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R-(+)-perillyl alcohol-induced cell cycle changes, altered actin cytoskeleton, and decreased *ras* and p34^{cdc2} expression in colonic adenocarcinoma SW480 cells

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Monoterpenes as S-(-)-perillyl alcohol (PA) have been shown to inhibit the isoprenylation of such growth regulatory proteins as ras*. In this study, we investigated the effects of the R-(+) enantiomer of PA on cell cycle, signaling, and cytoskeletal control in the colonic adenocarcinoma cell line SW480, which carries a K-*ras *mutation. Cell cycle analysis by flow cytometry of SW480 cells treated with 1 mM PA for 24 hours demonstrated an increase in the number of cells in G0/G1 with a decrease in S phase, compared with untreated control cells. These cell cycle changes correlated with an inhibition of protein isoprenylation from ^{14}C-mevalonate and decreased expression of the cell cycle regulatory kinase p34^{cdc2}. Additionally, PA-treated cells acquired a flattened morphology with a condensation of cytoskeletal actin spikes to the periphery. This was in contrast to treatment with 15 μM mevinolin (MVN), a direct mevalonate synthesis inhibitor, which imparted to SW480 cells a more rounded and spindly morphology, associated with the depolymerization of actin microfilaments. Together, these data suggest that fluctuations in mevalonate and isoprenoid pools may involve different morphologic phenomenon. Because* ras *mediated signaling is related to the organization of the actin cytoskeleton, we investigated the effects of PA on the isoprenylation of* ras*. Although MVN treatment inhibited* ras *farnesylation, PA treatment decreased the expression of total* ras *protein. In summary, R-(+)-PA-induced cell signaling events correlated with alterations in the organization of cytoskeletal actin and decreased protein expression of growth regulatory proteins, such as* ras *and* cdc2 *kinase. These effects may contribute to the growth inhibitory activity of R-(+)-PA.* (J. Nutr. Biochem. 10:19–30, 1999)

Keywords: monoterpenes; protein isoprenylation; actin; *ras*; *cdc2*; colon cancer

Introduction

The cytoskeletal system is central in the maintenance of cell morphology and transduction of growth factor signals from cell surface to initiate DNA synthesis.[1] Compounds that block mevalonate synthesis by inhibiting the activity of 3-hydroxy,3-methylglutarylCoenzymeA reductase (HMGCoA R) inhibit cell proliferation and induce morphologic changes, including differentiation.[2] Schmidt et al.[3] showed a general correlation with compactin-treated fibroblasts between compactin-induced cell rounding and decreased cellular proliferation. They suggested that an active factor derived from mevalonate might influence either the organization of the cytoskeleton or the attachment of cells to the substrate. However, Maltese et al.,[2] in examining the relationship of isoprenoid production to cell proliferation and cell shape, noted that neurite outgrowth (characteristic of differentiated murine neuroblastoma cells) was found to commence before any changes in cell cycling were observed. Despite these temporal dissimilarities, a common mechanism was sug-

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gested by these investigators to underlie both morphologic and cycle related phenomena.[2,3]

Cell cycling and mevalonate synthesis have been the focus of numerous studies.[2,4–7] Cell cycling requirements for mevalonate were not effectively replaced by dolichol, ubiquinone, or isopentenyladenine, but a close temporal correlation between the decline of DNA synthesis and the decline of protein isoprenylation was observed. This implied that nonsterol isoprenoids may play a role in cell cycling. However, the activity of HMGCoA R remains constant throughout the cell cycle, even when alterations occur in the utilization of farnesyl pyrophosphate in various branches of the isoprenoid pathway.[8] It has been suggested that the effects of mevalonate deprivation on DNA synthesis, and subsequent G1 arrest, may be due to the inactivation of *ras*.[9] Conversely, inhibition of cell growth due to mevalonate depletion may be independent of *ras* function[10] and inhibition of tumors dependent on mutated *ras* for growth may require more specific inhibitors of the farnesyl transferases than mevinolin (MVN).[11–17] Only mevalonate was found to fully reverse MVN-induced G1 arrest; thus, it is possible that the action of one or more proteins modified by an early intermediate of the pathway is prevented by the addition of MVN.[7]

Limonene, the predominant monoterpene in orange peel oil, possesses chemopreventive and chemotherapeutic activity against certain cancers.[18,19] Perillyl alcohol (PA), a metabolic derivative, is better tolerated and more effective. Such activities include the induction of hepatic phase II detoxifying enzymes and growth suppression of chemically initiated and transplantable tumors.[18,19] In addition, monoterpenes have been shown to suppress hepatic HMGCoA R activity,[20] regress mammary carcinomas,[21] and inhibit both the growth of tumors and the posttranslational isoprenylation of cellular proteins, including *ras*.[22–28]

Although recent literature refers only to S-(-)-PA, to our knowledge, no studies have been reported investigating the activity of the R-(+) enantiomer of PA against tumor growth. In this study, we investigated the effects of R-(+)-PA on morphologic and cell cycle changes in the colonic adenocarcinoma cell line SW480, which carries a K-*ras* mutation.[29] R-(+)-PA-treated SW480 cells exhibited cell cycle changes that correlated with decreased expression of the cell cycle regulatory kinase $p34^{cdc2}$. Additionally, cells treated with this monoterpene acquired a flattened morphology with an altered actin cytoskeleton, which was accompanied by a decrease in total *ras* protein expression.

Methods and materials

Tissue culture techniques

The human colonic cancer cell line SW480 was obtained from American Type Culture Collection (Rockland, MD USA). Stock cultures were grown in monolayer in plastic 75 cm^2 flasks (Costar, Cambridge, MA USA or Corning, NY USA) and incubated at 37°C in a 5% carbon dioxide (CO_2)/95% air atmosphere. Culture medium consisted of Eagle's minimal essential medium (MEM), which contained antibiotics (penicillin 200 units/mL; streptomycin 0.2 mg/mL), glutamine 0.2 mg/mL, and nonessential amino acids and vitamins, both at 10 mL of 100× solution (GIBCO, Grand Island, NY USA), per 500 mL MEM and supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO USA). Cells were plated at a density of 10^6 cells per flask, and cultures divided weekly (1:20) using 0.05% trypsin/0.02% disodium ethylenediaminetetraacetic acid (EDTA). After removal of media, cells were rinsed with isotonic phosphate buffered saline (PBS; pH 7.4), and 4 mL of trypsin/EDTA was added. Flasks were incubated at 37°C until cells began to detach, after which 4 to 6 mL of fresh medium was added. Cells were pelleted at 800 rpm in a Beckman centrifuge (Beckman Instruments Inc., Palo Alto, CA USA). Cells used in experiments were grown in plastic 35- or 60-mm dishes. Cell cultures were assayed for the presence of mycoplasma using the Gen-Prove Mycoplasma T.C.11 Rapid Detection System (San Diego, CA USA) with negative outcome.

Preparation of MVN

MVN was kindly provided by A. Alberts of Merck Sharp & Dohme (Rahway, NJ USA) in the lactone form and prepared as described elsewhere.[30]

Protein determinations

Protein was determined by the bicinchoninic acid reagent method (Pierce Chemical Company, Rockford, IL USA) as described elsewhere.[30]

Inhibition of cell growth with MVN and PA combinations

Cells were seeded in triplicate in 12 well dishes (Costar) at a density of 1×10^4 cells/well in MEM + 10% FBS. (R)-(+)-PA stocks (Fluka, Ronkonkoma, NY USA) were made up in FBS before addition to the media. Equivalent amounts of ethanol were added to control groups. The final volume per dish was 0.5 mL. Medium was replaced every 2 days. Cell growth was determined by trypsinizing the wells with trypsin/EDTA and resuspending with medium containing serum. An aliquot was then stained with trypan blue and viable cells counted in a hemocytometer on days 2, 4, and 6.

Protein isoprenylation assay

Protein isoprenylation was measured as described by Crowell et al.[22] Briefly, cells were seeded in MEM + 10% FBS at a density of 5×10^5/35-mm dish (Costar). When cells reached 70% confluence, they were treated with 30 μM MVN for 24 hours. Following this incubation period, cells were treated for 3 additional hours in MEM + 10% FBS with 30 μM MVN, 15 μCi ^{14}C-mevalonate at 54.1 mCi/mmol (NEN), and PA 1 mM. Control cells received the same supplemented media without PA. Following treatments, cells were harvested with trypsin, centrifuged at 3,000 × g, and washed twice with PBS. Pellets were then suspended in 150 μL of electrophoresis sample buffer and equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) as described by Laemmli,[31] using 12% separating and 4% stacking polyacrylamide gels. Instruments were from Hoeffer Scientific Instruments (San Francisco, CA USA) and reagents from Biorad Life Science (Hercules, CA USA). Gels were then stained with Coomassie blue, permeated with fluorographic enhancer (En^{3}Hance, New England Nuclear, Boston, MA USA), and dried with a Biorad Gel Dryer for 2.5 hours at 70°C. They were then left to cool for an additional 30 minutes, followed by exposure to preflashed X-OMAT AR film (Eastman-Kodak Co., Rochester, NY USA) at −70°C for 14 days. Film was then developed using an x-ray film processor (Fuji Photofilm, Elmsford, NY USA) and fluorograms were scanned with a phosphorimager:SI (Molecular Dynamics, Sunnyvale, CA USA).

Table 1 Effects of mevinolin and R-(+)-perillyl alcohol on cell cycle progression* in SW480 cells

Treatment	%GO/G1 (±SEM)†	%S (±SEM)	%G2/M (±SEM)
FBS control	55.0 ± 3.7	37.0 ± 2.7	7.6 ± 1.8
2 MM mevinolin	63.0 ± 2.5	30.4 ± 6.0	6.6 ± 3.3
1 mM perilly alcohol	69.6 ± 4.8[a]	26.1 ± 3.9[a]	4.2 ± 1.3
2 μM mevinolin + 1mM perillyl alcohol	74.2 ± 3.5[a]	23.0 ± 4.1[a]	2.8 ± 1.0[b]

*SW480 cells were untreated or treated with 2 μM mevinolin, 1 mM perillyl alcohol, or 2 μM mevinolin + 1 mM perillyl alcohol for 24 hours. Cells were harvested, stained with propidium iodide in the presence of RNAse, and DNA content determined by flow cytometric analysis. The percentage of cells in cell cycle phases was calculated by the Cellfit multicycle analysis program.
†Results are mean ± SEM of duplicate determinations of $N = 4$, analyzed by analysis of variance.
[a]Indicates groups significantly different from the fetal bovine serum (FBS) control ($P < 0.05$).
[b]Indicates group significantly different from the mevinolin group ($P < 0.05$).

Cell cycle analysis

After trypsinization, cells were seeded in 60-mm dishes (Falcon labware, VWR Scientific, Philadelphia, PA USA) and grown to 50% confluency in MEM supplemented with 10% FBS for a period of 24 hours. They were then subjected to the following supplements for 24 hours in MEM + 10% FBS: (1) FBS alone; (2) MVN 2 μM; (3) PA 1 mM; and (4) MVN + PA. At the end of the incubation period, the monolayer was washed twice with PBS. Cells were trypsinized with trypsin/EDTA, neutralized with an appropriate amount of serum, and collected into labeled 50-mL polypropylene tubes (Falcon labware, VWR Scientific). After pipetting repeatedly to separate cells, the single cell suspension was vortexed and centrifuged for 10 minutes at 1,000 rpm (400 × g) at 4°C. The supernatant was poured off without disturbing the cell pellet, which was vigorously vortexed for 10 seconds in the remaining fluid to avoid excessive clumping. While vortexing, 1 to 3 mL of ice-cold 70% ethanol was added very slowly dropwise. Cells were then counted and 1.5 to 3 × 10^6 cells were transferred in duplicate to 12- × 75-mm plastic tubes and allowed to fix overnight in the ethanol at 4°C on a shaker. After 24 hours, samples were vortexed, centrifuged for 5 minutes at high speed (3,000 rpm), and the ethanol discarded. They were then gently vortexed to resuspend the cells in residual ethanol and 1 mL of a propidium iodide (PI; Sigma) staining solution was added. This solution consisted of 0.5 mL of a PI stock solution [100 mg PI dissolved in 100 mL H_2O filtered through 0.22-μ filter (Millipore, Bedford, MA USA) and stored in a brown glass container at 4°C], 10^6 units of RNase A (100 U/mL final), and sample buffer (1 g glucose, 1 L PBS without Ca^{+2} or Mg^{+2}, filtered through a 0.22-μ filter, and stored at 4°C) to a final volume of 10 mL, mixed together just before use. Samples were incubated for more than 30 minutes at room temperature in the dark (to avoid degradation of the PI), in a rocker to ensure the degradation of RNA by RNase. Cell fluorescence was measured within 24 hours by flow cytometry using a Facscan (Becton Dickinson, San Jose, CA USA). DNA analysis was performed using the Cellfit software for data accumulation and for cell cycle analysis. This software adjusts and controls the fluorescence activated cell sorter (FACS) for measuring cellular DNA content expressed in terms of DNA fluorescence intensity. The program utilizes chicken erythrocyte nuclei from a DNA Quality Control particle kit (Becton Dickinson) to calibrate the machine and adjust the settings to provide instrument linearity and resolution. Data is obtained as total number of cells in each channel of increasing fluorescence intensity, and the resulting histogram was analyzed using the Cellfit multicycle analysis program. Results, summarized in *Table 1*, were statistically evaluated by analysis of variance.

Western blotting of c-fos *and* cdc2 *proteins*

SW480 cells were seeded at a density of 5 × 10^5 cells/60-mm dish (Falcon, Lincoln Park, NJ USA), in MEM + 10% FBS for 24 hours until they reached 50% confluency. They were then subjected to the following treatments in MEM + 10% FBS for 24 hours: (1) FBS alone, (2) MVN 2 μM, (3) PA 1 mM, and (4) PA + MVN. After rinsing twice with ice-cold PBS, cells were lysed for 20 minutes on ice in two to three times their cell pellet volume with prechilled detergent lysis buffer consisting of 1% Triton X-100, 1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 25 mM Tris, pH 7.4, and protease inhibitors (1 mM PMSF, 50 mM NaF, 100 mM Na_3VO_4, and 2 μg/mL of aprotinin). Monolayers were scraped using a cell scraper (Costar) and disruption aided by passing the lysate three times through a 21-gauge syringe needle (Becton Dickinson and Company, Franklin Lakes, NJ USA) to sheer the DNA. The lysate was transferred to Eppendorf tubes (VWR labware), and centrifuged for 10 minutes in a benchtop centrifuge (Brinkmann, Westbury, NY USA) at 4°C, 14,000 rpm, to pellet the debris. Equal amounts of protein from whole cell extracts were analyzed by SDS/PAGE on a 12% polyacrylamide gel using the Biorad protocols and reagents (Biorad Life Science). Proteins were blotted onto immobilon P membranes (Millipore), using a trans-blot cell apparatus (Biorad Life Science). Blots were then equilibrated in TBST (Tris buffered saline with 0.1% tween) for 10 minutes and blocked overnight in TBST with 5% milk at 4°C. All subsequent steps occurred with constant agitation at room temperature following the procedures provided by Oncogene Science (Cambridge, MA USA). Blots were washed twice with TBST for 10 minutes each and incubated for 1 hour with the primary antibodies mouse anti-$p34^{cdc2}$ or rabbit anti-*cfos* (Oncogene Science) at a 1:10 dilution in TBST 5% milk. Following incubations, they were rinsed three times in TBST 5% milk and incubated for 45 minutes with the secondary antibodies (peroxidase conjugated goat anti-mouse or goat anti-rabbit; Pierce Chemical Company) at 1:10,000 in TBST 5% milk. Antibody bound proteins were detected by chemiluminescence with an ECL Western blotting detection kit (Amersham Life Science Inc., Arlington Heights, IL USA) and exposed to Kodak X-Omat LS film (Eastman-Kodak Co.).

Visualization of cytoskeletal filaments by fluorescence microscopy

Cells were grown on poly d-lysine (Sigma) coated coverslips (12 × 12 mm; Bradford Scientific, Epping, NH USA) that had been previously washed in double distilled H_20, then once in 95%

ethanol, and finally in MEM before use. Treatments were as indicated for 48 hours in MEM 10% + FBS: (1) FBS alone; (2) MVN 15 μM; (3) PA 1 mM; (4) PA 1 mM + MVN at 2 μM. All solutions were previously heated at 37°C before use. Medium was then aspirated and the cells were washed with PBS and PM2G, a microtubule stabilizing buffer containing 0.1 PIPES, 1.0 mM $MgSO_4$ (Fisher Scientific, Fairlawn, NJ USA), 2.0 mM EGTA, and 2.0 M glycerol, pH 6.9. Cells were then fixed with 3.7% formaldehyde (Aldrich Chemicals Co. Inc., Milwaukee, WI USA) in PM2G, followed by a 5-minute rinse in PBS. The monolayer was then rinsed with PBS containing 0.1 M glycine at pH 7.4 for 5 minutes to quench the remaining formaldehyde. Cells were made permeable using a non-ionic detergent Nonidet (NP40) 0.3% in PBS for 10 minutes, and rinsed twice with PBS before staining. If not stained immediately, plates were kept in PBS at 4°C. The cytoskeleton was stained using a double labeling technique. Microfilaments were stained directly by using a fluorescent probe NBD-PHALLACIDIN (Molecular Probes, Eugene, OR USA) that specifically labels F-actin. Microtubules were stained by using a polyclonal rabbit anti-tubulin primary antibody (Polysciences, Warrington, PA USA) followed by a rhodamine conjugated sheep anti-rabbit secondary antibody (Organon-Teknika Cappel, Durham, NC USA). Bodipy-phallacidin in methanol (2.5 μL per coverslip) was evaporated to dryness and reconstituted in the appropriately diluted anti-tubulin antibody solution. Before staining, coverslips were carefully removed with tweezers from the dishes and put in a staining chamber, where they were covered with 20 μL of the antibody solution diluted 1:40. Staining was carried out for 30 minutes in the dark in a humidified chamber at 37°C. Coverslips were rinsed five times by dipping them repeatedly in PBS-filled beakers, and the secondary antibody added for another 30 minutes in the incubator. Stained coverslips were rinsed seven times in PBS and once in double distilled H_2O, and mounted, cell side down, onto glass microslides (25 × 75 mm) with a solution of Gelvatol (Monsanto Chemical Co., Indian Orchard, MA USA) consisting of 20 g of Gelvatol 20/30 in 80 mL of PBS plus 40 mL of glycerol. Slides were kept flat in the dark at 4°C overnight before examination. Stained cytoskeletal elements were examined with a Nikon optiphot fluorescence microscope equipped with a 100 W high pressure mercury lamp. The magnification was usually 400×, obtained by using a Plan Apochromat 40× oil immersion objective lens together with a 10× eyepiece lens. Microscope filters used were of the excitation types B2 (blue) and G (green) to detect fluorescein and rhodamine derivatives, respectively. Photographs were taken with a Nikon FX-35A 35-mm camera and a UFX automatic exposure photomicrographic system on Kodak Tri-X or T-Max black and white films (ASA 400). Films were exposed to 800 ASA and developed at 1600 ASA.

Membrane fractionation and Western analysis of ras

Membranes were isolated as described by Courtneidge et al.[32] Briefly, cells were grown to 50% confluency in 150-mm dishes (Falcon) in MEM supplemented with 10% FBS serum. They were then subjected to the following treatments for 48 hours: (1) FBS alone, (2) MVN 2 μM, (3) PA 1 mM, and (4) PA + MVN. Monolayers were washed twice with PBS, trypsinized, and pelleted at 1,000 rpm in a Beckman Centrifuge (Beckman). Pellets were swollen on ice for 15 minutes with 1 mL of a hypotonic lysis buffer consisting of 10 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4, and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 100 mM Na_3VO_4, and 2 μg/mL of aprotinin]. After homogenizing in a tight fitting Dounce Tissue Homogenizer (VWR Scientific, Bridgeport, NJ USA), the lysate was centrifuged at 3,000 × g in a benchtop microfuge (Brinkmann) for 10 minutes at 4°C. Membranes were collected as a pellet by centrifugation at 100,000 × g for 1 hour in Nalgene polycarbonate ultracentrifuge tubes (VWR Scientific) using a fixed angle type 40 rotor at 4°C. Supernatants were collected as cytosolic fractions, and membranes were dissolved in detergent buffer consisting of 1% Triton 100, 1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 25 mM Tris pH 7.4, and protease inhibitors. Aliquots were then frozen at −70°C. Equal amounts (150 μg) of membrane and cytosolic proteins that had been concentrated by Centricon 10 filters (Amicon Inc., Beverly, MA USA) were analyzed by SDS/PAGE on 12% polyacrylamide gels as above. Proteins were then blotted into immobilon P membranes (Millipore) and blocked for 3 hours in TBST with 5% milk at 4°C. Transfer was carried out using a Transblot Cell Apparatus (Biorad Life Science) according to the manufacturer's instructions. Immunoblotting followed the protocols from Oncogene Science with a slight modification from Xiang et al.[33] Briefly, blots were washed twice with TBST for 5 minutes each and incubated overnight in a rocker (Lab Industries Inc., Berkeley, CA USA) in the cold with the primary antibody Y13-259, a rat monoclonal to v-H *ras* that reacts with all forms of *ras*, at 1:20 dilution in TBST with 5% milk (Oncogene Science). All subsequent steps were at room temperature with constant agitation on a platform rocker. Following incubation, blots were rinsed three times in TBST with 5% milk and incubated for 45 minutes with the secondary antibody, peroxidase conjugated goat anti-rat antiserum (Pierce Chemical Company) at 1:2,500 in TBST with 5% milk. This was followed by six extensive rinses for 5 minutes each in TBST. Antibody reactive proteins were detected by chemiluminescence using an ECL Western blotting detection kit (Amersham Life Science Inc.) following the manufacturer's instructions, and exposed to Kodak X-Omat LS film (Eastman-Kodak Co.). The ECL kit uses the horseradish peroxidase catalyzed reaction of luminol (a cyclic diacylhydrazide) with hydrogen peroxide to produce a chemiluminescent product that is detectable by X-Omat LS film. Film was then developed using an x-ray film processor (Fuji, Photofilm USA Inc.) and fluorograms were scanned with a phosphorimager:SI (Molecular Dynamics).

Immunoprecipitation of ^{14}C-mevalonate labeled p21-ras

SW480 cells were seeded at a density of 10^6 cells/100-mm dish (Falcon) in MEM + 10% FBS until they reached 70% confluency. They were subjected to the following treatments in the presence of 1 μCi/mL of ^{14}C-mevalonate 54.1 mCi/mmol (NEN) for 33 hours. Treatments were added in MEM + 10% FBS and consisted of: (1) FBS alone; (2) MVN 2 μM; (3) PA 1 mM; and (4) MVN + PA. Immunoprecipitation was done as described by Crowell et al.[22] Briefly, cells were rinsed twice with ice-cold PBS and lysed in 1 mL volume of prechilled detergent lysis buffer consisting of 1% Triton X-100, 1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 25 mM Tris, pH 7.4, and protease inhibitors (1 mM PMSF, 50 mM NaF, 100 mM Na_3VO_4, and 2 μg/mL aprotinin). Lysis proceeded for 20 minutes on ice. Monolayers were scraped using a cell scraper (Costar) and disruption aided by passing the lysate three times through a 21-gauge syringe needle (Becton Dickinson and Company) to sheer the DNA. Lysate was transferred to Eppendorf tubes (VWR labware) and centrifuged for 10 minutes in a benchtop centrifuge (Brinkmann) at 4°C, 12,000 rpm, to clarify and pellet debris. Equal aliquots of total cell extract at a concentration of 3 mg/mL were incubated with 10 μL/mL of the anti-v-Ha-*ras* monoclonal antibody Y13-259 conjugated to protein A agarose beads (Oncogene Science) on a rocker at 4°C for 16 to 18 hours. A separate control group was immunoprecipitated with non-immune rat serum at 1 μg/mL in 20 μL of protein G plus/protein A agarose. The final pellet was collected by centrifugation at 12,000 rpm, washed four times in detergent buffer, and dissolved in 20 μL of electrophoresis sample buffer. Samples were

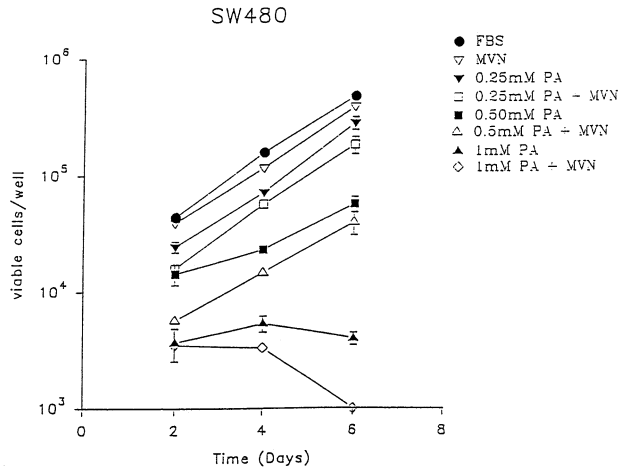

Figure 1 Inhibition of cell growth in SW480 cells in response to mevinolin (MVN) and R-(+)-perillyl alcohol (PA) treatments. Cells were grown in 12-well dishes in triplicate in minimal essential medium + 10% fetal bovine serum (FBS). The following treatments were added on day 0: (1) no treatment (FBS); (2) MVN 2 μM; (3) PA at 0.25-mM, 0.5-mM, or 1-mM concentrations; and (4) PA at the previous concentrations + MVN 2 μM. The final volume per well was 0.5 mL. Cells received fresh medium every 2 days. Cells were removed from the wells at the times indicated with trypsin/ethylenediaminetetraacetic acid (EDTA), stained with trypan blue, and counted by hemacytometer. This figure shows a representative of one of three different experiments. Each point represents the mean of three samples plus SD (standard deviations may be occluded by the symbols at each time point). Results were analyzed by analysis of variance on days 4 and 6. All groups were significantly different from the FBS control and from each other at the level $P < 0.01$.

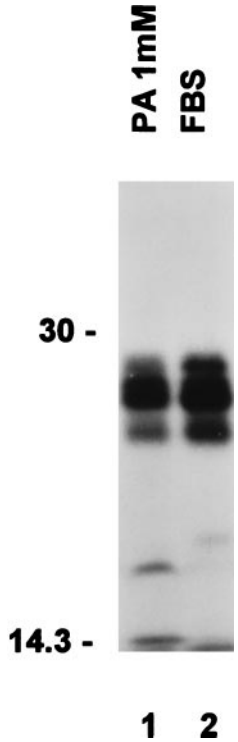

Figure 2 Inhibition of protein isoprenylation in SW480 under mevinolin (MVN) and R-(+)-perillyl alcohol (PA) treatments. SW480 cells were seeded at a density of 5×10^5/35-mm dish and cultured for 2 days in minimal essential medium (MEM) + 10% fetal bovine serum (FBS). At 70% confluence, cells were subjected to treatment with 30 μM MVN for 24 hours. Afterward, cells were treated for 3 additional hours in MEM + 10% FBS with 30 μM MVN, 15 μCi of ^{14}C-mevalonate at 54.1 mCi/mmol (NEN), and PA 1 mM. Control cells received the same supplemented media without PA. Cells were harvested after trypsin treatment, centrifuged and washed twice with phosphate buffered saline, and suspended in electrophoresis buffer. Equal aliquots of protein from whole cell extracts were then analyzed by SDS-PAGE in 12% polyacrylamide gels, stained, and enhanced. Incorporation of label into 21- to 28- kD proteins was analyzed by densitometry. Results are depicted in lanes 1 (PA) and 2 (FBS).

boiled for 2 minutes and centrifuged at 12,000 rpm. Supernatant was analyzed by SDS-PAGE as above, dried, and detected by fluorography with presensitized film 21 days later. Fluorograms were scanned by densitometry with a phosphorimager:SI (Molecular Dynamics).

Results

Inhibition of cell growth with MVN and R-(+)-PA combinations

MVN and S-(-)-PA have been previously demonstrated to inhibit the growth and proliferation of tumor cell lines. In this study, the effects of MVN and R-(+)-PA on the growth of the SW480 cell line were investigated (*Figure 1*). Significant decreases in cell numbers were demonstrated in all PA-treated groups on days 4 and 6 relative to the FBS control ($P < 0.01$). MVN treatment inhibited growth of SW480 cells such that at day 4 there were only 70% as many as those growing in the FBS medium without other additives ($P < 0.01$). A greater effect was observed by PA, which demonstrated significant dose related inhibition at concentrations of 0.25, 0.5, and 1 mM, compared with the FBS control group ($P < 0.01$). A concentration of 1 mM PA growth arrested the cells. Combinations of this drug with 2 μM MVN significantly enhanced the growth inhibition over that of PA alone ($P < 0.01$).

Effects of R-(+)-PA on the incorporation of ^{14}C-mevalonate into isoprenylated proteins

S-(-)-PA has been previously shown to inhibit protein isoprenylation in a number of tumor cell lines.[19,21–23] In this assay, we investigated the effects of the R-(+) enantiomer of PA on protein isoprenylation in the adenocarcinoma cell line SW480. Protein isoprenylation was assessed by measuring the incorporation of ^{14}C-mevalonate into proteins, as described in the Materials and Methods section, using similar methodology of previous investigations. *Figure 2* shows an autoradiogram of ^{14}C-mevalonate labeled proteins in SW480 cells treated with either FBS or PA. Densitometric analysis of ^{14}C-mevalonate incorporation into the 21 to 28 kD proteins showed a 25% decrease in label incorporation in 1 mM PA-treated cells compared with the FBS control (lane 1 vs. lane 2), suggesting that the R-(+) enantiomer of PA inhibited protein isoprenylation in SW480 cells.

Alterations in the organization of the actin cytoskeleton due to R-(+)-PA treatment

Numerous studies have suggested that cell shape is determined by the organization of actin microfilaments and their associated proteins.[1] Compounds that block mevalonate

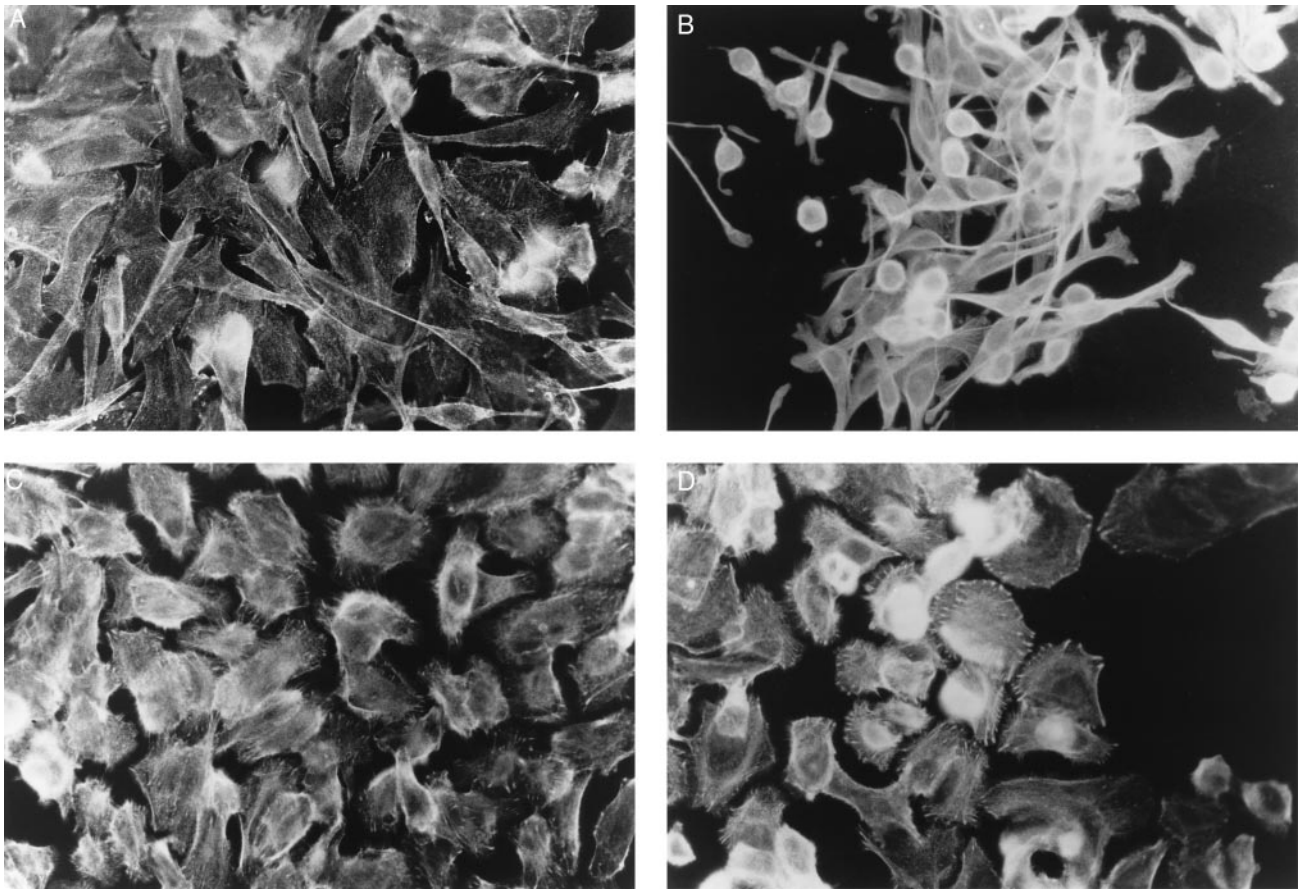

Figure 3 Effects of mevinolin (MVN) and R-(+)-perillyl alcohol (PA) treatments on cytoskeletal filaments by fluorescence microscopy. SW480 cells were grown and treated on coverslips, washed with phosphate buffered saline (PBS) and PM2G, a microtubule stabilizing buffer, fixed with 3.7% formaldehyde in PM2G, and extracted with 0.3% NP-40 in PBS. Microfilaments were stained directly by using a fluorescent NBD-PHALLACIDIN that specifically labels F-actin. Stained coverslips were mounted on glass slides, examined, and photographed using a Nikon fluorescence microscope. Photographs depict SW480 cells stained for F-actin after treatment with the following compounds: *Figure 3A*, fetal bovine serum; *Figure 3B*, MVN 15 μM; *Figure 3C*, PA 1 mM; and *Figure 3D*, PA, taken at a higher magnification.

synthesis have been shown to inhibit cell proliferation and induce morphologic changes.[2] In this assay, SW480 cells were treated with the isoprenylation inhibitors MVN and R-(+)-PA and stained with different probes to examine cytoskeletal elements, as indicated in the Materials and Methods section. MVN, a mevalonate synthesis inhibitor, altered the shape of SW480 cells to a more spindly character, accompanying a loss of actin microfilaments, as opposed to the FBS control (*Figure 3A* vs. *Figure 3B*). On the other hand, PA imparted a flattened morphology to the cells with a loss of intercellular contacts and condensation of actin in the periphery (*Figures 3C and 3D*). No changes were observed in the microtubular network with MVN or PA treatment alone or in combination (data not shown).

Cell cycle alterations due to R-(+)-PA and MVN treatments in SW480 cells

Monoterpenes and MVN have been shown to arrest cells in the early G1 phase of the cell cycle.[7,24] This inhibition of DNA synthesis is thought to be due to the depletion of mevalonate derived isoprenoids, or isoprenylated protein(s), which play important roles in controlling the cell cycle progression of cells.[2,8] In this assay, SW480 cells treated with 1 mM R-(+)-PA and MVN combinations were examined by flow cytometry. Results in *Table 1* are mean percentages ($N = 4$) of the total population of cells in each of the three phases of the cell cycle (G0/G1, S, G2/M). PA and PA + MVN groups had significantly higher percentages of cells in G0/G1 phase and significantly lower percentages of cells in S phase compared with the FBS control groups, respectively. The percentage of cells in G0/G1 in the PA + MVN group were also significantly higher than in the MVN-treated group. No groups differed significantly from the FBS control in terms of their percentage of cells calculated to be in G2/M. However, PA + MVN treatment was found to significantly reduce the percentage of cells in G_2/M compared with MVN alone.

Analysis of cell cycle arrest by evaluating the expression of c-fos *and p34^{cdc2}*

Expression of the protooncogene *c-fos*[34,35] was then evaluated in SW480 cells treated with MVN and R-(+)-PA to investigate the point of arrest in G0 or G1 phases of the cell cycle seen by FACS. *Figure 4* shows a western blot of

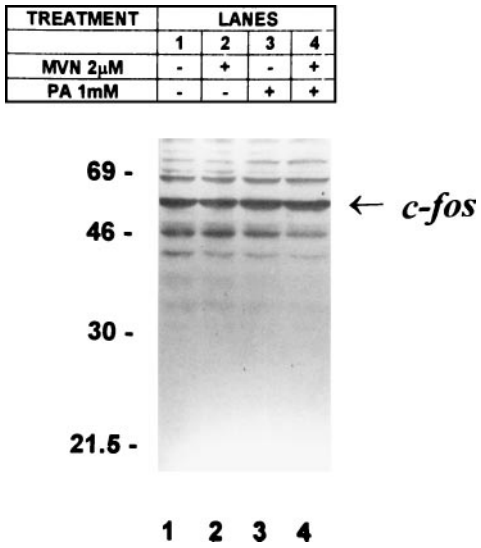

Figure 4 Western blot analysis of *c-fos* protein in SW480 cells treated with mevinolin (MVN) and R-(+)-perillyl alcohol (PA). Cell extracts were prepared from cells treated with the following in minimal essential medium + 10% fetal bovine serum (FBS) for 24 hours: (1) FBS alone; (2) MVN 2 μM; (3) PA 1 mM; and (4) MVN + PA. Aliquots of protein (150 μg) were analyzed per lane by SDS/PAGE in 12% polyacrylamide gels, blotted into immobilon membranes, and immunoblotted with a rabbit polyclonal antibody specific for *c-fos*. Antibody reactive peptides were developed by chemiluminescence. This antibody cross-reacted with other proteins.

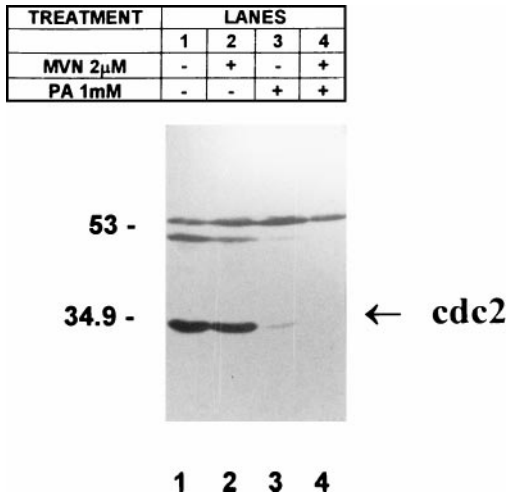

Figure 5 Western blot analysis of $p34^{cdc2}$ protein in SW480 cells treated with mevinolin (MVN) and perillyl alcohol (PA). Cell extracts were prepared from cells treated with the following in minimal essential medium + 10% fetal bovine serum (FBS) for 24 hours: (1) FBS; (2) MVN 2 μM; (3) PA 1 mM; and (4) MVN + PA. Aliquots of protein (150 μg) were analyzed per lane by SDS/PAGE in 12% polyacrylamide gels, blotted into immobilon membranes, and immunoblotted with a monoclonal antibody specific for $p34^{cdc2}$. Antibody reactive peptides were developed by chemiluminescence.

SW480 cell extracts, immunoblotted with a rabbit polyclonal antibody to *c-fos*. Treatments did not affect the expression of *c-fos*. Western blots of the *c-fos* protein indicated equal expression of this early G1 marker for all cells regardless of treatment, suggesting a mechanism of action by PA in G1.

To confirm these observations, protein expression of the cell cycle regulator $p34^{cdc2}$, which plays a role in S and M phases of the cell cycle,[36,37] was measured in cells treated with MVN and/or R-(+)-PA. *Figure 5* shows that the expression of $p34^{cdc2}$ in PA-treated cells was minimal, whereas expression of this kinase in untreated and MVN-treated cells remained high. Additionally, the combination of the two treatments resulted in no detectable expression of $p34^{cdc2}$. These results suggest that PA affected SW480 cells by arresting growth in early to mid-G1 of the cell cycle, a finding that was not supported by the pattern of *c-fos* expression alone.

Effects of R-(+)-PA on the processing of pro-$p21^{ras}$ in SW480 cells

Ras is a protooncogene that plays a significant role in the growth of cells in the mid-G1 phase of the cell cycle.[38] To investigate if the observed changes in the cell cycle and the cytoskeleton could be linked to an inhibition of *ras* farnesylation, we examined the effects of R-(+)-PA treatment in relation to the isoprenyl processing of *ras* in SW480 cells, which are known to express K-*ras* mutations.[29] *Figure 6* shows a western blot of subcellular cytosolic (S_0) and membrane (P_0) fractions of SW480 treated cells for 48 hours, using a monoclonal antibody to *ras*.[39] PA treatment decreased the amount of both nonmembrane bound pro-*ras* (nonisoprenylated, 21 kD upper band) and mature membrane bound *ras* (isoprenylated, 2 kD lower) by 80% compared with the FBS control (lane 7 vs. lane 3, respectively). Additionally, PA + MVN treatment exhibited a

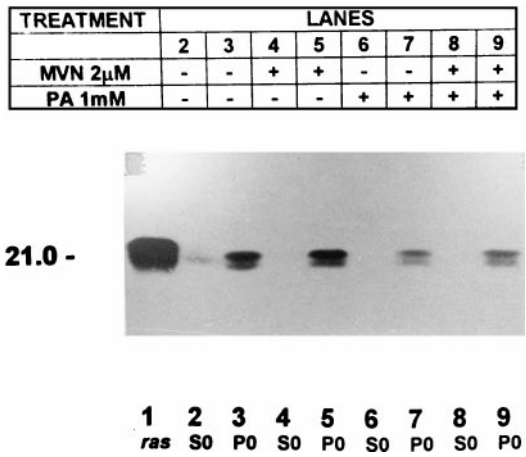

Figure 6 Effects of mevinolin (MVN) and R-(+)-perillyl alcohol (PA) treatments on the processing of pro-$p21^{ras}$. SW480 cells were seeded at a density of 6×10^6 in 150-mm dishes and cultured for 3 days in minimal essential medium + 10% fetal bovine serum (FBS). They were then subjected to the treatments shown for 48 hours as described for each lane: (1) contains purified $p21^{H\text{-}ras}$ protein; (2 and 3) no treatment (FBS); (4 and 5) MVN 2 μM; (6 and 7) PA 1 mM; and (8 and 9) MVN + PA. Cells were lysed in hypotonic lysis buffer and the postnuclear supernatants separated into membrane (P_0), and cytosolic (S_0) fractions by ultracentrifugation at 100,000 × g. Western blotting was performed with a monoclonal antibody to *ras* (Y13-259). Equal amounts of protein (150 μg) were electrophoresed in separate lanes of a 12% polyacrylamide gel, immunoblotted with a monoclonal antibody to *ras* (Y13-259), and detected by an ECL western blotting kit.

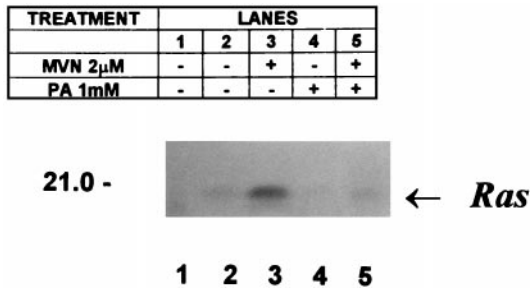

Figure 7 The effect of mevinolin (MVN) and R-(+)-perillyl alcohol (PA) on the incorporation of ^{14}C-mevalonic acid into farnesylated *ras*. SW480 cells were seeded in 100-mm dishes and cultured to 80% confluency. They were then treated in minimal essential medium + 10% fetal bovine serum (FBS) for 33 hours in the presence of 1 μCi ^{14}C-mevalonic acid as described for each lane: (1) a control group immunoprecipitated with rat non-immune serum; (2) no treatment (FBS); (3) MVN 2 μM; (4) PA 1 mM; and (5) PA + MVN. Cells were harvested and p21^{ras} was immunoprecipitated with the rat monoclonal antibody (Y13-259) conjugated to protein A-agarose beads. Immunoprecipitates were dissolved in electrophoresis sample buffer and analyzed by SDS-PAGE in 12% polyacrylamide gels and fluorography. The same amount of immunoprecipitated material from equal amounts of protein was loaded onto each lane.

similar effect. Both membrane bound *ras* and pro-*ras* were decreased compared with the MVN treatment alone (lane 9 vs. lane 5). These data indicate that R-(+)-PA inhibited the expression of total *ras* protein. Interestingly, cytosolic (S_0) *ras* was basically undetectable in any of the treatments except for the FBS control group (lanes 4, 6, and 8 vs. lane 2, respectively), suggesting that these treatments affected *ras* processing.

Effects of R-(+)-PA on the farnesylation of p21^{ras} in SW480 cells

To determine if R-(+)-PA inhibited *ras* farnesylation, p21^{ras} was immunoprecipitated from whole cells, which were pretreated in the presence of ^{14}C-mevalonate. MVN treatment increased the incorporation of radiolabelled mevalonate into *ras* compared with untreated control cells (*Figure 7*; lanes 3 vs. 2, respectively). In contrast, PA-treated cells decreased the amount of labeled immunoprecipitated *ras* by 50% (lane 4). Additionally, treatment with PA + MVN increased the amount of farnesylated *ras* compared with PA treatment alone (lanes 4 and 5). Although these data suggest that R-(+)-PA treatment inhibits *ras* farnesylation, one cannot dismiss the possibility that this decrease may be due to a decrease in the amount of total *ras* due to PA treatment, which by 48 hours was up to 80% (see *Figure 6*).

Discussion

The mevalonate pathway leads to synthesis of cholesterol and polyisoprenoid lipids such as dolichol, ubiquinone, heme, and t-RNA.[40] Rapidly growing transformed cell lines are particularly sensitive to MVN, because this drug inhibits critical cellular processes during rapid cell proliferation.[41] Cells deprived of mevalonate, but not sterols, do not enter S phase within one cell cycle[42] and evidence has suggested a role of prelamin A in the mevalonate dependent cell cycle arrest.[43] Additionally, dolichol dependent N-linked glycosylation and coenzyme Q delayed the growth arrest in human fibroblasts[44] and breast cancer cells due to mevalonate deprivation.[45]

Although studies have demonstrated the antitumor activity of S-(-)-PA,[19,21–23] and it is in this form that clinical trials are based,[46] this work represents the first report investigating the activity of the R-(+) enantiomer of PA inhibiting tumor growth, and may thus contribute to the developing story of the antitumor activity of this monoterpene. Initially, the effect of R-(+)-PA on growth was characterized using the colonic adenocarcinoma cell line SW480 (*Figure 1*). Growth of this cell line was inhibited in a dose dependent manner, causing growth arrest at a 1-mM concentration. The addition of 2 μM MVN to PA-treated cells resulted in an additive inhibition of growth, with cytotoxicity at higher doses. Together, these results indicated an inhibitory action of these inhibitors on the growth of the colonic adenocarcinoma SW480, suggesting that the chemotherapeutic use of combinations of these inhibitors may be possible at nontoxic doses.

The effect of protein isoprenylation inhibition on cell cycle progression was subsequently investigated in SW480 cells. R-(+)-PA was used at a 1-mM concentration, which caused cell growth arrest of SW480 cells with no effect on cytotoxicity (*Figure 1*). Using conventional techniques, this concentration inhibited the incorporation of ^{14}C-mevalonate into the 21- through 28-kD proteins, implying that this monoterpene inhibited protein isoprenylation in SW480 cells (*Figure 2*). Additionally, cell cycle analysis of PA-treated colon tumor cells indicated a block at the G0/G1 phase of the cell cycle, with a decrease in the percentage of cells in S phase (*Table 1*). This is in agreement with previous work in lymphocytes where monoterpene treatment inhibited protein isoprenylation and caused cells to arrest in G0/G1.[24]

Because R-(+)-PA caused arrest of colonic adenocarcinoma cells at a point before initiation of DNA synthesis, and because PI staining is incapable of discriminating between G0 (resting) and G1 (cycling) cells, further analysis of the expression of two proteins involved in growth control was performed. The protein kinase p34^{cdc2} is a catalytic subunit of the M phase promoting factor, which is activated at the G2/M transition and controls the onset of mitosis.[47,48] In cycling cells, a constant presence of the protein kinase p34^{cdc2} is maintained throughout the cell cycle. The decrease in quantity of p34^{cdc2} protein seen as a result of PA treatment (*Figure 5*) is consistent with an arrest in G0, because this protein is not expressed in resting cells. However, this same treatment did not interfere with *c-fos* protein expression (*Figure 4*)—evidence indicative of an arrest in G1 rather than G0. Although expression of p34^{cdc2} is known to regulate the onset of both the S and M phases in yeast,[49] its role in regulating the G1/S transition in mammalian cells is unclear.[50,51] Treatment with R-(+)-PA could be consistent with a growth arrest of the cells in early G1, because *cdc2* mRNA levels increase markedly in late G1, and this correlates with the accumulation of newly synthesized *cdc2* protein before S phase.[36] It is suggested that the decrease in p34^{cdc2} protein is a consequence of (1)

growth arrest in early G1, at a point prior to enhanced expression of the *cdc*2 gene, and possibly (2) a more direct effect of PA on the ability of this protein to be processed, similar to the reduction seen with *ras* protein levels. The possibility that the cells are arrested in G0 cannot be discarded; however, this would require: (1) a constitutive generation of the c-*fos* protein or (2) that the treatments increase the number of cells in G0 (causing a reduction in $p34^{cdc2}$ protein), while not affecting the number of cells in G1 (which express the *c-fos* gene, and therefore the *c-fos* protein).

Cancer cells exhibit an altered cytoskeletal organization, which probably reflects the loss of stress fibers and consequently, cell contact growth inhibition, providing a more rounded morphology.[1] Bifulco et al.[52] have shown that MVN causes stress fibers to disassemble; this is followed by cortical actin fragmentation, and finally cell rounding. Both cytoskeletal and morphologic perturbations were found to be reversed by mevalonate, but not by dolichol, ubiquinone, isopentenyladenine, or cholesterol. Furthermore, microinjection of farnesyl-pyrophosphate or geranylgeranyl-pyrophosphate into MVN-treated cells also resulted in rapid morphologic reversion associated by the loss of actin cables.[53] With this information considered, we evaluated the extent of the cell cycle changes induced by R-(+)-PA on the morphology of SW480 cells. PA-induced G0/G1 arrest correlated with a flattened morphology in SW480 cells, as opposed to MVN treatment effects, where cells exhibited a rounded morphology with extended projections (*Figure 3*). These results suggest that fluctuations in mevalonate derived metabolite may be involved in the organization of the actin cytoskeleton. Changes in cell morphology were not accompanied by gross changes in the microtubular lattice (data not shown). Interestingly, in a control study using NIH 3T3 cells, we did not see any changes in the organization of the actin cytoskeleton after R-(+)-PA treatment (data not shown), which may be due to the fact that these are well spread cells with extended stress fibers from end to end.

Three isoprenylated proteins have been implicated in the signaling and organization of actin.[54] These are farnesylated *ras*, and the two geranylgeranylated *ras* related small GTP binding proteins *rac* and *rho*, which play a role in the assembly of the actin cytoskeletal network.[55,56] Additionally, *rac* and *rho* are thought to transmit signals for the assembly of the actin cytoskeleton through *ras*.[57] It has been suggested that the phosphorylation state of cytoskeletal-associated proteins contributes to the structural changes that occur in transformed cells.[1] Caldesmon is a protein that, when phosphorylated by $p34^{cdc2}$, contributes to the disassembly of microfilaments.[58] Because R-(+)-PA treatment imparted a flattened morphology to SW480 cells, and the expression of the $p34^{cdc2}$ kinase was impaired by this treatment (*Figure 5*), one can speculate that perhaps this kinase plays a role in phosphorylating this protein. A dephosphorylated state of this protein may contribute to an increase in actin polymerization, resulting in a more flattened morphology and a reorganization of the actin cytoskeleton to the periphery. Conversely, R-(+)-PA treatment could result in isoprenylation inhibition of an unknown regulatory protein that, when unisoprenylated, has phenotypic effects that are opposed to those of *rac* and *rho*.

Cholesterol synthesis inhibitors such as MVN and dehydroepiandrosterone interfere with isoprenylation of the $p21^{ras}$ gene product.[59–62] Such treatments are capable of arresting cells in the G1[7,63] or G2/M[64] phases of the cell growth cycle, as well as inhibiting overall tumor growth.[28,40,65–74] However, it is unclear whether the antiproliferative activity of HMGCoA R inhibitors is due to their interference with the posttranslational modifications of *ras* or to inhibition of other biochemical processes that depend on normal levels of mevalonate or one of its metabolites.[75] Although HMGCoA R inhibitors could be affecting a variety of cellular proteins, MVN has been shown to inhibit *ras* stimulated signal transduction processes at concentrations that did not affect other signal transduction pathways, suggesting that suppression of mevalonate synthesis may selectively inhibit *ras*-stimulated transcriptional events.[76] In fact, specific farnesyltransferase inhibitors were found to inhibit *ras* dependent signaling pathways in *ras*-transformed Rat-1 cells,[11] but not in untransformed cells.[11,77] These data, although limited, imply that the accumulation of unfarnesylated oncogenic *ras* exhibited a dominant negative effect on *ras*-dependent transformation.[11]

The effects of farnesyltransferase inhibitors, which induce morphologic reversion in *ras* transformed fibroblasts, support a relationship between cell morphology and farnesylation.[57] Cell enlargement and actin stress fiber formation accompanied this treatment. However, inhibition of *ras* processing did not correlate with this reverted phenotype. Additional studies have shown that oxygenated metabolites of limonene and S-(-)-PA inhibit farnesyltransferases, suggesting these compounds may act by inhibiting the farnesylation of certain proteins.[78] Although studies have suggested that monoterpenes affect the posttranslational processing of $p21^{ras}$ in mammary tumors,[22] other investigations using PANC-1 and *ras* transformed rat fibroblasts, rat liver epithelial tumor cells, and human leukemia cells did not indicate an inhibition of membrane bound *ras* by monoterpene treatments.[79–81] This may imply that the ability of monoterpenes to affect the isoprenylation of *ras* could change according to the cellular demands and/or tissue origin of these tumors. Monoterpenes also have prevented the formation of mammary carcinomas without activated *ras*, to the same extent as those with the activated oncogene, providing evidence that these agents may not selectively prevent carcinomas having activated *ras*.[21]

In this study, PA strongly inhibited the expression of *ras* protein, but did not significantly inhibit *ras* processing. Similar results were recently observed with another inhibitor of this pathway, fluoromevalonate.[82] Although R-(+)-PA treatment resulted in a 50% decrease in immunoprecipitated ^{14}C-mevalonate labeled *ras* (*Figure 7*), this decrease could not solely be credited to inhibition of *ras* protein farnesylation, because *ras* protein expression was inhibited by this treatment (*Figure 6*). A reduction in expression of this oncogene in PA-treated cells could explain the inhibition of growth induced by this compound in SW480 cells, although one cannot ignore the possibility that the effect of monoterpenes on *ras* expression may be a consequence of the growth arrest. It is of interest that it took 48 hours of treatment to see any effects of this treatment on

ras protein expression. This observation is consistent with both the 20-hour half-life observed for this protein,[83] and a recent study that suggested that mevalonate derived isoprenoids regulate *ras* expression at a transcriptional level.[84] This latter work suggested that R-(+)-PA may affect a regulatory isoprenoid responsible for the transcriptional regulation of *ras*. Because the *ras* oncogene is involved with signaling that can affect the actin cytoskeleton,[57] it is possible that agents that can influence *ras* expression may lead to cytoskeletal alterations in cells that depend on this oncogene for unrestricted growth as observed in this study.

Although PA is metabolized in vivo to two major circulating species, perillic acid and dihydroperillic acid,[85,86] we chose to work with this metabolite for our in vitro studies due to its greater potency than limonene and perillic acid in inhibiting in vitro cell proliferation and protein isoprenylation[87] and in vivo growth of mammary tumor models.[85] In addition, the greater potency of PA compared with limonene has made it possible to use less toxic doses of this monoterpene to achieve potentially effective systemic concentrations of the active principals in ongoing phase I clinical trials.[46] Although the specific metabolic fate of PA in human colonic cells has not been reported, this monoterpene would most likely be partially metabolized to perillic and dihydroperillic acids by cellular cytochrome p450 enzymes, as occurs with other monoterpenes in vivo.[88] This study demonstrates that R-(+)-PA may ultimately provide chemotherapeutic benefit by its significant impact on such fundamentally important cell functions as protein isoprenylation, proliferation, signaling, and cytoskeletal control.

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