

Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells

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Abstract

Substantial evidence from epidemiological studies supports the inverse association between the intake of fruits, vegetables and other plant products and cancer incidence. Cancer-preventive constituents of fruits and vegetables may inhibit carcinogen activation, enhance carcinogen detoxification, prevent carcinogens from interacting with critical target sites, or impede tumor progression. These activities, however, are achievable only when levels of individual bioactive constituents reach beyond those attainable from a normal balanced diet. Isoprenoids, a broad class of mevalonate-derived phytochemicals ubiquitous in the plant kingdom, suppress the proliferation of tumor cells and the growth of implanted tumors. A search for volatile isoprenoid constituents of food products spanning seven plant families identified 179 isoprenoids. Of these, 41 purchased from commercial sources were screened for efficacy in suppressing the proliferation of murine B16 melanoma cells. Individual isoprenoids suppressed the proliferation of B16 and HL-60 promyelocytic leukemia cells with varying degrees of potency. Cell cycle arrest at the G₀-G₁ phase and apoptosis account, at least in part, for the suppression. Blends of isoprenoids suppressed B16 and HL-60 cell proliferation with efficacies equal to the sum of the individual impacts. These findings suggest that the cancer-protective property of fruits, vegetables, and related products is partly conferred by the cumulative impact of volatile isoprenoid constituents. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Isoprenoids; Murine B16 melanoma; Human HL-60 leukemia

1. Introduction

The inverse association between cancer risk and fruit and vegetable consumption, evidenced by more than 250 epidemiological studies with rare disputable exceptions on certain types of cancer [1], has been well documented [2]. Numerous bioactive constituents have been hypothesized to act as cancer-preventing agents by inhi-

biting the activation of procarcinogens, enhancing the detoxification of carcinogens, preventing carcinogens from interacting with critical target sites, or impeding the progression of carcinogenesis [3–5]. These constituents further modulate the promoter-independent growth of tumors by affecting the expression of genes involved in cell signaling, particularly those regulating cell proliferation, the cell cycle, apoptosis and differentiation [3]. Alternatively, bioactive constituents may suppress enzymatic pathways that provide products essential for the post-translational processing and biological activity of proteins critical in cell proliferation [6,7].

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One of the enzymatic pathways that are crucial in cell proliferation is the mevalonate pathway that provides isoprenoids and sterols. Lovastatin, a competitive inhibitor of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, is widely prescribed for treating hypercholesterolemia. Lovastatin also suppresses the syntheses of dolichol phosphate, a substrate required for the glycosylation and membrane attachment of IGF-1 receptors [8], and farnesyl pyrophosphate, another substrate essential for the function of oncogenic ras [9] and the maturation of nuclear lamin B [9]. These actions contribute to the lovastatin-mediated cell cycle arrest at the G₀-G₁ phase [10] and induction of apoptosis [11]. The modest hypocholesterolemic effects of volatile [6] and other mevalonate-derived isoprenoids including lycopene [12], and three groups of vitamers, carotenoids [12,13], menaquinones [14] and tocotrienols [6], likely trace to the post-transcriptional suppression of HMG CoA reductase activity [7,12,13,15–17]. These actions have a modest impact on the sterol feedback-sensitive HMG CoA reductase activity in sterologenic tissue [18]. The aberrant, sterol feedback-resistant HMG CoA reductase activity characteristic of neoplastic cells retains a high sensitivity to isoprenoid-mediated modulation [7,18]. Suppression of HMG CoA reductase by isoprenoids in tumor cells, similar to the inhibition by lovastatin in both tumor and normal cells, keeps lamin B in a nascent state and leads to cell cycle arrest at the G₀-G₁ phase and initiation of apoptosis [19].

At levels obtained from dietary intake, individual isoprenoids probably do not have sufficient impact on the HMG CoA reductase to exert a cancer-protective effect. The protection conferred by plant-derived products may well be attributed to the cumulative effects of isoprenoid constituents on the dysregulated mevalonate pathway activity characteristic of tumor tissues. These considerations led us to catalogue the volatile isoprenoid constituents of fruits, vegetable and herbs representing seven plant families and to evaluate the individual and cumulative tumor-suppressive effects of assorted volatile isoprenoids.

2. Materials and methods

Fruits, vegetables and herbs representing seven

plant families (Table 1) were selected for this evaluation. Volatile isoprenoid constituents were catalogued through searches of Dr. Duke's Phytochemical and Ethnobotanical Databases (<http://www.ars-grin.gov/duke/index.html>), Agricola, Biological Abstracts, and FSTA indexing and abstracting databases (Table 2). Commercially available isoprenoids were contributed by Bedoukian Research Inc. (Danbury, CT) or were purchased from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO).

Murine B16 F10 melanoma cells [20] were grown in monolayer culture (35 × 10 mm tissue culture dishes) in 3 ml RPMI 1640 medium (Sigma Biosciences, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Sigma Biosciences) and 2% penicillin/streptomycin (Penicillin–Streptomycin Liquid, 1 × 10⁷ units penicillin and 1 × 10⁷ µg streptomycin/l of 0.85% saline, GIBCO BRL, Grand Island, NY). Cultures seeded with 3.3 × 10⁴ cells/ml were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. The medium was decanted and replaced with fresh medium containing the test agents dissolved in ethanol and incubations were continued for 48 h. All cultures contained 5 ml ethanol/l (80 mmol/l). The medium and detached (apoptotic) cells were decanted, and the monolayer was washed twice with Hanks' Balanced Salt Solution (HBSS, Sigma

Table 1
Fruits, vegetables and herbs selected for database search for volatile isoprenoid constituents

Family	Representative member
Anacardiaceae	Mango (<i>Mangifera indica</i> L.)
Ericaceae	Blueberry (<i>Vaccinium corymbosum</i> L.) Cranberry (<i>Vaccinium macrocarpon</i> L.)
Lamiaceae	Basil (<i>Ocimum basilicum</i> L.) Marjoram (<i>Origanum majorana</i> L.) Peppermint (<i>Mentha piperita</i> L.) Thyme (<i>Thymus vulgaris</i>)
Rosaceae	Apricot (<i>Prunus armeniaca</i> L.) Peach (<i>Prunus persica</i> L.) Plum (<i>Prunus Americana</i> L.) Raspberry (<i>Rubus idaeus</i> L.) Strawberry (<i>Fragaria x ananassa</i>)
Rutaceae	Grapefruit (<i>Citrus x paradisi</i>) Lemon (<i>Citrus limon</i> L.) Orange (<i>Citrus sinensis</i> L.)
Solanaceae	Tomato (<i>Lycopersicon esculentum</i>)
Vitaceae	Grape (<i>Vitis vinifera</i> L.)

Table 2
Volatile isoprenoid constituents of fruits, vegetables and herbs^a

Abscisic acid ^b	α -Cubebene ^d	β -Ionone ^{a-d}	Ocimenol ^c
Acorenone ^g	<i>p</i> -Cymene ^{a-g}	γ -Ionone ^a	Ocimino ^c
Alloaromadendrene ^f	<i>p</i> -Cymen-8-ol ^{a,b}	3,4-Didehydro- β -ionone ^b	Perillaldehyde ^d
Aromadendrene ^{f,g}	<i>p</i> -Cymen-9-ol ^b	Dihydro- α -ionone ^b	Perillyl alcohol ^d
<i>trans</i> - α -bergamotene ^g	Damascenone ^{a,b,c}	Dihydro- β -ionone ^b	α -Phellandrene ^{a,b,d,f,g}
Bisabolene ^d	3-Hydroxy- β -damascone ^b	Epoxy- β -ionone ^a	β -Phellandrene ^{a,b,d,g}
Borneol ^g	4-Hydroxy- β -damascone ^b	3-Hydroxy-7,8-dihydro- β -ionone ^b	α -Pinene ^{a-g}
Bornyl acetate ^g	β -Elemene ^{a,d,g}	3-Hydroxy-5,6-epoxy- β -ionone ^b	β -Pinene ^{b-d,f,g}
Isoborneol ^g	γ -Elemene ^a	3-Hydroxy- α -ionone ^b	α -Pinene oxide ^g
δ -Cadinene ^{d,f}	δ -Elemene ^a	3-Hydroxy- β -ionone ^b	Pinocamphone ^b
Cadinene ^g	Estragol ^g	4-Hydroxy- β -ionone ^b	Piperitol ^g
Cadinol ^g	Estragole ^g	β -Methyl- <i>n</i> -ionone ^b	Piperitone ^{d,g}
Camphene ^{a-d,g}	Eugenol ^{a-c,g}	4-Oxo- β -ionone ^b	Pristane ^a
Camphor ^{b,g}	Isoeugenol ^{a-c,g}	Pseudoionone ^a	Pulegone ^g
5-caranol ^g	Methyl eugenol ^g	Isopinocamphone ^b	Sabinene ^{d,g}
3-carene ^{a-d,f,g}	β -Farnesene ^{a,b,d,g}	Isopulegol ^d	Sabinene hydrate ^g
Carvacrol ^{a,c,g}	Farnesal ^a	Limonene ^{a-g}	Sabinol ^d
Carvacrol methyl ether ^g	Farnesol ^{a-c}	Limonene-1,2-epoxide ^d	Santalol ^g
Carvone ^{a,d,g}	Farnesyl acetate ^{a,c}	Linalool ^{a-g}	Selinadiene ^d
Pinocarvone ^g	Farnesyl acetone ^a	Dihydrolinalool ^b	β -Selinene ^f
1-Carveol ^{c,d,g}	α -Fenchol ^b	Linalool oxides ^{a-f}	γ -Selinene ^d
Pinocarveol ^g	Fenchone ^{b,g}	Linalyl acetate ^{a,b,d,g}	α -Sinensal ^d
Carvyl acetate ^b	Fenchyl acetate ^g	Longifolene ^d	β -Sinensal ^d
β -Caryophyllene ^{a-d,f,g}	Fenchyl alcohol ^g	<i>p</i> -Mentha-2,8,-dien-1-ol ^d	Spathulol ^g
Carophyllene oxide ^g	Geranial ^{a,b,d,e}	<i>trans-p</i> -menthen-9-ol ^d	Styrene ^b
α -Cedrine ^c	Geranic acid ^{a,e}	Menthol ^{a,b,f,g}	<i>cis</i> -1,8-terpin ^c
Cineole ^c	Geraniol ^{a-c,g}	Menthyl acetate ^g	<i>trans</i> -1,8-terpin ^c
1,4-Cineole ^e	Geranyl acetate ^{a,b,d,g}	Neomenthol ^g	α -Terpinene ^{b,d,e,g}
1,8-Cineole ^{c,g}	Geranyl acetone ^{a,b}	Menthone ^g	γ -Terpinene ^{b-g}
2-Hydroxy-1,8-cineole ^e	Geranyl butyrate ^a	Isomenthone ^g	Terpinen-1-ol ^c
Cinnamaldehyde ^b	Germacrene A ^a	α -Muurolene ^{f,g}	Terpinen-4-ol ^{a-c,g}
Ethyl cinnamate ^b	Germacrene B ^{a,g}	Myrcenol ^c	α -Terpineol ^{a-g}
Methyl cinnamate ^{b,g}	Germacrene C ^a	Myrcen-2-ol ^c	4-Terpineol ^{d,g}
Citral ^d	Germacrene D ^a	Myrcene ^{a-g}	Terpinolene ^{a-c,g}
Cyclocitral ^{a,b}	Bicyclogermacrene ^g	Myrtenol ^b	α -Terpinyl acetate ^{b,g}
Citronellal ^{a,d}	Hotrienol ^{b,c}	Neral ^{a,b,d,c}	<i>cis</i> -dihydro- α -terpinyl acetate ^g
Citronellol ^{a-c,g}	α -Humulene ^{a,d,f,g}	Nerol ^{a-c,g}	Thujene ^{c,d,g}
Citronellyl acetate ^{a,d}	α -Ionol ^b	Nerol oxide ^c	Thujone ^g
Citronellyl butyrate ^a	β -Ionol ^b	Neryl acetate ^{d,g}	Thymol ^{c,g}
Citronellyl propionate ^a	3-Hydroxy-7,8-dihydro- β -ionol ^b	Nerolidal ^{a,b}	Tricyclene ^g
Hydroxycitronellol ^c	3-Hydroxy- β -ionol ^b	α -Nerolidol ^{b,d,g}	Vanillin ^{b,d}
α -Copaene ^{f,g}	3-Oxo- α -ionol ^b	β -Nerolidol ^{b,g}	Valencene ^d
β -Copaene ^d	4-Oxo- β -ionol ^b	Nootkatone ^d	Verbenone ^{b,g}
<i>o</i> -Cresol ^c	3-Oxo-7,8-dihydro- α -ionol ^b	Ocimene ^{b,d-g}	Vitispirane ^c
<i>p</i> -Cresol ^{b,c}	α -Ionone ^{a-c}	Allo-ocimene ^{d,f,g}	

^a Solanaceae; ^b Rosaceae; ^c Ericaceae; ^d Rutaceae; ^e Vitaceae; ^f Anacardiaceae; ^g Lamiaceae.

Biosciences) and then incubated with a trypsin-EDTA solution (Sigma Biosciences) at 37°C for 2 min. Trypsin was inactivated by suspending the cells in medium containing 10% FBS. Trypsinized cells were harvested by centrifugation (250 × g) and resus-

pended in HBSS. Viable cells, cells that excluded 0.4% Trypan Blue (GIBCO BRL), were counted with a hemocytometer; 0-time (24 h) cell counts were deducted from final cell counts to provide an estimate of the net increase in cell number.

Human HL-60 acute promyelocytic leukemia cells (CCL-240, ATCC) were grown in suspension culture (25 cm² flasks) in 8 ml RPMI 1640 medium with 20% FBS and 2% penicillin/streptomycin. Cultures seeded with 1.25×10^5 cells/ml were incubated with test agents for 24 h at 37°C in a humidified atmosphere of 5% CO₂. The cells were then collected by centrifugation (250 × *g*) and resuspended in HBSS. Viable cells, cells that excluded 0.4% Trypan Blue, were counted with a hemocytometer; 0-time (seeding) cell counts were deducted from final cell counts to provide an estimate of the net increase in cell number.

The IC₅₀ value represents the concentration of an isoprenoid required to inhibit the net increase in the cell count by 50% at a time point within the linear growth period plotted for control cells. We determined the IC₅₀ value by plotting cell number against isoprenoid concentration. IC₅₀ values were calculated for isoprenoids that suppressed tumor cell proliferation with potency equal to or greater than that of *d*-limonene. All IC₅₀ estimates are based on two or more individual tests; SD values are presented for values obtained with three or more tests. Cumulative impacts of isoprenoids on B16 melanoma cell proliferation were examined using a blend of two cyclic isoprenoids, eugenol and β-ionone, a blend of two acyclic isoprenoids, farnesol and nerolidol, and a third blend of an acyclic (geraniol) and a cyclic (α-terpineol) isoprenoid. We further examined the impact of the farnesol/nerolidol blend on the growth of B16 melanoma and HL-60 leukemia cells and that of a more complex blend of cyclic and acyclic isoprenoids on the proliferation of HL-60 leukemia cells.

Farnesol/nerolidol-initiated changes in cell cycle distribution of B16 and HL-60 cells were monitored by flow cytometry. Cell pellets (>1 × 10⁶ cells), harvested as described above, were fixed in 1 ml 70% ethanol at 4°C for 60 min, washed in 1 ml PBS and resuspended in 400 μl PBS containing 0.5 mg RNase A (Sigma). After gentle mixing a 100 μl aliquot of propidium iodide (1 g/l PBS) (Sigma) was added [21]. The cells were incubated in the dark at room temperature for 15 min and then held at 4°C in the dark for flow cytometric analysis. For each sample, at least 1 × 10⁴ cells were analyzed for DNA content using a flow cytometer FACSCalibur (Becton Dickinson, San Jose, CA). The data acquisition software used for the DNA analysis and distribu-

tion of cells in sub-G₁, G₀-G₁, S, and G₂-M was CellQuest/ModFit (Verity, Topsham, ME). The sub-G₁ peak is an indicator of the onset of apoptosis [22].

StatView software (Abacus Concepts, Berkeley, CA) was used for the assessment (unpaired *t*-test) of treatment-mediated effects on tumor cell proliferation.

3. Results

Through searching indexing and abstracting databases, we identified 179 volatile isoprenoid constituents (Table 2) of fruits, vegetables and herbs spanning seven plant families (Table 1). We then screened, of the constituents above, 41 commercially obtained isoprenoids for tumor-suppressive potency in murine B16 melanoma and human HL-60 acute promyelocytic leukemia cells. Murine B16 melanoma and P388 leukemia cells were used for early NCI Drug Discovery screens [23,24]. In 1985, a major modification of the cancer screening strategy led to the adoption of test panels comprised of human tumor cell lines for novel drug discovery within the area of natural products. The HL-60 leukemia was found to respond to test agents with high sensitivity [23]. We present in Table 3 the IC₅₀ values calculated using the more resistant B16 melanoma cell line. *d*-Limonene (IC₅₀, 450 ± 43 μmol/l) was the first isoprenoid to be evaluated in human trials for tumor-suppressive activity [25]. The IC₅₀ value of *d*-limonene calculated for the more sensitive HL-60 cells is 180 μmol/l. Many of the other volatile isoprenoids available for screening, *trans*, *trans* farnesol (Fig. 1A) for example (IC₅₀, 28 ± 16 μmol/l), have much greater efficacy than *d*-limonene (Table 3).

We recognize that the IC₅₀ levels of even the more potent isoprenoids are not attainable within a dietary context. However, our earlier studies suggested that the growth-suppressive actions of diverse isoprenoids are cumulative [7,20]. The flavor/aroma profile of tomatoes reflects the spicy, cinnamon, clove contribution of eugenol (Fig. 1B), and the warm, woody, balsamic, floral contribution of β-ionone (Fig. 1B). Fig. 2 shows the cumulative effects of these volatile isoprenoids on the growth of B16 melanoma cells. The significant growth suppression imposed by 75 and 150 μmol/l eugenol (26 and 58%, respectively)

and 70 and 140 $\mu\text{mol/l}$ β -ionone (28 and 61%, respectively) are consistent with the IC_{50} values shown in

Table 3
 IC_{50} values reflecting the relative tumor-suppressive potencies of volatile isoprenoids

Isoprenoid	IC_{50} ($\mu\text{mol/l}$) ^a
<i>trans, trans</i> Farnesol	28 \pm 16 ^b
Citral ^c	30 \pm 10
Nerolidol	65 \pm 11
Thymol	120 \pm 15
Perillaldehyde	120 \pm 17
Carvacrol	120 \pm 15
Geraniol	139 \pm 27
β -Ionone	140 \pm 23
Geranyl butyrate	150
Eugenol	163 \pm 27
Geranyl acetone	171 \pm 35
Camphene	178
Geranyl acetate	185 \pm 35
β -Caryophyllene	190
α -Ionone	190
Cresol	200
Perillyl alcohol	250 \pm 28
Ocimene	250
Menthol	250
Carvyl acetate	267 \pm 42
Fenchol	300
Fenchone	>300
Menthone	>300
Myrtenol	300
Nerol	332 \pm 33
Geranic acid	338
α -Pinene	350
Citronellal	350
Cineole	400
Linalool	400
α -Terpineol	400
Citronellol	415
Citronellyl propionate	450
<i>d</i> -Limonene	450 \pm 43
<i>p</i> -Cymene	>500
<i>p</i> -Mentha-2,8-dien-1-ol	>500
Verbenone	>500
Neryl acetate	555
β -Farnesene	750
Myrcene	>1000
Cinnamaldehyde	>1000

^a The IC_{50} value is the concentration of an isoprenoid required to suppress the net 48 h increase in the B16 melanoma population by 50%.

^b Values represent the mean \pm SD; values lacking SD represent an average of two determinations.

^c Mixture of geraniol and nerol.

Table 3. The 60% suppression of growth, imposed by the blend of 75 $\mu\text{mol/l}$ eugenol and 70 $\mu\text{mol/l}$ β -ionone, approaches the 54% inhibition predicted by the sum of impacts (26 + 28%) of the two isoprenoids (Fig. 2). Other blends of eugenol and β -ionone suppressed growth with potencies close to the predicted values. The flavor/aroma profile of strawberries reflects the floral lilac contribution of α -terpineol (Fig. 1B) and the sweet, floral fruity rose contribution of geraniol (Fig. 1A). The inhibition of B16 melanoma growth (Fig. 3) effected by 190 and 380 $\mu\text{mol/l}$ α -terpineol (23 and 35%, respectively) and 70 and 140 $\mu\text{mol/l}$ geraniol (32 and 48%, respectively) is consistent with the IC_{50} values recorded in Table 3. Blends suppressed growth to the extent predicted by the individual actions.

The response of B16 cells to the acyclic isoprenoids, farnesol (a mixture of *trans, trans* and *cis, trans* isomers) and nerolidol (Fig. 1A), is shown in Fig. 4. Plotted on the upper graph are growth values, % of control, on isoprenoid concentration. Data plotted on

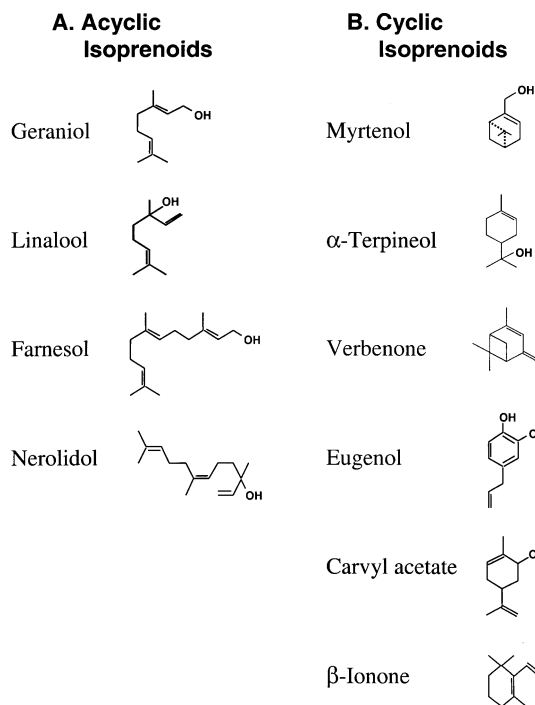


Fig. 1. Structures of acyclic (A) and cyclic (B) volatile isoprenoids evaluated for their individual and cumulative impacts on the proliferation of B16 melanoma and HL-60 leukemic cells.

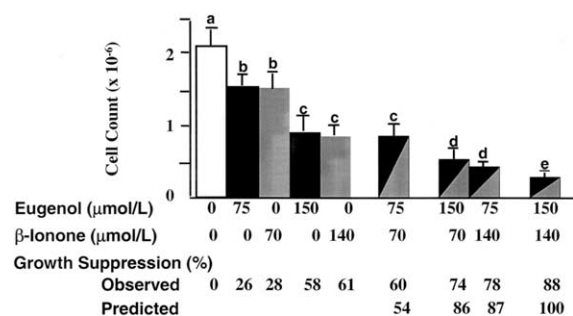


Fig. 2. Cumulative impacts of two cyclic isoprenoid constituents of tomatoes, eugenol and β -ionone, on the proliferation of B16 melanoma cells. Cultures, seeded with 3.3×10^4 cells/ml, were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . The medium was decanted and replaced with fresh medium containing the test agents and incubations were continued for 48 h. Values with different letters (a–e) are significantly different ($P < 0.05$).

the lower graph illustrate the cumulative impacts of the two isoprenoids on the growth of B16 melanoma cells. At low concentrations, farnesol (10 $\mu\text{mol/l}$) and nerolidol (25 $\mu\text{mol/l}$) suppressed the growth of B16 cells by 10 and 18%, respectively. The combined treatment suppressed growth by 36%, slightly exceeding the 28% predicted by the sum of the individual suppression (Fig. 4, Table 4). Concomitant with this isoprenoid-mediated suppression of cell proliferation is a substantial impact on the distribution of cells in

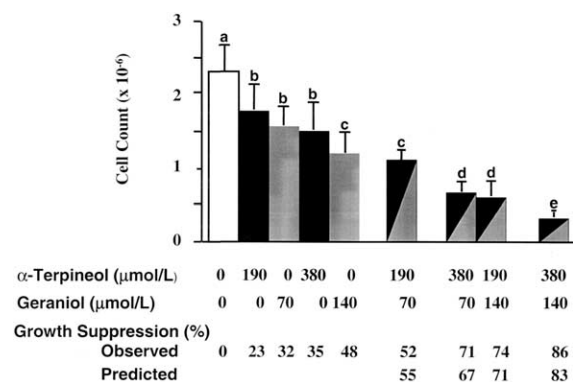


Fig. 3. Cumulative impacts of a cyclic (α -terpineol) and acyclic (geraniol) constituent of strawberries on the proliferation of B16 melanoma cell. Cultures, seeded with 3.3×10^4 cells/ml, were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . The medium was decanted and replaced with fresh medium containing the test agents and incubations were continued for 48 h. Values with different letters (a–e) are significantly different ($P < 0.05$).

the cell cycle. The isoprenoids effected increases and decreases, respectively, in the proportions of cells in the G_0 - G_1 and S phases of the cell cycle. The G_0 - G_1 /S ratio, a marker of the arrest of cells at the G_0 - G_1 phase of the cell cycle, increased from 0.89 (control) to a maximum of 2.0 for cells incubated with the combined agents (Table 4).

Responses of HL-60 cells to the acyclic isoprenoids, the farnesol isomers and nerolidol, are also shown in Fig. 4. Plotted on the upper graph are growth values, % of control, on isoprenoid concentration. The IC_{50} values calculated for the farnesol isomers and nerolidol are 30 and 25 $\mu\text{mol/l}$, respectively. A comparison with the corresponding IC_{50} values for B16 cells, 45 ± 7 and 100 ± 11 $\mu\text{mol/l}$, confirms the greater sensitivity of HL-60 leukemic cells to these isoprenoids. The lower plot shows the cumulative impact of the two isoprenoids on the growth of HL-60 cells (Fig. 4). At low concentrations, farnesol isomers (2.5 $\mu\text{mol/l}$) and nerolidol (5 $\mu\text{mol/l}$) suppressed the growth of HL-60 cells by 4 and 9%, respectively. The combined treatment suppressed growth by 20%, slightly exceeding the 13% predicted by the sum of the individual suppression (Fig. 4, Table 4). Similar cumulative effects were observed with blends of these two isoprenoids at various concentrations (Table 4). Concomitant with this isoprenoid-mediated suppression of cell proliferation is a substantial impact on the distribution of cells in the cell cycle. The isoprenoids effected dose-dependent increases and decreases, respectively, in the proportions of cells in the G_0 - G_1 and S phases of the cell cycle. The G_0 - G_1 /S ratio increased from 0.87 (control) to a maximum of 1.30 (Table 4). The suppressed growth was also accompanied by increases in the proportion of apoptotic cells.

We next tested a panel of volatile isoprenoid constituents of strawberries, the consumption of which is reported to be strongly associated with a decreased incidence of prostate cancer [26]. Each of the eight constituents was evaluated at a concentration predicted to suppress the growth of HL-60 cells by 10–20% (Fig. 5). A blend of five cyclic isoprenoid constituents (Fig. 1B) suppressed cell growth by 30%; another blend of three acyclic isoprenoids (Fig. 1A) suppressed cell growth by 42%. These results predict a 72% reduction in response to the blend of eight isoprenoids. We observed an 81% suppression of growth (Fig. 5).

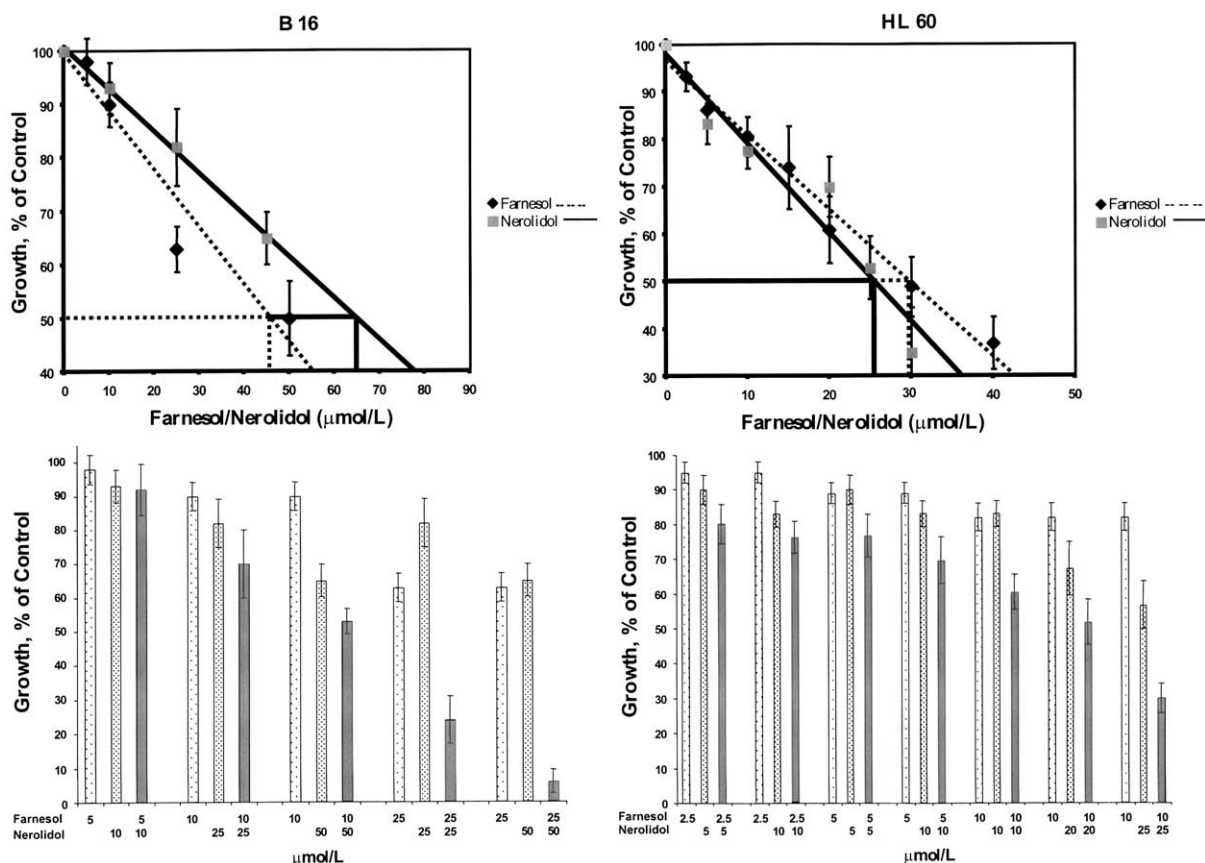


Fig. 4. Cumulative impacts of two acyclic isoprenoids, farnesol (isomers) and nerolidol, on the proliferation of murine B16 melanoma and human HL-60 leukemic cells. B16 cultures, seeded with 3.3×10^4 cells/ml, were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . The medium was decanted and replaced with fresh medium containing the test agents and incubations were continued for 48 h. HL-60 cultures, seeded with 1.25×10^5 cells/ml, were incubated with test agents for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . Plotted on the upper graph are growth values, % of control, on individual isoprenoid concentration. Data plotted on the lower graph point to the cumulative impacts of blends of the two isoprenoids on the growth of murine and human cancer cells.

4. Discussion

The B16 melanoma screen predicts the potential impact of isoprenoids on the growth of implanted B16 melanoma without the time-consuming expenditure of animal resources [20,27]. This screen does not apply to the evaluation of other chemopreventive phytochemicals that attenuate the potency of chemical carcinogens via induction of phase I and II detoxifying activities and oxygen scavenging actions [4]. Previous studies found that human HL-60 promyelocytic leukemia, MCF-7 mammary adenocarcinoma, and Caco-2 colon adenocarcinoma cells are more sensitive than normal human fibroblasts (CCD-

18Co) to the isoprenoid-mediated suppression of growth [6,19]. The differential sensitivities to isoprenoids between tumor and normal cells *in vitro* translate into, in animal feeding studies, isoprenoid-mediated suppression of the growth of implanted tumors without impact on the growth of the host [6,20,27–29]. The aforementioned studies addressed the impacts of individual isoprenoids at levels well beyond that attainable through the diet.

Our search identified 179 volatile isoprenoid constituents of fruits, vegetables and herbs representing seven plant families. Of the 41 isoprenoids screened in the aggressively-growing B16 melanoma, 16 had greater potency than perillyl alcohol and 33

Table 4
Cumulative impact of farnesol and nerolidol on the growth of B16 and HL-60 cells

Treatment ($\mu\text{mol/l}$) (farnesol/nerolidol)	Growth (% suppression) (observed/predicted)	Apoptosis (%)	Cell cycle distribution			
			G ₀ -G ₁ (%)	S (%)	G ₂ /M (%)	G ₀ -G ₁ /S
<i>Murine B16 melanoma cells</i>						
0/0	0	– ^a	47.2	52.8		0.89
10/0	10	–	61.1	38.9		1.57
0/25	18	–	61.4	38.6		1.59
10/25	36/28	–	67.3	32.7		2.06
<i>Human HL-60 promyelocytic leukemia cells</i>						
0/0	0	3	41	47	12	0.87
2.5/0	4	6	45	45	10	1.00
0/5	9	9	44	47	9	0.94
2.5/5	20/13	8	49	41	10	1.20
5/0	12	7	46	45	9	1.02
0/10	18	10	46	46	8	1.00
5/10	32/30	11	48	45	8	1.07
2.5/10	24/22	8	46	45	9	1.02
5/5	24/21	7	49	43	8	1.14
10/10	42/37	11	51	42	7	1.21
10/20	49/53	11	52	40	8	1.30
10/25	70/64	11	50	42	8	1.19

^a Apoptotic cells were decanted prior to harvest.

had greater potency than *d*-limonene, the two isoprenoids that have advanced to clinical trials [25,30].

We now record findings of the cumulative impacts of blends of isoprenoids on the growth of B16 and HL-60 cells. The growth suppressions were accompanied by the partial arrest of B16 and HL-60 cells at the G₀-G₁/S interface of the cell cycle and apoptotic cell death. The degree of apoptosis detected by flow cytometry was quantitatively greater in HL-60 cells, we suggest, because late-stage apoptotic B16 cells were decanted before analysis. These findings are consistent with our earlier report that an isoprenoid-initiated mevalonate starvation impeded the post-translational processing of lamin B, a process essential for the completion of mitosis [19].

In sterologenic tissues, HMG CoA reductase activity is primarily regulated by sterol at the transcriptional level. When needs for sterol and non-sterol products such as farnesyl pyrophosphate and dolichol phosphate are met, the reductase activity is post-transcriptionally modulated by the endogenous *trans*, *trans* farnesol diverted from the mevalonate pathway

[15,31–33]. The farnesol pool is then siphoned off by sequential cytosolic alcohol and aldehyde dehydrogenase activities and microsomal cytochrome P-450-dependent monooxygenase activities [34–41]. Tumor HMG CoA reductase resists sterol feedback regulation; the secondary regulation imposed by farnesol thus rises to be dominant [6,7]. The acyclic isoprenoids evaluated herein act as mimetics of the endogenous modulator of reductase activity. The cyclic isoprenoids may induce activities that reroute farnesol away from the mevalonate pathway [33,42]. A synergy between cyclic and acyclic isoprenoids may account for the potency of the isoprenoid blend in suppressing HL-60 growth shown in Fig. 5. Furthermore, synergistic interactions may reach beyond the boundaries of a specific class of phytochemicals. Our recent studies showed that genistein, the isoflavone postulated to be responsible for the hypocholesterolemic effect of soy products, synergized with farnesol in suppressing the proliferation of B16 melanoma cells [43]. These findings suggest that the lower cancer incidence in populations with high consumption of

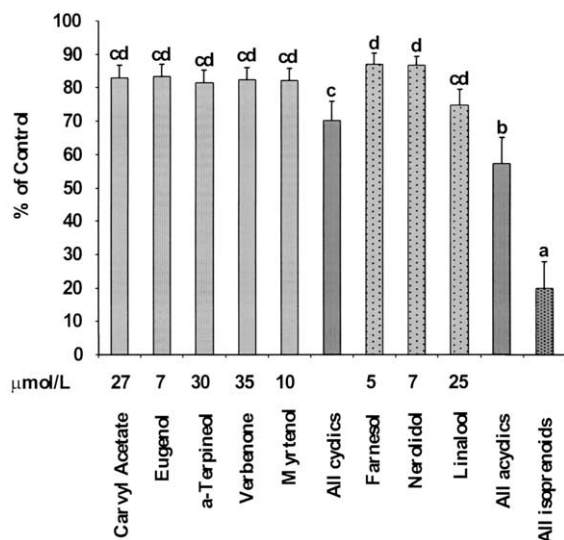


Fig. 5. Cumulative impacts of isoprenoid constituents of strawberries on the proliferation of human HL-60 leukemic cells. Cultures, seeded with 1.25×10^5 cells/ml, were incubated with constituent isoprenoids for 24 h at 37°C in a humidified atmosphere of 5% CO₂. Individual isoprenoids were evaluated at concentrations predicted to suppress cell proliferation by 10–20%. Blends consisting of all cyclic, all acyclic and all isoprenoids were evaluated. Values with different letters (a–d) are significantly different ($P < 0.05$).

plant food may be attributed in part to the cumulative or synergistic impact of assorted isoprenoids, and possibly other phytochemicals, present in fruits, vegetables and herbs.

Quantitation of dietary intake of individual isoprenoids is precluded by variety and variations in growing conditions, maturity, processing, and post-harvest storage of fruits and vegetables, as well as the undefined number of isoprenoid constituents in the food [44]. Given the indeterminate number of isoprenoids with tumor-suppressive potential, it is unlikely that the serum level of a specific isoprenoid will predict cancer risk. Nevertheless, we feel there may be a biological marker for monitoring the intake of volatile isoprenoids and for predicting cancer risk. We previously reported findings that three of the isoprenoids listed in Table 3, carvacrol, β -ionone and thymol, induced a prenyl pyrophosphate pyrophosphatase activity that, we speculated, diverted farnesol from the sterologenic pathway [42]. Providing this finding can be confirmed and expanded to include

responses to other isoprenoids, the urinary excretion of a farnesol metabolite may have potential as a marker of isoprenoid intake.

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