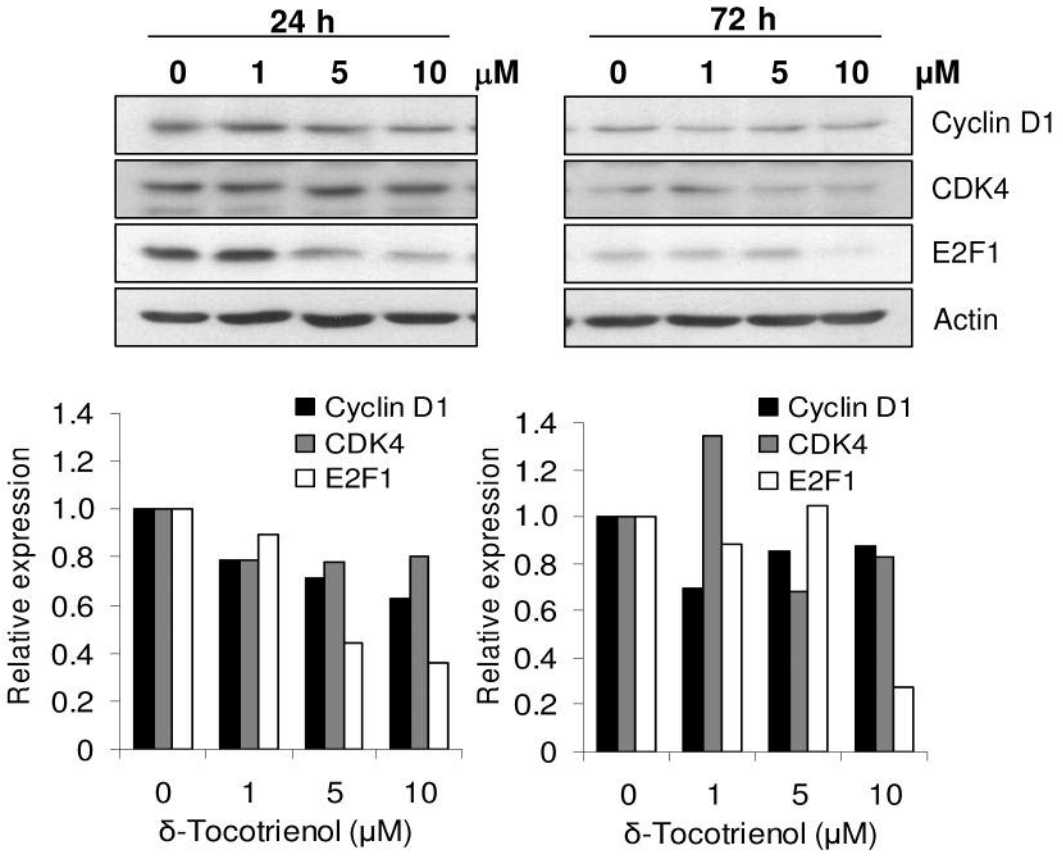


Figure 3. *Effect of δ-tocotrienol on the expression of cyclin D1, CDK4, E2F1 in MDA-MB-231 cells. Cells were exposed to 1, 5 or 10 μM concentrations of δ-tocotrienol for 24 or 72 h. Cell lysates were prepared, fractionated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed for cyclin D1, CDK4 and E2F1. The blots were stripped and reprobed with actin which served as loading control. Densitometric data provided under the immunoblots are normalized to a level of 1.0 present in control cells in relation to the expression of actin in each sample.*

Effect of δ-tocotrienol on cyclin B1/CDK1 expression. On the basis of our findings showing a loss of Rb phosphorylation at specific sites and corresponding alterations of G1 cell cycle regulators such as cyclin D1 and E2F1, the effect of δ-tocotrienol on the G2/M cell cycle regulators cyclin B1 and cdc2p34 (CDK1) was assessed. Similar δ-tocotrienol treatment of the MDAMB-231 cells resulted in a decrease in cyclin B1 and CDK1 protein levels (Figure 4). Compared with their respective controls, densitometry analysis of the blots for cyclin B1 induced a maximum of 23 and 84% decrease in protein levels after 24 and 72 h treatment at the 10 μM dose of δ-tocotrienol, respectively. Similarly, δ-tocotrienol also caused a strong decrease (69%) in CDK1 protein level after 72 h treatment at the 10 μM dose. δ-Tocotrienol was overall not effective in modulating cyclin E/CDK2 complex levels at any of the doses or time-points studied (data not shown).

Discussion

Understanding the regulatory mechanism controlling cell cycle progression and cell growth could play a critical role in the development of new agents that can prevent and treat cancer. The present study demonstrated that δ-tocotrienol reproducibly inhibited Rb phosphorylation in a dose- and time-dependent manner in human MDA-MB-231 cells. Rb is an archetypal tumor suppressor and cell cycle regulator which controls progression through the late G1-phase and thereby commitment to enter the S-phase and complete the cell cycle (21, 22). Rb contains 16 Ser/Thr pro-sequence sites that have been demonstrated to be phosphorylated *in vitro* (23). The kinases responsible for Rb phosphorylation *in vivo* are known to include members of the CDK family including CDK2 in association with cyclins E and A as well as CDK4 and CDK6 in association with D-type cyclin (24). During the cell cycle progression, Rb is

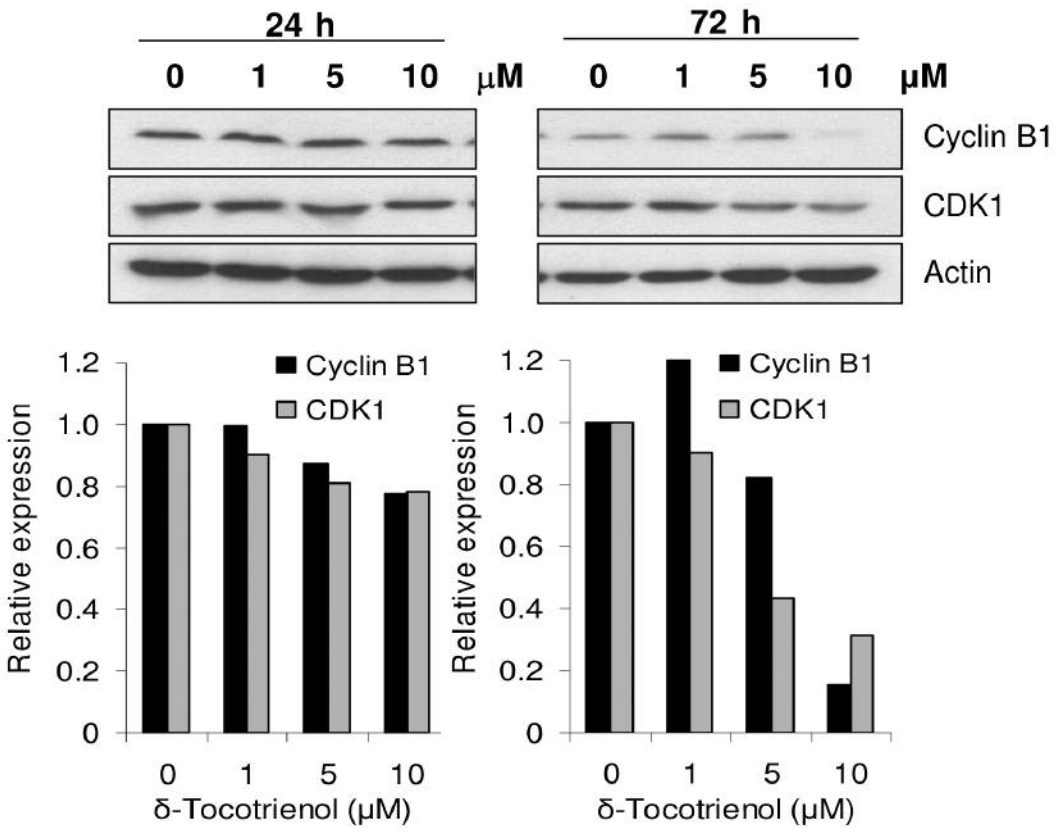


Figure 4. *Effect of δ-tocotrienol on the expression of cyclin B1/CDK1 in MDA-MB-231 cells. Cells were exposed to 1, 5 or 10 μM concentrations of δ-tocotrienol for 24 or 72 h. Cell lysates were prepared, fractionated on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed for cyclin B1 and CDK1. The blots were stripped and reprobed with actin which served as loading control. Densitometric data provided under the immunoblots are normalized to a level of 1.0 present in control cells in relation to the expression of actin in each sample.*

sequentially phosphorylated by different cyclin/CDK complexes at particular Rb residues (25). This suggests that different sites of Rb phosphorylation by distinct CDKs may result in differential regulation of downstream effector pathways. Among the phosphorylation sites in pRb, Ser780, Ser795, Ser 807/811 and Thr826 have been reported to be phosphorylated by CDK4/cyclin D, and Thr821 has been reported to be phosphorylated by CDK2/cyclin E (19, 26). This coincided with the present observation that δ-tocotrienol down-regulated the phosphorylation of Rb on Ser780, Ser795 and Ser 807/811 and Thr826, presumably because of a decrease in the cyclin D1/CDK4 complex. The observation that δ-tocotrienol had no effect on phosphorylation of Rb at Thr821 implied that δ-tocotrienol did not affect CDK2 activity (data not shown). In the hypophosphorylated state, the Rb associates with and inhibit the activity of E2F family transcription factors, which are involved in the transcription of key cell cycle regulatory proteins (20). Profound loss of cyclin D1, CDK4 and E2F1 was observed after 24 h exposure to the δ-tocotrienol, suggesting that these cancer cells would be unable to move to S-phase.

In addition to demonstrating that δ-tocotrienol inhibited cyclin D1 and CDK4, the δ-tocotrienol treatment led to the down-regulation of cyclin B1 and CDK1 that are required for the progression of the cell cycle through the G_2/M checkpoint. The negative regulation of these positive regulators of cell cycle progression would impair regulation of the cell cycle and contribute to the increase in G_1 and G_2/M arrest following addition of δ-tocotrienol.

δ-Tocotrienol suppresses the signaling pathway of cell proliferation in MDA-MB-231 cells by suppressing the expression of cyclin D1 and CDK4, leading to a decrease in site-specific phosphorylation of Rb. The findings support the premise that δ-tocotrienol is a potentially promising agent for development as a chemopreventive and/or chemotherapeutic agent against breast cancer.

Acknowledgements

This research was supported in part by a research grant from the Susan G. Komen Breast Cancer Foundation (BCTR0600943) to JMW.

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Received April 18, 2008
Revised June 5, 2008
Accepted June 17, 2008