Functions and Actions of Retinoids and Carotenoids: Building on the Vision of James Allen Olson

The Enigma of β-Carotene in Carcinogenesis: What Can Be Learned from Animal Studies1,2

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ABSTRACT β-carotene and other carotenoids have been thought to have anti-cancer activity, either because of antioxidant activity or because of their ability to be converted to vitamin A. Nevertheless, two large scale intervention studies in humans using high doses of β-carotene found that β-carotene supplementation resulted in more lung cancer rather than less lung cancer among smoking and asbestos exposed populations. Studies conducted in the ferret have elucidated molecular mechanisms behind this observation, in that high-dose β-carotene and smoke exposure in these animals leads to squamous metaplasia, a pre-cancerous lesion in the lung. High dose β-carotene in the smoke exposed animals was found to give rise to a number of transient oxidative metabolites, which include P450 enzymes that result in the destruction of retinoic acid, and diminished retinoid signaling, and enhanced cell proliferation. In addition, eccentric cleavage β-carotene metabolites facilitate the binding of smoke derived carcinogens to DNA. In other ferret studies low dose β-carotene smoke exposure provided mild protection against squamous metaplasia. Thus, it appears that the explanation of the apparent paradoxical effects of β-carotene on lung cancer is related to dose. The metabolism and breakdown of natural products should be thoroughly investigated in animal models before embarking on large scale intervention trials, particularly when using unusually high doses that greatly exceed normal dietary levels. J. Nutr. 134: 262S–268S, 2004.

KEY WORDS: • β-carotene • carotenoids • vitamin A • retinoids • lung cancer • smoking

Tobacco use in the United States is responsible for over 134,000 lung cancer deaths every year (1). Clearly the best protection against lung cancer is the avoidance of tobacco smoke. But the numbers of current smokers remains high, and one out of every five Americans continues to smoke (1). In addition, passive smokers are at risk, since more free radicals are generated from smoke as the smoke becomes aged. In seeking other ways to modify lung cancer risk, nutritional interventions have been proposed—specifically antioxidant interventions that might protect tissues against oxidative free radical damage.

A large body of observational epidemiologic studies has consistently demonstrated that individuals eating more fruits and vegetables (which are rich in carotenoids) and people having higher serum β-carotene levels have a lower risk of cancer, particularly lung cancer (2,3). Moreover, a number of animal and laboratory studies have shown that β-carotene can block certain carcinogenic processes and inhibit tumor cell growth (3,4). Some proposed mechanisms for these actions are that β-carotene may: 1) function as an antioxidant (5,6); 2) be a precursor for retinoic acid (7,8); 3) enhance gap junction communication (9,10); 4) increase immunologic function (11,12); and 5) induce carcinogen-metabolizing enzymes (13). However, in contrast to the results from the epidemiologic studies, high dose β-carotene supplements in humans were shown to increase the risk of lung cancer among smokers in two out of three intervention trials (14,15–19). In the ATBC trial, 20 mg of β-carotene and/or 50 mg of vitamin E were given daily to smokers. After a period of six years, there was found to be an 18% increase in lung cancer. In the CARET trial, 30 mg of β-carotene along with 25,000 IU of preformed retinol were given for a period of four years to smokers and/or asbestos exposed workers. In this period there was a 28% increase in lung cancer among those receiving β-carotene and vitamin A. In contrast to these two studies, the Physicians’ Health Study was conducted among mainly nonsmokers: 50 mg of β-carotene was given every other day and no effect was

1 Presented as part of the James Allen Olson Memorial Symposium, “Functions and Actions of Retinoids and Carotenoids” held at Iowa State University, June 21–24, 2001 to honor the memory of James Allen Olson. This conference was supported by the U.S. Department of Agriculture; National Institutes of Health; Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University (ISU); Department of Food Science and Human Nutrition, ISU; College of Liberal Arts and Sciences, ISU; F. Hoffmann-La Roche; Kemlin Foods, L.C.; Procter & Gamble Company; Lipton; Best Foods; BASF; SmithKline Beecham; Cognis Corporation; Allergen and INEXA. Guest editor for this symposium was Norman I. Krinsky, Department of Biochemistry, School of Medicine, and the Jean Mayer Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111–1837.

2 This research has been supported in part by the U.S. Department of Agriculture, under agreement number 1950–51000-048–01A. The contents of this publication do no necessarily reflect the views or policies of the United States Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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0022-3166/04 $8.00 © 2004 American Society for Nutritional Sciences.
found on lung cancer risk in either the smokers or nonsmokers (19). When considering these different results, it is of interest that the serum concentrations of \( \beta \)-carotene in the two trials where an increase of lung cancer incidence was demonstrated were markedly higher (210–300 \( \mu \)g/dL) than in the Physicians' Health Study (120 \( \mu \)g/dL), where no increase in lung cancer risk was seen from \( \beta \)-carotene supplementation. It should also be noted that in NHANES III blood, which primarily reflects dietary intake (not supplemental intake), the normal range of serum \( \beta \)-carotene is 5–50 \( \mu \)g/dL (20). There are many different possible explanations for the different results seen in the ATBC and CARET trials vs. the Physicians' Health Study. Most importantly, there were far fewer smokers in the Physicians' Health Study. But also, and possibly of some importance, are the much higher levels of serum and tissue \( \beta \)-carotene achieved in the ATBC and CARET trials.

Because so many questions have arisen about what happened in these different trials, we turned to an animal model (domesticated ferret) to try to reproduce the findings of the ATBC and CARET trials, and to study the mechanisms underlying the increase in lung cancer carcinogenesis due to \( \beta \)-carotene supplementation. The ferret is a uniquely appropriate model for use in studies involving cigarette smoke and carotenoids. The ferret and human are similar in: 1) the absorption of intact \( \beta \)-carotene (21); 2) the accumulation of \( \beta \)-carotene in lung tissue (22); 3) the formation of oxidative metabolites of \( \beta \)-carotene (22, 23); 4) similar architecture in cigarette smoke-induced pathology (22, 24–26); and 5) DNA sequence of several genes. Although no animal model perfectly replicates the human situation, the ferret model is an outstanding model that can yield critical insights into whether and how to pursue the use of the antioxidant nutrients to prevent cancer.

We initially showed that ferrets fed \( \beta \)-carotene at 4 or 20 mg/Kg body weight achieved high serum levels (15 and 41 \( \mu \)g/dL) as well as appreciable levels in liver and adipose tissue (23). This is in contrast to the rat which, when fed the same levels of dietary \( \beta \)-carotene, achieved minimal serum concentrations with no detectable \( \beta \)-carotene in either the liver or adipose tissue. In order to study the absorption of \( \beta \)-carotene further in this animal model, we cannulated the mesenteric lymph ducts while perfusing a micellar solution of \( \beta \)-carotene through the proximal small bowel (21). During the period of \( \beta \)-carotene perfusion, the concentration of \( \beta \)-carotene in lymph increased with time; after perfusion was stopped, the concentration of \( \beta \)-carotene decreased back to baseline levels.

We carried out a smoke inhalation study using the ferret model (22). The ferrets were divided into two groups, \( \beta \)-carotene fed or non-\( \beta \)-carotene fed. Each of these groups was either exposed to smoke or not exposed to smoke for a period of six months. \( \beta \)-carotene was administered to the animals in doses calculated to be equivalent to 30 mg/d in the humans (as was fed in the CARET trial). All trans-\( \beta \)-carotene over and above that contained in the basal diet was dissolved into 1 mL of corn oil and fed orally, not gavaged, to the ferrets every morning. The ferrets in the control group were fed the basal diet plus 1 mL of corn oil only. Ferrets like oil and lap it up. Smoke exposure was carried out twice in the morning and twice in the afternoon (10 cigarettes over a 30 min period each time while being contained in a special chamber connected to a smoking device). Urinary cotinine in the smoke-exposed animals (14.3 ± 0.6 \( \mu \)g/ml) was equivalent to levels that would be found in human smokers smoking 1½ packs of cigarettes a day. After six months, the animals were killed.

The results of this study showed that smoke exposure caused mild aggregation and proliferation of macrophages in the lung tissues of ferrets. However, localized proliferation of alveolar cells and alveolar macrophages with keratinized squamous epithelium was observed in all six ferrets given the high dose \( \beta \)-carotene supplement alone. But the most severe focal proliferation of alveolar cells, squamous metaplasia and severe destruction of alveolar walls were observed in all six ferrets that were given the combination of high dose \( \beta \)-carotene supplement and exposed to smoke (Fig. 1). Keratinized squamous metaplasia was confirmed by immunohistochemistry with antikeratin antibody in the lung sections of all ferrets given either high dose \( \beta \)-carotene alone or high dose \( \beta \)-carotene with additional smoke exposure. Nuclear protein extracts were prepared from lung tissues for proliferating cell nuclear antigen (PCNA)\(^4\) analysis. PCNA is a marker for epithelial cell proliferation, which can be quantified by Western blot analysis by using monoclonal or polyclonal antibodies against this antigen. We did this analysis because the lesions are focal and it is hard to get a true picture of the amount of proliferation going on in the lung without an overall measure of cell proliferating activity. There was a 1.8-fold increase in PCNA expression in the lungs of \( \beta \)-carotene supplemented ferrets who were not exposed to smoke, and a 3.7-fold increase in the lungs of \( \beta \)-carotene supplemented ferrets who were exposed to smoke.

As expected, lung tissue concentrations of \( \beta \)-carotene were markedly increased in the animals supplemented with \( \beta \)-carotene; however, if they were also exposed to smoke, the levels dropped precipitously, although remaining higher than in the control animals (Table 1). Lung retinol levels were not different in the four groups of animals, whereas lung retinyl palmitate levels were higher in the \( \beta \)-carotene supplemented (alone) animals. Perhaps of greatest interest is the fact that retinoic acid levels were lower in all three groups of animals, (smoke-exposed, \( \beta \)-carotene supplemented, or smoke-exposed plus \( \beta \)-carotene supplemented) vs. controls. In fact, retinoic acid levels were unable to be detected in either of the smoke-exposed groups.

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\(^4\) Abbreviations used: AP-1, activated protein 1; CYP, cytochrome P450; PCNA, proliferating cell nuclear antigen; RAR, retinoic acid receptor.
We hypothesized the cigarette smoke in animals exposed to high dose β-carotene would give rise to a number of transient oxidative metabolites of β-carotene which would explain the lower lung levels of the intact β-carotene upon smoke exposure. Further, these oxidative metabolites might perhaps cause a lowering of lung retinoic acid levels. In order to demonstrate that the dramatic decrease in β-carotene was due to enhanced breakdown of the molecule, we conducted in vitro incubations of all trans-β-carotene with post-nuclear fractions from lung tissue of ferrets either exposed or nonexposed to smoke. The results showed that the formation of β-apo-14’, β-apo-12’, β-apo-10’, and β-apo-8’ carotenals was threefold higher when using lung extracts from ferrets exposed to smoke than when using lung extracts from ferrets that were not exposed to smoke (22). These data indicate that the free radical rich atmosphere in the lungs of cigarette smoke exposed-ferret leads to conditions that modify β-carotene metabolism to form an abundance of excentric cleavage metabolites that are structurally similar to retinoids. We wondered if these structurally similar metabolites could somehow interfere with the metabolism of retinoic acid and thus retinoid signaling.

We and others tested whether the lower level of retinoic acid found in ferret lungs after smoke exposure along—or after high dose β-carotene supplementation with or without smoke exposure—could be due to a stimulation of cytochrome P450 (CYP) activity. Gradelet et al. had reported that β-apo-8’-carotenal, an excentric cleavage product of β-carotene, but not intact β-carotene itself, was a strong inducer of CYP 1A1 in rats (27). Cytochrome P450 1A1 is a predominant cytochrome P450 enzyme in the lung. In our experiments we showed that the formation of β-apo-8’-carotenal from β-carotene was 2.5-fold higher in lung extracts of smoke-exposed ferrets than in lung extracts of nonsmoke-exposed ferrets. Moreover, Paolini et al., in 1999, showed a significant increase in several cytochrome P450 enzymes including (CYP 1A1) in the lungs of rats that had been supplemented with very high doses of β-carotene (500 mg/kg body weight) (28). It is not known if this increase in enzyme was due to a high dose of β-carotene itself, however, or due to breakdown products of β-carotene.

From these data, we hypothesized the P450 enzymes induced by excentric cleavage breakdown products of β-carotene are involved in the destruction of retinoic acid. To test this, we incubated retinoic acid with the microsome fractions of normal ferret lung, in the presence or absence of the cytochrome P450 inhibitor, liarozole. Polar metabolites of retinoic acid including 18 hydroxy-retinoic acid and 4 oxo-retinoic acid were extracted and analyzed after the incubation by HPLC. As seen in Figure 2A, the formation of 4-oxo-retinoic acid was increased significantly over controls after incubation with microsomes from smoke-exposed ferrets, high dose β-carotene supplemented ferrets or ferrets exposed to both. However, this enhancement of microsomal retinoic acid catabolism was inhibited with liarozole, the nonspecific inhibitor of CYPs. Similarly, 18-hydroxy-retinoic acid increased after incubation with microsomes from smoke exposed ferrets, but this increase was inhibited by liarozole (Fig. 2B). Thus, these observations provide an explanation for why retinoic acid levels are lower in the lungs of smoke-exposed and high dose β-carotene supplemented animals.

We reasoned that the decrease in lung concentrations of retinoic acid would result in diminished retinoid signaling, and thereby enhanced cell proliferation and squamous metaplasia. One of the proposed mechanisms for the antiproliferative effect of the retinoic acid receptors (RAR and RXR) is through an interaction with the API complex made up of c-Fos and c-Jun (29). c-Fos and c-Jun form a complex in the nucleus termed activated protein 1 (AP-1), which binds to a DNA sequence motif not recognized by retinoid receptors, and which is referred to as the AP-1 binding site. AP-1 sites are DNA sequence motif not recognized by retinoid receptors, and tumor promoters, usually resulting in cell proliferation and squamous metaplasia. c-Fos and c-Jun are responsible for certain of the antiproliferative and anticancer properties attributed to retinoic acid.

We found that the expression of c-Fos was up-regulated 3- to 4-fold in β-carotene supplemented ferrets that were exposed to smoke compared with the control animals. Similarly, we found c-Jun to be upregulated in a like manner. This overexpression of AP-1 elements was associated with squamous metaplasia and correlated with the increased expression of PCNA.

Further, we found that RAR β expression in ferret lung was down-regulated in the smoke exposed group, the β-carotene exposed group, and most profoundly in the β-carotene plus smoke exposed group. Several lines of evidence have demonstrated that RAR β plays an important role in normal lung development (30–32). Primary lung tumors and lung cancer cell lines lack RAR β expression and such loss of expression may be an early event in lung carcinogenesis (30,33–35). Conversely, restoration of RAR β-2 in an RAR β negative
A lung cancer cell line has been reported to inhibit tumorigenicity in nude mice (36).

Thus, we feel that excentric cleavage products of β-carotene are responsible for inducing P450 enzyme activity resulting in lowered retinoic acid levels, which results in an interference with retinoid signaling and a cascade effect (1c-Fos, 1c-Jun, 2RARβ expression) with increased cell proliferation and carcinogenesis.

The question arises as to how an excess of excentric cleavage products are formed in the smoke exposed lung: is the process enzymatic or oxidative? Two pathways have been proposed for conversion of β-carotene to retinoids in mammals: central cleavage resulting in retinaldehyde and excentric cleavage resulting in a number of β-apo carotenals and carotenoid acids (21,37–43). To study β-carotene cleavage in human, monkey, ferret and rat tissue, whole tissue homogenates were prepared (40). After incubation of these homogenates with β-carotene in the presence of NAD and dithiothreitol, significant amounts of β-apo-12',10', and-8'-carotenals, retinal and retinoic acid were found. There were no β-apo-carotenals or retinoids detected in the control incubations without tissue homogenates. Moreover, the amounts of β-apo-carotenals and retinoids formed were markedly reduced when NAD was replaced by NADH, or when dithiothreitol and cofactors were deleted from the incubation. Both β-apo-carotenals and retinoid production were inhibited completely by adding disulfiram, an inhibitor of sulfhydryl containing enzymes. Two major cleavage products were identified by mass spectroscopy as β-apo-13-carotenone and β-apo-14'-carotenal (44). Thus, these data supported the existence of an enzymatic excentric cleavage mechanism for β-carotene. However, subsequent work by Yeum showed that similar metabolites could also be explained by a cooxidation mechanism (45).

Recently, Kiefer et al. in the laboratory of von Lintig demonstrated the identification of a cDNA from BALB/c mice, encoding a carotene oxygenase which catalyzes exclusively the asymmetric oxidative cleavage of β-carotene at the 9',10' double bond of β-carotene—resulting in the formation of β-apo-10' carotenol and β-ionone (46). The investigators identified 15,15'-dioxygenase in the small intestines, liver, and kidneys of mice, humans and zebra fish and the β-9',10' dioxygenase in the small intestines, brain, kidneys, heart, liver, lungs and testes of mice, zebra fish and humans. We examined whether this enzyme (which is termed as β,β-carotene-9',10'-dioxygenase) exists in the ferret. RT-PCR analysis was performed with total RNA isolated from ferret lung tissue, using primer sets derived from β,β-carotene-9',10'-dioxygenase cDNA sequence of the mouse. A specific RT-PCR product, of a size similar to that in mouse, was detected in ferret lung tissue. RT-PCR products of the β,β-carotene-9',10'-dioxyge-
nase where also detected in liver and intestine tissues of the ferret. Thus, an enzyme which is similar to mouse \( \beta_2 \)-carotene-9',10'-dioxygenase is also expressed in several tissues of ferret.

Another possible explanation for the generation of \( \beta \)-carotene excentric cleavage products in the lung is that of simple oxidation. It is interesting that in studies of tissue incubations, the investigators who were able to show central cleavage of \( \beta \)-carotene exclusively, without any evidence of excentric cleavage whatsoever, used \( \alpha \)-tocopherol or other antioxidants such as BHT in their systems (Table 2). However, the system used by Wang et al., who showed considerable excentric cleavage, had no additional antioxidant added (40). In addition, it should be noted that in the studies of Wang et al. the postnuclear fraction was used in the incubations, whereas most other studies used post mitochondrial or cytosolic fractions. Yeum et al. performed intestinal incubations with \( \beta \)-carotene using the postmitochondrial fraction of rat intestine and examined the effects of added \( \alpha \)-tocopherol on the route of \( \beta \)-carotene cleavage (47). In the presence of \( \alpha \)-tocopherol, \( \beta \)-carotene was converted exclusively to retinal, whereas in the absence of \( \alpha \)-tocopherol, both retinal and \( \beta \) apo carotenoids were formed. These data suggest that in the presence of adequate antioxidant such as \( \alpha \)-tocopherol, \( \beta \)-carotene is converted almost exclusively to retinal by central cleavage; however, in the absence of adequate antioxidant \( \beta \)-carotene can be cleaved \( \beta \) apo carotenoids. In the free radical rich environment of the lungs of cigarette smokers, decreased levels of antioxidants such as ascorbate and \( \alpha \)-tocopherol are highly probable (48,49). These molecules normally would have a stabilizing effect on the unoxidized form of \( \beta \)-carotene, and in their absence, high levels of oxidized excentric cleavage products may appear.

We have seen that oxidative excentric cleavage products of \( \beta \)-carotene induce P450 enzymes, resulting in a local deficiency of retinoic acid. In addition, it has recently been shown by Salgo et al. that fractions containing \( \beta \)-carotene metabolites, but not \( \beta \)-carotene itself, facilitate the binding of the smoke-derived carcinogen (benzo[\( \alpha \)]pyrene) metabolites to DNA (50). Moreover, Perocco et al. have shown that the induction of BALB/C 3T3 cell transformation by benzo[\( \alpha \)]pyrene is due to \( \beta \)-carotene itself, as compared to using pharmacologic doses, lung cell proliferation and squamous metaplasia could be ameliorated. More specifically, we wanted to know whether a physiologic dose of \( \beta \)-carotene would provide antioxidant protection, while not giving rise to undesirable metabolic by-products and thus possibly afford protection against lung carcinogenesis. We therefore carried out a study wherein ferrets were divided into \( \beta \)-carotene supplemented versus nonsupplemented groups, and were either smoke-exposed or not exposed to smoke. Doses of \( \beta \)-carotene were either physiologic (low) dose or pharmacologic (high) dose equivalent to 6 mg versus 30 mg/d in the human which would be the amounts contained in a reasonably high fruit and vegetable diet versus pharmacologic supplementation (52). Alveolar cell proliferation and keratinized squamous metaplasia were observed in the lung tissue of all ferrets supplemented with high dose \( \beta \)-carotene with and without smoke exposure. As seen before, the pathology and squamous metaplasia was worse in the lungs of the animals given high dose \( \beta \)-carotene and exposed to smoke. In contrast, we did not observe any pathological lesions in the ferrets given low dose \( \beta \)-carotene alone with or without smoke exposure. In fact, in this study low dose \( \beta \)-carotene appeared to alleviate the keratinized squamous metaplasia caused by smoke-exposure alone, since keratinized squamous metaplasia was found in none of the smoke-exposed animals receiving low dose \( \beta \)-carotene, whereas it was seen in 2 of 6 animals exposed to smoke alone. The same findings as before were seen in the high dose \( \beta \)-carotene supplemented animals with or without smoke exposure; reduced lung retinoic acid levels and RAR \( \beta \) gene expression, and increased expression of AP-1 and PCNA.

Thus, from these two sets of smoke inhalation experiments it appears that the decreased lung retinoic acid concentration results from increased P450 activity; this causes a depression of AP-1 activity, and thereby increased PCNA expression and squamous metaplasia. Recently it has been reported that c-Jun is required for progression through the G1 phase of the cell cycle by a mechanism that involves direct transcriptional control of the cyclin D1 gene (53). An increased expression of cyclin D1 was seen in our experiments in the lungs of the ferrets supplemented with high dose \( \beta \)-carotene in the absence or presence of smoke exposure.

A question that needs to be answered is whether lung carcinogenesis caused by high-dose \( \beta \)-carotene and smoke can

### TABLE 2

**Metabolism of \( \beta \)-Carotene**

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<th>Incubation conditions</th>
<th>Central cleavage</th>
<th>Excentric cleavage (4 Ref 43)</th>
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be mitigated or abolished by simply maintaining high levels of other antioxidants in the lung (for example, vitamins C and E). Both vitamins C and E can inhibit cytochrome P450-mediated lipid peroxidation and carcinogen activation (54). Further, vitamin C is a strong reducing agent, and diets high in vitamin C have been associated with a lower risk of cancers of the lung (55,56). Smokers are known to have significantly lower plasma levels of vitamin C as compared to nonsmokers, and similarly, passive smokers have reduced ascorbic acid concentrations in their plasma (48,49). It is known that vitamin C can regenerate vitamin E from the vitamin E radical during lipid peroxidation (57), and recent studies have also shown that vitamin C can convert the β-carotene radical back to β-carotene, maintaining β-carotene in its unoxidized form (58). Conversely, it has been shown that oxidized vitamin E can be recycled by β-carotene (59). The combination of β-carotene and α-tocopherol results in the inhibition of free radical induced lipid peroxidation, which is significantly greater than the sum of the individual effects (60).

We are now carrying on experiments in smoke exposed ferrets using low or high dose β-carotene in combination with two antioxidants; α-tocopherol and ascorbate. The simple combination of β-carotene and vitamin E were not found to be protective against smoke-related lung cancer in the ATBC study (14). However, vitamin C, which will facilitate both vitamin E recycling and β-carotene stability, was not used in the ATBC study. It may be particularly important to have broad antioxidant protection when using high doses of β-carotene in order to prevent the production of β-carotene excincent cleavage products and the associated cascade of events that result from them.

So what have we learned so far from the animal experiments? Firstly, we have learned that taking a natural product at any dose is not wise. Dose does count. High dose antioxidant supplementation in the animal models clearly results in lung pathology with or without smoke exposure, although the pathology is far worse when the combination of high dose β-carotene and smoke is used. Secondly, we have learned a great deal about the possible molecular mechanisms involved: the breakdown products of β-carotene can induce P450 enzyme activity resulting in lower tissue retinoic acid levels and diminished retinoid signaling producing a cascade of events leading to lung cell proliferation. Thirdly, and perhaps most importantly, we have learned that we had better understand the metabolism and breakdown of natural products, and have thoroughly tested them at various doses in appropriate animal models, before embarking on large-scale intervention trials, particularly when using unusually high doses that greatly exceed normal dietary levels of the product. Our studies show how important preliminary animal studies can be before embarking on large-scale interventions or before making public health pronouncements.

LITERATURE CITED


