British Journal of Nutrition (2005), **94**, 540–543
DOI: 10.1079/BJN20051484

Efficiency of a few retinoids and carotenoids *in vivo* in controlling benzo[*a*]pyrene-induced forestomach tumour in female Swiss mice

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(Received 17 May 2004 – Revised 11 March 2005 – Accepted 14 March 2005)

The anticarcinogenic effect of vitamin A_2 (dehydroretinol and 3-hydroxyretinol) compounds was studied and compared with that of vitamin A_1 (retinoic acid, retinol and retinal) and carotenoids (lutein and β-carotene) in the benzo[*a*]pyrene (B(a)P)-induced forestomach tumour model of female Swiss mice *in vivo*. Tumour growth and gross tumour incidence observed after the administration of B(a)P (eight doses of 1 mg, twice weekly for 4 weeks) and retinoids/carotenoids (2·5 and 4·7 μM per animal per d, 2 weeks before, during and 2 weeks after B(a)P) showed that the groups supplemented with lutein and 3-hydroxyretinol produced the best results in inhibiting tumour growth and had low tumour incidence compared with the control group given B(a)P only ($P<0·05$). Weights recorded after the different treatments showed that the β-carotene-supplemented group exhibited maximum weight gain, followed by retinal, retinol, retinoic acid, lutein, dehydroretinol and 3-hydroxyretinol. These results indicate that the anticarcinogenicity of the compounds is not related to the vitamin A biopotencies. Vitamin A_2 compounds having half the biopotency of the vitamin A_1 compounds were seen to be anticarcinogenic. Again, among the carotenoids, lutein, having 50 % less biopotency, showed more significant results than β-carotene. Thus it is imperative to conclude that the low animal growth achieved with these compounds has a correlation with the highest suppression of tumour occurrence in the present experiment. Therefore, the daily consumption of foods having high content of lutein and vitamin A_2 should be given due importance and weight in further studies.

Vitamin A: Carotenoids: Dehydroretinol: 3-Hydroxyretinol: Carcinogen: Benzopyrene: Cancer: Stomach tumour

Growing evidence suggests that retinoids and carotenoids act as significant potent inhibitors of many natural oncogenic agents present in the environment. Examples are the inhibition of methyl-*N*-nitrosourea-induced mammary cancer by retinyl acetate (Moon & Itri, 1984); the inhibition by 13-*cis*-retinoic acid of mouse skin carcinoma induced by phorbol ester (Verma *et al.* 1979); and the inhibition in rats of 7,12-dimethylbenz[*a*]anthracene-induced mammary cancer (Rettura *et al.* 1984) and salivary gland cancer (Alam *et al.* 1984) by β-carotene. Saloi (1995) has reported the anticarcinogenic effect of a few carotenoids such as canthaxanthin, β-carotene and 8′-apo-β-carotenal in some experimental models.

The present study aimed to examine the effects of some carotenoids (lutein and β-carotene), vitamin A_1 compounds (retinol, retinoic acid and retinal) and vitamin A_2 compounds (dehydroretinol and 3-hydroxyretinol) in an *in vivo* bioassay commonly used to assess the anticarcinogenicity of compounds. To date, no information is available regarding the effect of vitamin A_2 on cancer. Hence, this type of comparative study easily helps to assess the efficacy of vitamin A_2 compounds in the prevention of cancer. Benzo[*a*]pyrene (B(a)P), the most commonly occurring natural carcinogen responsible for a significant percentage of cancer cases (International Agency for Research on Cancer, 1973), was used in the study to induce forestomach tumours in female Swiss mice.

The results indicate that supplementation with lutein, the precursor of dehydroretinol, resulted in the least number of tumours following induction with B(a)P. The efficiency of the other tested compounds vis-à-vis B(a)P as seen in our experiment was 3-hydroxyretinol, β-carotene, dehydroretinol, retinoic acid, retinal and retinol. Dehydroretinol, which has less biopotency (−40 %) than retinol (Shantz & Brinkman, 1950), was more effective in inhibiting tumour growth than most of the retinoids, and did not promote weight gain in the mice.

Materials and methods

Animals and diets

Female Swiss mice were obtained and housed in the animal colony of the Cancer Research Centre, Mumbai, India in metal cages (five mice per cage). The colony was maintained at 21 ± 1°C and 55 % relative humidity under a 12 h light/12 h dark cycle. Food and water were supplied *ad libitum*. The composition of the diet is shown in Table 1. At the start of the experiment the mice were 6–7 weeks old and weighed 20–25 g.

Induction of forestomach tumours

The anticarcinogenic effects of various retinoids/carotenoids were evaluated in the B(a)P-induced forestomach tumour model. Tumour formation in the mouse forestomach was induced as described by Wattenberg (1972) and Wattenberg *et al.* (1980) with some modifications.

Abbreviation: B(a)P, benzo[*a*]pyrene.

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Table 1. Composition of the mice's diet (Santhanam *et al.* 1987)

Composition	g/100 g
Wheat	70
Bengal gram	20
Fishmeal*	5
Yeast	4
Groundnut oil	0·95
Shark-liver oil	0·05

* The fishmeal was prepared from the flesh of the freshwater catfish, *Wallago attu*, which was powdered and sun-dried for eight consecutive days (8 h/d). This resulted in the loss of both carotenoids or vitamin. A negligible amount of shark-liver oil was added to prevent mortality of the mice during the experimental period, as previous experience using the diet without such addition showed a mortality rate of 50–60 %.

For details of diets and procedures, see p. 540.

The 6–7-week-old Swiss mice were given the chemopreventive agent (at a concentration of 2·5 or 4·7 μM per animal per d). From the third week onwards the mice also received eight doses (twice weekly for 4 weeks) of 1 mg B(a)P in 0·1 ml peanut oil by intragastric intubation. Administration of the test compounds was continued for two more weeks after cessation of the carcinogen treatment. The following treatment groups, with twenty animals per group, were set up:

(1) B(a)P-treated positive control: mice received only B(a)P twice weekly for 4 weeks.
(2) B(a)P + tested compounds: retinoids/carotenoids at daily concentration of 2·5 and 4·7 μM for 2 weeks prior to, during and 2 weeks after the carcinogen administration.

The treatment schedule is presented in Table 2.

After completion of the treatment, the animals were kept under observation and killed under anaesthesia using diethyl ether, at the age of 180 d. The stomach was fixed by injection of 10 % formalin. After 24 h the stomach was cut open longitudinally and forestomach papillomas were counted under a dissecting microscope.

Authentic samples of retinoid and carotenoid compounds (β-carotene, lutein, retinol, retinal, retinoic acid and dehydroretinol) were obtained from F. Hoffman La-Roche, Basel, Switzerland. They were further purified through HPLC as described earlier (Goswami, 1984; Guillou *et al*. 1993). 3-Hydroxyretinol was isolated (Barua *et al.* 1979) from the freshwater siluroid fish *Wallago attu*, which is rich in dehydroretinol (Goswami & Barua, 1981; Goswami, 2005) and later purified following Guillou *et al.* (1993). B(a)P was a product of Sigma-Aldrich (St. Louis, MO, USA).

Table 2. Treatment schedules of various test groups of animals

Group	Treatment
1	Benzo[*a*]pyrene (B(a)P) alone
2	B(a)P + β-carotene (BC); 2·5 μM
3	B(a)P + BC, 4·7 μM
4	B(a)P + lutein (LU), 2·5 μM
5	B(a)P + LU, 4·7 μM
6	B(a)P + retinol (ROL), 2·5 μM
7	B(a)P + ROL, 4·7 μM
8	B(a)P + retinal (RAL), 2·5 μM
9	B(a)P + RAL, 4·7 μM
10	B(a)P + retinoic acid (ROC), 2·5 μM
11	B(a)P + ROC, 4·7 μM
12	B(a)P + dehydroretinol (DROL), 2·5 μM
13	B(a)P + DROL, 4·7 μM
14	B(a)P + 3-hydroxyretinol (3HDROL), 2·5 μM
15	B(a)P + 3HDROL, 4·7 μM

For details of diets and procedures, see p. 540.

Statistical analysis

ANOVA followed by a multiple comparison test was conducted to determine the statistical significance of the differences between all groups with regard to the effect of treatment on tumour growth, gross tumour incidence and weight gain.

The exact binomial test was applied to determine if the first treatment, B(a)P alone, differed significantly from each of the other treatments in the percentage of animals affected with cancer.

Results and discussion

Table 3 presents analyses on the occurrence of forestomach tumours and their incidence. From the present experiment, the following observations can be made.

(1) Tumour growth. Among the compounds tested, the lutein-supplemented group showed the greatest inhibition of tumour growth (tumours per mouse: 3·6 (SE 0·7) and 2·5 (SE 0·5) at 2·5 and 4·7 μM, respectively), compared with the control group ($P<0·05$), in the forestomach of female Swiss mice. This was followed by 3-hydroxyretinol, β-carotene, retinoic acid, retinal, dehydroretinol and retinol, respectively.
(2) Gross tumour incidence. Regarding gross tumour incidence, both the lutein- and the 3-hydroxyretinol-supplemented

Table 3. Effect of carotenoids and retinoids on benzo[*a*]pyrene-induced forestomach tumours in the different groups of mice

	Weight gain (g)		Gross tumour incidence	Tumours per mouse	
Group*	Mean	SE	No. of animals with tumour/ total no. in group (%)	Mean	SE
1	4·80[a]	0·38	20/20 (100)	8·5[a]	0·4
2	4·35[a]	0·16	5/20 (25)	5·4[b]	1·0
3	5·90[b]	0·16	4/20 (20)	3·25[b]	0·6
4	2·74[b]	0·21	3/20 (15)	3·6[b]	0·7
5†	4·53[b]	0·16	2/20 (10)	2·5[b]	0·5
6	4·94[a]	0·13	9/20 (45)	7·7[a]	1·0
7	5·42[a]	0·25	8/20 (40)	6·0[b]	1·0
8	4·73[a]	0·21	8/20 (40)	6·25[b]	1·0
9	5·62[a]	0·25	7/20 (35)	5·0[b]	1·0
10	3·52[b]	0·22	8/20 (40)	6·5[a]	1·1
11	5·00[a]	0·17	7/20 (35)	4·4[b]	0·8
12	2·51[b]	0·16	5/20 (25)	7·0[a]	1·4
13	2·75[b]	0·14	5/20 (25)	5·0[b]	1·0
14	2·24[b]	0·24	3/20 (15)	3·0[b]	0·6
15	2·37[b]	0·11	2/20 (10)	4·0[b]	0·8

[a,b] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$) at 95 % confidence interval; the calculation includes all animals in a tested group.

* For details of animals and procedures, see p. 540. See also Table 1 for corresponding treatment.

† Group showing the lowest tumour growth.

groups showed low tumour incidence (each 15 % at 2·5 μM and 10 % at 4·7 μM). Tumour incidence increased gradually with the supplementation of β-carotene, dehydroretinol, retinoic acid, retinal and retinol. All treatments differed significantly from the control in the percentage of animals affected with cancer.

(3) Weight gain. In the case of weight gain, the group supplemented with β-carotene showed the highest results (4·35 (SE 0·16) g at 2·5 μM, 5·90 (SE 0·16) g at 4·7 μM), which was followed by retinal, retinol, retinoic acid, lutein, dehydroretinol and 3-hydroxyretinol, respectively.

The experimental results provide evidence for the inhibition of B(a)P-induced forestomach tumours in female Swiss mice by the tested carotenoids/retinoids. The relative potency of these compounds was not found to be the same and showed differences in inhibiting the tumours.

The carotenoids tested in the present experiment were β-carotene and lutein, which have pro-vitamin A characteristics. β-Carotene is the main precursor of vitamin A (Moore, 1957) and has the highest pro-vitamin A activity. Carotene is an effective antioxidant (Burton & Ingold, 1984; Burton, 1989; Kennedy & Liebler, 1992; Palozza & Krinsky, 1992) and thus has a specific anticarcinogenic action. Lutein, the precursor of dehydroretinol (Goswami & Barua, 1981; Goswami & Bhattacharjee, 1982; Goswami, 1984, 2005), has a biopotency less than 50 % that of β-carotene (Isler, 1971), but was found to be significantly more active than β-carotene in inhibiting B(a)P-induced forestomach tumours *in vivo*. Similar results were also found by Saloi (1995), that lutein is more active than β-carotene in inhibiting the formation of AFB_1–DNA adducts *in vitro*. With regard to weight gain, however, it was found in the present study that β-carotene-supplemented mice showed better growth than lutein-supplemented mice. From these findings it can be inferred that the intrinsic chemopreventive properties of carotenoids do not depend so largely on their conversion into vitamin A. It may be because of their antioxidant property that they are effective quenchers of singlet oxygen (Burton & Ingold, 1984; Krinsky, 1989) and free radicals (Krinsky & Deneka, 1982; Burton & Ingold, 1984; Santamaria *et al.* 1988), which play important roles in the inhibition of carcinogenesis (Krinsky, 1974; Cerutti, 1985; Kensler & Taffe, 1986; Bendich, 2004; Cooper, 2004).

Among the retinoids in the present experiment, 3-hydroxyretinol supplementation showed effective results in inhibiting tumour growth and the lowest occurrence of tumour incidence, with a minimum weight gain. This was followed by the dehydroretinol-supplemented group, which also showed the same trend. Considering available information on the biopotency of dehydro vitamin A compounds, 3-hydroxyretinol has the lowest biopotency in the regulation of growth. It is imperative to conclude that the low growth observed in the mice supplemented with these compounds has a correlation with the highest suppression of tumour occurrence in the present experiment. Moreover, from our present findings along with some other earlier reports (Mayer *et al.* 1978; Bollag & Matter, 1981; Bollag, 1983; Ong & Chytil, 1983), it has been observed that retinoic acid is an anticarcinogen and is more active than retinol or retinal in numerous *in vitro* test systems (Strickland & Mahdevi, 1978; Breitman *et al.* 1980; Lotan, 1980; Sporn & Newton, 1981).

In our earlier studies (Goswami & Bhattacharjee, 1982; Goswami, 1984), we showed that lutein is metabolised into dehydroretinol through anhydrolutein and 3-hydroxyretinol. Several studies have shown that dietary lutein consistently inhibits the growth of mammary tumours in mice (Chew *et al.* 1996; Brown *et al.* 2001; Chew & Park, 2004). Dietary lutein was also seen to increase mRNA expression of the pro-apoptotic gene *p53*, possibly because of its involvement in apoptosis (Chew & Park, 2004). β-Carotene and other carotenoids have been thought to have anticancer activity because of either their antioxidant activity or their ability to be converted into vitamin A (Krinsky, 1993; Goswami *et al.* 1995). Nevertheless, two large-scale intervention studies in man using high doses of β-carotene showed that β-carotene supplementation resulted in greater lung cancer among the smoking population and in those exposed to asbestos (Russel, 2004). It has been found that high-dose β-carotene gives rise to a number of transient oxidative metabolites, which include P-450 enzymes that result in the destruction of retinoic acid, diminish retinoid signalling and enhance cell proliferation. In addition, excentric cleavage metabolites facilitate the binding of smoke-derived metabolites to DNA, while low-dose β-carotene provides protection against squamous metaplasia.

Thus it can be concluded from the present study that, apart from carotenoids, modification of any part of the retinoid molecule has a tremendous effect on the compound's anticancer activity. The molecular architecture of the dehydro compounds, with their extra double bond in the 3 and 4 position in the case of dehydroretinol and OH group in the β-ionone ring of 3-hydroxyretinol, showed significantly different activities than those of retinol. Similarly, the COOH group-bearing retinoid showed itself to be more effective than the compounds bearing CHO and CH_2OH groups. Finally, from these *in vivo* studies it has been seen that dehydroretinol, having less biopotency than the retinols, is more anticarcinogenic than most of the retinoids.

Acknowledgements

We express our gratitude to the late Dr S. V. Bhide, Head of the Carcinogenesis Division, Cancer Research Institute, Mumbai, for allowing us to conduct the present experiment in the animal house facilities of the institute. Thanks are due to Dr Magnus Azunie for taking care of the animals. The financial assistance obtained by one of us (N. S.) from the University Grants Commission, New Delhi, is gratefully acknowledged.

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