Combined Treatment of γ-Tocotrienol with Statins Induce Mammary Tumor Cell Cycle Arrest in G1

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Statins and γ-tocotrienol (a rare isoform of vitamin E) both inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase activity and display anticancer activity. However, clinical application of statins has been limited by high dose toxicity. Previous studies showed that combined statin and γ-tocotrienol treatment synergistically inhibits growth of highly malignant +SA mammary epithelial cells in culture. To investigate the mechanism mediating this growth inhibition, studies were conducted to determine the effect of combination low dose γ-tocotrienol and statin treatment on +SA mammary tumor cell cycle progression. Treatment with 0.25 μM simvastatin, lovastatin, mevastatin, 10 μM pravastatin or 2.0 μM γ-tocotrienol alone had no effect, while combined treatment of individual statins with γ-tocotrienol significantly inhibited +SA cell proliferation during the 4-day culture period. Flow cytometric analysis demonstrated that combined treatment induced cell cycle arrest in G1. Additional studies showed that treatment with 0.25 μM simvastatin or 2 μM γ-tocotrienol alone had no effect on the relative intracellular levels of cyclin D1, CDK2, CDK4 and CDK6, but combined treatment caused a large reduction in cyclin D1 and CDK2 levels. Combined treatments also caused a relatively large increase in p27, but had no effect on p21 and p15 levels, and resulted in a large reduction in retinoblastoma (Rb) protein phosphorylation at ser780 and ser807/811. Similar effects were observed following combined treatment of γ-tocotrienol with low doses of lovastatin, mevastatin and pravastatin. These findings demonstrate that combination low dose statin and γ-tocotrienol treatment induced mammary tumor cell cycle arrest at G1, resulting from an increase in p27 expression, and a corresponding decrease in cyclin D1, CDK2, and hypophosphorylation of Rb protein. These findings suggest that combined treatment of statins with γ-tocotrienol may provide significant health benefits in the treatment of breast cancer in women, while avoiding myotoxicity associated with high dose statin monotherapy. Exp Biol Med 234:639–650, 2009

Key words: γ-tocotrienol; statins; breast cancer; G1 arrest; cyclin D1; p27; Rb

Introduction

Statins are potent inhibitors of HMGCoA reductase, the rate limiting enzyme in the mevalonate pathway for cholesterol biosynthesis (1). In addition to sterol synthesis, the mevalonate pathway produces pools of farnesyl and other phosphorylated products, which isoprenylate various cellular proteins such as small G-proteins, nuclear lamins and heteromeric G-protein γ-subunit (1). These proteins are vital for cellular growth, and isoprenylation is needed for their activation, localization and function (1). Statins inhibit mevalonate synthesis and induce cell cycle arrest in various tumor cells types in both *in vitro* and *in vivo* experimental model systems (1–7). However, clinical application of statin use has been limited due to high dose toxicity that can result in rhabdomyolysis and even death (8). In contrast, γ-tocotrienol also reduces HMGCoA reductase activity by causing post-transcriptional down-regulation of the enzyme (9–13). Previous studies demonstrated that low doses of γ-tocotrienol and statins act synergistically to inhibit growth of highly malignant +SA mammary epithelial cells in culture, and these antiproliferative effects were found to be strongly cytostatic, but not cytotoxic (14).

The cell cycle comprises a series of controlled events that drive DNA replication and cell division. In eukaryotes, cells prepare for DNA replication in the Gap phase 1 (G1), synthesize DNA in the S-phase (S), prepare for division in the Gap phase 2 (G2), and divide during mitosis (M) phase of the cell cycle (15, 16). The transition between these phases is tightly regulated by a family of kinases called cyclin-dependent kinases (CDKs) and their regulatory binding proteins called cyclins (17). Cyclin D1 is a critical modulator of G1 to S transition. Cyclin D-CDK4/CDK6 and

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cyclin E-CDK2 complexes regulate cell cycle entry from G1 to S phase. These complexes phosphorylate and inactivate the retinoblastoma (Rb) protein, which upon phosphorylation, dissociates from E2F family of transcription factors and allows for E2F-dependent transcription to occur (17). Activity of cyclin-CDKs is also modulated by CDK inhibitors (CKIs). The INK4 family of CKIs includes proteins such as p15 that specifically inhibit the catalytic subunits of CDK4 and CDK6 (18). In contrast, the activity of Cip/Kip CKI family members such as p21 and p27 is relatively nonspecific and can attenuate the activity of all CDKs.

The cyclin D1-CDK-CKI-Rb pathway is universally disrupted in human cancer (19). Cyclin D1 is overexpressed, primarily due to gene amplification, in approximately 50% of breast cancers (17), and cyclin D1 overexpression in transgenic mice leads to mammary hyperplasia and carcinoma (20). The major growth factor dependent signal transduction pathways that regulate the expression of cyclin D1 are the Akt and MAPK mitogenic pathways. Previous studies have shown that combined statin and γ-tocotrienol treatment suppressed the activation of Akt and MAPK signaling in +SA mammary tumor cells (14). These findings lead to the hypothesis that the growth inhibitory effects of combined statins and γ-tocotrienol treatment may be due to a reduction in cyclin D1 levels and subsequent induction of cell cycle arrest in G1. Therefore, studies were conducted to determine the effects of combination low dose statin and γ-tocotrienol treatment on cell cycle progression in malignant mouse +SA mammary epithelial cells *in vitro*. Additional studies were also conducted to determine the effects of combined statin and γ-tocotrienol treatment on intracellular levels of CDK2, CDK4, CDK6, p15, p21, p27, and hyperphosphorylated Rb protein.

Materials and Methods

Sources of Reagents and Chemicals. All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated. Purified γ-tocotrienol was generously provided by Carotech Bhd. (Ipoh, Malaysia). Simvastatin, lovastatin, mevastatin, pravastatin (sodium salt), antibody for α-tubulin, and propidium iodide were purchased from EMD Biosciences (La Jolla, CA, USA). Antibodies for cyclin D1, CDK2, CDK4, CDK6, p27, p15, phospho-Rb (ser780), and phospho-Rb (ser807/811) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for p21 and total Rb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Biosciences (Boston, MA, USA).

Cell Line and Culture Conditions. All the experiments in the present study were conducted using the highly malignant mouse +SA mammary epithelial cell line. The neoplastic +SA cell line was derived from a mammary adenocarcinoma that developed spontaneously in a BALB/c female mouse (21–23). The +SA cell line is characterized as being highly malignant, estrogen-independent, and displays anchorage-independent growth when cultured in soft agarose gels. When +SA cells are injected back into the mammary gland fat pad of syngeneic female mice, they form rapidly growing anaplastic adenocarcinomas that are highly invasive and metastasize to the lung. Since previous studies demonstrated the synergistic antiproliferative activity of statins and γ-tocotrienol (14), experiments were conducted to further elucidate the mechanism of growth arrest in the same cell line. Cell culture conditions have been previously described in detail (9, 23–25). Briefly, +SA cells were maintained in serum-free defined DMEM/F12 media containing 10 mg/ml insulin and 10 ng/ml epidermal growth factor (EGF) as a mitogen (9, 23–26).

Experimental Treatments. The highly lipophilic γ-tocotrienol was conjugated with bovine serum albumin (BSA) to form an aqueous stock solution as described previously (9, 24). Simvastatin, lovastatin and mevastatin were activated prior to addition to treatment media as described previously (14). Briefly, these statins were dissolved in 70% ethanol solution containing 0.1 N NaOH and incubated at 50°C for 1 h. The alkaline solution was then neutralized by the addition of 70% ethanol solution containing 0.1 N HCl to form a stock solution that was used to prepare fresh treatment media. Pravastatin was directly dissolved in 70% ethanol to form a stock solution as it does not require activation. The final ethanol concentration in media was maintained as the same in all the groups within a given experiment and never exceeded 0.05%.

In growth studies, cells were plated at a density of 5×10^4 cells/well (6 wells/group) in 24-well culture plates and allowed to attach overnight. Cells were divided into different groups and media was replaced with fresh serum-free defined control or treatment media containing simvastatin (S), lovastatin (L), mevastatin (M), or pravastatin (P) alone or their respective combination with γ-tocotrienol (γT^3). Cells were fed fresh control or treatment media everyday for 4 days. To study treatment effect on +SA cell cycle progression, cells in the various groups were synchronized (i.e. cells were induced to accumulate in the same phase of cell cycle) to prevent phase variation between different groups before mitogen (EGF) treatment. The method of mitogen starvation was employed by using mitogen-free media to synchronize all the cells in G1 phase of cell cycle. +SA cells were initially plated at 1×10^6 cells/100 mm culture plate and allowed to attach overnight. Control defined media (contains EGF as a mitogen) was removed, and cells were washed with PBS to remove any traces of mitogen, followed by 48 h exposure to mitogen-free control and treatment media to allow cells to synchronize in the G0/G1 phase of the cell cycle. Afterwards, media was removed and replaced with fresh control and treatment media containing EGF to initiate simultaneous cell cycle progression in all groups.

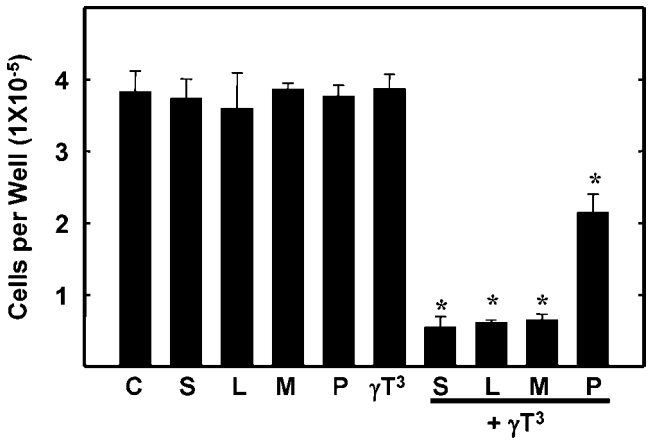

Figure 1. Effects of combination low dose statin and γ-tocotrienol treatment on growth of highly malignant +SA mammary epithelial cells in culture. Cells were plated at a density of 5×10^4 cells/well (6 wells/group) in 24-well culture plates and exposed to 0.25 μM simvastatin (S), 0.25 μM lovastatin (L), 0.25 μM mevastatin (M), or 10 μM pravastatin (P) alone or their respective combination with 2 μM γ-tocotrienol (γT^3) for a 4-day period. Afterwards, viable cell number was determined by MTT assay. Vertical bars indicate the mean cell count ± SEM in each treatment group. * $P < 0.05$ as compared with vehicle-treated control group (C).

Measurement of Viable Cell Number. +SA cell count was determined by using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (9, 25). Briefly, cells were incubated at 37°C for 4 h with fresh control media containing 0.42 mg/ml MTT. Then, media was removed and MTT crystals were dissolved in 1 ml isopropanol, and the optical density of each sample was measured at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company). The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometry, at the beginning of each experiment.

Cell Cycle Analysis by Flow Cytometry. Cells were initially plated at a density of 1×10^6 cells/100 mm culture plate in serum-free defined control media and allowed to attach overnight. Cells were then divided into different groups, washed with phosphate buffered saline (PBS) and incubated with mitogen-free control or treatment media for 48 h to synchronize them in G1 phase. Afterwards, cells were exposed for 24 h to their respective control and treatment media containing 100 ng/ml EGF, and then cells were harvested for cell cycle analysis. Cells in all the groups were pelleted, washed with PBS and fixed with 70% ethanol. This was followed by incubation with hypotonic DNA staining buffer (sodium citrate 1 mg/ml, triton-X 100 3 μl/ml, propidium iodide 100 μg/ml, ribonuclease A 20 μg/ml) at 4°C in the dark for 30 min before the acquisition on the flow cytometer, according to the method of Krishan (27). Cell cycle histograms were generated after the acquisition of PI-stained cells by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For each sample, at least 1×10^4 events were recorded and all analyses were performed using CellQuest software (BD Biosciences, San Jose, CA).

Western Blot Analysis. +SA cells were plated at a density of 1×10^6 cells/100 mm culture plate and incubated in control and treatment EGF-free media for 48 h. Afterwards, cells were washed with PBS and fed fresh defined control or treatment media containing 100 ng/ml EGF, and isolated cells were collected at various time points over a 24 h period. Cells were isolated with trypsin and the whole cell lysates were prepared as previously described (25, 26). Protein concentration in each sample was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amount (25 μg total protein/lane) of each sample was subjected to electrophoresis through 7.5% or 15% SDS-polyacrylamide minigels. All minigels within a given experiment were run simultaneously using AccuPower model 500 power supply unit (VWR, Suwanee, GA). Typically, proteins from four minigels (Figs. 2, 4 and 6) or three minigels (Figs. 3, 5 and 7) were transblotted at 30 V for 12–16 h at 4°C onto a single $5'' \times 7''$ polyvinylidene fluoride (PVDF) membrane (PerkinElmer Life Sciences, Wellesley, MA) in a Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA) according to the methods of Towbin, and these $5'' \times 7''$ PVDF membranes were used for subsequent Western blot and scanning densitometric analysis as previously described (14, 25, 26). Briefly, the membranes were blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then, incubated with specific primary antibodies against cyclin D1, CDK2, CDK4, CDK6, p27, p21, p15, Rb, or α-tubulin, diluted 1:5,000 to 1:10,000 in TBST/2% BSA for 2 h. However, primary antibodies against phospho-Rb (ser780) and phospho-Rb (ser807/811) were incubated with transblotted membranes for a longer period (12 h) to optimize their binding to the corresponding protein. Membranes were washed five times with TBST and then incubated with respective horseradish peroxide-conjugated secondary antibodies diluted 1:5,000 in TBST/2% BSA for 1 h followed by rinsing with TBST. Chemiluminescence (Pierce, Rockford, IL) was used to visualize the antibody bound proteins. Images of protein bands from all treatment groups within an experiment were acquired on a single $8'' \times 10''$ film and the densitometric analysis was performed with Metamorph Analysis software (Universal Imaging Corp.). The visualization of α-tubulin was used to ensure equal sample loading in each lane. All experiments were repeated at least three times and a representative Western Blot image from each experiment is shown in Figures 2 to 7. For quantification, the values obtained from densitometry of Western blot images for various treatment groups were normalized to their respective α-tubulin- and control-densitometric values to clearly visualize the differences between treatment groups.

Statistical Analysis. Differences among the various treatment groups in +SA cell growth study were determined by analysis of variance (ANOVA) followed by Dunnett's *t*

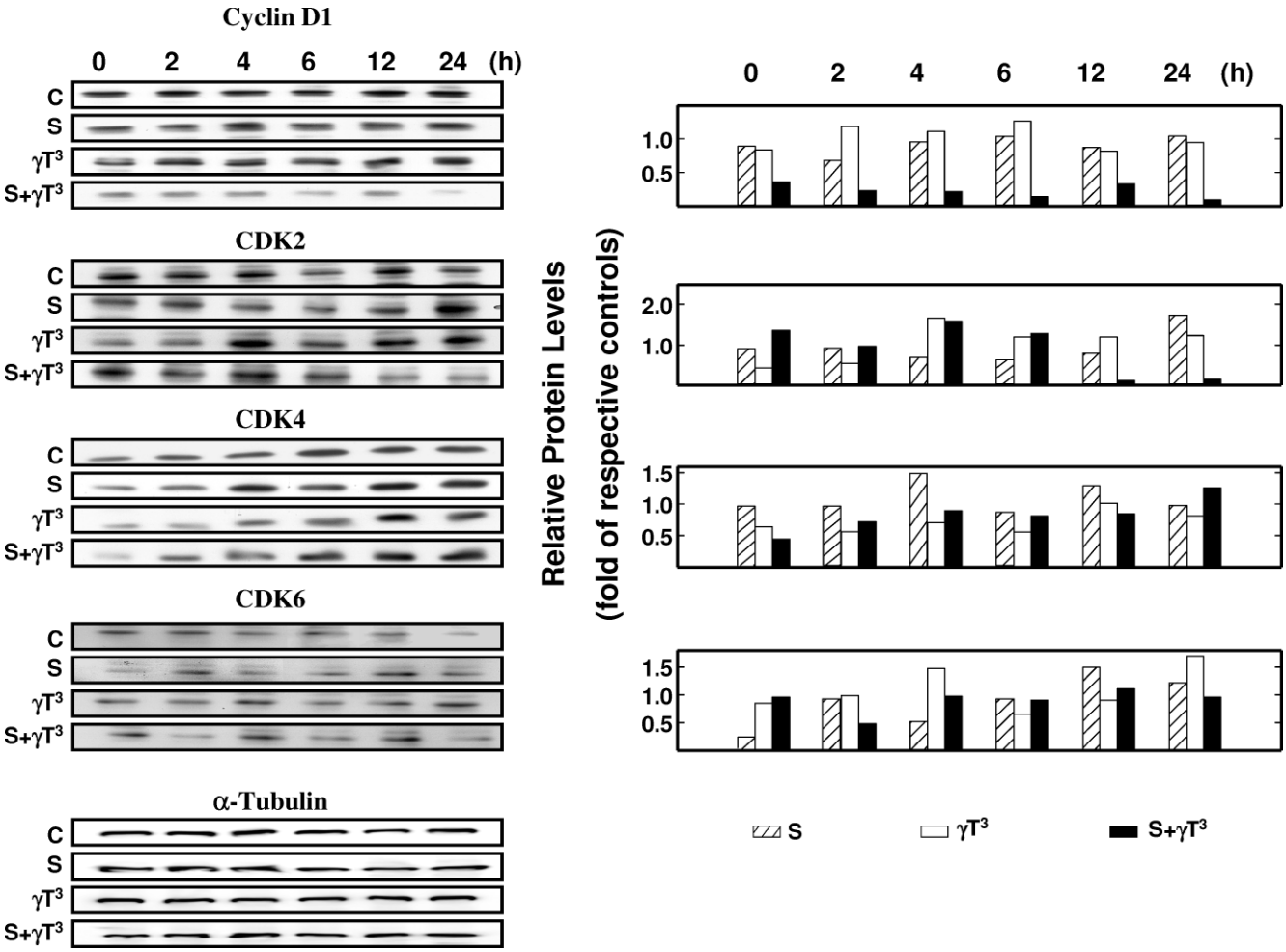

Figure 2. Effects of simvastatin (S) and γ-tocotrienol (γT^3) on cyclin D1 and CDK levels in highly malignant +SA mammary epithelial cells. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes in defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C), 0.25 μM simvastatin (S), 2 μM γ-tocotrienol (γT^3), or combined 0.25 μM S and 2 μM (γT^3) media in order to establish cell cycle synchronization in all groups. After a 48 h incubation period, the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were then collected at 0, 2, 4, 6, 12, 24 h after mitogen stimulation and whole cell lysates were prepared for Western blot analysis of intracellular levels of cyclin D1, CDK2, CDK4 and CDK6. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and the proteins from 4 minigels were transblotted to a single PVDF membrane, followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and the integrated optical density of each band was normalized with corresponding α-tubulin and control, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate the fold-change in protein levels in various treatment groups as compared with their respective controls.

test. The difference of $P < 0.05$ was considered to be statistically significant as compared with vehicle-treated controls.

Results

Growth Inhibition by Combined Low Dose Statins and γ-Tocotrienol Treatment. The effects of individual statins and γ-tocotrienol given alone or in combination on the growth of neoplastic +SA mammary epithelial cells over a 4-day culture period are shown in Figure 1. Exposure to individual treatments with 0.25 μM simvastatin, lovastatin, mevastatin, 10 μM pravastatin, or 2 μM γ-tocotrienol had no effect on +SA cell proliferation, as compared with the untreated control group. However, when the similar subeffective doses of individual statins were administered in combination with the same subeffective dose of γ-tocotrienol, there was a significant inhibition in the growth of +SA cells as compared with the untreated control group. The more lipophilic statins (simvastatin, lovastatin and mevastatin) did not differ significantly in their antiproliferative potency. However, a relatively much higher dose (10 μM) of the less lipophilic pravastatin was required in combination with γ-tocotrienol to significantly inhibit +SA cell growth.

Effects of Statin and γ-Tocotrienol Treatment on Cell Cycle Progression. Cell cycle analysis as determined by flow cytometry showed that exposure to individual statins or γ-tocotrienol given alone had little or no effect on the distribution of cells in different cell cycle

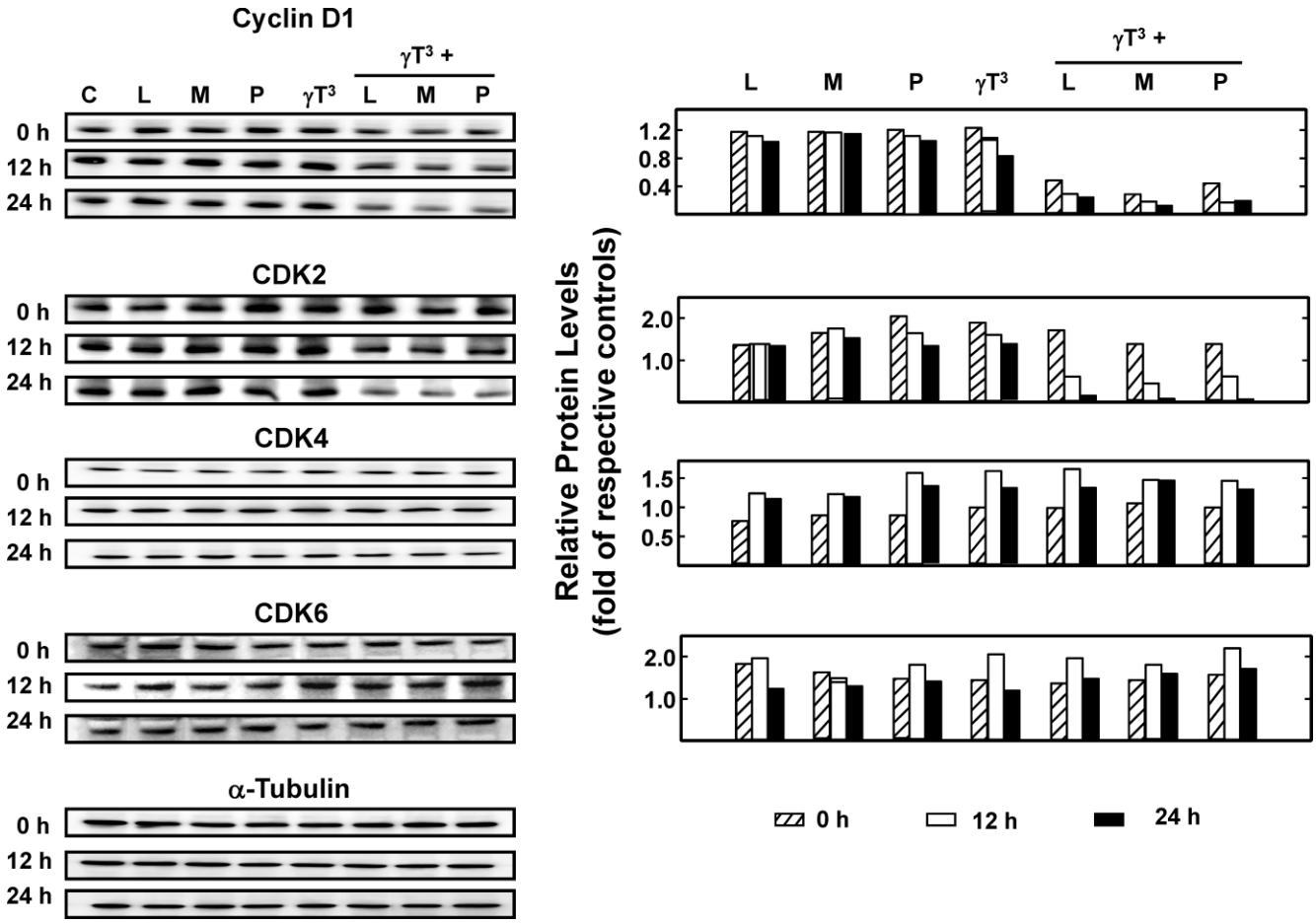

Figure 3. Effects of lovastatin (L), mevastatin (M), and pravastatin (P) and γ-tocotrienol (γT^3) on cyclin D1 and CDK levels in highly malignant +SA mammary epithelial cells. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C), 0.25 μM L, 0.25 μM M, 10 μM P or 2 μM γT^3 media, either alone or in combination, in order to establish cell cycle synchronization in all groups. After a 48 h incubation period, the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at various times over the next 24 h period and whole cell lysates were prepared for Western blot analysis of intracellular levels of cyclin D1, CDK2, CDK4 and CDK6. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and the proteins from 3 minigels were transblotted to a single PVDF membrane, followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and the integrated optical density of each band was normalized with corresponding α-tubulin and control, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate the fold-change in protein levels in various treatment groups as compared with their respective controls.

phases as compared with cells in the untreated control group (Table 1). However, combined treatment of individual statins with γ-tocotrienol resulted in +SA tumor cell cycle arrest in G1 phase, as indicated by a higher percentage of cells in G1 as compared with cells in the untreated control group (Table 1). Similarly, the height of the G1 peak in DNA histograms was higher, while the height of the G2 peak was barely visible in combination treatment groups as compared with untreated controls. Among the combination treatment groups, G1 arrest was relatively less pronounced in the P+ γT^3 group than others, consistent with the results observed in growth studies (Fig. 1 and Table 1). Exposure to 100 ng/ml EGF following a 48 h serum starvation period caused +SA cell cycle progression into subsequent phases in both control and single drug treatment groups, whereas +SA cell cycle progression was prevented in combined statin and γ-tocotrienol treatment groups (Table 1). Furthermore, the appearance of a peak to the left of the G1 peak (sub-G0/G1) corresponds to fragmented DNA and represents cells undergoing apoptosis. However, no prominent peak was observed in any of treatment group DNA histograms, indicating that combined statin and γ-tocotrienol treatment induced cell cycle arrest (cytostatic), but did not initiate programmed cell death (Table 1).

Effect of Statins and γ-Tocotrienol on Cyclin D1 and CDK Levels. The relative intracellular levels of cyclin D1 and CDKs in the various treatment groups as determined by Western blot analysis are shown in Figures 2 and 3. Treatment with 0.25 μM simvastatin or 2 μM γ-tocotrienol alone had no effect on relative cyclin D1 levels, but combined treatment with these compounds resulted in a large reduction in cyclin D1 levels in a time-dependent

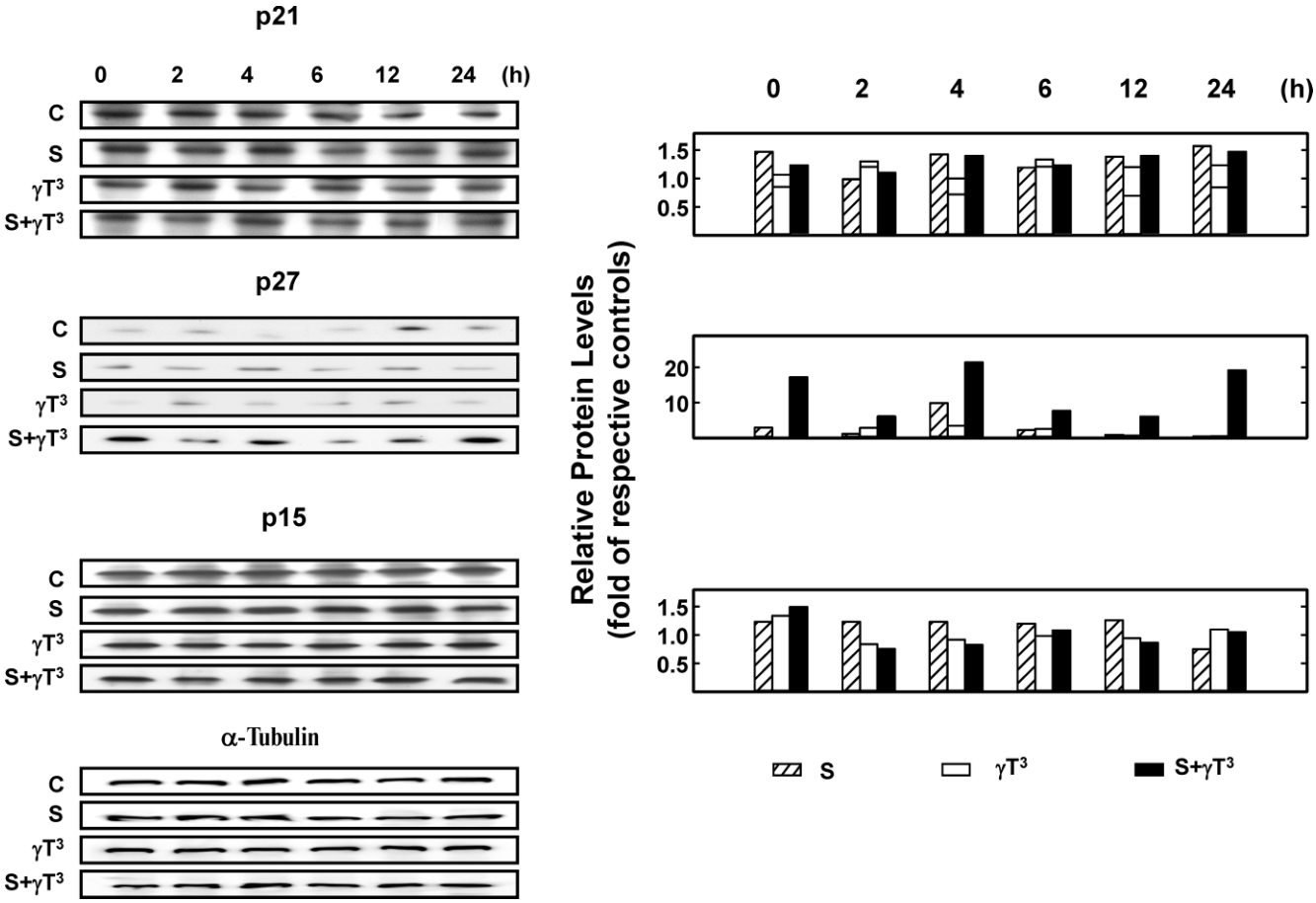

Figure 4. Effects of simvastatin (S) and γ-tocotrienol (γT^3) on CKI levels highly malignant +SA mammary epithelial cells. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C) and treatment mitogen-free media containing 0.25 μM S, 2 μM γT^3 or combination of both in order to establish cell cycle synchronization in all groups. After a 48 h incubation period, the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at various times over the next 24 h period and whole cell lysates were prepared for Western blot analysis of p21, p27 and p15 proteins. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and the proteins from four minigels were transblotted to a single PVDF membrane, followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and the integrated optical density of each band was normalized with corresponding α-tubulin and control, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate the fold-change in protein levels in various treatment groups as compared with their respective controls.

manner, as compared with cells in the untreated control group (Fig. 2). Similarly, combined treatment of mevastatin, lovastatin or pravastatin with γ-tocotrienol also decreased the relative intracellular levels of cyclin D1 in +SA mammary tumor cells (Fig. 3). Additional studies showed that combined statin and γ-tocotrienol treatment caused a relatively large reduction in CDK2 levels within 12–24 h after EGF exposure as compared to their respective untreated control groups (Figs. 2 and 3). CDK4 levels in +SA cells were found to gradually increase over a 24 h period, reaching a peak at 12 h and then progressively declined thereafter. This relative pattern of CDK4 expression was similar in all control and treatment groups (Figs. 2 and 3). Relative CDK6 levels remained unchanged following treatment with individual statins, γ-tocotrienol, or their combination as compared with their respective untreated control groups (Figs. 2 and 3).

Effects of Statins and γ-Tocotrienol on the Expression of Endogenous CDK Inhibitors. Figures 4 and 5 show treatment effects on the intracellular levels of endogenous CDK inhibitors, p21, p27 and p15. Results show that there was little or no difference in p21 or p15 levels throughout the 24 h period following treatment with various statins and γ-tocotrienol alone or in combination as compared to their respective controls (Figs. 4 and 5). However, combined treatment of statins with γ-tocotrienol caused a marked increase in p27 levels as compared to cells in the untreated control group (Figs. 4 and 5).

Effects of Statins and γ-Tocotrienol on Hyperphosphorylation of Rb Protein. Figures 6 and 7 show the relative levels of hyperphosphorylated Rb protein in +SA cells during the 24 h period following EGF exposure in control and treatment groups. Combined treatment with 0.25 μM simvastatin and 2 μM γ-tocotrienol caused a relatively

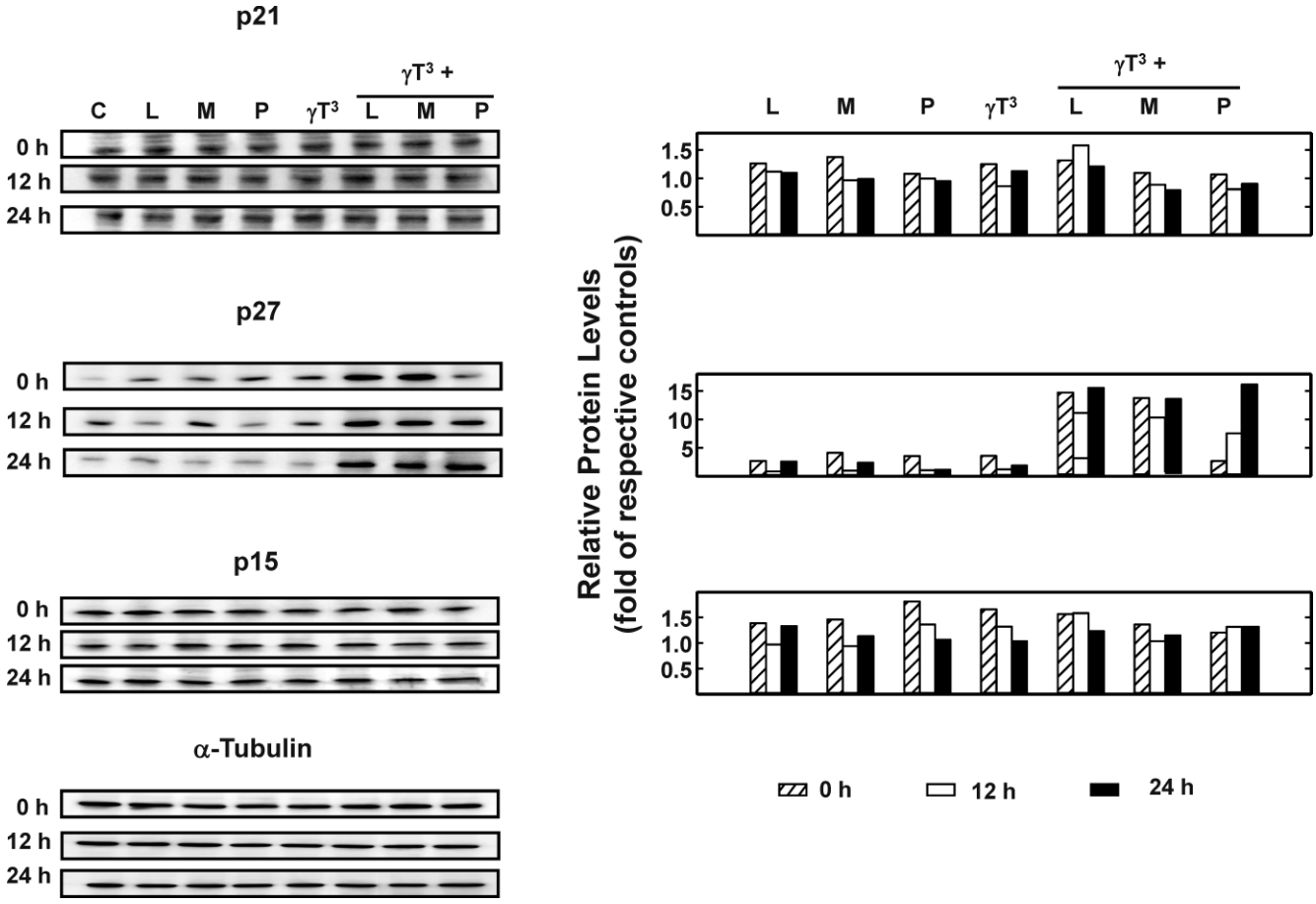

Figure 5. Effects of lovastatin (L), mevastatin (M), and pravastatin (P) and γ-tocotrienol (γT^3) on CKI levels in highly malignant mammary epithelial cells. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C), 0.25 μM L, 0.25 μM M, 10 μM P or 2 μM γT^3 media, alone or in combination, in order to establish cell cycle synchronization in all groups. After a 48 h incubation period the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at various times over the next 24 h period and whole cell lysates were prepared for Western blot analysis of intracellular levels of p21, p27, and p15. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and the proteins from three minigels were transblotted to a single PVDF membrane, followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and the integrated optical density of each band was normalized with corresponding α-tubulin and control, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate the fold-change in protein levels in various treatment groups as compared with their respective controls.

large reduction in Rb protein phosphorylation at ser780 and ser807/811 (Fig. 6). Treatment with either simvastatin or γ-tocotrienol alone had no effect on Rb protein phosphorylation as compared with cells in the untreated control group (Fig. 6). Likewise, treatment with lovastatin, mevastatin, or pravastatin alone had no effect, while combined treatment of these individual statins with γ-tocotrienol suppressed Rb protein phosphorylation in +SA cells at both ser780 and ser807/811 throughout the 24 h period following EGF exposure (Fig. 7). In contrast, the relative levels of total Rb were not altered by any single or combined drug treatment, as compared with respective controls.

Discussion

The results in this present study demonstrate that combined treatment with low doses of statins and γ-tocotrienol induces cell cycle arrest in G1 in highly malignant +SA mammary epithelial cells in culture. Additional studies showed that these cytostatic effects were associated with a large decrease in the relative levels of cyclin D1, CDK2, and hyperphosphorylated Rb protein, and a corresponding increase in p27 levels. Since similar effects were observed when γ-tocotrienol was combined with simvastatin, lovastatin, mevastatin, or pravastatin, these effects appear to be common to all statins and not a unique characteristic displayed by an individual statin family member.

The systemic bioavailability of statins is very low due to intestinal and hepatic metabolism. Administration of high treatment doses (25 mg/kg daily) of statins in humans results in plasma drug concentrations of approximately 3.9 μM or less (8). Various *in vitro* studies have revealed that circulating statin levels of 3.9 μM or higher are required for inducing cell cycle arrest growth in many cancer cell lines

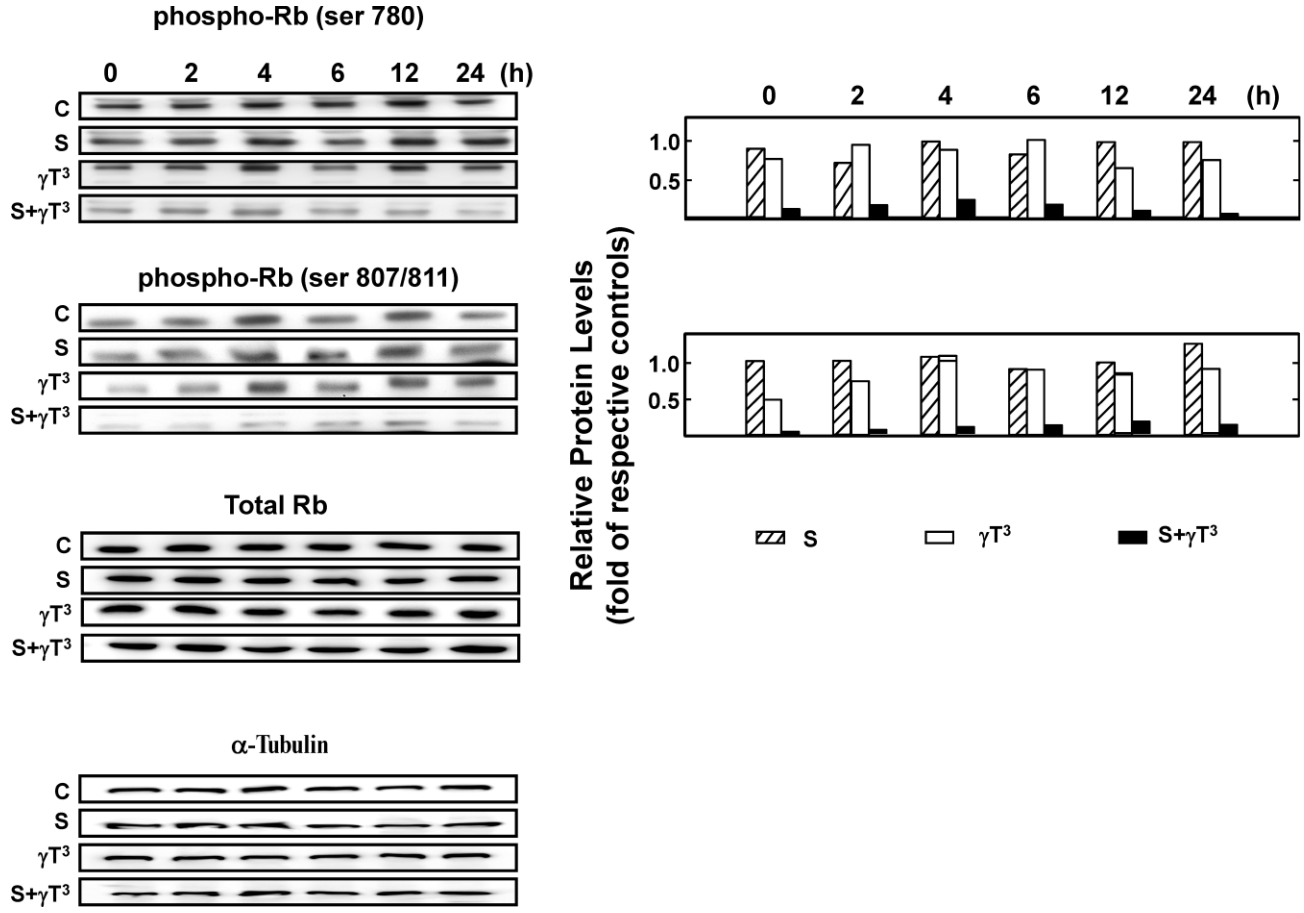

Figure 6. Effects of simvastatin (S) and γ-tocotrienol (γT^3) on retinoblastoma (Rb) phosphorylation in highly malignant +SA mammary epithelial cells. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C) and treatment mitogen free media containing 0.25 μM S, 2 μM γT^3 or the combination of 0.25 μM S and 2 μM γT^3 in order to establish cell cycle synchronization in all groups. After a 48 h incubation period, the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at various times over the next 24 h period and whole cell lysates were prepared for Western blot analysis of intracellular levels of phospho-Rb (ser780), phospho-Rb (ser807/811) and total Rb. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and the proteins from four minigels were transblotted to a single PVDF membrane, followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and the integrated optical density of each band was normalized with corresponding total Rb, α-tubulin and control, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate the fold-change in protein levels in various treatment groups as compared with their respective controls.

(3, 8). Unfortunately, treatment doses of statins greater than 25 mg/kg in humans are associated with severe rhabdomyolysis and even death (8). Combined treatment with subeffective doses of individual statins (0.25 μM simvastatin, lovastatin, and mevastatin or 10 μM pravastatin) and a subeffective dose (2 μM) of γ-tocotrienol acted in a synergistic manner to inhibit malignant +SA mammary epithelial cell growth. These findings confirm and extended previous findings that reported the cytostatic activity of combined low dose γ-tocotrienol and statin treatment (14). It is believed that pravastatin is less potent than the other statins because it is less lipophilic and thereby is less able to be taken up and accumulate in tumor cells. This characteristic may explain why pravastatin, even at high doses, is significantly less potent in suppressing +SA growth in combination with γ-tocotrienol.

Cell cycle analysis by flow cytometry showed that combined γ-tocotrienol and statin treatment induced cell cycle arrest without inducing apoptosis. These findings are in agreement with previous studies that showed that similar treatments with statins and γ-tocotrienol were found to be cytostatic, but not cytotoxic to these cells (14). These results strongly suggest that the unwanted toxicity associated with high dose statin monotherapy, might be circumvented by using low dose statin therapy in combination with another anticancer agent, such as γ-tocotrienol. Since statins act directly to inhibit HMGCoA reductase activity and γ-tocotrienol induces the post-transcriptional down-regulation of this enzyme (13), studies are currently underway to determine if the antiproliferative effects of combined statin and γ-tocotrienols treatment result solely from the inhibition

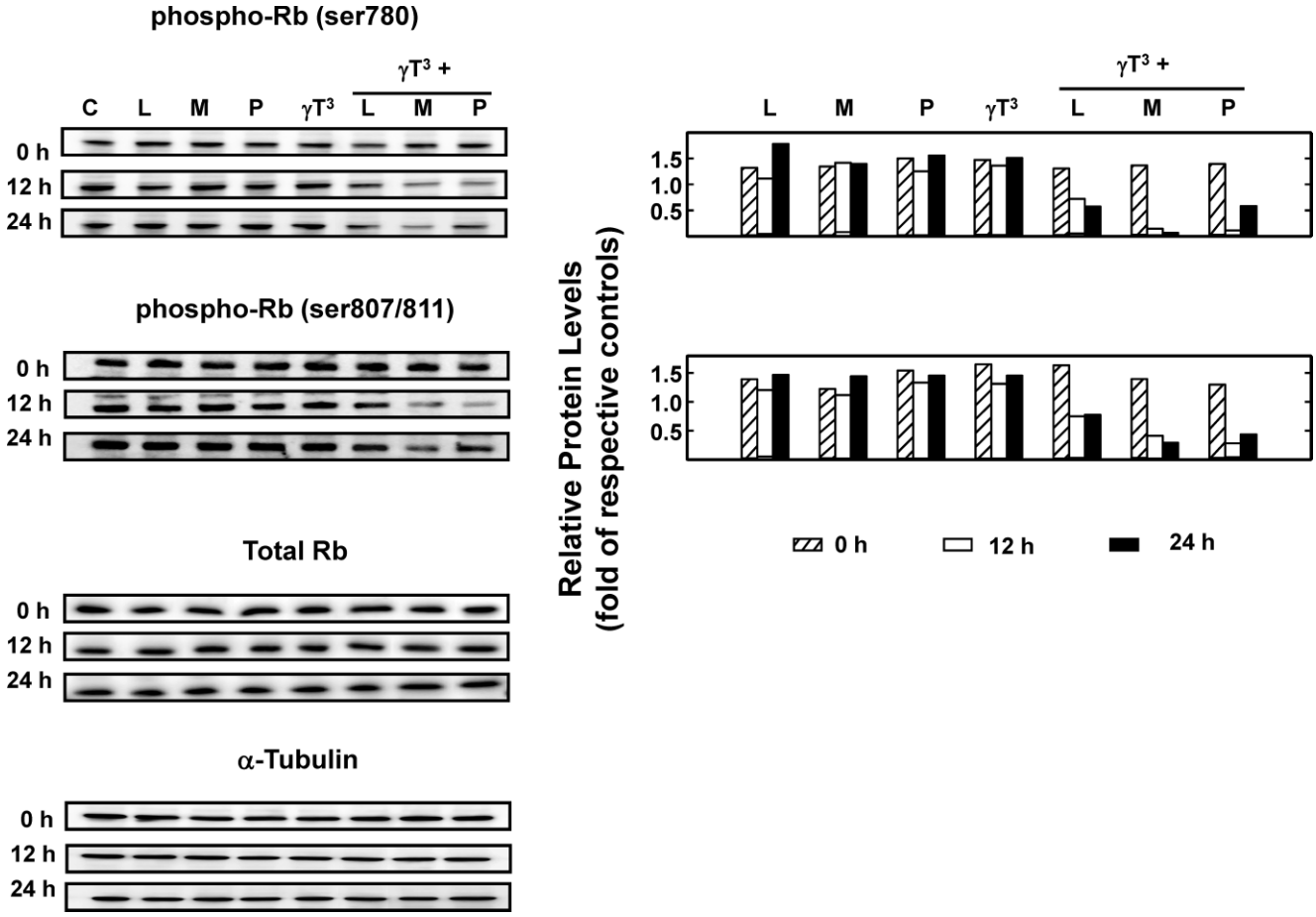

Figure 7. Effects of lovastatin (L), mevastatin (M), and pravastatin (P) and γ-tocotrienol (γT^3) on retinoblastoma (Rb) phosphorylation in highly malignant +SA mammary epithelial cells. +SA cells were plated at a density of 1×10^6 cells/100 mm culture dishes and fed defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C), 0.25 μM L, 0.25 μM M, 10 μM P or 2 μM T^3 media, alone or in combination, in order to establish cell cycle synchronization in all groups. After a 48 h incubation period the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF. Cells were collected at various times over the next 24 h period and whole cell lysates were prepared for Western blot analysis of intracellular levels of phospho-Rb (ser780), phospho-Rb (ser807/811) and total Rb. For each experiment, samples were electrophoresed through SDS-polyacrylamide minigels run simultaneously and the proteins from three minigels were transblotted to a single PVDF membrane, followed by Western blot analysis. Scanning densitometric analysis was performed for each blot and the integrated optical density of each band was normalized with corresponding total Rb, α-tubulin and control, as shown in bar graphs adjacent to their respective Western blot images. Vertical bars in the graph indicate the fold-change in protein levels in various treatment groups as compared with their respective controls.

of HMGCoA reductase or also involve additional HMGCoA reductase independent mechanisms.

Cell cycle transition from G1 to S phase is primarily regulated by cyclin D1 in complex with CDKs. Mitogenic stimuli increase the intracellular levels of cyclin D1, which then form an assembly with CDK4 or CDK6, and ultimately lead to the hyperphosphorylation of Rb protein (28, 29). Exogenous cyclin D1 has been shown to elevate cyclin D1/CDK4 activity and cause the hyperphosphorylation of Rb protein. Studies have shown that blockade of cyclin D1 activation with siRNA results in a significant reduction in the hyperphosphorylation of Rb protein and is associated with G1 cell cycle arrest in MCF-7 cells (30). Overexpression of cyclin D1 has been observed in approximately 50% of all breast cancers (31–34), while suppression of cyclin D1 activity has been shown to significantly reduce mammary tumorigenesis (35, 36). In addition, cyclin D1 knockout mice are resistant to HER2 and Ras induced tumorigenesis (37, 38). In the present study, intracellular cyclin D1 levels progressively increased over the 24 h period following EGF exposure in control, statin, and γ-tocotrienol alone treated groups. However, combined statin and tocotrienol treatment caused a relatively large reduction in cyclin D1 expression in malignant +SA mammary epithelial cells. Active cyclin D1-CDK4/6 and cyclin E-CDK2 complexes sequentially phosphorylate and inactivate the Rb protein to initiate E2F dependent transcription, a requirement for progression into S-phase. Results showed a marked reduction in the levels of CDK2 in +SA cells that received combined γ-tocotrienol-statin treatment. Furthermore, Rb phosphorylation was markedly suppressed by all the statin-γ-tocotrienol combinations. This hypophosphor-

Table 1. Flow Cytometry Analysis of Statins and γ-Tocotrienol Effects on Cell Cycle Progression in Highly Malignant +SA Mammary Epithelial Cells[a]

Cell cycle phase	Treatment groups									
	C	S	L	M	P	γT^3	S+ γT^3	L+ γT^3	M+ γT^3	P+ γT^3
sub-G0/G1 %	3.92	2.29	2.89	3.27	4.20	3.08	2.22	3.43	2.01	4.19
G1 %	38.89	42.79	42.56	48.99	43.50	43.44	56.16	61.20	62.48	51.54
S %	25.31	25.39	22.83	20.20	22.28	24.69	26.06	21.12	21.02	27.16
G2 %	28.63	25.89	27.58	23.37	28.40	25.13	11.94	11.51	12.16	15.01

[a] Neoplastic +SA cells were initially plated at a density of 1×10^6/100 mm culture dishes and fed defined control media in order to allow cells to attach. The next day, cells were divided into control and treatment groups, media was removed, the cells washed, and then fed fresh mitogen-free control (C), 0.25 μM simvastatin (S), 2 μM γ-tocotrienol (γT^3), or combined 0.25 μM S and 2 μM (γT^3) media in order to establish cell cycle synchronization in all groups. After a 48 h incubation period, the media was removed and cells in all groups were fed their respective control or treatment media containing 100 ng/ml EGF, and 24 h later, cells were collected for DNA content analysis by flow-cytometry. At that time, cells were fixed with 70% ethanol followed by incubation with nuclear DNA staining buffer. The propidium iodide-stained cells were detected and histograms generated by FACSCalibur flow cytometer. CellQuest software was used to determine the percentage of cells in sub-G0/G1, G1, S and G2 phases.

ylation of Rb appears to be caused by the unavailability of active cyclin-CDK complexes due to decrease in cyclin D1 and CDK2 levels. Additional studies are required to determine the treatment effects of statin-γ-tocotrienol combinations on E2F responsive gene expression and Rb-E2F complex association.

Previous studies showed that combined statin and γ-tocotrienol treatment inhibited phosphorylation and activation of Akt and MAPK mitogenic signaling in +SA cells (14). Activation of these pathways has been shown to stimulate the progression from G1 to S phase by stimulating cyclin D1 activity and subsequent hyperphosphorylation of Rb protein (17, 39). Specifically, Akt has been shown to enhance cyclin D1 transcription and protein expression (40), and increase glycogen synthase kinase 3β (GSK3β) phosphorylation, and thereby preventing GSK3β from phosphorylating and destabilizing the cyclin D1 protein (17). In addition, FOXO transcription factors, which repress cyclin D1 expression and induce p27 expression, are also phosphorylated and inhibited by Akt activation (41). Similarly, MAPK activation leads to the phosphorylation and stabilization of c-myc, a transcription factor that induces cyclin D1 and decreases CKI expression (42, 43). Taken together, these findings indicated that combined statin and γ-tocotrienol treatment induced changes in endogenous cyclin, and CDK expression most likely results from treatment-dependent inhibition of Akt and MAPK mitogenic signaling. Therefore, it would be of great interest to identify the exact transcriptional and/or post-translational mechanisms involved in the reduction of cyclin D1 expression through Akt or MAPK suppression by statin-γ-tocotrienol combination.

The present study also showed that combination low dose statin and γ-tocotrienol treatment induced a relatively large increase in intracellular levels of p27, apparently resulting from treatment-dependent inhibition of Akt and MAPK mitogenic signaling. It is well established that another important mechanism that prevents cell cycle progression from G1 to S phase is the inhibition of CDK activity by CKIs. CKIs belonging to the INK4 family of related compounds have been shown to bind specifically to CDK4 and CDK6 and inhibit their forming a complex with cyclin D1 (44). In addition, CKIs, such as p27 and p21, are members of CIP/KIP family and can bind to and stabilize cyclin D1-CDK4/6 complexes (18, 45–47). Studies have also shown that p27 exerts an inhibitory effect on the activity of CDK2 complexes during early G1 (29). In addition, activation of mitogen receptors removes p27 inhibition of CDK2 by suppressing transcription, translation or nuclear localization of p27, and by increasing p27 sequestration by cyclin D-CDK4/6 complexes (45, 48, 49). Activated CDK2 can also phosphorylate p27, which leads to the polyubiquitination and degradation of p27 and further facilitating cell cycle progression from G1 to S (47, 50, 51). Normal human mammary duct epithelial cells show nuclear immunoreactivity for p27, whereas mammary cancers often exhibit lower p27 expression or localization of p27 in the cytosol (52). Enhanced MAPK signaling in breast cancer cells decreases the half-life of p27, and these effects show a positive correlation with increased tumor malignancy and poor prognosis (53).

In summary, these studies demonstrated that γ-tocotrienol acts synergistically with statins to induce cell cycle arrest by causing a large relative decrease in intracellular levels of cyclin D1, CDK2, and Rb protein phosphorylation. Since combined treatment of statins and γ-tocotrienol has also been shown to significantly inhibit Akt and MAPK mitogenic signaling and these signaling pathways stimulate cell cycle progression by increasing cyclin D1, CDK2 activation while at the same time reducing p27 expression, these data provide new insights into the intracellular mechanisms involved in mediating the cytostatic effects of combined statin and γ-tocotrienol treatment. These findings also suggest that combined low dose statin and γ-tocotrienol therapy may provide significant health benefits in the treatment of breast cancer in women, while

avoiding myotoxicity, such as rhabdomyolysis, that is associated with high dose statin monotherapy.

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