Tocotrienols Potentiate Lovastatin-Mediated Growth Suppression *In Vitro* and *In Vivo*

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3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase is the rate-limiting enzyme in the mevalonate pathway that provides essential intermediates for the membrane anchorage and biologic functions of growth-related proteins. Contrary to preclinical studies showing the growth-suppressive activity of statins, competitive inhibitors of HMG CoA reductase, clinical application of statins in cancer is precluded by their lack of activity at levels prescribed for the prevention of cardiovascular disease and by their dose-limiting toxicities at high doses. The dysregulated and elevated HMG CoA reductase activity in tumors retains sensitivity to the isoprenoid-mediated posttranscriptional down-regulation, an action that complements the statin-mediated inhibition and may lead to synergistic impact of blends of isoprenoids and lovastatin on tumor HMG CoA reductase activity and consequently tumor growth. *d*-γ- and *d*-δ-tocotrienols, vitamin E isomers containing an isoprenoid moiety, and lovastatin-induced concentration-dependent inhibition of the 48-hr proliferation of murine B16 melanoma cells with IC50 values of 20 ± 3, 14 ± 3, and 1.5 ± 0.4 μM respectively. A blend of lovastatin (1 μ*M*) and *d*-γ-tocotrienol (5 μ*M*) totally blocked cell growth, an impact far exceeding the sum of inhibitions induced by lovastatin (12%) and *d*-γ-tocotrienol (8%) individually. Synergistic impact of these two agents was also shown in human DU145 prostate carcinoma and human A549 lung carcinoma cells. C57BL6 mice were fed diets supplemented with 12.5 mg lovastatin/kg body weight, 62.5 mg *d*-δ-tocotrienol/kg body weight, or a blend of both agents for 22 days following B16 cell implantation; only the latter had significantly lower tumor weight than those with no supplementation. Co-administration of isoprenoids that posttranscriptionally down-regulate tumor reductase may lower the effective dose of statins and offer a novel approach to cancer chemoprevention and/or therapy. Exp Biol Med 232:523–531, 2007

Key words: lovastatin; tocotrienol; HMG CoA reductase; B16 melanoma; synergy

Introduction

A recent meta-analysis of long-term, large-scale, randomized, active- or placebo-controlled clinical trials for the prevention of cardiovascular disease showed that statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, have a neutral effect on cancer and cancer death risk (1). This observation stands in contrast to retrospective analyses suggesting that lovastatin at clinically relevant levels reduces the risk of developing cancer (2).

This inconsistency may have been reflected in earlier phase 1 and 2 clinical trials that showed that clinically significant responses can only be achieved by high-dose (>25 mg/kg body weight/day) statins (3, 4) with dose-limiting toxicities including myalgia, muscle weakness, elevated creatine phosphokinase activity, anorexia, ulcerative lesions, rhabdomyolysis, nausea, diarrhea, and fatigue, which unfortunately preclude their long-term application in cancer therapy.

These toxicities trace to the statin-mediated inhibition of HMG CoA reductase activity that deprives cells of essential components including cholesterol, a component of cell membranes; ubiquinone, a component of the electron transport chain; isopentenyl adenine, which is required for tRNA function; and mevalonate-derived components, which are required for posttranslational processing—farnesylation, geranylgeranylation, dolichylation, and N-linked glycosylation—and for the membrane localization and biologic functions of an estimated 1% of all cellular proteins (5–8). Of relevance are the signaling proteins, nuclear lamins and growth factor receptors, which play critical roles in cell cycle progression and cell proliferation. Cells treated with lovastatin are arrested in G1 phase of the cell cycle; those

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passing through G1 undergo apoptotic death (5–11). Both events are reversed by the simultaneous addition of mevalonate (5, 12).

Preclinical trials suggest the chemotherapeutic potential of high-dose (15–70 mg/kg body weight/day) statins in the treatment of lung (11), prostate (13), mammary (13), and pancreatic tumors (14) and melanoma (15). Achieving clinical efficacy through targeted mevalonate starvation in tumors, distinctive from the indiscriminate inhibition and the associated toxicity of high-dose lovastatin, remains a prospective approach to cancer therapy.

We recently proposed that dysregulated HMG CoA reductase, an anomaly widely present in tumors, offers a novel and specific target for chemotherapeutic agents (8, 16). HMG CoA reductase activity in tumor tissues is elevated and is resistant to sterol feedback regulation, the degree of resistance correlated to the virulence of the cancer. This dysregulation ensures that the pools of the aforementioned mevalonate-derived products, primarily farnesyl pyrophosphate and geranylgeranyl pyrophosphate, remain optimal to meet the needs for tumor growth. Nevertheless, tumor HMG CoA reductase activity retains sensitivity to the posttranscriptional down-regulation triggered by endogenous *trans, trans* farnesol (16). Farnesol (17), the *trans, trans* farnesol mimetics, the *d*-γ-, *d*-δ-, and less potent *d*-α-tocotrienol (18) and other farnesyl mimetics (19), geraniol (20, 21), and geraniol mimetics (22) suppress HMG CoA reductase activity (17–22) and suppress tumor cell proliferation (16, 23–33), the latter being manifested by an accumulation of cells in G1 phase of the cell cycle and the onset of apoptotic cell death (23, 26–29, 33). Supplemental mevalonate reverses the geraniol-mediated arrest of P388 leukemic cells (29). Differing from lovastatin, dietary *d*-γ-tocotrienol suppresses the growth of implanted tumors but not those of host and tumor-free animals (25, 28). Similar findings have been reported for farnesol (30–32), other farnesol mimetics (33), and geraniol (29, 31).

Both the statins and farnesol mimetics impose limits on the pools of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, the former by competitively inhibiting HMG CoA reductase (5), the latter by posttranscriptional actions that decrease reductase mass (17–21). The inhibitory action of the statins triggers a compensatory increase in reductase mass through increased transcriptional and translational activities and decreased degradative activity (5). The suppression of reductase activity imposed by farnesol and farnesol mimetics (17–21) occurs at posttranscriptional levels, namely decreasing translational activity and increasing degradative activity. Activities with the potential of initiating G1 arrest and apoptosis that are down-regulated or up-regulated subsequent to the suppression of mevalonate synthesis by statins and isoprenoids were catalogued in our recent review (8). Therefore, we hypothesize that a blend of a statin and a farnesol mimetic, suppressors of HMG CoA reductase through complementary mechanisms, will have a synergistic impact on tumor cell proliferation as has been demonstrated on HMG CoA reductase activity in HepG2 cells (18).

We have reported that the concomitant application of lovastatin and a farnesol mimetic (e.g., *d*-γ-tocotrienol [26] or farnesyl-*O*-acetylhydroquinone [34]) yielded a significantly greater than additive impact on the proliferation of melanoma B16 cells. We now confirm and extend this observation of synergy by testing the impact of lovastatin and *d*-δ-tocotrienol individually and in a blend on the proliferation of murine B16 melanoma cells and of lovastatin and *d*-γ-tocotrienol individually and in a blend on human DU145 prostate carcinoma and A549 lung carcinoma cells. Preclinical studies demonstrated the significant impacts of high-dose (15–70 mg/kg body weight) lovastatin (11, 13, 15) and high-dose *d*-γ-tocotrienol (20–160 mg/kg body weight) (25, 28) via diet (25) or ip injection (28) on the growth of implanted tumors. We now evaluate the impact of diets estimated to provide either 12.5 mg lovastatin/kg body weight or 60 mg *d*-δ-tocotrienol/kg body weight and the impact of a diet estimated to provide 12.5 mg lovastatin and 60 mg *d*-δ-tocotrienol per kg body weight on the growth of implanted B16 melanomas.

Materials and Methods

Chemicals. Dr. Abdul Gapor, Palm Oil Research Institute of Malaysia, provided chromatographically pure (>98%) *d*-γ-tocotrienol that was isolated from palm oil. Dr. Barrie Tan, American River Nutrition, Inc. (Hadley, MA) provided chromatographically pure (>99%) *d*-δ-tocotrienol that was isolated from annatto. Lovastatin was a gift from Merck. The tocotrienols were dissolved in ethanol, and lovastatin was dissolved in dimethyl sulfoxide (DMSO).

Cell Growth Assays. We first evaluated the impact of the agents individually and in combination on the proliferation of murine B16F10 melanoma cells. Cell growth was measured according to the CellTiter 96 Aqueous One Solution (Promega, Madison, WI) procedure, an updated MTT-based assay suitable for substitution of [^{3}H]thymidine incorporation assay (35) and previously corroborated by manual cell counting (34). B16 cells were grown in monolayer culture (96-well tissue culture plate; Fisher Scientific, Waltham, MA) in 0.1 ml of RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 80 mg/l gentamicin (Sigma-Aldrich). Cultures, seeded in escalating densities (0–8000 cells/well with a 500-cell interval), were incubated at 37°C for 72 hrs in a humidified atmosphere of 5% CO_2. At 24 hrs the medium was decanted from each well, cells were rinsed with 0.1 ml of Hank's balance salt solution (HBSS), and 0.1 ml of fresh medium was added. The 72-hr cell populations were determined by adding 20 μl of CellTiter 96 Aqueous One Solution to each well; the plate was held in the dark at 37°C for 2 hrs and then read at 490 nm with a SPECTRAmax 190 multiplate reader with SOFTmax PRO version 3.0 (Molecular Devices, Sunnyvale,

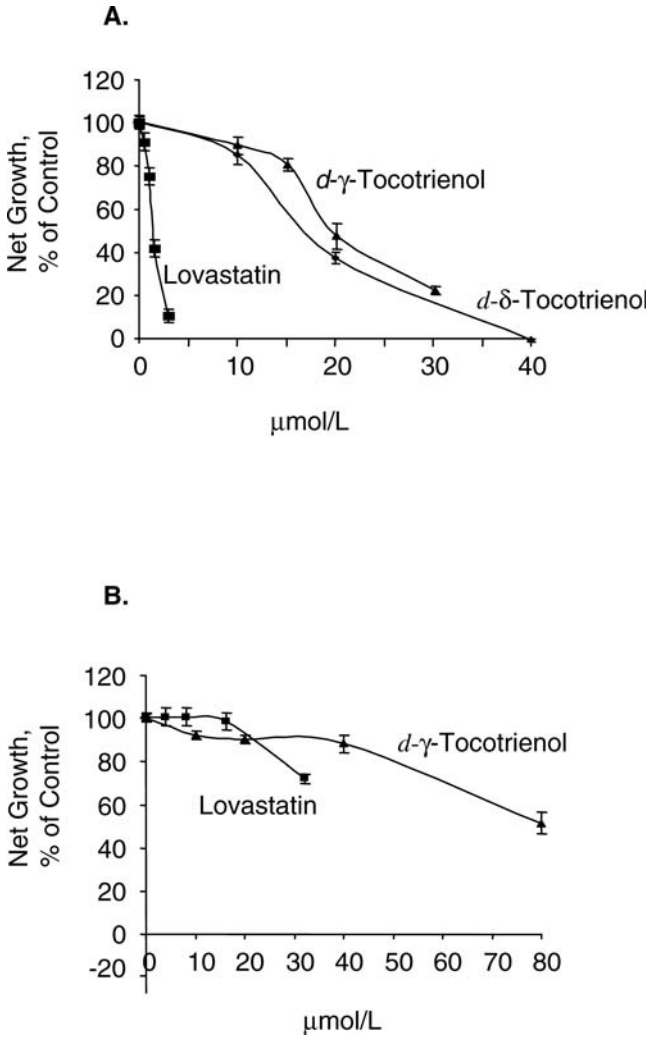

Figure 1. Dose-response plots of murine B16 melanoma (A) and human A549 lung carcinoma (B) cells to lovastatin and *d*-γ- and *d*-δ-tocotrienols. Cells were inoculated at 1000 cells/well (B16) or 1500 cells/well (A549) and cultured in 96-well plates. After a 24-hr incubation the medium was replaced with media containing the test agents. After an additional 48-hr incubation the cell population was measured using the CellTiter 96 Aqueous One Solution procedure as described in Materials and Methods. Net growth represents the difference in A_{490} between final and 24-hr measurements. Values are mean ± SEM, $n = 8$.

CA). The absorbance was plotted against cell-seeding density. The absorbance had a linear correlation with cell-seeding density for up to 2000 B16 cells/well. The midpoint of cell inoculation density within the linear curve, 1000 cells/well, was chosen for the B16 cell proliferation assay. In the proliferation assay B16 cells were incubated in 96-well plates for 24 hrs. The medium was decanted from each well, and cells were rinsed with 0.1 ml of HBSS. A pretreatment absorbance baseline (0-time value) was established by adding 0.1 ml of fresh medium and 20 μl of CellTiter 96 Aqueous One Solution to each well; 0-time values were measured at 490 nm following a 2-hr incubation in the dark at 37°C. For the rest of the wells, 0.1 ml of fresh medium containing the test agents was added. All cultures contained 1 ml/l each of ethanol and DMSO. Incubation continued for an additional 48 hrs. Cell populations were determined according to the CellTiter 96 Aqueous One Solution procedure as described above. Net absorbance, the difference in absorbance between final and 0-time values, represents net growth.

Human A549 lung carcinoma cells (CCL-185; ATCC, Manassas, VA) were grown in Kaighn's modification of Ham's F12 medium modified by ATCC to contain 1.5 g/l sodium bicarbonate, supplemented with 10% FBS and 80 mg/l gentamicin. Human DU145 prostate carcinoma cells (HTB-81; ATCC) were grown in Eagle's minimal essential medium modified by ATCC to contain 1.0 m*M* sodium pyruvate, 0.1 m*M* nonessential amino acids, and 1.5 g/l sodium bicarbonate, supplemented with 10% FBS and 80 mg/l gentamicin. Cultures of these human tumor cells, seeded in 0.1 ml of medium with 1000 cells/well (DU145) or 1500 cells/well (A549) in a 96-well plate, were incubated at 37°C for 24 hrs in a humidified atmosphere of 5% CO_2. At 24 hrs the medium was decanted from each well; cells were rinsed with 0.1 ml of HBSS, and 0.1 ml of fresh medium containing the test agents was added. All cultures contained 1 ml/l each of ethanol and DMSO. Incubation continued for an additional 48 hrs (A549) or 72 hrs (DU145). Cell populations were determined using the CellTiter 96 Aqueous One Solution procedure as described above.

Microscopy. Photomicrographs of representative fields of washed monolayers of B16 melanoma cells were produced with a Nikon Eclipse TS 100 microscope (Nikon

Table 1. Estimates of the Concentrations of Evaluated Agents Required to Suppress by 50% the Proliferation of Cells Derived from a Murine Melanoma (Lovastatin, *d*-γ- and *d*-δ-tocotrienols) and Human Prostate and Lung Carcinomas (Lovastatin and *d*-γ-tocotrienol)

	IC_{50} (μ*M*)		
Cell line	Lovastatin	*d*-γ-tocotrienol	*d*-δ-tocotrienol
Murine B16 melanoma	1.5 ± 0.4[a]	20 ± 3	14 ± 3
Human DU145 prostate carcinoma	20.5 ± 2.4	19.4 ± 3.8	
Human A549 lung carcinoma	>43	>80	

[a]Values are mean ± SD, $n \geq 3$.

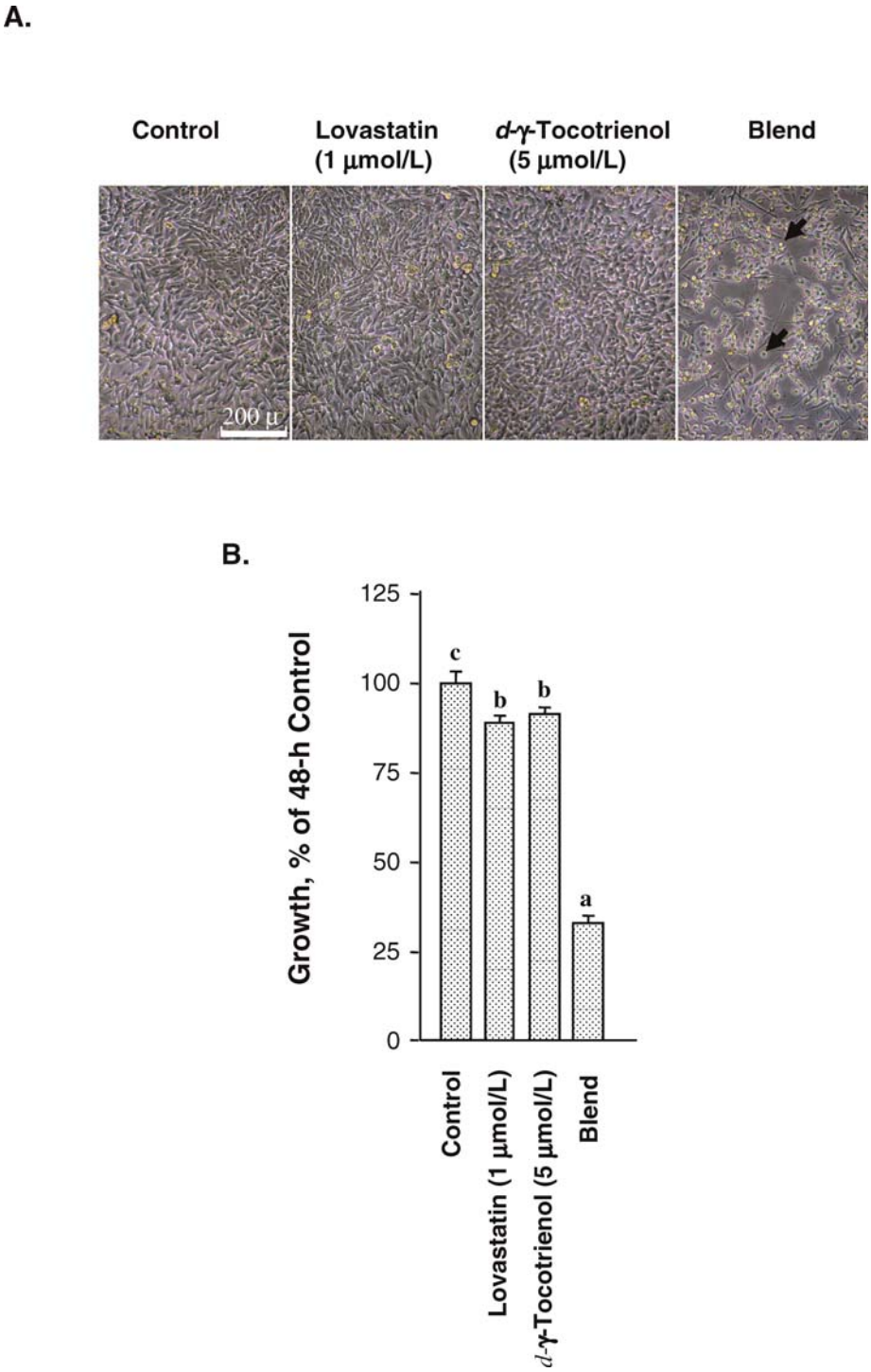

Figure 2. Synergistic impact of blends of lovastatin and *d*-γ-tocotrienols on the proliferation of B16 murine melanoma cells following a 48-hr treatment as shown in photomicrographs (A) and growth plots (B). Arrows (A) mark apoptotic cells with significant morphologic changes including cell shrinkage and elongation, marked cytoplasmic and chromatin condensation, and plasma membrane blebbing, changes reminiscent of apoptotic cell death. The apoptotic cells eventually rounded up and detached from the monolayer. B16 cells were grown and growth measured using the CellTiter 96 Aqueous One Solution procedure as described in Materials and Methods. Absorbance values representing cell growth for cells following 48-hr treatments are shown as percentages of that of 48-hr untreated cells (control). Values are mean $\pm$ SD, $n = 3$. Values not sharing a letter are significantly different ($P < 0.01$) (B). Color figure available in on-line version.

Corp., Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corp.).

Animal Studies. Fifty 4-week-old female C57BL/6 mice (Harlan-Sprague Dawley, Houston, TX) housed at 72°F and 70% humidity in groups of 5 on wood shavings in plastic cages with free access to water were fed AIN-93G diet (Teklad Test Diets, Madison, WI) for 10 days. Then 4 cages were assigned to the control group and 2 each to the 3 experimental groups (lovastatin, *d*-δ-tocotrienol, and blend). In sequence, 2 mice in the control group and 1 mouse in each of the experimental groups received subcutaneous injections of B16 cells as previously described (25, 33); the sequence was repeated 10 times. The mice were continued on the AIN-93G diet for 2 days. For the remainder of the study mice were fed the control and experimental diets as described below. The AIN-93G diet was fed to the control and lovastatin groups; the diet fed to the latter group contained an additional 50 ppm lovastatin (125 μmol/kg). Based on findings that *dl*-α-tocopherol attenuates the impact of tocotrienols on HMG CoA reductase activity (36), we prepared vitamin E–free diets for the tocotrienol and blend groups as previously described (25), then added 250 ppm (630 μmol/kg) *d*-δ-tocotrienol to the former and a blend of lovastatin (125 μmol/kg) and *d*-δ-tocotrienol (630 μmol/kg) to the latter. This level of *d*-δ-tocotrienol, doubling that of *dl*-α-tocopherol in the AIN-93G diet, was two-thirds of the level of a less potent *d*-γ isomer employed in a previous study (25). The diets were administered *ad lib* for 22 days, and diet intake was monitored daily; mice were weighed at 2-day intervals. The mice were euthanized (CO_2 overdose) at termination, and the tumors and livers were excised and weighed. The animal protocol was approved by Texas Woman's University Institutional Animal Care and Use Committee.

Statistical Methods. SPSS version 11.01 for Windows (SPSS Inc., Chicago, IL) was used for the assessment (one-way analysis of variance, Tukey post test) of treatment-mediated effects; $P < 0.05$ was considered significant.

Results

Representative dose-response plots for *d*-γ-tocotrienol and lovastatin obtained with murine B16 melanoma cells are shown on Figure 1. The IC_{50} values, concentrations of compounds required to suppress cell proliferation by 50%, estimated using values from 3 or more plots for *d*-γ-tocotrienol (20 ± 3 μ*M*) and lovastatin (1.5 ± 0.4 μ*M*) confirm previously reported values for the respective agents (25, 26). A comparison of the tocotrienol isomers revealed *d*-δ-tocotrienol to have greater growth-suppressive potency in the B16 model than *d*-γ-tocotrienol (IC_{50} values 14 ± 3 and 20 ± 3 μ*M*, respectively). Confirming earlier work (26), IC_{50} values for diverse tumor cell lines differed substantially with A549 lung carcinoma cells having considerable resistance to both agents (Table 1). Physiologically attainable levels of lovastatin (3) and *d*-γ-tocotrienol (37) were substantially lower than their respective IC_{50} values.

To demonstrate synergy, we incubated B16 melanoma cells with a minimal concentration of lovastatin or γ-tocotrienol, concentrations predicted to have little impact on cell growth, and with a blend of the 2 agents. The photomicrographs (Fig. 2A) and bar graph (Fig. 2B) recorded minor decreases of the 48-hr population by lovastatin (1 μ*M*) and γ-tocotrienol (5 μ*M*). Pairing of lovastatin and *d*-γ-tocotrienol yielded synergy as manifested in the total arrest of cell growth ($P < 0.01$). Morphologic changes consistent with apoptotic cell death, cell shrinkage and elongation, cytoplasmic and chromatin condensation, and plasma membrane blebbing, are suggested in the photomicrograph (Fig. 2A). The impact of a lower concentration of lovastatin (0.5 μ*M*) alone and in combination with *d*-γ-tocotrienol (5 μ*M*) further demonstrates the synergy attained with the blended agents; at this concentration lovastatin had no impact on cell growth. When blended with *d*-γ-tocotrienol, growth was limited to 32% ± 4% of the control. Pairing of lovastatin and the more potent *d*-δ-tocotrienol, each at one-half the respective IC_{50} value (Table 1), yielded an 81% ± 10% suppression of B16 growth.

DU145 cells also responded to the synergy attained with a blend of the agents. Growth of cells treated with lovastatin (1.5 μ*M*), *d*-γ-tocotrienol (5 μ*M*), and the blend was 100% ± 8%, 92% ± 12%, and 67% ± 3%, respectively, of that of untreated cells (Fig. 3). Also tested were the two agents at 2X concentrations. Cell growth with lovastatin (3 μ*M*), *d*-γ-tocotrienol (10 μ*M*), and the blend treatment were 100% ± 10%, 73% ± 4%, and 22% ± 4%, respectively, of that of untreated cells.

Consistent with the aforementioned resistance to lovastatin, the growth of A549 cells was not suppressed by a relatively high concentration of lovastatin (12 μ*M*; Fig. 1); an unanticipated finding was that a blend of lovastatin (12 μ*M*) and *d*-γ-tocotrienol (5 μ*M*) markedly suppressed the growth of A549 lung carcinoma cells (Fig. 4).

Following up on studies that demonstrated the efficacy of high-dose lovastatin (11–13, 15) and *d*-γ-tocotrienol (25, 28) in suppressing the growth of implanted tumors, we next evaluated the impact of a blend of the 2 agents, each at a more modest level than those previously employed, on the growth of implanted B16 melanomas. As noted above, mice were fed experimental diets formulated to provide, per kg body weight per day, 12.5 mg lovastatin, 62.5 mg *d*-γ-tocotrienol, or a blend of both agents. The diets were fed for a 22-day period initiated 2 days following the implant of B16 cells. The study was terminated with the death of a mouse in the lovastatin group. During the intervening period diet intakes ranged from 4.47 ± 0.64 (lovastatin group) to 5.39 ± 1.26 (blend group) g/mouse/day; intake by the lovastatin group was significantly lower than that of the tocotrienol and blend groups ($P = 0.022$; Table 2); tocotrienol reversed the impact of lovastatin on diet

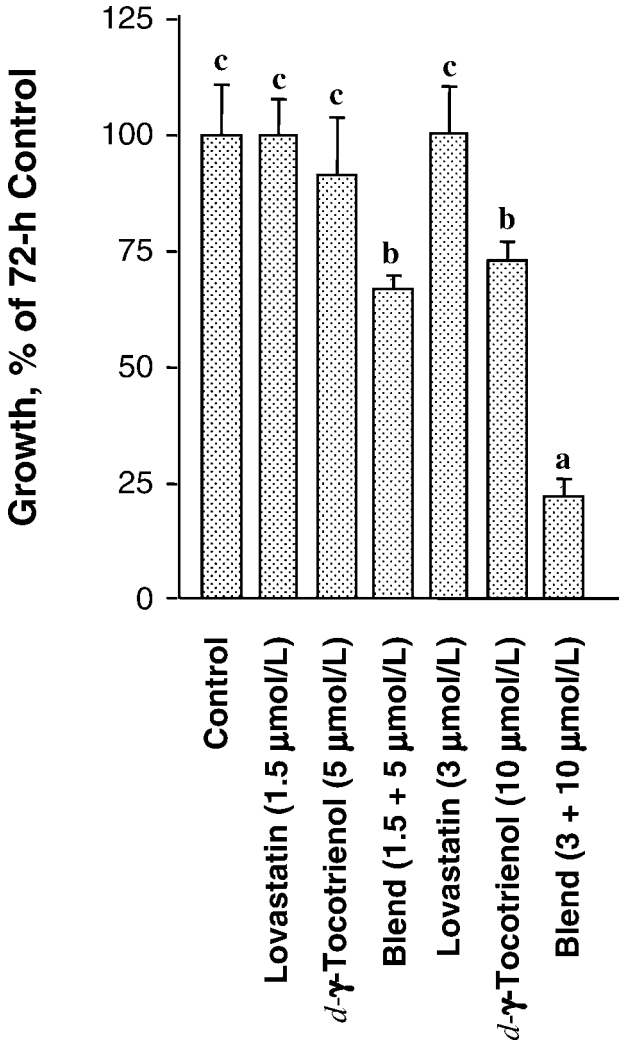

Figure 3. Synergistic impact of lovastatin and *d*-γ-tocotrienol on the proliferation of DU145 human prostate carcinoma cells following a 72-hr treatment. DU145 cells were grown and growth measured using the CellTiter 96 Aqueous One Solution procedure as described in Materials and Methods. Absorbance values representing cell growth for cells following 72-hr treatments are shown as percentages of that of 72-hr untreated cells (control). Values are mean ± SD, $n = 3$. Values not sharing a letter are significantly different ($P < 0.01$).

consumption. Mice in the lovastatin group consumed 11.5 ± 1.6 mg lovastatin/kg body weight/day, mice in the tocotrienol group consumed 66.8 ± 7.6 mg *d*-δ-tocotrienol/kg body weight/day, and mice in the blend group consumed 14.2 ± 2.7 mg lovastatin and 71.2 ± 13.4 mg *d*-δ-tocotrienol/kg body weight/day. Terminal weights fell within the range of 20.58 ± 0.58 to 23.40 ± 0.61 g (Table 2). The tocotrienol group had a significantly greater body weight than those of the control and blend groups ($P = 0.002$). Final liver weights fell within the range 0.81 ± 0.04 to 0.95 ± 0.05 g; liver weights as percent body weight fell within the range 3.85%–4.07%. The differences between treatment groups were not significant.

Tumors metastasized in 1 mouse in the control and tocotrienol groups, and 1 mouse was lost from the lovastatin group. Mean weights of solid tumors excised from the flanks of mice in the control, lovastatin, tocotrienol, and blend groups were 1.23 ± 0.20, 0.99 ± 0.29, 0.61 ± 0.29, and 0.49 ± 0.27 g, respectively (Table 2). Only the latter weight was significantly lower than that of the control ($P = 0.034$). Tumors weighing less than 10 mg were excised from 5%, 0%, 22%, and 30%, respectively, of the mice in the control, lovastatin, tocotrienol, and blend groups. By another measure, tumors weighing more than the average weight of tumors excised from control mice (1.23 ± 0.20 g) were detected in 22%, 11%, and 10%, respectively, of mice in the lovastatin, tocotrienol, and blend groups. We must note that the difference in weight between the tumors excised from mice fed the diet that provided 66.8 ± 7.6 mg *d*-δ-tocotrienol/kg body weight and controls approached significance ($P < 0.084$). When corrected for the weight of the excised tumors, group weights fell between 20.12 g (control group) and 22.79 g (tocotrienol group).

Discussion

As reported elsewhere (26), the sensitivity to the *d*-γ-tocotrienol-mediated and lovastatin-mediated inhibition of growth differs substantially among tumor cell lines. Within the cell lines tested to date, human HL-60 cells are the most sensitive to both agents, IC_{50} values being 0.5 µ*M* for lovastatin and 4 ± 4 µ*M* for *d*-γ-tocotrienol (26); A549 human lung carcinoma cells are the most resistant (Table 1). The IC_{50} values previously recorded for the *d*-γ- and *d*-δ-tocotrienol isomers in the B16 melanoma model (25), 20 ± 3 and 10 ± 3 µ*M*, respectively, are consistent with the values recorded in Table 1. The finding that the *d*-α isomer has substantially lower tumor growth suppressive potency ($IC_{50} = 110 \pm 23$ µ*M*) than the *d*-γ and *d*-δ isomers (25) parallels findings of the relative potencies of the isomers in suppressing HMG CoA reductase activity (38). An early comparison of the relative potencies of the *d*-α-, *d*-γ-, and *d*-δ-tocotrienol isomers for the suppression of HMG CoA reductase activity in HepG2 cells, each evaluated at a 10 µ*M* concentration, recorded a 15% inhibition by the *d*-α isomer and 64%–65% by the *d*-γ and *d*-δ isomers. Confirming the unique sensitivity of HMG CoA reductase in tumor cells to posttranscriptional down-regulation (8, 16), IC_{50} values for the suppression of acetate incorporation into digitonin-precipitable sterols, reflecting HMG CoA reductase activity, in primary rat hepatocytes for the *d*-α, *d*-γ, and *d*-δ isomers fell in the ranges of >1000–1300, 270–360, and 260–340 (~650–850 µ*M*) µg/ml, respectively (38). These data show the reductase activity in isolated primary rat hepatocytes to be 100-fold less sensitive than that in HepG2 cells to down-regulation by *d*-δ-tocotrienol.

Novel synthetic tocotrienol analogs and natural desmethyl isomers having greater potency in suppressing HMG CoA reductase activity (22) and tumor growth may have greater potential for development as a therapeutic intervention. The IC_{50} value determined for one desmethyl

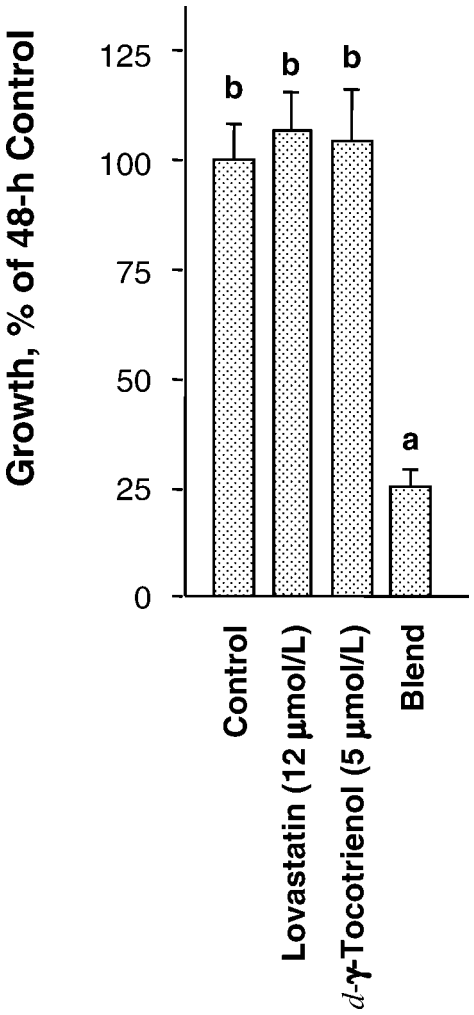

Figure 4. Synergistic impact of 12 μ*M* lovastatin and 5 μ*M* *d*-γ-tocotrienol on the proliferation of A549 human lung carcinoma cells following a 48-hr treatment. A549 cells were grown and growth measured using the CellTiter 96 Aqueous One Solution procedure as described in Materials and Methods. Absorbance values representing cell growth for cells following 48-hr treatments are shown as percentages of that of 48-hr untreated cells (control). Values are mean ± SD, $n = 3$. Values not sharing a letter are significantly different ($P < 0.01$).

isomer, *d*-2-desmethyl tocotrienol, using the B16 model was substantially lower (0.9 ± 0.2 μ*M*) than those recorded for the *d*-γ and *d*-δ isomers (25). Consistent with the suggestion that the aberrant HMG CoA reductase offers a unique target for therapeutic intervention (8, 16) and findings that the tocotrienols block the lovastatin-mediated increase in HMG CoA reductase mass (18), we found that the pairing of lovastatin with *d*-γ- or *d*-δ-tocotrienol yielded significant synergy in suppressing the growth of the very resistant A549 cells and moderately sensitive B16 and DU145 cells.

When administered intraperitoneally (50 mg/kg body weight on alternate days), lovastatin inhibited metastasis but not the growth of B16 melanomas following implantation in nude mice (15). When administered at a lower dosage, lovastatin (5 mg/kg body weight/day) injected either intraperitoneally (39) or intratumorally (40) failed to attenuate the growth of B16 melanomas implanted in the foot pads of C57BL/6xDBA/2 mice.

We now report findings that the mean weight of melanomas excised from the flanks of C57BL/6 mice fed a diet including 11.5 mg lovastatin/kg body weight was approximately 20% lower (NS) than that of tumors excised from control mice. Our estimate of a minimally effective dietary level of *d*-δ-tocotrienol for this study was based on findings gained from a study employing the less potent *d*-γ isomer (92 mg/kg body weight) (25). In that study the 28-day growth of implanted melanomas was suppressed by 50%.

We now report that the mean weight of tumors in mice fed a diet including 66.8 mg *d*-δ-tocotrienol/kg body weight/day was about one-half that of controls, the difference in weight approaching significance ($P < 0.082$). Blending of the agents to provide 14.2 mg lovastatin and 71.2 mg *d*-δ-tocotrienol/kg body weight yielded a 60% suppression of tumor growth when compared with the control ($P < 0.034$). The suppression of tumor growth in this group was not uniform. On one hand, the weight of 1 tumor in this experimental group, 2.34 g, was greater than that of 85% of the control tumors. On the other, tumors in 3 of the experimental mice were minimally detectable, weighing less than 10 mg. The latter responses and, to a lesser degree, findings that tumors weighing less than 50% of the mean weight of the controls were excised from 3, 6, and 8 mice of the mice receiving lovastatin, *d*-δ-tocotrienol, and the blend, respectively, appear to be consistent with the *in vitro* findings of the synergy attained with the blended agents.

Our study has shown for the first time that lovastatin and

Table 2. Treatment-Mediated Impact on Diet Intake, Body Weight, Treatment Dose, and Tumor Weight

Treatment	*n*	Body weight (g)	Tumor weight (g)	Liver (% body weight)	Diet (g/day)	Lovastatin (mg/kg body weight/day)	Tocotrienol (mg/kg body weight/day)
Control	19	21.35 ± 0.42[a,1]	1.23 ± 0.20[1]	4.07 ± 0.001	5.15 ± 0.80[1,2]		
Lovastatin	9	21.90 ± 0.61[1,2]	0.99 ± 0.29[1,2]	3.85 ± 0.002	4.47 ± 0.64[2]	11.5 ± 1.6	
Tocotrienol	10	23.40 ± 0.61[2]	0.61 ± 0.29[1,2]	4.04 ± 0.002	5.38 ± 0.98[1]		66.8 ± 7.6
Blend	10	20.58 ± 0.58[1]	0.49 ± 0.27[2]	3.96 ± 0.002	5.39 ± 1.26[1]	14.2 ± 2.7	71.2 ± 13.4
P		0.002	0.034	>0.05	0.022		

[a]Values are mean ± SD. Values in columns not sharing a superscript number are different.

d-δ-tocotrienol, two suppressors of HMG CoA reductase, synergistically suppress tumor growth *in vivo*, a concept that stemmed from our earlier findings *in vitro* (8, 26). A recent study suggested that both *d*-γ- and *d*-δ-tocotrienols down-regulate HMG CoA reductase activity by accelerating the ubiquitination and degradation of reductase, whereas only *d*-δ-tocotrienol may block the processing of sterol regulatory element binding protein, a transcriptional factor of HMG CoA reductase (41). This observation appears to be consistent with the higher potency of *d*-δ-tocotrienol (Table 1).

Our report supports the theses that HMG CoA reductase is a viable target for cancer chemoprevention and therapy and that blends of agents augmenting each other in suppressing HMG CoA reductase activity via complementary mechanisms have therapeutic potential (8, 16).

1. Dale KM, Coleman CI, Henyan NN, Kluger J, White CM. Statins and cancer risk: a meta-analysis. JAMA 295:74–80, 2006.
2. Lovastatin Study Groups I through IV. Lovastatin 5-year safety and efficacy study. Arch Intern Med 153:1079–1087, 1993.
3. Thibault A, Samid D, Tompkins AC, Figg WD, Cooper MR, Hohl RJ, Trepel J, Liang B, Patronas N, Venzon DJ, Reed E, Myers CE. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin Cancer Res 2:483–491, 1996.
4. Kim WS, Kim MM, Choi HJ, Yoon SS, Lee MH, Park K, Park CH, Kang WK. Phase II study of high-dose lovastatin in patients with advanced gastric adenocarcinoma. Invest New Drugs 19:81–83, 2001.
5. Brown MS, Goldstein JL. Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res 21:505–517, 1980.
6. Jakobisiak M, Golab J. Potential antitumor effects of statins. Int J Oncol 23:1055–1069, 2003.
7. Wong WW, Dimitroulakos J, Minden MD, Penn LZ. HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis. Leukemia 16:508–519, 2002.
8. Mo H, Elson CE. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. Exp Biol Med (Maywood) 229:567–585, 2004.
9. Jones KD, Couldwell WT, Hinton DR, Su Y, He S, Anker L, Law RE. Lovastatin induces growth inhibition and apoptosis in human malignant glioma cells. Biochem Biophys Res Commun 205:1681–1687, 1994.
10. Perez-Sala D, Mollinedo F. Inhibition of isoprenoid biosynthesis induces apoptosis in human promyelocytic HL-60 cells. Biochem Biophys Res Commun 199:1209–1215, 1994.
11. Hawk MA, Cesen KT, Siglin JC, Stoner GD, Ruch RJ. Inhibition of lung tumor cell growth in vitro and mouse lung tumor formation by lovastatin. Cancer Lett 109:217–222, 1996.
12. Marcelli M, Cunningham GR, Haidacher SJ, Padayatty SJ, Sturgis L, Kagan C, Denner L. Caspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. Cancer Res 58:76–83, 1998.
13. Shibata MA, Kavanaugh C, Shibata E, Abe H, Nguyen P, Otsuki Y, Trepel JB, Green JE. Comparative effects of lovastatin on mammary and prostate oncogenesis in transgenic mouse models. Carcinogenesis 24:453–459, 2003.
14. Kusama T, Mukai M, Iwasaki T, Tatsuta M, Matsumoto Y, Akedo H, Inoue M, Nakamura H. 3-hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors reduce human pancreatic cancer cell invasion and metastasis. Gastroenterology 122:308–317, 2002.
15. Jani JP, Specht S, Stemmler N, Blanock K, Singh SV, Gupta V, Katoh A. Metastasis of B16F10 mouse melanoma inhibited by lovastatin, an inhibitor of cholesterol biosynthesis. Invasion Metastasis 13:314–324, 1993.
16. Elson CE, Peffley DM, Hentosh P, Mo H. Isoprenoid-mediated inhibition of mevalonate synthesis: potential application to cancer. Proc Soc Exp Biol Med 221:294–311, 1999.
17. Meigs TE, Roseman DS, Simoni RD. Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo. J Biol Chem 271:7916–7922, 1996.
18. Parker RA, Pearce BC, Clark RW, Gordon DA, Wright JJ. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J Biol Chem 268:11230–11238, 1993.
19. Bradfute DL, Simoni RD. Non-sterol compounds that regulate cholesterogenesis. Analogues of farnesyl pyrophosphate reduce 3-hydroxy-3-methylglutaryl-coenzyme A reductase levels. J Biol Chem 269:6645–6650, 1994.
20. Peffley DM, Gayen AK. Plant-derived monoterpenes suppress hamster kidney cell 3-hydroxy-3-methylglutaryl coenzyme a reductase synthesis at the post-transcriptional level. J Nutr 133:38–44, 2003.
21. Houten SM, Schneiders MS, Wanders RJ, Waterham HR. Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients. J Biol Chem 278:5736–5743, 2003.
22. Pearce BC, Parker RA, Deason ME, Dischino DD, Gillespie E, Qureshi AA, Volk K, Wright JJ. Inhibitors of cholesterol biosynthesis. 2. Hypocholesterolemic and antioxidant activities of benzopyran and tetrahydronaphthalene analogues of the tocotrienols. J Med Chem 37: 526–541, 1994.
23. Adany I, Yazlovitskaya EM, Haug JS, Voziyan PA, Melnykovych G. Differences in sensitivity to farnesol toxicity between neoplastically- and non-neoplastically-derived cells in culture. Cancer Lett 79:175–179, 1994.
24. Rioja A, Pizzey AR, Marson CM, Thomas NS. Preferential induction of apoptosis of leukaemic cells by farnesol. FEBS Lett 467:291–295, 2000.
25. He L, Mo H, Hadisusilo S, Qureshi AA, Elson CE. Isoprenoids suppress the growth of murine B16 melanomas in vitro and in vivo. J Nutr 127:668–674, 1997.
26. Mo H, Elson CE. Apoptosis and cell-cycle arrest in human and murine tumor cells are initiated by isoprenoids. J Nutr 129:804–813, 1999.
27. Odysseos AD, Vraka P, Bouralexis S, Frindlay DM, Papas A, Evdokiou A. Gamma-tocotrienol triggers caspase-mediated induction of apoptosis in androgen-responsive, but not androgen unresponsive prostate cancer cells in vitro. Free Radical Res 36:116–117, 2002.
28. Komiyama K, Iizuka K, Yamaoka M, Watanabe H, Tsuchiya N, Umezawa I. Studies on the biological activity of tocotrienols. Chem Pharm Bull (Tokyo) 37:1369–1371, 1989.
29. Shoff SM, Grummer M, Yatvin MB, Elson CE. Concentration-dependent increase of murine P388 and B16 population doubling time by the acyclic monoterpene geraniol. Cancer Res 51:37–42, 1991.
30. Burke YD, Ayoubi AS, Werner SR, McFarland BC, Heilman DK, Ruggeri BA, Crowell PL. Effects of the isoprenoids perillyl alcohol and farnesol on apoptosis biomarkers in pancreatic cancer chemoprevention. Anticancer Res 22:3127–3134, 2002.
31. Burke YD, Stark MJ, Roach SL, Sen SE, Crowell PL. Inhibition of pancreatic cancer growth by the dietary isoprenoids farnesol and geraniol. Lipids 32:151–156, 1997.
32. Rao CV, Newmark HL, Reddy BS. Chemopreventive effect of farnesol and lanosterol on colon carcinogenesis. Cancer Detect Prev 26:419–425, 2002.
33. Mo H, Tatman D, Jung M, Elson CE. Farnesyl anthranilate suppresses the growth, in vitro and in vivo, of murine B16 melanomas. Cancer Lett 157:145–153, 2000.
34. McAnally JA, Jung M, Mo H. Farnesyl-O-acetylhydroquinone and geranyl-O-acetylhydroquinone suppress the proliferation of murine

B16 melanoma cells, human prostate and colon adenocarcinoma cells, human lung carcinoma cells, and human leukemia cells. Cancer Lett 202:181–192, 2003.

35. Riss TL, Moravec RA. Comparison of MTT, XTT, and a novel tetrazolium compound for MTS for in vitro proliferation and chemosensitivity assays. Mol Biol Cell (Suppl.) 3:184a, 1992.
36. Qureshi AA, Pearce BC, Nor RM, Gapor A, Peterson DM, Elson CE. Dietary alpha-tocopherol attenuates the impact of gamma-tocotrienol on hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in chickens. J Nutr 126:389–394, 1996.
37. Mustad VA, Smith CA, Ruey PP, Edens NK, DeMichele SJ. Supplementation with 3 compositionally different tocotrienol supplements does not improve cardiovascular disease risk factors in men and women with hypercholesterolemia. Am J Clin Nutr 76:1237–1243, 2002.
38. Pearce BC, Parker RA, Deason ME, Qureshi AA, Wright JJ. Hypocholesterolemic activity of synthetic and natural tocotrienols. J Med Chem 35:3595–3606, 1992.
39. Feleszko W, Mlynarczuk I, Olszewska D, Jalili A, Grzela T, Lasek W, Hoser G, Korczak-Kowalska G, Jakobisiak M. Lovastatin potentiates antitumor activity of doxorubicin in murine melanoma via an apoptosis-dependent mechanism. Int J Cancer 100:111–118, 2002.
40. Feleszko W, Zagozdzon R, Golab J, Jakobisiak M. Potentiated antitumour effects of cisplatin and lovastatin against MmB16 melanoma in mice. Eur J Cancer 34:406–411, 1998.
41. Song BL, DeBose-Boyd RA. Insig-dependent ubiquitination and degradation of 3-hydroxy-3-methylglutaryl coenzyme a reductase stimulated by delta- and gamma-tocotrienols. J Biol Chem 281: 25054–25061, 2006.