

2010

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## Recommended Citation

Fernandes N, Guntipalli PK, Mo H. (2010) d- $\beta$ -Tocotrienol-mediated cell cycle arrest and apoptosis in human melanoma cells. *Anticancer Research* 30:4937-4944.

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## ***d*- $\delta$ -Tocotrienol-mediated Cell Cycle Arrest and Apoptosis in Human Melanoma Cells**

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**Abstract.** *Background: The rate-limiting enzyme of the mevalonate pathway, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, provides essential intermediates for the prenylation or dolichylation of growth-related proteins. *d*- $\delta$ -Tocotrienol, a post-transcriptional down-regulator of HMG CoA reductase, suppresses the proliferation of murine B16 melanoma cells. Dietary *d*- $\delta$ -tocotrienol suppresses the growth of implanted B16 melanomas without toxicity to host mice. Materials and Methods: The proliferation of human A2058 and A375 melanoma cells following a 72 h incubation in 96-well plates was measured by CellTiter 96<sup>®</sup> Aqueous One Solution. Cell cycle distribution was determined by flow cytometry. Fluorescence microscopy following acridine orange and ethidium bromide dual staining and procaspase-3 cleavage were used to detect apoptosis. Western-blot was employed to measure protein expression. Results: *d*- $\delta$ -Tocotrienol induced dose-dependent suppression of cell proliferation with 50% inhibitory concentrations (IC<sub>50</sub>) of 37.5 $\pm$ 1.4 (A2058) and 22.3 $\pm$ 1.8 (A375)  $\mu$ mol/l, respectively (data are reported as mean $\pm$ standard deviation). *d*- $\delta$ -Tocotrienol-mediated cell cycle arrest at the G<sub>1</sub> phase was accompanied by reduced expression of cyclin-dependent kinase 4. Concomitantly, *d*- $\delta$ -tocotrienol induced caspase-3 activation and apoptosis. The impact of *d*- $\delta$ -tocotrienol on A2058 cell proliferation was potentiated by lovastatin (IC<sub>50</sub>=3.1 $\pm$ 0.5  $\mu$ mol/l), a competitive inhibitor of HMG CoA reductase. Conclusion: *d*- $\delta$ -Tocotrienol may have potential application in melanoma chemoprevention and/or therapy.*

The mevalonate pathway provides essential intermediates, namely farnesyl- and geranylgeranyl-pyrophosphates, for the

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*Key Words:* HMG CoA reductase, mevalonate, tocotrienol, statin, Cdk4, caspase-3, Ras, cell cycle, apoptosis.

post-translational modifications and biological activities of growth-related proteins including Ras (1), nuclear lamins (2) and insulin-like growth factor I receptor (3) and supports cell cycle progression and cell proliferation (4, 5). The tocotrienols are vitamin E isomers that post-transcriptionally down-regulate 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity, the rate-limiting activity of the mevalonate pathway (4). A recent publication reviewed the parallel impact of the tocotrienols and the statins, competitive inhibitors of HMG CoA reductase, on cell cycle progression, apoptosis, and cell proliferation consequent to tocotrienol- and statin-mediated mevalonate deprivation (6).

The dysregulation of HMG CoA reductase in tumors offers a unique target for tumor suppression. Distinctive from the multivalent regulation of the reductase activity consisting of sterol-mediated transcriptional feedback inhibition and non-sterol-mediated post-transcriptional ablation in sterologenic tissues, tumor reductase is resistant to sterol-feedback regulation but retains a unique sensitivity to the non-sterol- and tocotrienol-mediated down-regulation. As a consequence of the tocotrienol-mediated tumor-specific suppression of reductase (7), tocotrienols suppress the growth of cells isolated from breast, liver, prostate, skin, colon, blood, lung, lymph gland, cervix and nerve tumors; these actions are attributable to tocotrienol-mediated cell cycle arrest and initiation of apoptosis. Dietary tocotrienols suppress the growth of chemically initiated mammary, lung and liver carcinogenesis, that of implanted melanoma, prostate and mammary tumors and that of spontaneous liver tumors (reviewed in (6)).

It was previously shown that *d*- $\gamma$ -tocotrienol (8-10) and the more potent *d*- $\delta$ -tocotrienol (8, 9) suppress the proliferation of murine B16 melanoma cells. Cell cycle arrest at the G<sub>1</sub> phase and initiation of apoptosis contribute to the impact of *d*- $\gamma$ -tocotrienol (10). The growth of implanted B16 melanomas in mice was suppressed by dietary *d*- $\gamma$ -tocotrienol (9), and a blend of *d*- $\delta$ -tocotrienol and lovastatin (8); the latter impact was synergistic.

The current study evaluated the impact of *d*- $\delta$ -tocotrienol, the tocotrienol with the highest tumor-suppressive activity among the tocotrienol isomers (8), on the proliferation of

human A375 malignant melanoma cells and A2058 metastatic melanoma cells. In both cell lines, *d*- $\delta$ -tocotrienol mediated concentration-dependent suppression of the proliferation, which was accompanied by cell cycle arrest at the G<sub>1</sub> phase and initiation of apoptosis. Lovastatin potentiated the *d*- $\delta$ -tocotrienol-mediated growth suppression.

## Materials and Methods

**Chemicals.** Lovastatin and *d*- $\delta$ -tocotrienol were gifts from Merck Research Laboratories (Rahway, NJ, USA) and American River Nutrition, Inc. (Hadley, MA, USA), respectively.

**Cell proliferation assay.** Human melanoma cell proliferation was measured by using CellTiter 96<sup>®</sup> Aqueous One Solution (Promega, Madison, WI, USA) as previously described (11). Briefly, A375 and A2058 melanoma cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) modified by ATCC to contain 4 mM L-glutamate, 1.5g/l sodium bicarbonate, and 4.5 g/l glucose and supplemented with 10% fetal bovine serum (FBS; Hyclone Lab Inc., Logan, UT, USA) and 1% penicillin-streptomycin (MP Biomedicals, Solon, OH, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, were seeded at 3,000 cells/0.1 ml medium/well in 96-well tissue culture plates (Fisher Scientific Company LLC, Houston, TX, USA). At 24 h, the medium was decanted from each well and replaced with 0.1 ml fresh medium containing *d*- $\delta$ -tocotrienol and lovastatin that were pre-dissolved in ethyl alcohol and dimethyl sulfoxide (DMSO), respectively. All cultures including the controls contained 1 ml/l of ethyl alcohol or DMSO. Cells were incubated for additional 72 h. The 96-h cell populations were determined by adding 20  $\mu$ l of CellTiter 96<sup>®</sup> Aqueous One Solution to each well; plates were placed in the dark at 37°C for 2 h then read at 490 nm with a SPECTRAmax<sup>®</sup> 190 multi-plate reader with SOFTmax<sup>®</sup> PRO version 3.0 (Molecular Devices, Sunnyvale, CA, USA). Absorbances from wells containing cell-free medium were used as baselines and deducted from absorbances of other cell-containing wells. The IC<sub>50</sub> value was determined as the concentration of an agent required to suppress the net increase in cell number by 50%.

**Microscopy.** Photomicrographs of representative fields of monolayers of melanoma cells were produced with a Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corporation).

**Cell cycle analysis.** A375 and A2058 cells were seeded in 75 cm<sup>2</sup> flask (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 3 $\times$ 10<sup>6</sup> cells/flask with 10 ml medium/flask and incubated for 24 h. Medium was then decanted and cultures replenished with medium containing *d*- $\delta$ -tocotrienol or lovastatin. Following additional 48 h incubation, adherent cells were harvested by trypsinization and pelleted by low speed centrifugation. Cell pellets were fixed in 70% ethanol (1 $\times$ 10<sup>6</sup> cells/ml) at 4°C overnight, washed in 1 ml phosphate-buffered saline (PBS) and re-suspended in 500  $\mu$ l PBS containing 0.5 mg RNase A (Sigma, St. Louis, MO, USA) at 37°C for 30 min. Following gentle mixing, 500  $\mu$ l PBS and 100  $\mu$ l aliquot of propidium iodide (Sigma, 1 g/l in PBS containing 0.1% Triton X-100) were added. The cells were incubated in the dark at room temperature for 15 min and then placed in the dark at 4°C for flow cytometric analysis. Aliquots of

1 $\times$ 10<sup>4</sup> cells were analyzed for DNA content using a BD FACSCaliber<sup>™</sup> Flow Cytometer (BD Biosciences, San Jose, CA, USA). The distribution of cells in the G<sub>1</sub>, S, and G<sub>2</sub>/M phases of the cell cycle was determined using MultiCycle AV software (Phoenix Flow Systems, San Diego, CA, USA).

**Acridine orange and ethidium bromide dual staining assay for apoptosis.** A375 cells were inoculated in 6-well plates with 3 ml medium and 6 $\times$ 10<sup>4</sup> cells per well. Following a 48 h incubation with *d*- $\delta$ -tocotrienol, a dye mixture (15  $\mu$ l) containing 50  $\mu$ g/ml acridine orange (Becton, Dickinson and Company, Sparks, MD, USA) and 50  $\mu$ g/ml ethidium bromide (Sigma) was added to each well. Following a 1 min staining, the cells were observed under an Observer Z1 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) equipped with a X-CITE<sup>®</sup> series 120 Q lamp, an AxioCam MRm digital camera system and an AxioVision Rel. 4.7 program (all from Carl Zeiss MicroImaging, Inc.). The phase-contrast images of representative fields of each well and the green and red fluorescence emitted by acridine orange and ethidium bromide staining were captured by using bright field phase 2, fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate filters, respectively.

**Western blot.** Human A375 and A2058 melanoma cells cultured in 175 cm<sup>2</sup> flasks (BD Bioscience, Bedford, MA, USA) at 1.5 $\times$ 10<sup>6</sup> cells per flask were incubated with *d*- $\delta$ -tocotrienol for 48 h. Following the incubation, the growth medium was aspirated and cells washed with 15 ml ice-cold PBS twice, harvested by trypsinization, centrifuged and counted; 150  $\mu$ l of Laemmli buffer (Bio-Rad, Hercules, CA, USA) with 1% protease inhibitor cocktail (Sigma) freshly mixed was then added to pellets of 1.0 $\times$ 10<sup>6</sup> cells. Cells were subjected to homogenization for 45 s and placed in a dry bath incubator (Boekel Scientific, Feasterville, PA, USA) at 90°C for 20 min. Protein concentration of each sample was determined with Pierce 660 nm Protein Assay Reagent mixed with Ionic Detergent Compatibility Reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Samples containing 40  $\mu$ g proteins were mixed with  $\beta$ -mercaptoethanol (1:20) and boiled for 5 min before being loaded onto a Mini PROTEAN<sup>®</sup> 3 (Bio-Rad) electrophoresis unit with a 15% SDS-polyacrylamide gel and run at 150 V for 2-4 h. The proteins were then transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 10 V for 2 h or a Mini Trans-Blot<sup>®</sup> Cell (Bio-Rad) at 100 mA overnight. The immobilon-P transfer membranes were then incubated in blocking solution (5% fat-free dry milk in PBS containing 0.1% Tween-2; PBST) for 2 h at 4°C with shaking, rinsed with PBS, and then incubated with monoclonal antibodies to cyclin-dependent kinase (Cdk) 4 (Cell Signaling Technology, Beverly, MA, USA), procaspase-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and Ras (Cell Signaling Technology) at 4°C overnight. After rinsing with PBST for 10 min, the membrane was incubated with a secondary antibody (horseradish peroxidase linked; Cell Signaling Technology; 1:3,000 in PBST) for 20 min at room temperature, washed with PBST for 15 min, and reacted with SuperSignal West Pico Chemiluminescence Kit (Pierce) before being photographed at Chemi Doc XRS imaging system (Bio-Rad). Precision Strep Tactin-HRP (Bio-Rad) protein standards were used to identify the molecular weight of protein bands. A non-specific IgG (Cell Signaling Technology) was used for background control.

**Statistics.** One-way analysis of variance (ANOVA) was performed to assess the differences between groups using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Differences in means between control and treatment groups were analyzed by Dunnett's test. Levels of significance were designated as  $p < 0.05$ . Data are reported as the mean  $\pm$  standard deviation of three experiments.

## Results

*d*- $\delta$ -Tocotrienol and lovastatin (Figure 1A) elicited a concentration-dependent suppression of the proliferation of human A2058 and A375 melanoma cells. A375 cells were more sensitive than A2058 cells to *d*- $\delta$ -tocotrienol- and lovastatin-mediated growth suppression. The IC<sub>50</sub> values for *d*- $\delta$ -tocotrienol were 22.3  $\pm$  1.8 (A375) and 37.5  $\pm$  1.4 (A2058)  $\mu$ M, respectively. Lovastatin with IC<sub>50</sub> values of 0.5  $\pm$  0.1 (A375) and 3.1  $\pm$  0.5 (A2058)  $\mu$ M, respectively, was more potent than *d*- $\delta$ -tocotrienol in growth suppression.

The *d*- $\delta$ -tocotrienol-mediated growth suppression was accompanied by morphological changes. Photomicrographs of A2058 (Figure 1B, I - III) and A375 (Figure 1B, IV - VIII) melanoma cells incubated with *d*- $\delta$ -tocotrienol showed a concentration-dependent decrease in cell density, nuclear condensation, membrane blebbing and cell detachment from the monolayer; characteristics reminiscent of apoptosis. Cells remaining on the monolayer were harvested for cell cycle analysis because previous studies suggested that *d*- $\delta$ -tocotrienol-mediated cell cycle arrest contributes to *d*- $\delta$ -tocotrienol-mediated growth suppression (11). Following a 48 h incubation with 24  $\mu$ M (2/3  $\times$  IC<sub>50</sub> value) of *d*- $\delta$ -tocotrienol and 3  $\mu$ M (IC<sub>50</sub> value) of lovastatin, the percentages of cells in the G<sub>1</sub> phase reached 63.3  $\pm$  1.2% and 59.5  $\pm$  0.2%, respectively, both significantly ( $p < 0.01$ ) higher than that of control cells (46.7  $\pm$  1.2%) (Table I). Concomitantly, the percentages of cells in the S phase reached 20.3  $\pm$  0.9% and 22.4  $\pm$  1.7% for cells incubated with *d*- $\delta$ -tocotrienol and lovastatin, respectively; both values were significantly ( $p < 0.01$ ) lower than that of the control cells (35.1  $\pm$  1.1%). The G<sub>1</sub>/S ratio, an indicator of G<sub>1</sub> arrest, for cells incubated with *d*- $\delta$ -tocotrienol (3.1  $\pm$  0.2) and lovastatin (2.7  $\pm$  0.2) were 135% ( $p < 0.01$ ) and 100% ( $p < 0.01$ ), respectively, higher than that for the control cells (1.3  $\pm$  0.1%). The *d*- $\delta$ -tocotrienol- and lovastatin-mediated cell cycle arrest at the G<sub>1</sub> phase was also shown in A375 cells. Following a 48 h incubation with 33  $\mu$ M (1.5  $\times$  IC<sub>50</sub> value) of *d*- $\delta$ -tocotrienol and 0.5  $\mu$ M (IC<sub>50</sub> value) of lovastatin, the G<sub>1</sub>/S ratio was increased to 2.7  $\pm$  0.3 and 2.7  $\pm$  0.2, respectively, significantly ( $p < 0.05$ ) higher than that of the control cells (2.1  $\pm$  0.3). The G<sub>1</sub> arrest induced by *d*- $\delta$ -tocotrienol was accompanied by a decreased expression of Cdk4 (Figure 2), a key regulator in the G<sub>1</sub>/S transition (12). Following a 48 h incubation with 38  $\mu$ M (A2058) or 33  $\mu$ M (A375) *d*- $\delta$ -tocotrienol, the intensity of Cdk4 protein band decreased significantly in comparison to the control.

Table I. The impact of *d*- $\delta$ -tocotrienol and lovastatin on the 48-h cell cycle distribution of human A2058 and A375 melanoma cells. Values represent the mean  $\pm$  standard deviation of three experiments.

	G <sub>1</sub> (%)	S (%)	G <sub>2</sub> (%)	G <sub>1</sub> /S
<b>A2058</b>				
Control	46.7 $\pm$ 1.2	35.1 $\pm$ 1.1	18.1 $\pm$ 1.1	1.3 $\pm$ 0.1
<i>d</i> - $\delta$ -Tocotrienol (24 $\mu$ M)	63.3 $\pm$ 1.2*	20.3 $\pm$ 0.9*	16.3 $\pm$ 0.8	3.1 $\pm$ 0.2*
Lovastatin (3 $\mu$ M)	59.5 $\pm$ 0.2*	22.4 $\pm$ 1.7*	18.1 $\pm$ 1.6	2.7 $\pm$ 0.2*
<b>A375</b>				
Control	59.2 $\pm$ 2.9	28.9 $\pm$ 2.1	11.9 $\pm$ 1.0	2.1 $\pm$ 0.3
<i>d</i> - $\delta$ -Tocotrienol (33 $\mu$ M)	65.7 $\pm$ 1.0*	24.3 $\pm$ 2.4	10.0 $\pm$ 1.5	2.7 $\pm$ 0.3#
Lovastatin (0.5 $\mu$ M)	64.5 $\pm$ 1.1#	24.1 $\pm$ 1.5#	11.4 $\pm$ 0.3	2.7 $\pm$ 0.2#

\* $p < 0.01$ ; # $p < 0.05$ .

Previous studies have shown that *d*- $\delta$ -tocotrienol-induced apoptosis, suggested in the photomicrographs of A2058 and A375 cells (Figure 1B), often accompanies cell cycle arrest in mediating growth suppression (6, 11). Therefore, first the impact of *d*- $\delta$ -tocotrienol on apoptosis was examined in A375 cells using acridine orange and ethidium bromide dual staining and fluorescence microscopy. Viable cells have acridine orange staining with normal cell morphology. Acridine orange- and ethidium bromide-stained cells with condensed nucleus are in early and late apoptosis stages, respectively (11). In representative fields of view of the same size, the total number of A375 cells following a 48 h incubation with 33  $\mu$ M/l of *d*- $\delta$ -tocotrienol (Figure 3A, V-VIII) was lower than that of control cells (Figure 3A, I-IV), most clearly shown in phase-contrast images (Figure 3A, I and V); nevertheless, the total number and percentage of A375 cells with ethidium bromide staining emitting red fluorescence increased (Figure 3A, III and VII).

*d*- $\delta$ -Tocotrienol-induced apoptosis was confirmed by Western blot of procaspase-3 cleavage in A2058 cells (Figure 3B). *d*- $\delta$ -Tocotrienol initiated a concentration-dependent cleavage of procaspase-3 in A2058 cells as evidenced by the gradual decrease in the procaspase-3 protein following a 48 h incubation with 19 and 38  $\mu$ M *d*- $\delta$ -tocotrienol, despite a constant amount of total protein loading indicated by the  $\beta$ -actin band.

A key protein that plays important roles in melanoma growth and one that may be impacted by the tocotrienol-mediated down-regulation of the mevalonate pathway activity is the oncogenic Ras protein that undergoes a farnesylation process (13). The finding that  $\alpha$ -tocotrienol down-regulates the expression of *Ras* in human A549 lung carcinoma cells (14) and the fact that 20% of human melanomas harbor *Ras* mutation (15) prompted the evaluation of the impact of *d*- $\delta$ -tocotrienol on total cellular Ras protein. As shown in Figure 4, 33  $\mu$ M and 38  $\mu$ M *d*- $\delta$ -

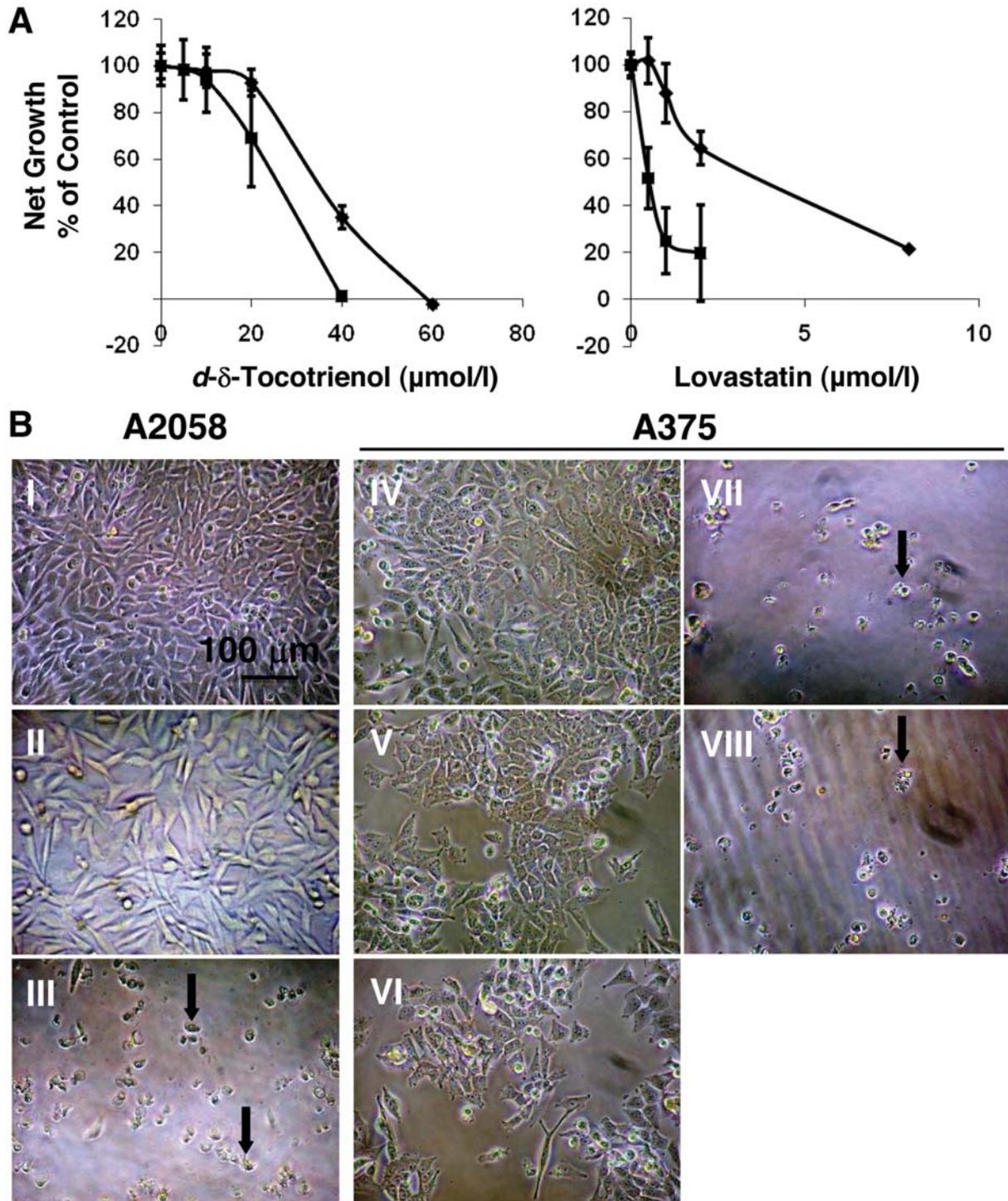


Figure 1. *d*- $\delta$ -Tocotrienol- and lovastatin-mediated suppression of the proliferation of human A375 and A2058 melanoma cells. A: Representative growth curves of human A375 (■) and A2058 (◆) melanoma cells showing the concentration-dependent suppression of cell proliferation by *d*- $\delta$ -tocotrienol and lovastatin. Cells were cultured and incubated with the compounds for 72 h before cell proliferation was measured by CellTiter 96<sup>®</sup> Aqueous One Solution as described in the Materials and Methods. Values represent the mean  $\pm$  standard deviation of four experiments. B: The concentration-dependent inhibition of human A2058 and A375 melanoma cell proliferation shown in photomicrographs of A2058 (I-III) and A375 (IV-VIII) cells following a 72-h incubation with 0 (I and IV), 30 (II), 60 (III), 11 (V), 22 (VI) and 33 (VII)  $\mu$ mol/l *d*- $\delta$ -tocotrienol and 0.75  $\mu$ mol/l lovastatin (VIII). The contact inhibition-disabled growth of the A2058 and A375 cells is shown in the control cultures (I and IV). Concentration-dependent decreases in cell density and changes in cell morphology occurred progressively as the concentration of *d*- $\delta$ -tocotrienol increased. Arrows mark indicators of apoptotic cell death (nuclear condensation and membrane blebbing) and changes in cell morphology.

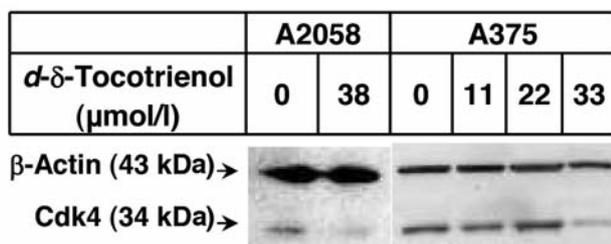


Figure 2. The impact of *d*- $\delta$ -tocotrienol on the expression of Cdk4 protein in human A2058 and A375 melanoma cells following a 4-h incubation. Cell lysates were subjected to Western blot procedures and blots were detected by chemiluminescence. Representative blots from four experiments are shown.

Table II. Synergistic effect of *d*- $\delta$ -tocotrienol and lovastatin on the growth of human A2058 melanoma cells. Values represent the mean $\pm$ standard deviation of four experiments. Means not sharing a superscript are significantly different ( $p < 0.01$ ).

	Net growth (% of control)
Control	100 $\pm$ 8 <sup>a</sup>
<i>d</i> - $\delta$ -Tocotrienol (24 $\mu$ M)	89 $\pm$ 7 <sup>a</sup>
Lovastatin (1.5 $\mu$ M)	70 $\pm$ 13 <sup>ab</sup>
Lovastatin (3 $\mu$ M)	50 $\pm$ 12 <sup>ab</sup>
<i>d</i> - $\delta$ -Tocotrienol (24 $\mu$ M)+lovastatin (1.5 $\mu$ M)	21 $\pm$ 4 <sup>ab</sup>
<i>d</i> - $\delta$ -Tocotrienol (24 $\mu$ M)+lovastatin (3 $\mu$ M)	3 $\pm$ 1 <sup>b</sup>

tocotrienol decreased the expression of Ras protein in A375 and A2058 cells, respectively, following a 48 h incubation.

The IC<sub>50</sub> values of *d*- $\delta$ -tocotrienol for the human melanoma cells, and particularly that for the A2058 cells, fall in the high end of the physiologically attainable peak plasma levels of *d*- $\delta$ -tocotrienol following oral administration (16). Previous studies have shown the synergy obtained with blends of *d*- $\delta$ -tocotrienol and lovastatin in suppressing the growth of murine B16 melanoma cells (8) and human MIA PaCa-2 pancreatic carcinoma cells (11). Next, the potential synergistic impact of these two agents on the more resistant A2058 cells was evaluated (Table II). The net growth of A2058 cells incubated with 24  $\mu$ M of *d*- $\delta$ -tocotrienol and 1.5  $\mu$ M of lovastatin for 72 h were 89 $\pm$ 7% and 70 $\pm$ 13%, respectively, of that of the control cells. A blend of the two agents suppressed cell growth by 79%, doubling the predicted impact suggested by the sum (41%) of individual actions. Synergy was also shown with a blend containing 24  $\mu$ M of *d*- $\delta$ -tocotrienol and a higher (3  $\mu$ M) but still physiologically attainable concentration (17) of lovastatin; the blend suppressed cell growth by 97%, far exceeding the predicted impact suggested by the sum (61%) of individual actions.

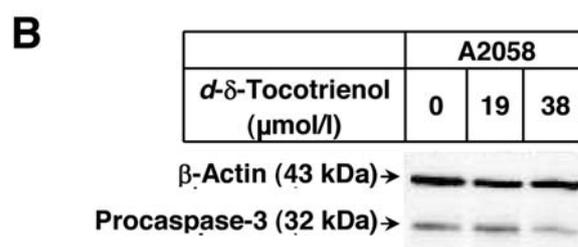
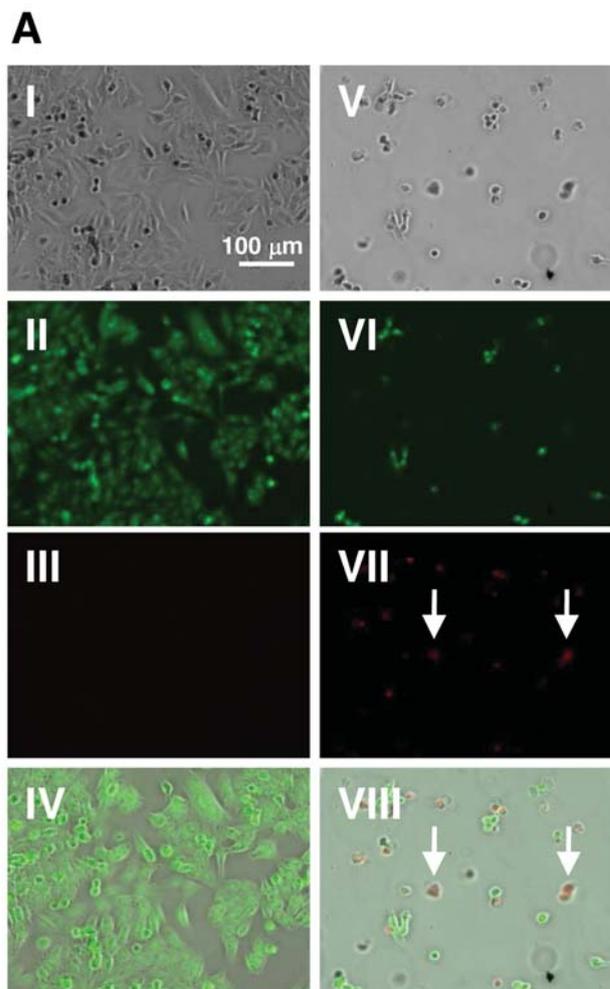


Figure 3. *d*- $\delta$ -Tocotrienol-mediated apoptosis in A375 (A) and A2058 (B). A: Photomicrographs of human A375 melanoma cells showing the *d*- $\delta$ -tocotrienol-initiated apoptosis detected by acridine orange and ethidium bromide dual staining. A375 cells were incubated with 0 (I-IV) and 33 (V-VIII)  $\mu$ M *d*- $\delta$ -tocotrienol for 48 h. Photomicrographs of the same fields were taken under phase-contrast microscope (I and V) and fluorescence microscope with a tetramethylrhodamine isothiocyanate (II and VI) or fluorescein isothiocyanate filter (III and VII) and then merged (IV and VIII). Arrows mark the red fluorescence emitted by ethidium bromide staining shown in late apoptotic and dead cells induced by *d*- $\delta$ -tocotrienol. B: The impact of *d*- $\delta$ -tocotrienol on the cleavage of procaspase-3 in human A2058 melanoma cells following a 48 h incubation. Cell lysates were subjected to Western blot procedures and blots were detected by chemiluminescence and quantitated. Representative blots from three experiments are shown.

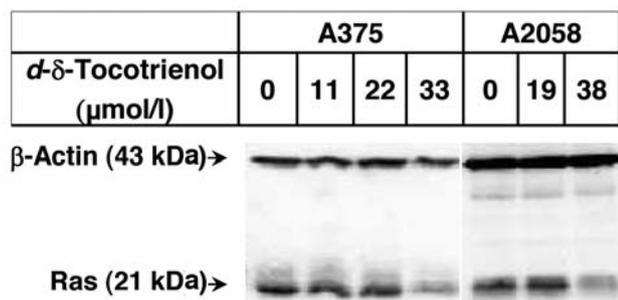


Figure 4. Representative blots showing the impact of *d*- $\delta$ -tocotrienol on the expression of Ras in human A375 and A2058 melanoma cells following a 48h incubation. Cell lysates were subjected to Western-blot procedures and blots were detected by chemiluminescence. Representative blots from four experiments are shown.

## Discussion

Building on the previous observation of the anti-proliferative activity of *d*- $\delta$ -tocotrienol ( $IC_{50}$ =10-14  $\mu$ M) in murine B16 melanoma cells (8, 9), the present study demonstrated the growth-suppressive activity of *d*- $\delta$ -tocotrienol, albeit with higher  $IC_{50}$  values, in two human melanoma cell lines. A recent study (18) with two human amelanotic and melanotic malignant melanoma cells, C32 and G361, and a shorter 24 h treatment period suggested  $IC_{50}$  values in the range of 40-60  $\mu$ M, consistent with the  $IC_{50}$  value for the A2058 cells. The  $IC_{50}$  value for lovastatin in A375 cells (0.5  $\mu$ M) was lower than that reported by Shellman *et al.* (approximately, 2.8  $\mu$ M) (19); modulating factors such as inoculating cell density (20) and assay methodology (21) may have contributed to such a difference. Nevertheless, the  $IC_{50}$  value for lovastatin in A2058 cells fell in the interval of reported  $IC_{50}$  values for human melanoma cell lines WM35, WM1552C, WM75, WM115, WM278, A375, WM1617, WM852, WM239A, HS294T (19), 1011 (22), SK-MEL-2 (23), HT144, M14, and SK-MEL-28, ranging from 1.2  $\mu$ M to 30  $\mu$ M (24).

Consonant with its impact on cell cycle distribution noted in previous studies with human pancreatic tumor cells (11), *d*- $\delta$ -tocotrienol effected cell cycle arrest at the  $G_1$  phase in A375 and A2058 cells. The loss of Cdk4 expression resembles the impact of *d*- $\delta$ -tocotrienol shown in MDA-MB-231 breast cancer cells (25).  $G_1$  arrest was also noted in murine B16 melanoma cells incubated with a less potent (8) *d*- $\gamma$ -tocotrienol (10), which, in a recent report (18), purportedly failed to induce  $G_1$  arrest in human C32 and G361 melanoma cells; the latter may be masked by a massive *d*- $\gamma$ -tocotrienol-induced sub-diploid peak, an indicator of apoptosis (26) that was confirmed by the

activation of caspases 3, 7 and 9 and the cleavage of poly (ADP-ribose) polymerase (PARP) (18). Consistent with its apoptotic activity shown in the human pancreatic tumor cells (11), *d*- $\delta$ -tocotrienol induced apoptosis in A375 and A2058 cells as shown by acridine orange and ethidium bromide dual staining and procaspase-3 cleavage; apoptosis and caspase-3 activation were also shown in lovastatin-treated A375 cells (19).

The Raf/mitogen-activated protein kinase (MAPK)/ extracellular signal regulated kinase (ERK) kinase (MEK)/ ERK/nuclear factor kappa B (NF- $\kappa$ B) and phosphoinositide 3-kinase (PI3K)/Akt/NF- $\kappa$ B pathways control cell cycle progression, up-regulation of Cdk4 (27), apoptosis and differentiation. The Ras protein (28) that plays critical roles in these pathways require mevalonate-derived farnesyl pyrophosphate for its post-translational modification and maturation (13). Ras mutation, mostly *N-Ras* mutation, is found in about 20% of human melanoma (15). Lovastatin has been shown to decrease Ras prenylation (3) and membrane attachment (29) in human melanomas. Consistent with a previous observation that  $\alpha$ -tocotrienol and a tocotrienol analog, 6-*O*-carboxypropyl- $\alpha$ -tocotrienol, inhibit the prenylation of Ras and reduce the level of Ras protein in human A549 lung carcinoma cells (30), *d*- $\delta$ -tocotrienol was shown to reduce Ras protein levels in A375 and A2058 cells (Figure 4). The decreased Ras protein expression likely reflects the dislodgment and enhanced degradation of unprenylated Ras, as seen in cells treated with farnesyl transferase inhibitors (FTIs) (31-33). The half-lives of total Ras decreased from >12 h in untreated human PANC-1 pancreatic carcinoma cells to 7-8 h in FTI-treated cells (31); FTI also reduced the half-life of H-Ras from 27 h to 10 h in H-Ra-transformed Rat1 cells (32). *d*-Limonene (1) and perillyl alcohol, the mevalonate-suppressive monoterpenes, decrease Ras protein expression; perillyl alcohol effected such a decrease in human myeloid THP-1 and lymphoid RPMI-8402 leukemia cells (34, 35), human SW480 colonic adenocarcinoma cells (36), human K562 erythroleukemia cells (37) and 1984-1 melanoma cells (33). More studies are needed to delineate whether Ras degradation directly contributes to the *d*- $\delta$ -tocotrienol-mediated growth suppression in A375 (38) and A2058 (39) cells with a wild-type *N-Ras*. The finding that lovastatin-mediated suppression of A375 cell proliferation was reversed by supplemental geranylgeranyl pyrophosphate but not by farnesyl pyrophosphate (19) that is required for Ras prenylation suggests a Ras-independent mechanism for the HMG CoA reductase suppressors. Rac1, a member of the Rho GTPases and a downstream intermediate of Ras, mediates the malignant phenotypes of skin tumor cells (40); functional Rac1 requires geranylgeranylation (41). Other proteins including nuclear lamins and insulin-like growth factor I receptor that are post-translationally modified by mevalonate-derived intermediates, may also mediate the tocotrienol effect.

The IC<sub>50</sub> values of *d*- $\delta$ -tocotrienol for A375 and A2058 cells approached the high end of physiologically attainable peak plasma levels reported in one oral feeding study (16), but far exceed that reported in another study (42). Nevertheless, tocotrienols were found in high concentrations in the skin in feeding studies (43, 44). In addition, the parallel impacts of *d*- $\delta$ -tocotrienol, a post-translational down-regulator of HMG CoA reductase and lovastatin, a competitive inhibitor of reductase, on growth, cell cycle, apoptosis and Ras protein noted in the present and other studies (5, 8, 10, 11) and the same order of sensitivity of the A375 and A2058 cells in responding to these two agents, suggest the essentiality of the mevalonate pathway. The synergy between *d*- $\delta$ -tocotrienol and lovastatin (Table II) is consistent with prior observations in B16 melanoma cells (10), human DU145 prostate carcinoma cells (5), MIA PaCa-2 pancreatic carcinoma cells (11), and A549 lung carcinoma cells (8), murine mammary tumor cells (45) and implanted B16 tumors (8), suggesting that physiologically attainable *d*- $\delta$ -tocotrienol and lovastatin (17) may be effective in melanoma treatment or prevention. Isoprenoids with lesser mevalonate-suppressive activity than *d*- $\delta$ -tocotrienol such as citral (46), geraniol (47),  $\beta$ -ionone (9) and *d*- $\gamma$ -tocotrienol (9), suppress the growth of implanted and chemically initiated melanomas. Blends of *d*- $\delta$ -tocotrienol and assorted mevalonate suppressors may provide a novel approach in the chemoprevention and/or therapy of melanomas.

### Acknowledgements

The Authors thank Dr. Heather Conrad-Webb, Ms. Amruta Bhusari and Ms. Amruta Mahadik of the Biology Department of the Texas Woman's University for their assistance with the BD FACSCalibur and Mr. Sudheer Dhanireddy and Mr. Rajshekar Katuru for their technical assistance. This study was supported by the Texas Department of Agriculture, the Texas Woman's University Research Enhancement Program, a Summer Stipend Program and the Human Nutrition Research Fund.

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Received October 22, 2010

Revised November 12, 2010

Accepted November 15, 2010