Isoprenoids Suppress the Growth of Murine B16 Melanomas In Vitro and In Vivo[1,2]

Lei He, Huanbiao Mo, Susilowati Hadisusilo,* Asaf A. Qureshi† and Charles E. Elson[3]

*Department of Nutritional Sciences, University of Wisconsin, Madison, WI 53706; *Department of Chemistry, University of Indonesia, Depok, Jakarta, Indonesia; and †Advanced Medical Research, Madison, WI 53719*

ABSTRACT Sundry mevalonate-derived constituents (isoprenoids) of fruits, vegetables and cereal grains suppress the growth of tumors. This study estimated the concentrations of structurally diverse isoprenoids required to inhibit the increase in a population of murine B16(F10) melanoma cells during a 48-h incubation by 50% (IC_{50} value). The IC_{50} values for *d*-limonene and perillyl alcohol, the monoterpenes in Phase I trials, were 450 and 250 μmol/L, respectively; related cyclic monoterpenes (perillaldehyde, carvacrol and thymol), an acyclic monoterpene (geraniol) and the end ring analog of β-carotene (β-ionone) had IC_{50} values in the range of 120–150 μmol/L. The IC_{50} value estimated for farnesol, the side-chain analog of the tocotrienols (50 μmol/L) fell midway between that of α-tocotrienol (110 μmol/L) and those estimated for γ- (20μmol/L) and δ- (10 μmol/L) tocotrienol. A novel tocotrienol lacking methyl groups on the tocol ring proved to be extremely potent (IC_{50}, 0.9 μmol/L). In the first of two diet studies, experimental diets were fed to weanling C57BL female mice for 10 d prior to and 28 d following the implantation of the aggressively growing and highly metastatic B16(F10) melanoma. The isomolar (116 μmol/kg diet) and the Vitamin E-equivalent (928 μmol/kg diet) substitution of *d*-γ-tocotrienol for *dl*-α-tocopherol in the AIN-76A diet produced 36 and 50% retardations, respectively, in tumor growth ($P < 0.05$). In the second study, melanomas were established before mice were fed experimental diets formulated with 2 mmol/kg *d*-γ-tocotrienol, β-ionone individually and in combination. Each treatment increased ($P < 0.03$) the duration of host survival. Our finding that the effects of individual isoprenoids were additive suggests the possibility that one component of the anticarcinogenic action of plant-based diets is the tumor growth-suppressive action of the diverse isoprenoid constituents of fruits, vegetables and cereal grains. J. Nutr. 127: 668–674, 1997.

KEY WORDS: • *mice* • *3-hydroxy-3-methylglutaryl coenzyme A reductase* • *tocotrienols* • *monoterpenes* • *tumor implants*

Sundry mevalonate-derived constituents (isoprenoids) of fruits, vegetables and cereal grains suppress chemically initiated carcinogenesis. This action has been attributed to the isoprenoid-mediated induction of detoxifying activities and to the antioxidant activity of some isoprenoids. Neither action explains the potent effect of isoprenoids on the promotion/progression stage of chemically initiated carcinogenesis and on the growth of chemically established and implanted tumors (reviewed by Elson 1995, Elson and Yu 1994). Isoprenoids differ substantially in the effect they have on tumor growth. Isoprenoids suppress, via posttranscriptional actions (Correll et al. 1994, Parker et al. 1993, D. M. Peffley and A. K. Gayen, communication[4]), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[5] reductase activity, the activity deemed to be rate-limiting for the synthesis of cholesterol. Correlations between the late stage tumor-suppressive potency of diverse isoprenoids and their effect on HMG-CoA reductase activity approach unity. The reductase activity of tumors differs from that of liver in being resistant to sterol feedback regulation. The tumor activity, however, retains high sensitivity to posttranscriptional regulation as triggered by diverse isoprenoids. As a consequence of the isoprenoid-mediated suppression of HMG-CoA reductase activity, the pools of mevalonate pathway intermediates become limiting for the posttranslational processing of growth-associated proteins (reviewed by Elson 1995, Elson and Yu 1994).

Our recent review presented a list of structurally diverse isoprenoids with varying capacity to suppress mevalonate synthesis (Elson 1995). We now evaluate the tumor-suppressive potency of a number of these compounds in vitro and that of two, *d*-γ-tocotrienol and β-ionone, in vivo. A lingering question is whether a dietary pattern emphasizing fruit, vegetable and cereal grain can provide isoprenoids in quantities sufficient to be effective. We build on findings that isoprenoids bearing little commonality other than that of sharing a common precursor, isopentenyl pyrophosphate, suppressed melanoma cell

[1] Supported by Public Health Service grant CA 73418 and the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin.

[2] The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ''advertisement'' in accordance with 18 USC section 1734 solely to indicate this fact.

[3] To whom correspondence should be addressed.

[4] Department of Pharmacology and Molecular Biology, Finch University of Health Sciences/The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064.

[5] Abbreviations used: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IC_{50}, the concentration required to suppress the increase in the population of melanoma cells by 50%; TRF, tocotrienol-rich fraction of palm oil; TRF_{25}, oryzanol-free tocotrienol-rich fraction of rice bran oil.

Manuscript received 17 September 1996. Initial review completed 30 October 1996. Revision accepted 23 December 1996.

TABLE 1

The concentrations of selected isoprenoids required to suppress the increase in the population of melanoma cells by 50% during a 48-h incubation.[1] Also listed are representative sources and aroma characteristics of each isoprenoid

Isoprenoid class	*n*	IC_{50}	Representative source	Aroma
		μmol/L		
Monoterpenes				
d-Limonene	3	450 ± 43	Citrus peel, mint	lemon
Perillyl alcohol	3	250 ± 28	Citrus peel, mint, sage, lavender	lilac
Geraniol	3	150 ± 19	Citrus peel, basil, rosemary	fruit
Perillaldehyde	3	120 ± 17	Citrus, basil, rosemary	fruit
Carvacrol	3	120 ± 15	Thyme, marjorum, mint, dill	mint
Thymol	3	120 ± 15	Thyme, oregano, tangerine peel	thyme
Sesquiterpenes				
Farnesol	2	50	Rose, chamomile, lavender, lilac	lilac
β-Ionone	5	140 ± 23	Grapes, corn, apricots, prunes	woody
Tocols				
d-*α*-Tocotrienol	4	110 ± 15	Barley, rice, oat palm, olive oils	oily
d-*γ*-Tocotrienol	6	20 ± 3	Barley, rice, oat bran, palm oils	oily
d-*δ*-Tocotrienol	3	10 ± 3	Barley, rice, oat bran, palm oils	oily
d-2-Desmethyl tocotrienol	3	0.9 ± 0.2	Rice bran	oily
dl-*α*-Tocopherol	4	>1600	Soybean, corn, wheat, barley, rice, oats, palm oils	oily

[1] Values are means ± SD.

proliferation with the demonstration that the effects of individual isoprenoids tested in binary mixtures are additive. We further report that a dietary-relevant intake of *d*-*γ*-tocotrienol suppressed the growth of implanted tumors.

MATERIALS AND METHODS

Isoprenoids. *d*-Limonene (97%), perillyl alcohol (99%), perillaldehyde (92%), carvacrol (98%), thymol (98%), *β*-ionone (96%), geraniol (98%), farnesol (96%) and *dl*-*α*-tocopherol (97%) were purchased from Aldrich Chemical, Milwaukee, WI. An abridged list of concentrated natural sources and aroma characteristics of these isoprenoids appears in **Table 1**. A preliminary study revealed the very potent tumor-suppressive action of the oryzanol-free tocotrienol-rich fraction of rice bran oil (TRF_{25}) prepared by molecular distillation (Dr. Laxman Singh, Vitamins, Chicago, IL). The fraction consisted of 6% *d*-*α*-tocopherol, 12.5% *d*-*α*-tocotrienol, 21% *d*-*γ*-tocotrienol, 10% *d*-*δ*-tocotrienol, 4.5% *d*-tocotrienol, 17% *d*-2-desmethyl tocotrienol, 18% unidentified tocotrienol isomers and 10% sterols and triglycerides (Qureshi et al., unpublished data). The major constituents, *d*-*α*-tocotrienol, *d*-*γ*-tocotrienol, *d*-*δ*-tocotrienol and *d*-2-desmethyl tocotrienol, were isolated by Advanced Medical Research, Madison, WI. A chromatographic procedure was developed to separate *d*-*γ*-tocotrienol from the tocotrienol-rich fraction (TRF) of palm oil (36% *d*-*γ*-tocotrienol, 18% *d*-*α*-tocotrienol, 12% *d*-*δ*-tocotrienol and 22% *d*-*α*-tocopherol), a gift of the Palm Oil Research Institute of Malaysia, Kuala Lumpur, Malaysia. Silica gel (Merck, 60 μm, 150 g) suspended in hexane was poured into a 350-mL glass funnel with a fritted disc. The gel was washed with 1 L hexane prior to being loaded with 5 g of the TRF in 20 mL hexane. The tocols were eluted with the sequential applications of 500-mL mixtures of diethyl ether (5, 10, 12, 14, 16, 18, 20, 22, 25 and 30%) in hexane. The elution of each application of solvent into a filter flask was speeded by the application of vacuum produced by water aspiration. The eluates were dried under vacuum, the residues redissolved in hexane and identified according to retention time and absorption profile using an analytical HPLC system. The fraction eluted with 18% diethyl ether was predominantly (98%) *d*-*γ*-tocotrienol.

IC_{50} determinations. Murine B16(F10) melanoma cells, a tumor cell line with high metastatic potential (Tsukamoto et al. 1991) obtained from Dr. William B. Ershler,[6] were grown in monolayer culture (35 × 10 mm flasks) in 3 mL RMPI 1640 media (Sigma, St. Louis, MO) supplemented with 10% newborn calf serum (GIBCOBRL, Grand Island, NY) and 80 mg/L gentamycin (Sigma). Cultures, seeded with $1–1.5 \times 10^5$ cells, were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. Isoprenoids, dissolved in absolute ethanol, were added at 24 h (0 time); all cultures contained 5 mL ethanol/L (85 μmol/L). The cultures were incubated for an additional 48 h. The medium was removed and the monolayers were washed twice with Hanks' balanced salt solution (Sigma) and then incubated with a trypsin-EDTA solution (Sigma) at 37°C for 2 min. Trypsin was inactivated by suspending the cells in medium containing 10% fetal bovine serum (Sigma). The cells were pelleted at 250 × *g* and resuspended in Hanks' balanced salt solution. Viable cells, cells that excluded 0.4% trypan blue (Gibco BRL), were counted with a hemocytometer; 24-h cell counts were deducted from final cell counts to provide an estimate of the net increase in cell number. The calculation of the concentration of an isoprenoid required to inhibit the net increase in the 48-h cell count by 50% (IC_{50}) is based on plots of data from three or more evaluations.

Animal studies. This evaluation of the tumor-suppressive potency of diverse isoprenoids was extended with two dietary studies. The first examined the effect of a diet-relevant intake of *d*-*γ*-tocotrienol on the growth of B16(F10) melanomas in host mice. The second study evaluated the effect of pharmacological intakes of *d*-*γ*-tocotrienol and *β*-ionone on the postimplant survival of melanoma-bearing mice. We first prepared a basal diet mix, patterned after the AIN-76A formulation (AIN 1977) but free of corn oil and *dl*-*α*-tocopherol. These ingredients and vitamin E-stripped corn oil were purchased from Teklad Test Diets, Madison, WI. Stock solutions of *dl*-*α*-tocopherol (80 μmol/g) and *d*-*γ*-tocotrienol (80 μmol/g) were prepared with vitamin E-stripped corn oil. Stock solutions of the tocols, diluted with vitamin E-stripped corn oil, were mixed with the basal diet to provide finished diets containing 5% corn oil and specified tocol concentrations. Where noted, *β*-ionone was added to the oil. The diets, mixed weekly, were stored under refrigeration. Food cups were cleaned and refilled daily.

Experiment 1. Weanling C57BL female mice (Harlan Sprague Dawley, Madison, WI) were housed in groups of four on wood shavings in plastic cages and maintained at 25°C with a 12-h light:dark cycle. The four groups of mice (20/group) were fed experimental diets for 10 d prior to and 28 d following the implantation of B16 melanoma cells. The split-plot design consisted of four treatments comprised of two tocols, *dl*-*α*-tocopherol and *d*-*γ*-tocotrienol, each presented at two levels, 116 and 924 μmol/kg diet. This design permitted two

[6] Department of Medicine, University of Wisconsin-Madison, 600 North Highland Avenue, Madison, WI 53792.

comparisons of diets equal in tocol content and one comparison of diets about equal in *d*-α-tocopherol equivalents. Lacking a definitive estimate of the biological activity of *d*-γ-tocotrienol, we considered reports that the 2, 5, 8 (*d*-β-) and 2, 7, 8 (*d*-γ-) trimethyl tocopherols have similar oxygen scavenging activity [maximally 66% that of the 2, 5, 7, 8 (*d*-α-) tetramethyl tocopherol] and that as a class, the tocotrienols have 5–30% of the biological activity of the tocopherols (Kamal-Eldin and Appelqvist 1996) in developing the rough estimate that *d*-α-tocopherol has, minimally, sixfold the biological activity of *d*-γ-tocotrienol. We then corrected for the biological activity of the *dl*-α-tocopherol mixture (70% of the activity of *d*-α-tocopherol) in arriving at the 8:1 tocotrienol/tocopherol ratio used in formulating the experimental diets. Melanoma cells, cultured and harvested as previously described (Shoff et al. 1991), were washed twice with RPMI 1640 containing 10% fetal bovine sera (Gibco BRL). The pelleted cells were suspended in RMPI 1640 and counted (98% viable) after a 1:20 dilution in 0.4% trypan blue. The cells were further diluted in RPMI 1640 (1×10^8 cells/L), and 0.1 mL of the suspension (1×10^4 cells) was injected subcutaneously into the flank of each mouse. The study was terminated d 28 when the first mouse, a mouse in the control group, died. The mice were killed by CO_2 overdose and the tumors were excised and weighed. The protocol was reviewed and approved by the College of Agricultural and Life Sciences Animal Care Committee.

Experiment 2. Weanling female C57BL female mice (n = 60, Harlan Sprague Dawley) were acclimated to the housing conditions and AIN-76A diet (116 μmol *dl*-α-tocopherol/kg diet) as described above. Tumors were implanted and the mice continued to receive the AIN-76A diet. Beginning on d 8 postimplant, the mice were palpated daily for the presence of a tumor. Tumors were first detected on d 14. Random numbers were generated for assigning each mouse in a sequential subset of five to a diet. Experimental treatments provided 2 and 4 mmol *d*-γ-tocotrienol/kg diet. We additionally tested the effect of β-ionone (2 mmol β-ionone/kg AIN-76A diet) and that of a blend of the two isoprenoids (2 mmol each/kg diet) on the survival of the mice. The mice continued to receive the respective experimental diets; moribund mice, identified by the Research Animal Resource-trained supervisor who was unaware of the experimental design, were killed by CO_2 overdose. The protocol was reviewed and approved by the College of Agricultural and Life Sciences Animal Care Committee.

Statistical methods. StatView and SuperANOVA software (Abacus Concepts, Berkeley, CA) were used for the assessment of treatment-mediated effects. Treatment-mediated differences in body and tumor weights, days to tumor appearance and days to morbidity were identified with split-plot ANOVA and pairwise t tests of least-squares means. Treatment-mediated differences in days to morbidity were also assessed using parametric (paired t test) and nonparametric (Wilcoxon Signed Rank) tests (Haycock et al. 1992).

RESULTS

Figure 1 shows a representative plot of the dose-dependent effect of five tocols on the growth of B16 melanoma cells. *dl*-α-Tocopherol had no effect on cell number. The growth-suppressive potency of the individual tocotrienols was inverse to the number of methyl groups on the 6-chromanol ring: *d*-α-tocotrienol (methyl groups at carbons 5, 7, 8 and 2) $\ll$ *d*-γ-tocotrienol (methyl groups at carbons 7, 8 and 2) $<$ *d*-δ-tocotrienol (methyl groups at carbons 8 and 2) $\ll$ *d*-2-desmethyl tocotrienol (no methyl groups) (Table 1). Within this series of tocotrienols, the IC_{50} value was inversely related to the number of methyl groups on the 6-chromanol ring. Similarly, the IC_{50} values calculated from plots for the more polar monoterpenoid alcohols, perillyl alcohol ([r]-4-isopropenyl-1-methanol-cyclohexene), perillaldehyde ([r]-4-isopropenyl-1-carboxaldehyde cyclohexene), thymol (5-methyl-2 isopropylphenol), carvacrol (5-isopropyl-2-methylphenol) and geraniol (*trans*-3,7-dimethyl-2,6-octadien-1-ol) were much lower than that for *d*-limonene ([r]-4-isopropenyl-1-methyl-1-cyclohexene). The IC_{50} value for β-ionone, the end ring analog of β-carotene, matched those of the more polar monoterpenes (Table 1). The IC_{50} for farnesol (*trans, trans* 3,7,11-trimethyl-2,6,10 dodecatrien-1-ol), a sesquiterpene and a structural analog to the side chain of the tocotrienols, fell midway between those of *d*-α-tocotrienol and *d*-γ- and *d*-δ-tocotrienol.

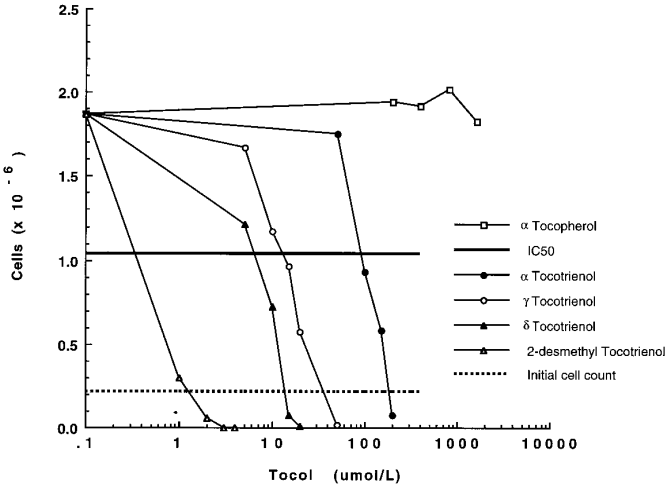

FIGURE 1 A representitive evaluation of the dose-dependent effect of tocols on the proliferation of melanoma B16 cells. Cultures (3 mL) seeded with $1–1.5 \times 10^5$ cells were incubated for 24 h prior to the introduction of the tocols. Viable cells were counted at 48 h following the addition of the tocols. The cell count at 24 h (0 time) is shown by the dashed line. The intersection of the solid horizontal line and the line plotted for each tocol indicates the concentration at which the tocol suppressed by 50% the increase in cell number during the incubation (IC_{50} value). IC_{50} values (mean, SD and n) for all isoprenoids tested are listed in Table 1.

The first dietary study evaluated the effect of *d*-γ-tocotrienol and *dl*-α-tocopherol on days to detection of a solid tumor and growth of implanted B16 melanomas. As noted above, the design permitted two comparisons of treatments providing equal tocol concentration (116 and 928 μmol/kg diet) and one comparison of treatments providing about 80 μmol *d*-α-tocopherol equivalents (35 mg)/kg diet. At the time of tumor implant, the body weight of mice receiving the high tocopherol diet was significantly lower than that of mice receiving the low tocol diets (**Table 2**). At 28 d postimplant, the melanomas accounted for 15% of the weight of mice receiving the AIN-76A diet. Two tocols were tested, each at two levels. The split-plot ANOVA showed that the effects of tocols ($P < 0.001$) and levels ($P < 0.04$) on 28-d tumor weight were significant; there was no evidence of an interaction between the two factors ($P = 0.77$). Least mean squares analyses confirmed the tumor growth-suppressive action of γ-tocotrienol (Table 2). The other measure of tumor growth, days postimplant to tumor detection, showed tocol ($P < 0.03$), level ($P < 0.01$) and the interaction ($P < 0.02$) to be significant. The least mean squares analysis showed that the effect of the treatment providing 928 μmol *d*-γ-tocotrienol/kg diet on this measure of tumor growth differed significantly from the effects of the other treatments.

We next determined that the effects of the isoprenoids on the growth of B16 melanoma cells in culture are additive. B16 melanoma cells were incubated with β-ionone and *d*-γ-tocotrienol in one test (**Fig. 2***a*). *d*-γ-Tocotrienol (7.5 μmol/L) and β-ionone (50 μmol/L) reduced the 48-h cell count by 19 and 10%, respectively (Fig. 2*a*). The 34% reduction in cell number achieved with the paired isoprenoids exceeded the 29% reduction predicted by the sum of the individual effects

TABLE 2

Effect of d-γ-tocotrienol on days to detection and 28-d growth of B16 melanomas implanted into the flanks of mice[1]

Tocol	μmol/kg diet	Body weight		Tumor		
		10 d	38 d	Detection[2]	Weight	Liver weight
		g	*g*	*d*	*g*	*g*
dl-α-Tocopherol	116	17.33[a]	23.30	19.55[b]	3.59[a]	1.18[a]
d-γ-Tocotrienol	116	17.02[a]	22.43	19.68[b]	2.31[bc]	1.12[ab]
dl-α-Tocopherol	928	16.41[b]	22.53	19.75[b]	2.89[ab]	1.06[b]
d-γ-Tocotrienol	928	16.83[ab]	22.56	22.90[a]	1.78[c]	1.03[b]
Pooled SEM		0.10	0.19	0.35	0.17	0.02

[1] Values are means, $n = 20$. [a–c] Means not sharing a superscript are different ($P < 0.05$).
[2] Days postimplant for appearance of a palpable tumor.

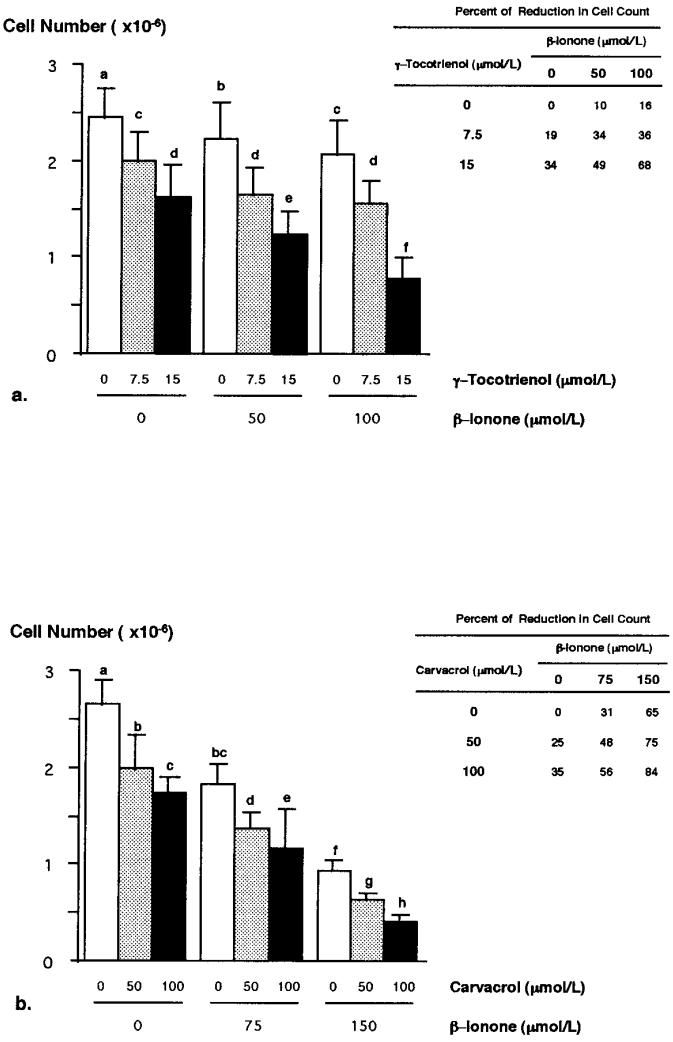

FIGURE 2 Assessment of growth suppression imposed by individual isoprenoids and equivalent blends of paired isoprenoids. (*a*) Additive effects of γ-tocotrienol and β-ionone on B16 melanoma cell populations. Values are means ± SD, $n = 28$; pooled SEM = 36 ($\times 10^4$). Cell counts calculated to show the decreased population relative to the control are listed in the inset table. [a–f]Means not sharing a superscript are different ($P < 0.001$). (*b*) Additive effects of carvacrol and β-ionone on B16 melanoma cell populations. Values are means ± SD, $n = 28$; pooled SEM = 62 ($\times 10^4$). Cell counts calculated to show the decreased population relative to the control are listed in the inset table. [a–h]Means not sharing a superscript are different ($P < 0.001$).

(Fig. 2*a*, *inset*). At higher concentrations *d-γ*-tocotrienol (15 μmol/L) and β-ionone (100 μmol/L) reduced the 48-h cell count by 34 and 16%, respectively; the additive effect, a 68% reduction in cell number, was also greater than the sum of the individual effects (59%) (Fig. 2*a*, *inset*). The foregoing results pointed to a possible synergistic action of *d-γ*-tocotrienol and β-ionone, whereas the study pairing carvacrol and β-ionone revealed only an additive effect (Fig. 2*b*). Carvacrol (50 μmol/L) and β-ionone (75 μmol/L) reduced the 48-h cell count by 25 and 31%, respectively; the additive effect, a 48% reduction in cell number, was less than the 56% reduction predicted by the sum of the individual effects. Carvacrol (100μmol/L) and β-ionone (150 μmol/L) reduced the cell count by 35 and 65%, respectively; the additive effect, an 84% reduction in cell number, again was less than the 100% reduction predicted by the sum the individual effects (Fig. 2*b*, *inset*). The reduction in cell number predicted by the sum of the individual effects of the isoprenoids was highly correlated with that achieved with the paired isoprenoids ($r = 0.91$, $n = 8$, $P < 0.01$).

We then asked if either dietary *d-γ*-tocotrienol or β-ionone would prolong the survival of mice bearing implanted melanomas. Dietary treatments were initiated following the detection of solid tumors. We also asked whether these structurally diverse isoprenoids would have an additive effect on survival. The experimental groups were constructed by random assignment of each member of successive subsets of five mice to one of the experimental diets. Time to tumor detection did not differ between groups (**Table 3**). Treatments increased median duration of sur-

TABLE 3

Effect of isoprenoid-enriched diets on the duration of survival of host mice bearing established melanomas[1]

Group	*d-γ*-Tocotrienol	β-Ionone	Days to tumor	Days survival	
				Mean	Median
	mmol/kg diet				
Control	0	0	15.00	13.83[b]	15.0
d-γ-Tocotrienol	2	0	14.92	18.67[a]	20.0
d-γ-Tocotrienol	4	0	14.92	18.46[a]	22.0
β-Ionone	0	2	14.92	18.27[a]	23.0
Blend	2	2	14.92	19.50[a]	20.5
Pooled SEM				0.547	

[1] Values are means, $n = 12$. [a–b] Means not sharing a superscript are different ($P < 0.03$).
[2] Each mouse was asigned to a treatment group following the detection of a solid tumor.

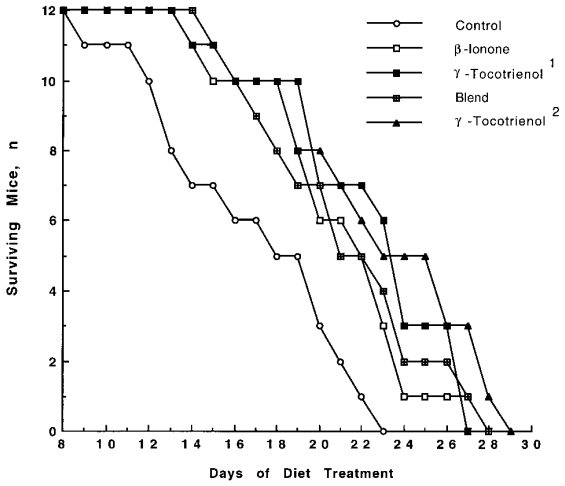

FIGURE 3 Survival curve for host mice receiving isoprenoid-enriched diets following the detection of a solid implanted B16 melanoma. Mean survival durations are presented in Table 4. β-Ionone and γ-tocotrienol* treatments provided 2 mmol of the isoprenoid/kg diet; the blend provided 2 mmol of each isoprenoid/kg diet; and γ-tocotrienol** provided 4 mmol γ-tocotrienol/kg diet.

vival by 42 ± 4.5% and the mean duration of survival by 30% ($P < 0.005$); differences between duration of survival means of the experimental groups were not significant (Table 3, **Fig. 3**). According to the experimental design, each mouse within a subset of five could be paired with another member of the subset for testing. *P*-values for the pairwise comparisons, assuming normal and abnormal distributions, are listed in **Table 4**. All analyses revealed significant differences between the control and treatment group means; differences among the treatment group means were not significant. Lending credibility to the results of the in vitro analysis showing an additive effect of the two isoprenoids is the trend shown in Table 4.

DISCUSSION

The B16(F10) melanoma provides a rigorous model for assessing, in vitro and in vivo, the potency of pharmacological agents (Gruber et al. 1992, Kuwashima et al. 1990, Mac Neil et al. 1992, Shoff et al. 1991, Tsukamoto et al. 1991). We now confirm our finding (Shoff et al. 1991) that the IC_{50} concentration of geraniol, like that of similar oxygenated monoterpenes, falls around 150 μmol/L (Table 1). Cyclic oxygenated monoterpenes tended to be somewhat more potent, whereas the hydrocarbon monoterpene, *d*-limonene, was considerably less potent. As we reported elsewhere (Crowell et al. 1991), the hepatic metabolite of *d*-limonene, perillyl alcohol, is more potent than the parent compound. β-Ionone, the end-ring analog of β-carotene, suppressed the growth of melanoma cells with a potency similar to that of the oxygenated monoterpenes. The tumor cell growth-suppressive action of farnesol (Adany et al. 1994) is confirmed. The tocotrienols, essentially tocol analogues of this sesquiterpenoid, very effectively suppressed the net increase in B16 melanoma cell population.

Each of these isoprenoids suppresses HMG-CoA reductase activity, an activity essential to the provision of isoprenoid anchors for the attachment of nuclear lamins and ras proteins to cellular membranes (see Elson 1995). The posttranscriptional down-regulation of HMG-CoA reductase activity by farnesol (Correll et al. 1994, Meigs et al. 1996), the tocotrienols (Parker et al. 1993), geraniol, perillyl alcohol and *d*-limonene (D. M. Peffley and A. K. Gayen, communication[4]) involves both the

TABLE 4

Pairwise comparisons of isoprenoid effects on the duration of survival following detection of a B16 melanoma in the flanks of mice

	Paired *t* test		Wilcoxon Signed Rank	
Group comparison	*t*-value	*P*-value	*Z*-value	*P*-value
Control vs.				
d-γ-Tocotrienol[1]	5.74	0.01	−2.93	0.01
d-γ-Tocotrienol[2]	2.70	0.02	−2.22	0.03
β-Ionone[3]	2.81	0.02	−2.18	0.03
Blend[4]	3.27	0.01	−2.45	0.01
d-γ-Tocotrienol[1] vs.				
d-γ-Tocotrienol[2]	0.46	0.66	−0.47	0.64
β-Ionone[3]	0.06	0.95	−0.15	0.88
Blend[4]	0.46	0.66	−0.47	0.64
d-γ-Tocotrienol[2] vs.				
β-ionone[3]	0.11	0.92	−0.06	0.95
Blend[4]	1.36	0.21	−1.42	0.16
β-Ionone[3] vs.				
Blend[4]	0.93	0.37	−0.83	0.41

[1] 2 mmol *d*-γ-tocotrienol/kg diet.
[2] 4 mmol *d*-γ-tocotrienol/kg diet.
[3] 2 mmol β-ionone/kg diet.
[4] 2 mmol *d*-γ-tocotrienol + 2 mmol β-ionone/kg diet.

induction of an HMG-CoA reductase-specific cysteine protease (Correll et al. 1994, Meigs et al. 1996, Parker et al. 1993) and an apparent decrease in reductase mRNA translational efficiency (Parker et al. 1993, D. M. Peffley and A. K. Gayen, communication[4]). A general ordering of the effect of these isoprenoids on B16 cell population matches that of the ordering of their effect on HMG-CoA reductase activity. Mevalonate deprivation instituted by lovastatin suppresses cell proliferation (DeClue et al. 1991) and initiates apoptosis (Perez-Sala and Mollinedeo 1994). Our preliminary findings indicate that the isoprenoid effect on net cell number involves both actions, the suppression of proliferation and the initiation of apoptosis (H. Mo and C. E. Elson, unpublished).

The efficacy of dietary isoprenoids in the prevention of chemically initiated carcinogenesis has been reviewed (Elson and Yu 1994). The growth of Ehrlich carcinomas implanted in mice was suppressed by 1 μmol *d*-γ-tocotrienol administered by intraperitoneal injection (Komiyama et al. 1989). The growth of P388 leukemia and B16 melanoma cells implanted in mice was significantly suppressed by dietary intakes of 20–25 μmol geraniol/d (Shoff et al. 1991, Yu et al. 1995). We now report that a diet providing an intake of 0.4 μmol *d*-γ-tocotrienol/d suppressed the growth of the B16 melanoma implanted in the flank of host mice. In our more rigorous test, a diet providing 7 μmol *d*-γ-tocotrienol or β-ionone/d suppressed the growth of established B16 melanomas. In vitro tests provided evidence of the additive effects of these two isoprenoids (Fig. 2*a, b*). The diet providing an intake of 7 μmol *d*-γ-tocotrienol/d increased the duration of survival by 35%. Doubling the intake of *d*-γ-tocotrienol to 14 μmol/d did not further increase the duration of survival (−0.21 d, $P = 0.95$), whereas adding an intake of 7 μmol of β-ionone to that of 7 μmol *d*-γ-tocotrienol/d may have increased the duration of survival (+0.83 d, $P = 0.65$, Tables 3, 4). This study evaluated the responses of established implanted melanomas to β-ionone and *d*-γ-tocotrienol, two isoprenoids with structural relationships to the two antioxidant nutrients recently discounted as having tumor-suppressive ac-

tions (Greenberg and Sporn 1996). Both isoprenoids significantly increased survival time (Table 4). We note findings that a massive intake of ascorbic acid (733 μmol/d) suppressed the growth of implanted B16 melanomas (Meadows et al. 1991). Our studies showed that a diet providing an eightfold elevation in *d*-α-tocopherol equivalents had a marginal effect on tumor growth, whereas a *d*-γ-tocotrienol diet, providing only the *d*-α-tocopherol-equivalent of the AIN-76A diet, yielded a significant suppression of tumor growth.

We propose that the answer to the question why HMG-CoA reductase activity in sterol feedback-resistant tumor cells is more sensitive than that of sterol feedback-sensitive cells (Kawata et al. 1990) to suppression by diverse isoprenoids (Elson and Yu 1994, Elson 1995) might be forthcoming from studies of the aberrant methylation pattern (Counts and Goodman 1995, Laird and Jaenisch 1994) in the 5′-flanking region of the reductase gene in tumor tissues (Coni et al. 1992, Rossiello et al. 1994, Vasudevan et al. 1994). Pharmacological targets relevant to the mevalonate pathway include HMG-CoA reductase (Thibault et al. 1996), mevalonate kinase (Cuthbert and Lipsky 1995, Thibault et al. 1995) and the farnesyl protein transferase (Kohl et al. 1995). Lovastatin (Thibault et al. 1996), sodium phenylacetate (Thibault et al. 1995) and perillyl alcohol (Kelloff et al. 1994), respectively, are being evaluated in Phase I and II clinical trials.

On the basis of extrapolations from animal tumor regression studies (13 mmol perillyl alcohol/kg diet), the protocol for the clinical evaluation of perillyl alcohol incorporates doses in excess of 50 mmol/d (Gould 1995), a level of intake not attainable through the diet. An extrapolation based on data presented in Table 2 predicts that a dose of *d*-γ-tocotrienol approaching 1 mmol/d will be sufficient to alter the course of tumor growth in humans. Although tocotrienol research has not progressed sufficiently to justify testing for use in human chemotherapy, we have reported that an intake of 0.5 mmol/d *d*-γ-tocotrienol is sufficient to lower cholesterol levels of hypercholesterolemic humans (Qureshi et al. 1995).

Can isoprenoids in sufficient quantity to be health protective be obtained from the diet? Isoprenoids are ubiquitous constituents of higher plants. Geranyl pyrophosphate and farnesyl pyrophosphate are the precursors of monoterpenes (Croteau 1981) and sesquiterpenes (Crane 1981), respectively, numbering in the hundreds. Geranylgeranyl pyrophosphate is the precursor of diterpenes, carotenoids and terpenoids of mixed biogenic origin including the tocotrienols (West 1981). However, data banks offer little information concerning the isoprenoid constituents of foods. Isoprenoids differ substantially in the potency of their prospective tumor suppressive action; within the group of 12 isoprenoids evaluated for this report, there was a 500-fold range in potency (Table 1). Determination of the dietary relevancy of the antitumor action of isoprenoids requires estimates of dietary intake, an expansion of the list of tumor-suppressive isoprenoids and their potencies, and confirmation of our finding that the isoprenoids have an additive tumor-suppressive effect (Fig. 2). Our findings are consistent with the consensus finding that populations consuming diets rich in fruits, vegetables and cereal grains, the dietary sources of isoprenoids, have a reduced cancer risk (Block et al. 1992).

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