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Inhibition of growth and cholesterol synthesis in breast cancer cells by oxidation products of β-carotene

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We have isolated and chemically characterized a polar oxidation product of β-carotene and tested the effect of a highly enriched fraction containing this compound on the growth and metabolism of breast cancer (MCF-7) cells. This fraction strongly inhibits cell growth and cholesterol synthesis in MCF-7 cells. Pretreatment of the cells with mevalonate overcomes inhibition of cell growth by the oxidized fraction. Addition of the antioxidant butylated hydroxytoluene protects against inhibition of the growth of MCF-7 cells by β-carotene but not by the oxidized fraction. Pretreatment of cells with mevalonate overcomes inhibition of cell growth by oxidation products of β-carotene but not by retinoic acid. The oxidized fraction neither stimulates activity nor inhibits binding of retinoic acid to its nuclear receptors (RXR-α, RXR-β, RXR-γ, RAR-α, RAR-β, RAR-γ, and peroxisome proliferation receptors) in transfection assays. Mevalonate does not protect retinoic acid-induced growth inhibition of MCF-7 cells. The major compound in the inhibitory fraction was identified by mass spectrometry and nuclear magnetic resonance spectroscopy as 5,8-endoperoxy-2,3-dihydro-β-apocarotene-13-one. Our data suggest that the β-carotene oxidation products we have isolated represent a class of compounds not previously described with potential antineoplastic activity. (J. Nutr. Biochem. 9:567–574, 1998) © *Elsevier Science Inc. 1998*

Keywords: β-carotene; oxidation; MCF-7 cells; cholesterol; mevalonate; cancer

Introduction

Long-term dietary consumption of fruits and vegetables high in β-carotene is associated with a lowered risk for cancer and heart disease.[1,2] In addition, in rats, mice, and hamsters, supplementation with carotenoids prior to initiation by ultraviolet (UV) or chemical carcinogens prevents tumorigenesis.[3,4] In cultured cells, carotenoids promote the synthesis of transmembrane proteins and inhibit cell division and growth of transformed cells.[5,6] However, β-carotene supplementation does not protect against human heart disease or cancer.

An hypothesis that has not previously been explored is that oxidation products of β-carotene are responsible for its anticancer activities. To test this hypothesis, we have oxidized β-carotene under controlled chemical conditions. From this mixture we have isolated, and chemically characterized by mass spectral and nuclear magnetic resonance (NMR) spectroscopy, a major oxidation product of β-carotene. Using a fraction highly enriched in this product, we have assessed its effect on growth and cellular metabolism in breast cancer (MCF-7) cells.

Materials and methods

Reagents

β-Carotene (>97%) was obtained from Fluka Biochemical Co. (Ronkonkoma, NY USA); 3-chloroperoxybenzoic acid (*m*CPBA)

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and 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) were from Aldrich Chemical Co. (Milwaukee, WI USA); dichloromethane and acetonitrile (ACN) were from Mallinckrodt Specialty Chemical Co. (Paris, KY USA); methanol (MeOH), tetrahydrofuran (THF), and hexane were from Baxter Health Care Co. (Muskegon, MI USA); ethyl acetate was from EM Science (Gibbstown, NJ USA); fetal bovine serum was from HyClone Lab Inc. (Logan, UT USA); and ^{14}C acetate was from Amersham Corporation (Arlington Heights, IL USA). All other reagents were analytical grade or better and were purchased from Sigma Chemical Co. (St. Louis, MO USA).

High performance liquid chromatography analysis

High performance liquid chromatography (HPLC) analysis was performed using a Waters Associates chromatographic pump (model 510, Waters Corp., Milford, MA USA) equipped with a C_{18}-120A-ODS 10 × 250 mm reversed phase columns (4.6 × 250 mm, 10 × 250 mm or 20 × 250 mm) (YMC, Morris Plains, NJ USA), and a Sperisorb-CN 5μ (4.6 × 250 mm) normal phase column (Alltech, Deerfield, IL USA). Detection was at 325 or 280 nm using a photodiode array detector (Model 1040A, Hewlett-Packard Corp., Avondale, PA USA).

Mass spectral analysis

HPLC-mass spectrometry spectra were obtained using a Finnigan TSQ 7000 mass spectrometer (San Jose, CA USA) equipped with an atmospheric ionization source and electrospray and atmospheric pressure chemical ionization probes. Samples were introduced into the HPLC-MS system using the HPLC column conditions outlined above. The mass spectrometer was scanned from 100 to 1000 daltons in 1 second. The collision induced dissociation (CID) MS-MS spectra were obtained by connecting the mass spectrometer to the HPLC system with the column removed. The mobile phase used for these flow injection experiments was methanol at a flow rate of 0.5 mL/min. The precursor ions were selected in the first mass analyzer of the TSQ 7000. CID was accomplished in a radio frequency (RF)-only collision cell with a gas pressure of 1 mTorr of argon and a collision energy of 25 eV. The scan range on the last mass analyzer was from m/z 50 to 10 amu above the precursor mass at a scan rate of 3 seconds per scan. The data were co-averaged for 45 scans to improve signal-to-noise ratio.

Nuclear magnetic resonance analyses

NMR spectra were acquired on a Bruker AM-500 spectrometer operating at a ^{1}H frequency of 500.13 MHz, using an inverse broadband 5 mm probe with temperature regulation at 30°C. Two-dimensional (2D) spectra were acquired in time proportional phase incrementation (TPPI) mode[7] with 2048 complex data points in t_2 and 750 real datapoints in t_1. For heteronuclear experiments, no ^{13}C decoupling was used during acquisition. A skewed 45 degree-shifted sine-bell window was used for heteronuclear 2D spectra and an unshifted sine-bell was used for the double quantam filtered correlation spectroscopy (DQF-COSY) spectrum. The residual $CHCl_3$ peak was used as a reference for ^{1}H (7.24 ppm) and ^{13}C (77.0 ppm), and as a quantitation standard (calibrated with a sample of methyl nicotinate).

Oxidation of β-carotene and analysis of products

Varying the time, temperature, and *m*CPBA:β-carotene ratio (1:2 to 5:1, w/w) revealed that incubation with a ratio of 2:1 and *m*CPBA:β-carotene (w/w) for 60 minutes at 25°C provided the optimal yield of the fraction of interest (approximately 0.02% of starting material). β-Carotene (typically 500 mg/reaction) was oxidized with *m*CPBA in methylene chloride (1:2:12 w/w/v) for 40 minutes at 25°C. Following the reaction, 2 g sodium carbonate was added and the mixture held at room temperature for 30 minutes and separated by centrifugation (20,000 × g, 2 min). The supernatant was removed, evaporated to dryness under nitrogen, and suspended in 100% methanol (MeOH). Following a second centrifugation step to remove hydrophobic compounds, the supernatant was fractionated on HPLC with a C_{18} 120A-ODS 10 × 250 mm reversed phase column using a mobile phase of MeOH: H_20(80:20, v/v), eluted at 2.5 ml/min and detected at 325 nm (*Figure 1A*). The major peak was collected and its UV maximum was determined to be 280 nm. This product then was fractionated on the same column at 0.5 ml/min in 100% ACN to four fractions detected at 280 nm (*Figure 1B*).

Following elution from the column, UV profiles of the isolated fractions were determined so that further HPLC elutions could be monitored at the UV maxima of the compounds of interest. Oxidation of β-carotene produces a large number of hydrophobic products, which have been described by others.[8–10] In these studies, however, only the polar fraction of interest (*Figure 1A*) was collected. Injection of fraction 2 (*Figure 1B*) on HPLC in MeOH:isopropanol (80:20, v/v) and ACN:aqueous ammonium acetate 4.0 mM (97:3, v/v), using a Sperisorb-CN 5μ 4.6 × 250 mm normal phase column at 1 ml/min, yielded fraction 2A as a single peak (*Figure 1C*). Homogeneity of fraction 2A was verified on three additional reversed phase systems and one normal phase [hexane:ethyl acetate (95:5, v/v)] HPLC system. Because of the time and expense required to produce large quantities of fraction 2A, this fraction was reserved for NMR and MS analyses. Fraction 2 was used for routine experiments, but results of all experiments were verified at least once using fraction 2A.

A sample of mobile phase, designated "solvent control," collected at baseline adjacent each test fraction was carried through all subsequent phases of the experiment simultaneously with the test fractions. Products and control samples were evaporated to dryness with nitrogen and stored at –20°C until use (within 2 weeks of preparation). Relative HPLC migration (k') and UV spectra of fractions prepared and stored in this manner were unchanged after 1 month.

For the purpose of comparisons with other test compounds, concentrations of impure fractions were estimated using the molar extinction coefficient of β-ionone ($\epsilon_{M\ 290}$ = 8700), a β-carotene oxidation product with a UV absorption spectrum similar to that of fraction 2.[8]

Cell culture

MCF-7 cells from human breast cancer tissue (Arizona Cancer Center, Tucson, AZ USA), were maintained in tissue culture in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U of both penicillin and streptomycin. Cells were incubated at 37°C under 5% CO_2 in air. Exponentially growing cells (1×10^4) were seeded into flasks. After incubation for 24 hours, the medium was replaced with fresh medium containing test compounds. The final solvent concentration in the media (THF or MeOH) was 0.1% or less. Cells were released by trypsin treatment and cell viability verified using Trypan blue dye exclusion.[11] For a single experiment, the number of cells was determined by counting and averaging four separate microscopic fields on each of three plates. Unless otherwise indicated, results are the average of three experiments performed in this manner on separate days.

Protein concentrations were determined from cell lysates using a modification of the Bradford dye-binding procedure.[12]

Cholesterol synthesis

Cholesterol synthesis in MCF-7 cells was assayed using a modification of a previously described method.[13] Following incubation of MCF-7 cells with ^{14}C acetate for 2 to 4 hours, cholesterol and cholesteryl esters were extracted and esters hydrolyzed with

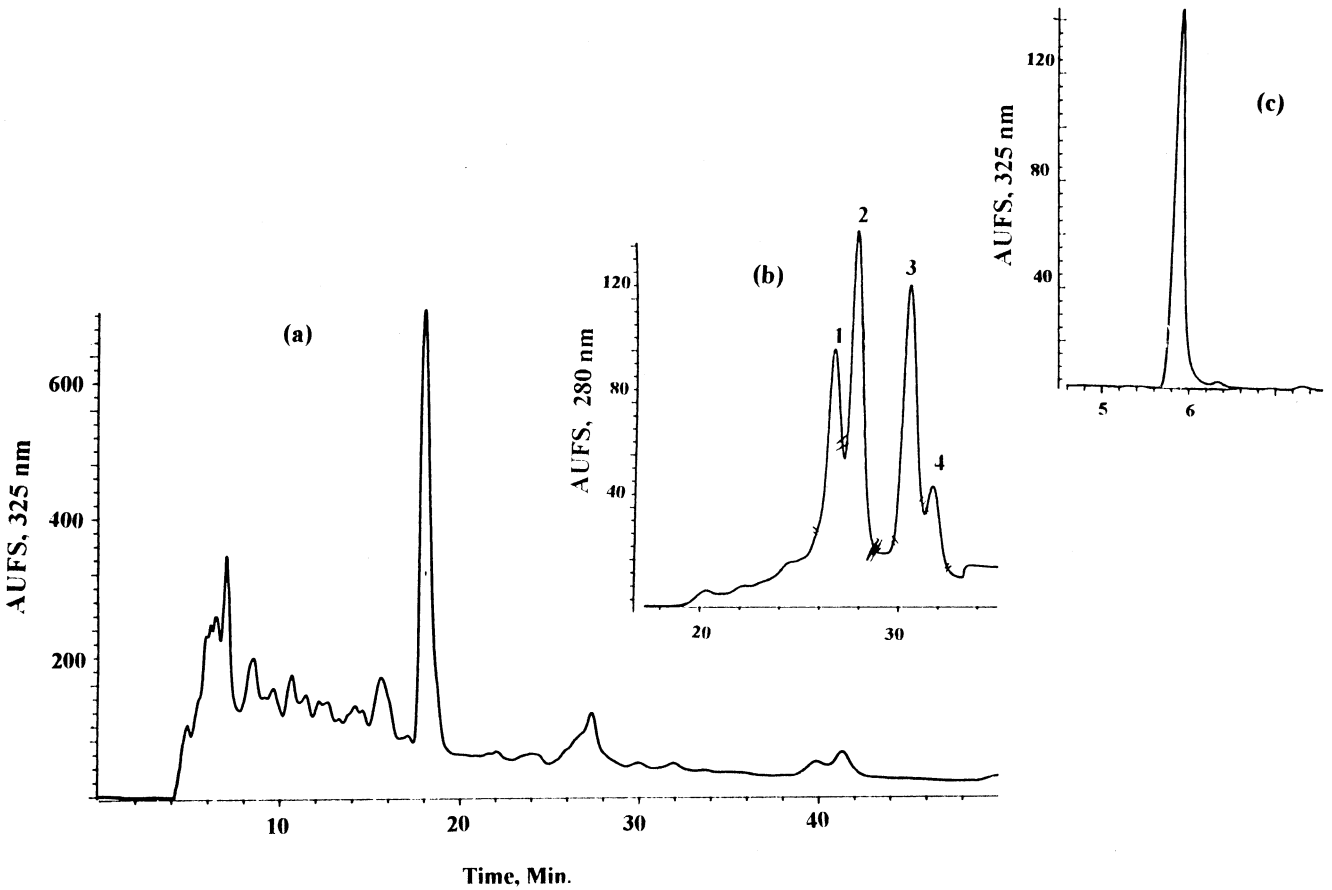

Figure 1 High performance liquid chromatography profile of oxidation products of β-carotene. Following oxidation with *m*CPBA in methylene chloride for 40 minutes at 25°C, the reaction mixture was separated in (a) MeOH/H_2O, 80:20 (v/v) and purified using (b) 100% ACN and (c) hexane:ethanol (95:5, v/v) as described in Materials and Methods. Hash marks on (b) indicate the area of the peak that was collected.

cholesterol esterase. To provide a chromophore for UV detection on HPLC, free cholesterol was oxidized to cholest-4-ene-3-one using cholesterol oxidase. The fraction co-eluting with authentic standard was collected and radiolabelled products were assayed by scintillation spectrometry. Results were standardized to cellular protein.

Nuclear receptor cotransfection assay

Expression plasmids for nuclear receptors were cotransfected with luciferase reporter plasmids into CV-1 cells as previously described.[14] After transfection, cells were treated with solvent (ethanol) or fraction 2. Transactivation results were expressed as relative light units (RLU) normalized for transfection efficiency using an internal β-galactosidase marker as a control.

Results and Discussion

Chemical characterization

Using the five HPLC systems described in Materials and Methods, we could identify only a single compound in fraction 2. Liquid chromatography/mass spectral analysis (HPLC-APCI-MS-MS) again detected only a single compound, yielding a collision-induced product ion of m/z at 291. The atmospheric pressure chemical ionization (APCI) mass spectrum confirmed a molecular weight of 290 daltons by the observation of MH+ at m/z 291. The CID MS-MS results for the MH+ (m/z 291) are consistent with the presence of three oxygen atoms in a β-apocarotene structure.

However, NMR analysis (heteronuclear multiple-quantam correlation [HMQC], heteronuclear multiple-bond correlation [HMBC]) revealed three compounds (*Figure 2*). NMR analysis by one-dimensional ^{1}H-NMR, DQF-COSY, [^{1}H-^{13}C] HMQC, and HMBC identified a major component (0.75 mg, approximately 70% of total retinoids on a molar basis) as 5,8-endoperoxy-2,3-dihydro-beta-apocarotene-13-one (Structure **1**) (*Figure 2* and *Table 1*). The HMQC spectrum shows 13 protonated carbon resonances: five methyl groups (s), three upfield methylene groups, a monooxygenated methine group (d, JHH = 4 Hz, 1JCH = 141 Hz), and four olefinic methine groups (d, J = 4 Hz; d, J = 16 Hz; br d, J = 11 Hz; dd, J = 11, 16 Hz). The ^{1}H-^{1}H coupling information reveals two fragments: C═CH-CH(OH)-C and C═CH-CH═CH-C, where in both cases the first and last carbons are quaternary, and in the second case the double bond CH═CH is trans. The presence of the unsaturated ketone is suggested by the downfield-shifted beta-methine (H-11) and terminal methyl (13-Me) proton signals at 7.44 and 2.27 ppm, respectively. Finally, long-range (two to three bond) ^{1}H-^{13}C correlations from the

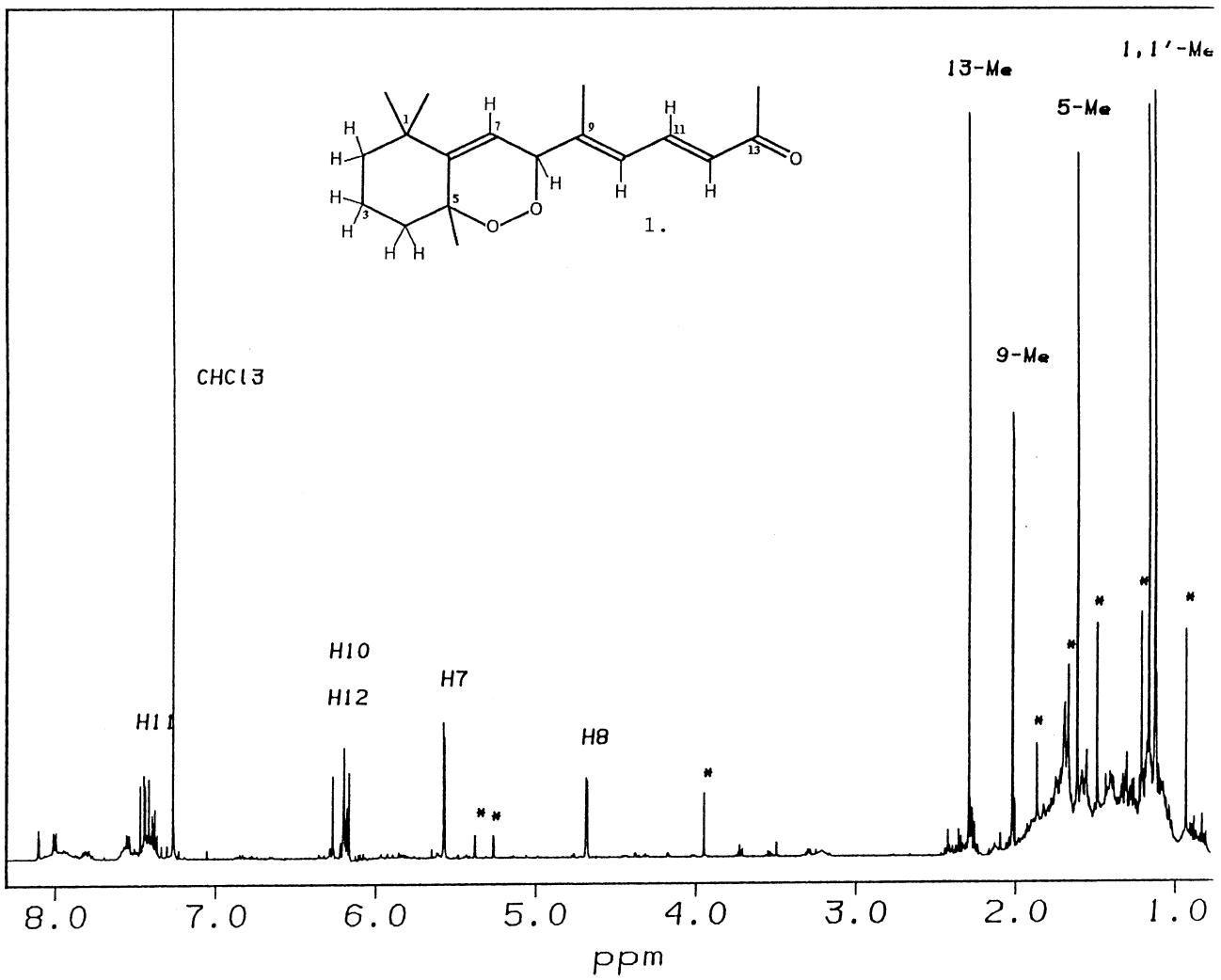

Figure 2 One-dimensional proton nuclear magnetic resonance spectrum of fraction 2A in $CDCl_3$. Peaks marked with an asterick (*) are due to a minor (16%) component. Inset: Structure of **(1)**.

HMBC spectrum connected these fragments to each other, to the remaining protonated carbons, and to five quaternary carbon resonances including the ketone carbonyl carbon at 198.4 ppm (*Table 1*). The oxygen functionality at carbons 5 and 8 was assumed to be a cyclic peroxide because no OH resonances were observed in the ^1H spectrum, and because the mass spectrum showed a molecular ion of m/z 290 (a 5,8-diol would give m/z 292). In addition, reaction with neither bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% chlorotrimethylsilane (TCMS) nor diazomethane, common procedures for derivatization of hydroxyl functionalities, produce the expected products using fraction 2A as substrate. The proposed structure **(1)** would result from 1,4 addition of a singlet oxygen equivalent to the 5,6,7,8 diene and oxidative cleavage of the 13-olefin of β-carotene. The trans geometry of the 11-12 double bond was established by the large (16 Hz) H-11/H-12 coupling.

Based on the analyses described above, the major compound (approximately 70% by mass) was identified as 5,8-endoperoxy-2,3-dihydro-β-apocarotene-13-one **(1)** (*Figure 2*). The minor components have chemical shifts typical of oxidized retinoids, but structural identification awaits final separation of the mixture. Thus, although **(1)** (*Figure 2*) is the major compound and fraction 2 could not be further separated by HPLC analysis, we can unambiguously assign the biological effects observed here only to oxidized Fraction 2 and not specifically to **(1)**.

Oxidation of retinol and retinoic acid

Retinoic acid is formed enzymatically in vivo from β-carotene[15] but also can be produced by chemical oxidation.[8,16] Thus, it was important to determine whether the oxidation products we isolated originated from β-carotene or from retinoic acid. To test this, in separate experiments, retinoic acid was oxidized and the products fractionated using the methods described above for chemical oxidation and fractionation of β-carotene. No compound migrating in the region of the chromatogram where fraction 2 eluted was observed on oxidation of retinoic acid. Thus, we conclude that the oxidation products we have identified and retinoic acid are formed by different oxidation pathways.

Table 1 Chemical shifts and coupling constants for **(1)**

^1H (^{13}C)	δ^1H	Multiplicity[1]	J(Hz)	$\delta^{13}C$	HMBC crosspeaks
1-Me	1.14	s		30.47	C (1-Me′), C1, C6
1-Me′	1.11	s		27.52	C (1-Me), C1, C6, C2
2a	1.29	dd	14, 17	40.84	
2b	1.55	br d	17	40.84	
3a	1.66	m		18.62	C2
3b	1.71	t	16	18.62	C4
4a	1.16	dt	6, 16	35.34	
4b	1.66	br d	16	35.34	
(5)	—	—		79.80	
5-Me	1.59	s		25.39	C4, C5, C6
(6)	—	—		149.90	
7	5.55	d	4.0	114.72	C1, C5, C6, C8
8	4.66	d	4.0	82.08	C5, C6, C7, C10
(9)	—	—		147.77	
9-Me	2.00	d	1.5	15.14	C8, C9, C10
10	6.16	dq	11.4, 1.5	125.48	C8, C(9-Me), C11, C12
11	7.42	dd	11.4, 15.7	138.48	C13
12	6.15	d	15.7	130.72	C9, C10, C13
(13)	—	—		198.35	
13-Me	2.27	s		27.37	C13

[1] s, singlet; d, doublet; dd, double doublet; t, triplet; dq, double quartet; m, multiplet; br, broad.

Effects of β-carotene oxidation products on retinoic acid receptors

To further compare actions of β-carotene oxidation products with those of retinoids, we tested the effect of fraction 2 on retinoic acid receptors in COS-1 cells transfected with retinoic acid receptors. Fraction 2 had no agonist or antagonist effect on RXR-α receptors (*Figure 3*). The same results were observed for RXR-β, RXR-γ, RAR-α, RAR-β, RAR-γ, or peroxisome proliferation (PPAR) receptors (data not presented). Thus, inhibition of growth of MCF-7 cells by retinoic acid and by oxidation products of β-carotene is apparently mediated by separate mechanisms.

Inhibition of growth and metabolism of MCF-7 cells by oxidation products of β-carotene

Samples of fraction 2 used for the experiments described in *Figure 4* were obtained from at least two oxidation mixtures and the results were confirmed independently by at least one other investigator in our laboratory. Fraction 2 strongly inhibited the growth of MCF-7 cells (*Figure 4A*). Inhibition of growth of MCF-7 cells also was inhibited strongly by retinoic acid and was comparable (IC_{50} of approximately 2 μM) to that reported in other breast cancer cell lines.[17,18] β-Ionone, another oxidation product of β-carotene,[8] showed only weak inhibition of growth of MCF-7 cells. Growth of MCF-10A cells, an estrogen-negative breast cancer cell line, was similarly inhibited by fraction 2 ($IC_{50apparent}$ of 20 ± 2 μM, $n = 6$).

BHT strongly protected MCF-7 cells against inhibition of growth by β-carotene. When low concentrations of BHT (2.5×10^{-5} %) were added to cells in the presence of 50 μM β-carotene, growth was inhibited by only 10% (*Figure 4B*) as opposed to 50% inhibition by β-carotene alone. BHT had no effect on the inhibition of growth by fraction 2 or on the growth of control cells. In fact, growth curves for cells treated with fraction 2 alone or fraction 2 with BHT were essentially superimposable (*Figure 4B*). These data demonstrate that oxidation of β-carotene greatly enhances its

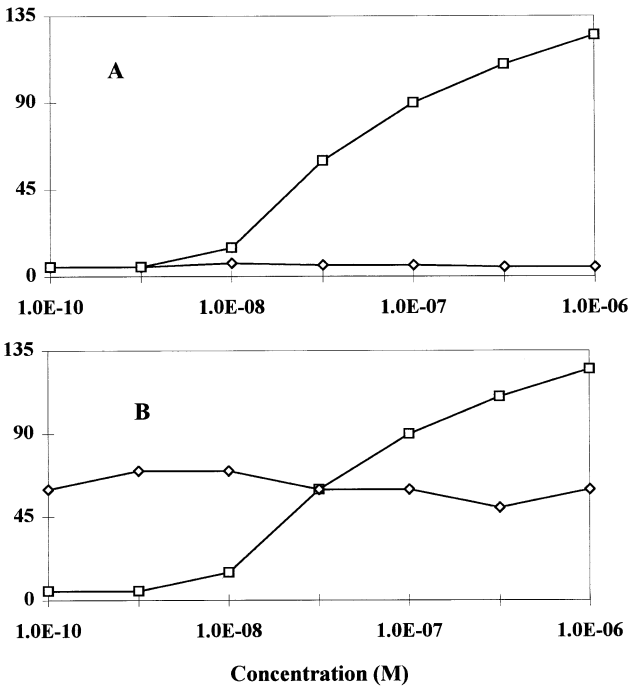

Figure 3 Effect of fraction 2 on RXR-α receptor activity. (*Figure 3A*) Agonist activity. CV-1 cells tranfected with an RXR expression plasmid (RSV-hRXRα) and a luciferase RXRE reporter (CRBPII-tk-LUC) were treated with increasing concentations of fraction 2 (◇) or 9-*cis* retinoic acid (□)as a control. (*Figure 3B*) Antagonist activity. CV-1 cells were transfected as in *Figure 3A* and treated with 10^{-7} M 9-*cis* retinoic acid plus increasing concentrations of fraction 2 (◇). Also shown is the dose response to 9-*cis* retinoic acid alone as a control (□).

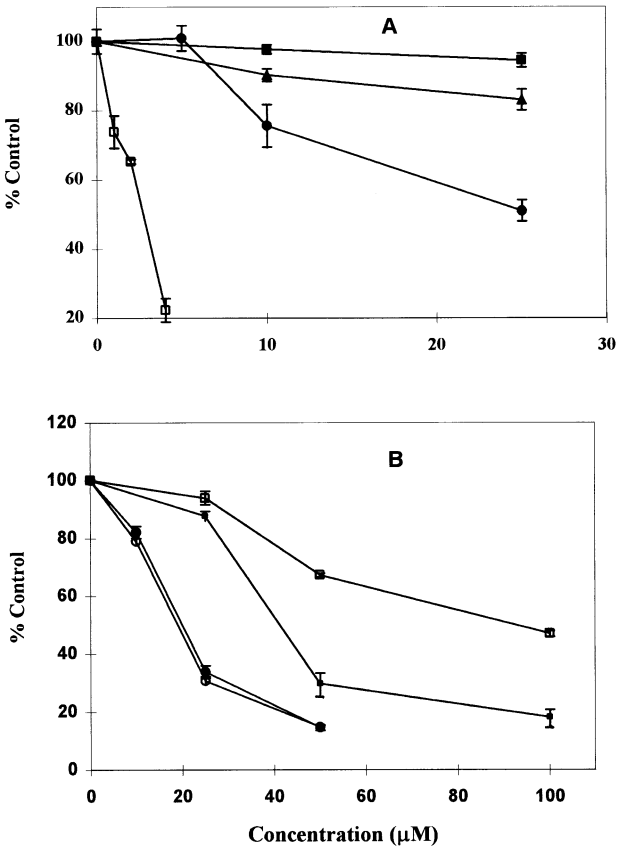

Figure 4 Inhibition of cell growth and metabolism by oxidation products of β-carotene compared with β-carotene and retinol. (*Figure 4A*) Inhibition of cell growth by oxidized products. Human breast cancer cells (MCF-7), grown as described in Materials and Methods, were harvested in their exponential growth phase (approximately 50% confluency), washed twice, and seeded at 30,000 cells/mL in RPMI medium containing (▲) β-carotene, (■) β-ionone, (●) fraction 2, or (□) retinoic acid. Cells were added to 96-well microtiter plates (200 μL per well), cultured for 72 hours, and cell growth determined as described in Materials and Methods. (*Figure 4B*) Protection by (BHT) of inhibition of cell growth by β-carotene but not by fraction 2 (% of control). (■) β-carotene, (□) β-carotene + BHT (250 ppb), fraction 2, (●) 20, (○) and fraction 2 + BHT (250 ppb). Control cells were treated with solvent eluted from the high performance liquid chromatography column adjacent to the fraction of interest and treated identically to fraction 2 as described in Materials and Methods. Date points are the average ±SDs of experiments performed in triplicate.

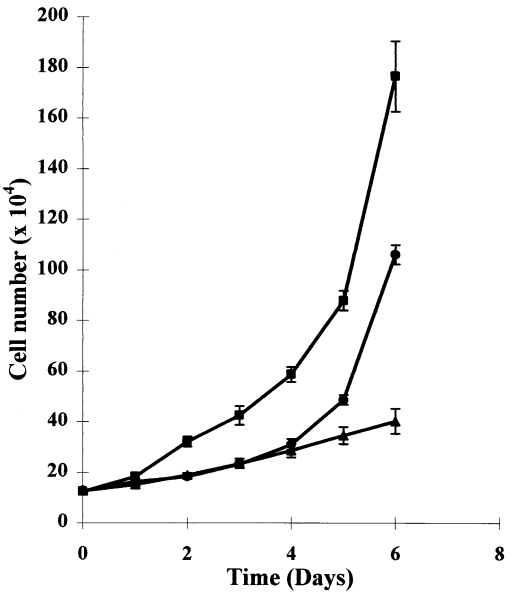

Figure 5 Reversal of growth inhibition after removal of fraction 2. Cells were incubated in the presence (●,▲) or absence (■) of fraction 2 (20 $\mu M_{apparent}$) as described in *Figure 4*. After 3 days, the medium was removed from one set of cells (●) and replaced with fresh media only. The remaining set (▲) was maintained in the presence of fraction 2. Control cells (■) were grown in the same media for the duration of the experiment and were treated with solvent eluted from the high performance liquid chromatography column adjacent to the fraction of interest, as described in Materials and Methods. Data points are the average ±SDs of experiments performed in triplicate.

ability to inhibit proliferation of MCF-7 cells and suggests that, in fact, oxidation products of β-carotene are the active compounds.

When media containing fraction 2 was replaced with fresh media, growth resumed to control levels within 48 hours (*Figure 5*). This evidence demonstrates that administration of fraction 2 did not result in death of the cells and is consistent with metabolic inhibition.

For analysis of the effects of fraction 2 on cholesterol synthesis, MCF-7 cells were labeled with ^{14}C acetate and free cholesterol and cholesteryl esters were extracted as described in Materials and Methods. Cholesteryl esters were hydrolyzed, fractionated on HPLC, collected, and quantitated by scintillation spectrometry. Addition of fraction 2 (approximately 20 μM) to MCF-7 cells inhibited synthesis of total cholesterol by 27.5% (±2.1%, $n = 6$) and growth by approximately 60%. As shown in *Figure 6*, growth inhibition by fraction 2 was prevented by pretreatment with mevalonic acid. In addition, protection by mevalonate was dose-dependent, with growth restored to control levels by the addition of 50 μM mevalonate. In preliminary experiments, addition of cholesterol did not protect against inhibition of cell growth by fraction 2 (data not presented). Addition of mevalonate did not protect against growth inhibition of MCF-7 cells by retinoic acid (1.7 μM) (*Figure 6*). These data corroborate the data reported above and suggest separate mechanisms of action for retinoic acid and the β-carotene oxidation products reported here.

We have not yet investigated the mechanism by which oxidation products of β-carotene inhibit cell growth and cholesterol synthesis. However, requirements for mevalonate, its metabolites, and hydroxy-methylglutaryl coenzyme A (HMGCoA) reductase activity for cell division[19–21] are well documented, as is the inhibition of HMGCoA reductase by β-carotene and its oxidation product β-ionone.[22–25] This evidence, taken together with our data, is consistent with the hypothesis that oxidation products of β-carotene inhibit mevalonate synthesis, which is required for DNA synthesis and cell proliferation.

There are well over 100 known oxidation products of β-carotene,[8] and most are produced in trace quantities. Therefore, identification of oxidized products of β-carotene in vivo will require more sensitive techniques such as recombinant technology or immunochemical assay. How-

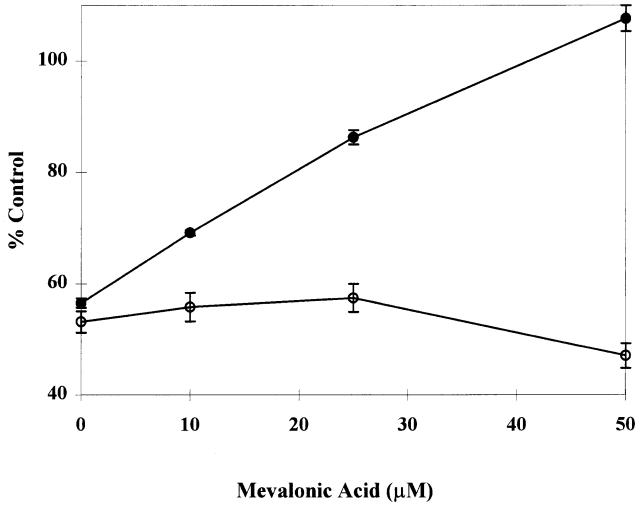

Figure 6 Effect of mevalonate on inhibition of growth of MCF-7 cells by oxidation products of β-carotene. Growth conditions are described in *Figure 4*. Mevalonic acid lactone was added at the indicated concentrations with (●) fraction 2 (20 $\mu M_{apparent}$) or (○) retinoic acid (1.7 μM) to cells in the exponential growth phase. After 72 hours, cells were harvested and cell growth determined as described in Materials and Methods. Control samples were treated with solvent eluted from the high performance liquid chromatography column adjacent to the fraction of interest, as described in the Materials and Methods. Data points are the average ±SDs of experiments performed in triplicate.

ever, assessment of potential anticancer actions of β-carotene oxidation products does not rely on identification of these compounds in physiologic quantities in vivo and should be further explored. Such pharmacologic benefits are well known for synthetic retinoids, with comparable chemical structures.[26–30]

In summary, we have isolated and chemically characterized a polar oxidation product of β-carotene and tested the effect of a highly enriched fraction containing this compound on the growth and metabolism of MCF-7 cells. This fraction inhibits cell growth and cholesterol synthesis in MCF-7 cells. BHT protects against inhibition of growth of MCF-7 cells by β-carotene. However, BHT does not protect against inhibition of growth by the oxidized fraction of β-carotene. Treatment of cells with mevalonate protects cells against inhibition of growth by the oxidized fraction of β-carotene. Conversely, mevalonate does not protect against inhibition of growth by retinoic acid. The oxidation products of β-carotene reported here are not produced by oxidation of retinol or retinoic acid and cannot substitute for retinoic acid in cotransfection assays. Collectively, the data suggest that oxidation products of β-carotene are members of a class of nonsteroidal isoprenes with potential antineoplastic activity.

Acknowledgments

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