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Effects of Epoxycarotenoids, β-Carotene, and Retinoic Acid on the Differentiation and Viability of the Leukemia Cell Line NB4 in Vitro

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Abstract: Three all-*trans* epoxides of β-carotene (β-Car), namely, 5,6-epoxy-β-carotene (5,6-EC), 5,8-epoxy-β-carotene (5,8-EC) and 5,6,5',6'-diepoxy-β-carotene (5,6,5',6'-DEC) were synthesized by treatment of β-carotene with 3-chloroperoxybenzoic acid, were purified chromatographically, and were characterized. The relative potencies (mean ± S.D.) of 1 μM compounds in inducing the differentiation of NB4 cells, a cell line that contains the chromosomal transposition t(15;17) characteristic of acute promyelocytic leukemia, after 4 days of incubation were: RA: 1.35 ± 0.16, 5,6-EC: 0.29 ± 0.01, 5,8-EC: 0.22 ± 0.05, 5,6,5',6'-DEC: 0.11 ± 0.02, βC: 0.09 ± 0.01, and the control: 0.06 ± 0.01. The same order of potencies existed at other concentrations tested and at other incubation times. P values for the differences between the inducing activities of successive pairs of compounds at 1 μM were: RA vs. 5,6-EC, < 0.001; 5,6-EC vs. 5,8-EC, < 0.01; 5,8-EC vs. 5,6,5',6'-DEC, < 0.01; 5,6,5',6'-DEC vs. β-Car, < 0.10; β-Car vs. control, < 0.005. Similar P values were also obtained for studies at other concentrations and at other incubation times. The viable cell mass at 4 days was inversely proportional to the extent of differentiation ($r_s = -1.0$). The inducing activities of all compounds were dose-dependent. Thus, the 5,6-monoepoxide of β-carotene, which has not previously been studied as an inducer, showed higher activity in NB4 cell differentiation than the 5,8-monoepoxide, the 5,6,5',6'-diepoxide, or β-carotene. Possible explanations of these observations are discussed.

Key words: Epoxycarotenoids, β-carotene, retinoic acid, 5,6-epoxyretinoic acid, cell differentiation, viability, NB4 cells

Introduction

Human acute promyelocytic leukemia (APL) is a hematopoietic malignancy that responds dramatically to treatment with all-*trans* retinoic acid (RA) [1]. APL is primarily associated with a specific chromosomal translocation t(15;17), in which the genes for the retinoic acid receptor alpha (RARα) and for the so-called promyelocytic leukemic protein (PML) are fused at the 15q chromosomal locus. Approximately 90% of APL patients achieve complete remission after treatment with RA [2], which induces the differentiation of APL cells to granulocyte-like cells [3]. However, the use of RA as a drug is limited both by the development of RA-resistant APL cells and by its toxicity [4].

In contrast to RA, carotenoids show little, if any, toxicity for humans [5]. Several carotenoids have been shown to induce the differentiation or to slow the growth of neoplastic cells, including the human acute myelocytic cell line, HL-60 [6–10], as well as of normal cells [11, 12]. Although hydrocarbon carotenoids, e.g., β-carotene, lycopene, and α-carotene, carotenoic acids, e.g. crocetin, and ketocarotenoids, e.g., canthaxanthin, have shown activity in these regards, epoxycarotenoids have not previously been examined. In this paper, monoepoxycarotenoids, but not 5,6,5',6'-diepoxy-β-carotene, showed significantly greater inducing activity than β-carotene towards the human leukemia cell line, NB4.

Materials and Methods

Chemicals: Chemicals and their purveyors were: all-*trans* RA (BASF Corp., Parsippany, NJ, USA); all-*trans* β-carotene, nitroblue tetrazolium (NBT), MTT, HEPES buffer, penicillin, streptomycin sulfate, RPMI 1640 medium, and silica gel plates, 250 μm × 20 cm × 20 cm (Sigma Chemical Co., St. Louis, MO, USA); fetal calf serum (Hyclone Laboratories, Logan, UT, USA); HPLC-grade organic solvents (Fisher Scientific Co., Fairhaven, NJ, USA); and 3-chloroperoxybenzoic acid (Aldrich Chemical Co., Milwaukee, WI, USA).

Abbreviations: β-Car, all-*trans*-β-carotene; 5,6-EC, all-*trans*-5,6-epoxy-β-carotene; 5,8-EC, all-*trans* 5,8-epoxy-β-carotene; 5,6,5',6'-DEC, all-*trans*-5,6:5',6'-diepoxy-β-carotene; RA, all-*trans* retinoic acid; 5,6-ERA, all-*trans* 5,6-epoxyretinoic acid; NBT, nitroblue tetrazolium, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]-ditetrazolium chloride; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); PML, a promyelocytic protein whose gene is located on chromosome 15 that forms a fusion protein with RARα; RARα, retinoic acid receptor type α; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

Synthetic Procedures: The epoxycarotenoids were prepared by stirring β-carotene (1 mmole) in diethyl ether with 3-chloroperoxybenzoic acid (1.2 mmole) for 2 h. After stopping the reaction with aqueous NaOH, the carotenoids were extracted 3× with diethyl ether. The extract was washed with water to remove traces of NaOH, and then was dried over anhydrous Na_2SO_4. The solvent was removed by rotoevaporation under reduced pressure, followed by thin layer chromatography (TLC) of the extract on silica gel plates with hexane:acetone (95:5).

Pure 5,6-EC (50 mg) in diethyl ether (10 ml) was stirred briefly (1 min) with traces of dilute HCl to make 5,8-EC. The solution of 5,8-EC was washed with water, dried over anhydrous Na_2SO_4, and evaporated under argon to dryness. The residue was dissolved in hexane. The R_f values on TLC plates for the carotenoids were: β-Car (0.92), 5,6-EC (0.73), 5,8-EC (0.64), and 5,6,5',6'-DEC (0.56). The λ_{MAX} and $E^{1\%}_{1\ cm}$ for these carotenoids in hexane were: 5,6-EC, 473, 445, 420 nm (2590); 5,8-EC, 455, 427, 405 nm (2440); 5,6,5',6'-DEC, 470, 440, 417 nm (2690); β-Car, 478, 450, 423 nm (2592) [13]. The purity of each carotenoid, as determined by use of a Waters 996 photodiode array detector linked to an HPLC system, was >98%.

5,6-Epoxyretinoic acid (5,6-ERA) was prepared in a similar way by stirring a cesium salt of RA in diethyl ether with 3-chloroperoxybenzoic acid for 2 h [14]. After stopping the reaction with aqueous NaOH and then acidifying with acetic acid, 5,6-ERA and unreacted RA were extracted 3× with diethyl ether. The pooled solvents were removed by rotoevaporation under reduced pressure followed by separation of the compounds by TLC on silica gel plates with hexane:diethyl ether (95:5). The R_f values of 5,6-ERA and RA were 0.58 and 0.35, respectively. The purity of 5,6-ERA, as determined by its retention time and PDA spectrum when analyzed by HPLC, was > 95%.

Cell culture: The NB4 cell line was provided by Dr. M. Lanotte (Hospital St. Louis, Paris, France) through Dr. R. E. Gallagher (Montefiore Medical Center, Bronx, NY). The cell line was maintained at 37°C in RPMI 1640 medium supplemented with 10 mM HEPES buffer (pH 7.4), antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin), and 10% fetal calf serum in a humidified incubator with 5% CO_2/air. The cells were subcultured every three days to maintain the cell density between 0.2×10^6 and 0.8×10^6 cells/ml.

Dissolution of test compounds: Although RA and 5,6-ERA are soluble in ethanol alone, carotenoids are not. Retinoids and carotenoids were consequently dissolved in dimethyl sulfoxide (DMSO):ethanol 1:3 v/v). All tested compounds in solution, whose concentrations were de-

termined spectrophotometrically, were then diluted with DMSO:ethanol (1:3 v/v) to give a concentration of 1 mM/L. All solutions were freshly prepared just before adding to the culture medium. In this regard, carotenoid solutions stored at –20°C often gave precipitates that were difficult to redissolve fully.

Incubation conditions: Subcultured NB4 cells, after being counted, were diluted with fresh warm (37°C) medium, as described above, to yield 10^5 cells/mL. Then 1.5×10^6 cells (15 mL of this suspension) were pipetted into a sterile flask. The following additions were then made: 1) Either 5, 15, or 50 µl of DMSO:ethanol (1:3 v/v) in the control incubations, or 2) the same volumes of various test compounds (1 mM/L) in DMSO:ethanol (1:3 v/v). Each flask was gently shaken to mix the compound with the cells in the medium. Aliquots were then transferred to the wells of 96 well plates. All experiments were done in triplicate. Racks containing the plates were closed, put inside a tray, and incubated at 37°C in a humidified incubator at 5% CO_2/air. Four racks were prepared; two racks were used on day 4, and the other two racks on day 7. One rack at each time was used for NBT and one for MTT tests.

Differentiation assay with NB4 cells: The differentiation of NB4 cells was measured by the reduction of nitroblue tetrazolium (NBT) to a dark formazan dye upon stimulation of the cells with phorbol ester [15]. To avoid the difficulty of taking aliquots of the NB4 cells, which adhere tightly to the culture vessel upon differentiation, the NBT assays were performed *in situ* in 96-well microplates [16]. The number of living cells present in those wells was determined on triplicate incubation mixtures in 96-well plates by a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay [17]. The resultant formazan was solubilized by adding a suspension of 3% SDS (sodium dodecyl sulfate; w/v) in 0.04 N HCl/isopropanol to the wells [17]. The intensity of the brown color was recorded in an ELISA reader at 570/630 nm. We verified in a control experiment that the MTT signal was proportional to the NB4 cell mass and was independent of the differentiation status of the cells. The extent of differentiation was expressed as the ratio of NBT/MTT.

Carotenoid and retinoid extraction from cells: Compounds were extracted from 1 ml cell suspensions grown in 96-well plates by 3 ml ethyl acetate (3×) and 3 ml hexane (3×). The pooled extracts were washed with 2 ml doubly distilled water, dried over sodium sulfate, and evaporated. The pellet was reconstituted in methanol:methylene dichloride (1:1, v/v) and injected onto an HPLC column [18, 19].

HPLC analysis: For simultaneous analysis of retinoids and carotenoids, a reversed-phase gradient system described recently [19] was used. A Waters 1996 photo-diode array detector scanned wavelengths from 300 to 530 nm. A Waters 5 µm C_{18} "Resolve" column (3.9 mm × 15 cm) at a flow rate of 1.2 ml/min was used. Under these conditions, the retention times and detection limits (in parentheses) were: RA, 10.8 min (0.2 ng) and ROL, 18.5 min (0.1 ng). For analysis of carotenoids only, a 3 µm C-18 Microsorb MV (3.9 mm × 10 cm) column was used with a mobile phase of acetonitrile/methylene dichloride/methanol/water (85:12:1:3, v/v) containing 0.1% ammonium acetate at a flow rate of 1 ml/min. Under these conditions, the retention times of the carotenoids were: 5,6,5',6'-DEC, 9.2 min; 5,6-EC, 17.2 min; 5.8-EC, 18.4 min, and β-Car, 32.5 min. The limit of detection of these carotenoids was in the range of 0.5–1 ng.

Statistics: Mean values, standard deviations, Students t-test, and the Spearman rank-correlation coefficient (r_s) were calculated by conventional procedures [20].

Results

Induction of differentiation: The relative extents of differentiation of NB4 cells induced by the studied compounds at 4 days are summarized in Table I. At 0.33 µM, RA clearly was 10-fold more active than any of the examined carotenoids ($P < 0.001$). Among the latter, however, the relative potencies at all concentrations were 5,6-EC > 5,8-EC > 5,6,5',6'-DEC ≅ β-Car > control. At 1 µM, P values between successive pairs of compounds were: 5,6-EC vs. 5,8-EC, < 0.01; 5,8-EC vs. 5,6,5',6'-DEC, < 0.01; 5,6,5',6'-DEC vs. β-Car, < 0.10; β-Car vs. control, < 0.005. Similar potencies and P values were obtained in other experiments conducted for 6 and 7 days. 5,6-Epoxyretinoic acid at 5 µM gave NBT-MTT ratios intermediate between those of RA and of 5,6-EC. In all cases, the responses of carotenoids were dose-dependent up

Table I: The extent of differentiation of NB4 cells, expressed as the NBT/MTT ratio, induced by all-*trans* retinoids and carotenoids at 4 days in serum-containing media[a]

Inducer	Initial concentration (µM) 0.33	1.0	3.3
Retinoic acid	1.25 ± 0.07	1.35 ± 0.16	1.23 ± 0.25
5,6-Epoxy-β-carotene	0.11 ± 0.01	0.29 ± 0.01	0.78 ± 0.06
5,8-Epoxy-β-carotene	0.10 ± 0.02	0.22 ± 0.05	0.57 ± 0.07
5,6,5',6'-Diepoxy-β-carotene	0.09 ± 0.02	0.11 ± 0.02	0.15 ± 0.01
β-Carotene	0.08 ± 0.02	0.09 ± 0.01	0.16 ± 0.01
None	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01

[a] Mean ± S.D. (n = 3)

Table II: The viable mass of NB4 cells, as determined by MTT values, in the presence of various inducers at 4 days in serum-containing media[a]

Inducer	Initial concentration (μM) 0.33	1.0	3.3
Retinoic acid	0.60 ± 0.04	0.57 ± 0.01	0.56 ± 0.02
5,6-Epoxy-β-carotene	1.08 ± 0.01	0.83 ± 0.01	0.66 ± 0.03
5,8-Epoxy-β-carotene	1.08 ± 0.05	0.88 ± 0.04	0.72 ± 0.02
5,6,5',6'-Diepoxy-β-carotene	0.93 ± 0.05	0.90 ± 0.01	0.80 ± 0.02
β-Carotene	1.12 ± 0.01	1.18 ± 0.03	1.04 ± 0.04
None	1.16 ± 0.05	1.19 ± 0.02	1.21 ± 0.03

[a] Mean ± S.D. (n = 3)

to a saturating value. The response to RA, however, was saturating at the lowest concentration tested (0.33 μM).

Effects on the viable cell mass: Values of the viable cell masses with various inducers, as determined by MTT measurements at 4 days, are summarized in Table II. These values are inversely related to the extent of differentiation induced by the added compounds. Indeed, at 1 μM and 3.3 μM, the Spearman rank-correlation coefficient (r_s) is –1.00 ($P < 0.001$).

Analyte recovery and product formation: The percent recovery for all analytes tested was > 40% after four days and > 25% after a seven-day incubation with cell suspensions at 37°C in a humidified incubator at 5% CO_2/air. The presence of fetal calf serum, with its lipoproteins and antioxidants, clearly enhanced carotenoid stability. Appreciable amounts of β-apo-8'-carotenoic acid (λ_{MAX}, 445, 470 nm; $E^{1\%}_{1\,cm}$ at 445 nm in hexane = 2500) [13], which was identified by HPLC using a photo-diode array detector, and traces of related compounds were present in the media after incubation with all carotenoids. No retinoids, however, could be detected under our assay conditions in the extracts from cells incubated with any of the tested carotenoids. Future studies should focus on an improvement in our assay sensitivity.

Discussion

RA, as anticipated, proved to be the most active compound tested in inducing the differentiation of NB4 cells to granulocyte-type cells. Even at the lowest tested concentration (0.33 μM), RA gave a maximal response. Thus, RA is *at least* 10-fold more active than the most active carotenoid (5,6-EC). Although all-*trans* β-carotene was also an active inducer in a concentration-dependent manner, the two monoepoxides, but not the diepoxide, were 2- to 3-fold more active. In keeping with the general observation that undifferentiated cells divide more rapidly than differentiated cells, the viable cell mass at any given time or concentration of inducer was inversely proportional to the extent of differentiation.

Carotenoids may be acting *per se* in inducing cell differentiation or only after their conversion to biologically active metabolites. Although RA and several of its derivatives are known to interact with nuclear transcription factors [21], no such nuclear receptors for intact carotenoids have yet been identified. Thus, it seems unlikely that carotenoids are acting *per se.* The most probable explanation for these effects is that β-carotene and its monoepoxides are centrally cleaved within cells by 15,15'-carotenoid dioxygenase to retinal and monoepoxyretinal, which in turn are oxidized to the active metabolites, RA and epoxy-RA. Inasmuch as the dioxygenase cleaves, 5,6-EC better than 5,8-EC and shows little, if any, activity on the diepoxide [22], the relative potency of the three epoxides in cell differentiation is related to their cleavage rates. In this regard, the action of canthaxanthin (4,4'-dioxo-β-carotene) in stimulating gap-junction communication also seems to result from its conversion to 4-oxoretinoic acid [23, 24]. Because very little RA or 5,6-ERA are required to induce cell differentiation and our HPLC system had

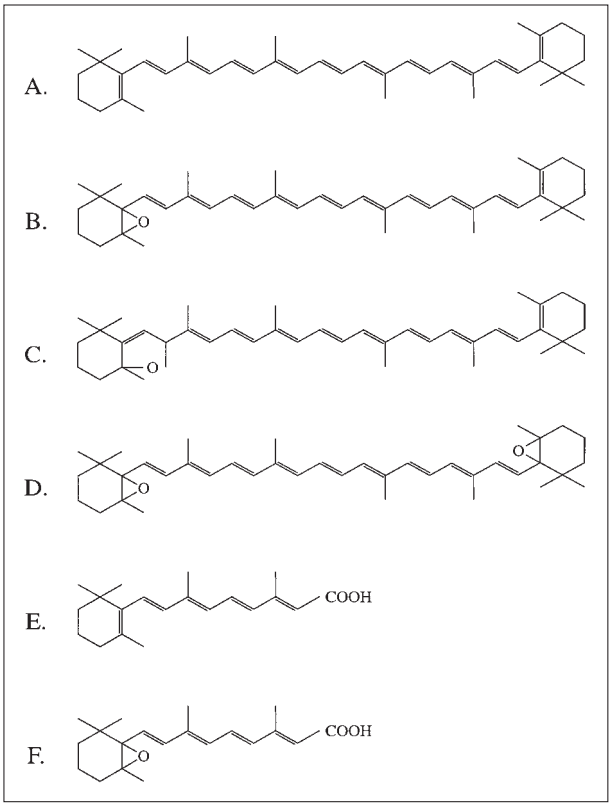

Figure 1: Formulas of all-*trans* isomers of β-carotene (A), 5,6-epoxy-β-carotene (B), 5,8-epoxy-β-carotene (C), 5,6,5',6'-diepoxy-β-carotene (D), retinoic acid (E), and 5,6-epoxyretinoic acid (F).

limited sensitivity, it is not surprising that we were unable to identify any retinoids in the cell cultures after incubation. On the other hand, retinol has been detected in cultured cells after incubation with β-carotene [25, 26], and carotenoids can also decompose to retinoid-like products in oxygenated media [27, 28]. The use of larger numbers of cells and more sensitive HPLC methods might well resolve this issue.

The reason for the relative inactivity of β-carotene is obscure. Quite possibly, β-carotene, because of its hydrophobicity, was taken up more slowly than the more polar carotenoid epoxides by NB4 cells. Furthermore, β-carotene was found to be somewhat less stable than the epoxycarotenoids during incubation, even though considerable amounts of intact carotenoids were recovered at the end of the incubation period. Finally, the different physical properties of hydrocarbon- and epoxy-carotenoids may play some role, e.g., 5,6-epoxy-lycopene and 5,6-EC are better antioxidants than their parent compounds in both liposomal and oil model system [29].

The apocarotenoic acids found in the cell cultures after incubation were most likely formed from the added carotenoids by chemical oxidation [28]. They do not seem to be biologically active in this system, inasmuch as they were formed in roughly equivalent amounts from most carotenoids, independent of the latter's inducing activity.

Epoxycarotenoids, although present in sizable amounts in many foods, e.g. mango, have not previously been studied as biologically active agents. The recent finding that 5,6-EC is absorbed by humans and converted to 5,6-epoxyretinyl palmitate in the serum [30] as well as the current study should further stimulate interest in their metabolism and possible biological activity.

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