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# Chemopreventive Effects of β-Carotene, α-Tocopherol and Five Naturally Occurring Antioxidants on Initiation of Hepatocarcinogenesis by 2-Amino-3-methylimidazo[4,5-*f*]quinoline in the Rat

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**Inhibitory effects of naturally occurring antioxidants on the initiation stage of hepatocarcinogenesis were studied. Group 1 rats were given a diet containing β-carotene (β-CT, 0.02%), α-tocopherol (α-TP, 1.5%), glutathione (GLT, 5%), vanillin (VNL, 1%), quercetin (QCT, 1%) or ellagic acid (ELA, 1%), or 3 doses of diallyl sulfide (DAS, 200 mg/kg, i.g.) over an 8-day period. On day 7, the animals received a single dose of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ, 100 mg/kg, i.g.), 12 h after two-thirds partial hepatectomy for initiation and 2 weeks thereafter, were placed on promotion regimen comprising phenobarbital (0.05% in diet) and a single dose of D-galactosamine (100 mg/kg, i.p.). Groups 2 and 3 were treated as described for Group 1, but without test material or IQ, respectively. Survivors were killed at week 11 and antioxidant influence was assessed by comparing values for preneoplastic glutathione S-transferase placental form-positive (GST-P$^+$) foci between Groups 1 and 2. All lesions larger than 70 μm in diameter consisting of approximately 5 cells in cross section were counted. Numbers of GST-P$^+$ foci/cm$^2$ in Group 1 were: β-CT, 7.99; α-TP, 8.21; GLT, 9.71; DAS, 10.37; VNL, 10.57; QCT, 11.1; ELA, 12.5 (n=11–15). All, except ELA, showed a significant decrease as compared with the Group 2 value of 14.54 (n=15). Only β-CT showed a significant decrease for the area value. This is the first report to show that β-CT, α-TP, GLT, DAS, VNL, QCT exert inhibitory effects on initiation of hepatocarcinogenesis by the food carcinogen IQ, suggesting that these antioxidants might find application as chemopreventive agents. Furthermore, the current protocol proved practical for the assessment of chemopreventive agents within 11 weeks, a relatively short period.**

Key words: Chemoprevention — Hepatocarcinogenesis — Initiation — Antioxidant

There is extensive evidence suggesting a preventive role for plant constituents against cancer development,[1–4] mainly from studies of fruit and vegetable intake and serum levels of specific micronutrients. For example, high consumption of fresh fruits and vegetables is associated with lowered cancer incidences, suggesting that some constituent compounds may participate in prevention of cancer.[1, 2] Plant foods contain various kinds of vitamins and phenolic compounds possessing antioxidant activity.[1, 2] Many studies regarding the preventive effects of such compounds have been based on experimental systems in which antioxidants were administered during the post-initiation stage of carcinogenesis.[5–10] For example, in the rat liver, several naturally occurring plant antioxidants have been shown to exert inhibitory effects on development of preneoplastic focal lesions when applied after appropriate initiation.[11, 12]

On the other hand, naturally occurring antioxidants are known to possess antimutagenic activity, which may be directly related to the initiation of carcinogenesis.[13, 14] However, there is only limited evidence that plant constituents can influence the first step of *in vivo* carcinogenesis, namely initiation. Since complete carcinogens by definition possess both initiation and promotion potential, it should also be possible to predict the extent of tumor development by assessing the initiation stage. It has been shown that not only liver carcinogens but also agents generally considered to be non-liver carcinogens may initiate the induction of enzyme-altered hepatocellular focal lesions or tumors when given as a single administration during a phase of replicative DNA synthesis caused by PH[5] or chemical agents.[15–18] Thus, it should be possible to create optimal conditions for assaying chemopreventive effects of compounds on initiation of hepatocarcinogenesis. In the present study, 2 vitamins, β-CT

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[5] Abbreviations: PH, partial hepatectomy; β-CT, β-carotene; α-TP, α-tocopherol; GLT, glutathione; DAS, diallyl sulfide; VNL, vanillin; QCT, quercetin; ELA, ellagic acid; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; GST-P$^+$, glutathione S-transferase placental form-positive; D-GA, D-galactosamine.

and $\alpha$-TP, and 5 naturally occurring antioxidants, GLT, DAS, VNL, QCT, and ELA, were studied for inhibitory effects on initiation by the food pyrolysate carcinogen IQ,[19] using preneoplastic GST-P$^+$ liver cell foci as end-point marker lesions.[20–22] For the purpose of promoting IQ-initiated hepatocytes to GST-P$^+$ foci of sufficient size to allow quantitative analysis, rats were subjected to administration of the hepatopromoter phenobarbital combined with a necrogenic dose of D-GA.[23–26]

## MATERIALS AND METHODS

A total of 162 male, 6-week-old Fischer rats (Charles River Japan Inc., Atsugi) were used. They were maintained on basal diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) *ad libitum* and housed in plastic cages in an air-conditioned room at 24±2°C and 60±5% humidity.

Group 1 rats were placed on basal diet containing $\beta$-CT (0.02%), $\alpha$-TP (1.5%), VNL (1%), QCT (1%), ELA (1%) (Tokyo Chemical Industry Co., Ltd., Tokyo) or GUT (5%) (Tokyo Chemical Industry Co., Ltd.) for 8 days, or DAS (Tokyo Chemical Industry Co., Ltd.) (200 mg/kg, dissolved in saline) given by gavage on days 0, 3 and 7. On day 7 the rats received a single dose of the food pyrolysate carcinogen, IQ (Nard Institute, Ltd., Amagasaki) (100 mg/kg, suspended in corn oil) by gavage at 12 h after two-thirds PH[27–29] for initiation of hepatocarcinogenesis. After 2 weeks on basal diet, the animals were placed on basal diet containing phenobarbital (0.05%) for 8 weeks. One week from the start of phenobarbital feeding, rats were injected with a single dose of D-GA (Tokyo Chemical Industry Co., Ltd.) (300 mg/kg, i.p.) to cause cell proliferation and enhance the promoting effect of phenobarbital.[26,27] Survivors were all killed at week 11 (15–17 rats for each compound). Groups 2, 3 and 4 were treated as described for Group 1, but without test material (Group 2) (15 rats), IQ (Group 3) (5 rats each) or both (Group 4) (5 rats each) (Fig. 1). Immediately after killing of the animals, the livers were excised. Slices 4–5 mm thick were cut with a razor blade, then immersed in ice-cold acetone for 4 weeks and embedded in paraffin. For immunohistochemical staining for GST-P$^+$ foci, paraffin sections were treated with polyclonal antibody to glutathione S-transferase placental form at a dilution of 1:5000.[20–22] Binding sites were demonstrated by the avidin-biotin peroxidase complex (ABC) method (Vectastain kit, Vector Laboratories Inc., Burlingame, CA, USA)[30] using diaminobenzidine-$H_2O_2$, and sections were then lightly counterstained with hematoxylin. Results were assessed by comparing number and area values for GST-P$^+$ foci larger than 70 $\mu$m in diameter (approximately 5 cells in cross section) (Fig. 2) between Groups 1 and 2 using a color video image processor (Olympus-Ikegami VIP-21C, Olympus Co., Ltd., Tokyo).[27,28] Statistical analyses were performed using Student's $t$ test.

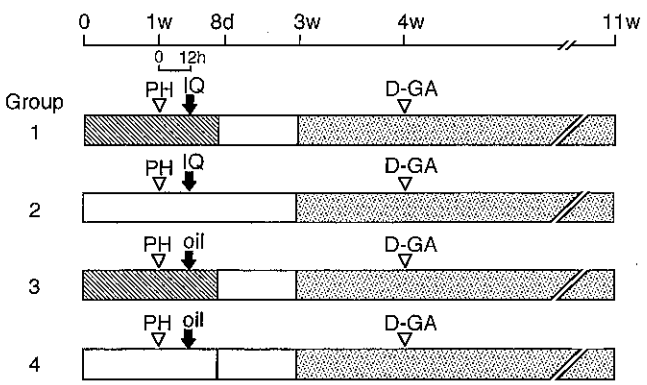


Fig. 1. Rats were divided into 4 groups. Group 1 rats were placed on a basal diet containing test compound (▨) then received a single dose of IQ (⬇) (100 mg/kg) by gavage, 12 h after PH (▽). After 2 weeks on basal diet (□), rats were fed on basal diet containing phenobarbital (0.05%) (▨) for 8 weeks, and at week 4, received an intraperitoneal injection of D-GA (▽) (300 mg/kg). Survivors were killed at week 11. Groups 2, 3 and 4 were treated as in Group 1 without test compound (Group 2), IQ (Group 3) or both (Group 4). Results were assessed by comparing number and area values for GST-P$^+$ foci larger than 70 $\mu$m in diameter (approximately 5 cells in cross section) between Groups 1 and 2.

## RESULTS

**Body and liver weights** Body weights of rats in Group 1 were significantly decreased ($P<0.05$) by $\beta$-CT and significantly increased ($P<0.05$) by QCT as compared to Group 2 values. Liver weights of rats in Group 1 fed

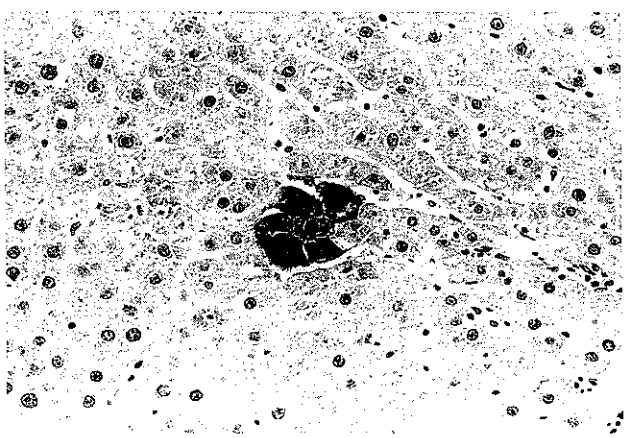

Fig. 2. Appearance of smallest GST-P$^+$ foci used for the quantitative analysis (immunostaining, ×80).

VNL and QCT were significantly decreased as compared to Group 2. Similarly, relative liver weights (% body weight) of rats fed β-CT, GLT, VNL and QCT were significantly increased. However, these differences were within approximately 10% of the absolute weights (Table I).

**GST-P$^+$ foci values** Numbers of GST-P$^+$ foci/cm$^2$ in Group 1 were: β-CT, 7.99±3.14 (n=13); α-TP, 8.21±4.42 (n=11); GLT, 9.71±3.55 (n=12); DAS, 10.37±3.57 (n=17); VNL, 10.57±3.82 (n=12); QCT, 11.18±2.20 (n=14); ELA, 12.59±5.23 (n=11). All, except ELA, showed a significant decrease as compared with the Group 2 value of 14.54±4.04 (n=15). Their area values ($\mu m^2/cm^2$) were: β-CT, 74,949±33,221; α-TP, 74,496±53,155; GLT, 83,763±34,120; DAS, 96,281±38,646; VNL, 109,945±56,878, QCT, 107,737±31,342; ELA,

Table I. Body and Liver Weight Data

| Group | Test compound | Initiation by IQ | No. of rats | Body weight (g) | Liver weight (g) | Relative liver weight (% body weight) |
|---|---|---|---|---|---|---|
| 1 | β-Carotene | + | 13 | 300.9±20.3* | 14.11±1.53 | 4.68±0.28** |
| | α-Tocopherol | + | 11 | 320.3±27.5 | 13.96±1.58 | 4.35±0.23 |
| | Glutathione | + | 12 | 311.9±28.1 | 14.52±1.08 | 4.67±0.35* |
| | Diallyl sulfide | + | 17 | 303.8±25.0 | 13.96±1.36 | 4.60±0.34 |
| | Vanillin | + | 12 | 327.3±14.9 | 15.71±1.20*** | 4.80±0.26*** |
| | Quercetin | + | 14 | 339.3±17.8* | 16.16±1.43*** | 4.76±0.31** |
| | Ellagic acid | + | 11 | 329.3±20.8 | 14.45±1.25 | 4.39±0.21 |
| 2 | Basal diet | + | 15 | 318.3±15.0 | 13.99±0.82 | 4.40±0.23 |
| 3 | β-Carotene | − | 5 | 313.8±12.6 | 13.58±0.95 | 4.33±0.26 |
| | α-Tocopherol | − | 5 | 319.2±6.4 | 13.17±0.49 | 4.12±0.10 |
| | Glutathione | − | 3 | 328.3±22.7 | 13.76±1.17 | 4.19±0.12 |
| | Diallyl sulfide | − | 4 | 336.4±13.9 | 14.24±0.35 | 4.23±0.08 |
| | Vanillin | − | 1 | 335.2 | 14.47 | 4.32 |
| | Quercetin | − | 5 | 317.7±6.0 | 13.13±0.50 | 4.13±0.17 |
| | Ellagic acid | − | 3 | 329.5±8.6 | 14.43±0.48 | 4.38±0.05 |
| 4 | Basal diet | − | 5 | 328.9±16.7 | 14.56±1.19 | 4.42±0.16 |

Significantly different from Group 2 at $P<0.05$ (*), 0.01 (**) or 0.001 (***).

Table II. Assay of Chemopreventive Effects of Naturally Occurring Antioxidants on Initiation of Rat Hepatocarcinogenesis by IQ

| Group | Test compound | Dose in diet (%) | Initiation by IQ | No. of rats | Values for GST-P$^+$-foci | |
|---|---|---|---|---|---|---|
| | | | | | No. (No./cm$^2$) | Area ($\mu m^2/cm^2$) |
| 1 | β-Carotene | 0.02 | + | 13 | 7.99±3.14*** | 74949±33221* |
| | α-Tocopherol | 1.5 | + | 11 | 8.21±4.42*** | 74496±53155 |
| | Glutathione | 5 | + | 12 | 9.71±3.55*** | 83763±34120 |
| | Diallyl sulfide | 200 mg/kg×3[a)] | + | 17 | 10.37±3.57* | 96281±38646 |
| | Vanillin | 1 | + | 12 | 10.57±3.82* | 109945±56878 |
| | Quercetin | 1 | + | 14 | 11.18±2.20* | 107737±31342 |
| | Ellagic acid | 1 | + | 11 | 12.59±5.23 | 149567±86059 |
| 2 | Basal diet | − | + | 15 | 14.54±4.04 | 107674±42178 |
| 3 | β-Carotene | 0.02 | − | 5 | 0.52±0.20 | 3972±1895 |
| | α-Tocopherol | 1.5 | − | 5 | 0.14±0.20 | 1536±2336 |
| | Glutathione | 5 | − | 3 | 0.45±0.28 | 4982±2957 |
| | Diallyl sulfide | 200 mg/kg×3[a)] | − | 4 | 0.10±0.20 | 4969±9939 |
| | Vanillin | 1 | − | 1 | 0.34 | 2892 |
| | Quercetin | 1 | − | 5 | 0.07±0.16 | 118±263 |
| | Ellagic acid | − | − | 3 | 0.53±0.23 | 6735±5929 |
| 4 | Basal diet | − | − | 5 | 0.27±0.38 | 8652±13724 |

*a*) i.g. at days 0, 3 and 7.
GST-P$^+$ foci were greater than 70 μm in diameter (approximately 5 cells in cross section).
Significantly different from Group 2 at $P<0.05$ (*), 0.01 (**) or 0.001 (***).

149,567±86,059. Only β-CT showed significant decrease as compared with the Group 2 value of 107,674±42,178. In Group 3, values ranged from 0.07 to 0.53 in numbers and 118 to 6,735 in areas (n=1–5) and did not show any significant differences as compared to Group 4 number values of 0.27±0.38 and area values of 8,652±13,724 (n=5) (Table II).

## DISCUSSION

The schedule described in the present study allowed the *in vivo* determination of inhibitory effects of naturally occurring antioxidants using GST-P$^+$ foci as end-point marker lesions, within a relatively short 11-week period. Furthermore, the duration of test compound administration was as short as 8 days. The numbers of GST-P$^+$ foci were clearly reduced, in line with previous findings, for example for β-CT inhibition of the initiation stage of liver carcinogenesis.[31,32] Since initiation is presumed to reflect interactions between carcinogens and individual liver cells, the number of focally derived altered populations is considered to represent the number of cells which underwent initiation. Thus, the current method is a practically applicable, new *in vivo* protocol for the screening of inhibitory agents of carcinogenesis.

It is generally accepted that cellular proliferation is critical for initiation of hepatocarcinogenesis and PH has been extensively utilized as one of the most effective methods to enhance initiation and carcinogenesis.[11,16,28,33] We therefore tested 7 compounds for inhibitory effects on initiation by IQ, utilizing our established model for the assay of initiation potential of known carcinogens, including IQ and liver carcinogens.[27] The results clearly indicate that β-CT, α-TP, GUT, DAS, VNL, QTN can all inhibit the induction of preneoplastic rat liver lesions when administered before and during IQ exposure. Since carcinogens can be defined as possessing initiation potential, blocking of this step corresponds to prevention of carcinogenicity. In addition to the reported inhibitory effect of α-TP when tested during the post-initiation phase, β-CT, α-TP, GUT, VNL, QTN could act as chemopreventive agents against the food carcinogen IQ, considered as one of the most probable cancer-causing agents in man. With regard to the earlier result suggesting that DAS may promote induction of GST-P$^+$ foci when administered during the post-initiation phase, this clearly did not extend to initiation. Further investigation using different initiating and promoting agents to establish how modification is achieved by different agents is required.[34]

Antioxidants induce phase II enzymes[32,35–37] which can detoxify IQ to species which do not bind to DNA.[38] Whether the present results reflect absolute differences in levels of IQ-DNA adducts needs elucidation.

There is increasing evidence that naturally occurring and synthetic antioxidants inhibit liver carcinogenesis in the rat.[8,39–42] One reason for such inhibition could be their lowering effect on oxygen radical levels.[43,44] Antioxidant vitamins such as ascorbic acid, β-CT, α-TP and retinols may neutralize free oxygen radicals which are produced by treatment with carcinogens and cause damage to macromolecules.[45] Although a direct causative role for oxygen radical interactions during the initiation stage in our protocol has not been confirmed, such a mechanism clearly deserves consideration. Interaction of these antioxidants with enzymes responsible for activation of IQ such as cytochrome P450IA2 may be involved in inhibition of the initiation potential.[3,46,47] Another possibility is that effects were exerted on gap junctional intercellular communication species, since carotenoids have been shown to up-regulate the expression of connexin 43 in carcinogen-treated fibroblastic cells.[48]

While naturally occurring and synthetic antioxidants inhibit liver carcinogenesis when administered during the post-initiation stage,[6,11,40,42] they were also found to promote tumor development or even be carcinogenic themselves in other organs. With this in mind, appropriate experimentation with wide-spectrum initiation protocols is necessary to clarify whether any particular compound could be practically usable as a chemopreventive agent.[39,40,49–52]

The current protocol is advantageous for the assay of inhibitory effects of compounds because of the short-term duration of treatment, with particular advantage when the availability of the test compound is limited. Financial considerations also are important with conventional long-term studies.

In conclusion, the present results indicate that β-CT, α-TP, GUT, DAS, VNL and QTN exert inhibitory effects on the initiation stage of hepatocarcinogenesis by IQ. Therefore, these naturally occurring plant antioxidants deserve further consideration as potential chemopreventive agents, especially in liver cancer development.

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