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Inhibitory Effects of Benzyl Isothiocyanate and Benzyl Thiocyanate on Diethylnitrosamine-induced Hepatocarcinogenesis in Rats

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The effects of two aromatic thiocyanates, benzyl isothiocyanate (BITC) and benzyl thiocyanate (BTC), on diethylnitrosamine (DEN)-induced hepatocarcinogenesis were examined in rats. A total of 108 male ACI/N rats, 5 weeks old, were divided into 6 groups (18 rats in each). Group 1 was given a single i.p. injection of DEN (200 mg/kg body weight) one week after the start of the experiment and then kept on the basal diet until the end of the experiment (1 year). Groups 2 and 3 were treated with DEN and received dietary BITC (100 ppm) or BTC (100 ppm), respectively, throughout the experimental duration. Groups 4 and 5 were not given the carcinogen and were fed the diet containing BITC or BTC, respectively. Group 6 was kept on the basal diet alone and served as a control. Liver neoplasms were seen in Groups 1, 2 and 3. Incidence and average number of liver neoplasms in Group 2 were significantly smaller than in Group 1 ($P<0.0005$ and $P<0.001$, respectively). The incidence of liver neoplasms in Group 3 was slightly lower than in Group 1, although the difference was not statistically significant. The numbers of glutathione S-transferase placental form (GST-P)-positive foci in Group 2 and γ-glutamyltranspeptidase (GGT)-positive foci in Groups 2 and 3 were significantly smaller than those in Group 1 ($P<0.001$). The average and unit areas of GST-P- or GGT-positive foci in Group 2 or 3 were also significantly smaller than those in Group 1 ($P<0.05$). These results suggest that BITC and BTC are chemopreventive agents for DEN-induced liver tumorigenesis.

Key words: Benzyl isothiocyanate — Benzyl thiocyanate — Chemoprevention — Hepatocarcinogenesis

Chemoprevention embraces the concept that non-carcinogenic synthetic chemicals or naturally occurring products can inhibit the process of carcinogenesis. A number of agents have proved effective against chemical carcinogenesis.[1-5] Benzyl isothiocyanate (BITC) and benzyl thiocyanate (BTC) are constituents of cruciferous vegetables, being present as their glucosinolate precursors.[6] BITC has been shown to inhibit, 9,10-dimethyl-1,2-benzanthracene(DMBA)-induced mammary carcinogenesis in rats[7,8] and to suppress the development of forestomach or lung tumors induced by DMBA, diethylnitrosamine (DEN) or benzo[*a*]pyrene (B[a]P) in mice.[7,9] BTC has also been reported to reduce DMBA-induced mammary carcinogenesis in rats.[7] In these studies, the aromatic thiocyanates were basically administered by oral intubation prior to carcinogen treatment.

Previously we examined the modifying effect of BTC on azoxymethane(AOM)-induced hepatocarcinogenesis in a rat model.[10] However, no clear inhibitory effect of BTC was found. This may be due to the low dose of BTC and the time of administration (only the initiation phase).

Recently, we demonstrated that BITC and BTC both reduce the levels of unscheduled DNA synthesis (UDS) in response to carcinogen-induced DNA damage and replicative DNA synthesis (RDS) generated by exposure to hepatocarcinogens including DEN in the rat hepatocytes/DNA repair assay.[11] These results raise the possibility that BITC and BTC are inhibitory agents against hepatocarcinogenesis. In the present study, the modifying effect of BITC or BTC was examined in DEN-induced rat hepatocarcinogenesis by analyzing glutathione S-transferase placental form(GST-P)-positive and γ-glutamyltranspeptidase (GGT)-positive foci in rats given the thiocyanates at a higher dose throughout the experimental duration.

MATERIALS AND METHODS

Animals and diets Inbred male ACI/N rats, maintained in our laboratory, were used. A total of 108 rats, 5 weeks of age, were divided into 6 groups (Group 1: 18 rats for DEN alone; Group 2: 18 rats for DEN and 100 ppm BITC; Group 3: 18 rats for DEN and 100 ppm BTC; Group 4: 18 rats for BITC alone; Group 5: 18 rats for BTC alone and Group 6: 18 rats for control). All animals were housed in wire cages (3 rats/cage) and were fed the basal diet or the diet mixed with BITC or BTC at a concentration of 100 ppm. CE-2 (CLEA Japan Inc., Tokyo) was used as a basal diet. The animals had free access to tap water and diet under controlled environmental conditions of humidity (50±10%), lighting

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(12 h light/dark cycle) and temperature (23±2°C). The experimental diets with BITC or BTC were prepared weekly and stored in a cold room.

Carcinogen and test chemicals DEN and BTC were purchased from Nacalai Tesque Inc., Kyoto, and BITC was supplied by Tokyo Kasei Kogyo Co., Ltd., Tokyo.

Experimental procedure Animals of Groups 1, 2 and 3 were given a single i.p. injection of DEN (200 mg/kg body weight) one week after the start of experiment. Rats of Group 1 were fed the basal diet alone throughout the experiment (53 weeks). Animals of Groups 2 and 3 were respectively given the diet containing BITC or BTC throughout the experiment. Animals of Groups 4, 5, and 6 were i.p. injected with normal saline instead of the carcinogen. Of them, Groups 4 and 5 were respectively given the diet containing BITC or BTC. Group 6 served as an untreated control. At the termination of the experiment, complete autopsies were performed after killing by ether anesthesia. At autopsy, the location, number and size of liver tumors were recorded. Liver tissues were sliced to obtain two pieces from each lobe. One set of slices was frozen in liquid nitrogen for preparation of frozen sections and the other set was fixed in 10% buffered formalin, embedded in paraffin blocks, and processed for routine histological observation with the use of hematoxylin and eosin stain. The liver sections from frozen tissues were stained for GGT-reaction according to the procedure of Ruttenburg *et al.*,[12] and an immunohistochemical staining for GST-P was also carried out using the avidin-biotin-peroxidase complex method[10] (Dako ABC kit, Dako Corp, Santa Barbara, CA). Anti-GST-P antibody was kindly provided by Prof. K. Sato, Hirosaki University School of Medicine, Hirosaki.

The areas of GST-P- and GGT-positive foci and the number of foci/cm^2 were measured by means of an image analyzer with a microscope (SP 500, Olympus Optical Co., Ltd., Tokyo). GST-P-positive lesions composed of more than 11 cells were recognized as altered liver cell foci.

Statistical analysis Differences of incidence or density of pathological lesions in the liver between groups were analyzed by the χ^2-test, Fisher's exact probability test or Student's *t* test.

RESULTS

General observation There was no clear evidence of toxicity in animals exposed to BITC and BTC diets (Table I). One rat in Group 2 and two rats in Group 3 died of pneumonia before termination of the experiment, and no neoplasm was found in them. Body weights of animals given the basal diet alone, and the diet containing BITC or BTC at termination were respectively 309±13 g, 320±10 g and 299±11 g, the differences being statistically significant. Liver weights were significantly decreased by BITC or BTC among the groups treated with DEN, but no significant difference could be found among vehicle-treated animals given the basal diet, BITC or BTC. As regards relative liver weights, statistically significant differences were observed between rats fed the experimental diets and basal diet with or without DEN treatment, except between vehicle-treated rats fed the basal diet and BITC.

Tumor incidence The liver tumors were recognized in the groups treated with DEN, but not in the rats without DEN treatment. The neoplasms were of hepatocellular origin (Table II). Hepatocellular adenomas and carcinomas were found in all DEN-treated groups except the group exposed to BITC, in which no carcinomas were seen. The incidences of hepatocellular carcinoma and adenoma, and the incidences of total liver neoplasms of Group 2 were much lower than those of Group 1

Table I. Body Weight, Liver Weight and Relative Liver Weight of Rats in Each Group

Group	Treatment	No. of rats (initial/ effective)	Body weight (g)	Liver weight (g)	Relative liver weight (g)
1	DEN	18/18	317±19[a]	10.4±0.7	3.28±0.20
2	DEN+BITC	18/17	292±13[b]	9.0±1.0[b]	3.07±0.24[c]
3	DEN+BTC	18/16	287±20[b]	8.0±1.4[b]	2.78±0.37[b]
4	BITC	18/18	320±10	9.0±1.5	2.82±0.09
5	BTC	18/18	299±11	7.5±0.5	2.51±0.08
6	Control	18/18	309±13[d, e]	8.9±3.7	2.88±0.46[f]

a) Mean±SD.
b, c) Significantly different from rats treated with DEN alone by Student's *t* test (*b*: $P<0.0001$, *c*: $P<0.01$).
d) Significantly different from rats given BITC alone by Student's *t* test ($P<0.01$).
e, f) Significantly different from rats given BTC alone by Student's *t* test (*e*: $P<0.025$, *f*: $P<0.01$).

($P<0.0005$). On the other hand, the incidences in Group 3 were lower than those of Group 1, but the differences were not statistically significant.

In this study, three types of hepatocellular foci (clear, eosinophilic and basophilic) which were positive for GST-P and GGT reaction, were found in all groups exposed to DEN. A few liver cell foci were also found in some animals in vehicle-treated animals, but no tumor could be observed. The results of quantitative analysis of the frequency of GST-P- and GGT-positive foci are summarized in Table III. There were no clear differences in the incidences (% animals) of GST-P- or GGT-positive foci among the DEN-treated animals. The incidence of GST-P-positive foci of Group 2 was significantly lower than that of Group 1 ($P<0.001$). The density of GST-P-positive foci of Group 3 was also rather lower than that of Group 1. However, the differences were not significant. A significant decrease in the average area of GST-P-positive foci was present in the animals fed the BITC or BTC diet ($P<0.01$ or $P<0.002$, respectively). The unit area of GST-P-positive foci was significantly decreased in the groups exposed to BITC or BTC ($P<0.001$ or $P<0.001$, respectively). Furthermore, BITC and BTC reduced the GGT-positive foci in density ($P<0.001$), average area ($P<0.05$) and unit area ($P<0.001$).

DISCUSSION

The results of the present study are of considerable interest since the glucosinolates, BITC and BTC, when given during the initiation and postinitiation phases, inhibited DEN-induced hepatocarcinogenesis in rats. These data indicate an anticarcinogenic effect of BITC and BTC.

In this study, BITC inhibited the occurrence of the liver neoplastic lesions more clearly than BTC. Results of quantitative analysis of altered liver cell foci using

Table II. Incidences of Preneoplastic and Neoplastic Lesions of the Liver in Rats of Each Group

Treatment	No. of rats examined	Liver neoplasms						Foci[c]
		Total		HCC[a]		Ad[b]		
		No. of rats (%)	Average no. of tumors (/rats)	No. of rats (%)	Average no. of tumors (/rats)	No. of rats (%)	Average no. of tumors (/rats)	No. of rats (%)
DEN	18	12 (67)	1.22±1.03[d]	6 (33)	0.33±0.47	10 (55)	0.88±0.87	18 (100)
DEN+BITC	17	1 (6)[e]	0.06±0.23[f]	0[g]	0[h]	1 (6)[i]	0.06±0.23[j]	17 (100)
DEN+BTC	16	6 (38)	0.63±0.99	2 (13)	0.13±0.33	6 (38)	0.50±0.71	16 (100)

a) Hepatocellular carcinoma.
b) Hepatocellular adenoma.
c) Enzyme-altered foci.
d) Mean±SD.
e, g, i) Significantly different from rats treated with DEN alone by Fisher's exact probability test (*e*: $P<0.0005$, *g*: $P<0.02$, *i*: $P<0.002$).
f, h, j) Significantly different from rats treated with DEN alone by Student's *t* test (*f*: $P<0.001$, *h*: $P<0.02$, *j*: $P<0.002$).

Table III. Quantitative Analysis of GST-P- and GGT-Positive Foci in Rats of Each Group

Treatment	Positive marker	Density (No. of foci/cm^2)	Average area of foci ($\times 10^{-2}$ mm^2)	Unit area of foci (%)
DEN	GST-P	11.0±3.5[a]	76.1±135.7	0.85±0.38
	GGT	17.2±3.6[a]	58.1±104.8	0.90±0.10
DEN+BITC	GST-P	6.2±2.2[b]	32.0±36.6[c]	0.19±0.06[b]
	GGT	6.2±3.9[b]	32.9±35.5[d]	0.20±0.10[b]
DEN+BTC	GST-P	8.5±5.3	35.6±39.3[e]	0.31±0.17[b]
	GGT	5.5±1.3[b]	33.8±45.6[c]	0.19±0.06[b]

a) Mean±SD.
b–e) Significantly different from rats treated with DEN alone by Student's *t* test (*b*: $P<0.001$, *c*: $P<0.01$, *d*: $P<0.05$, *e*: $P<0.002$).

phenotypic markers, GST-P and GGT, were also in agreement with the data on the incidences of liver neoplasms. These liver cell foci are generally recognized as preneoplastic lesions in the lineage of hepatocellular carcinoma development and reflect the carcinogenic potential due to the consistent manner in which liver cell foci appear during the postinitiation stage of hepatocarcinogenesis.[13]

From the results with the present experimental protocol, BITC and BTC are considered to be effective in the initiation phase as well as the promotion phase. The area of liver cell foci appears to be dependent on the growth activity in the tumor promotion, and the number of foci is considered to indicate the initiating potential.[14] In our previous study using azoxymethane in rats, dietary exposure to BTC (25 ppm) during the initiation phase only decreased the number of GST-P-positive liver cell foci, but it slightly enlarged the average area of foci.[10] Combining data from the present experiment and the previous study, BTC appears to act as a blocking as well as a suppressing agent for liver neoplasms.

The doses of experimental diets used in this experiment slightly retarded the body weight gain. Calorie intake and body weight have been reported to be related to tumor incidence. Albanes summarized the correlation of calories, body weights and tumor incidences in mice from 14 papers.[15] Adult body weight was highly correlated to caloric intake, while about 15.3% calorie restriction caused 20.2% tumor reduction and 11% weight loss reduced tumor incidence by 3.7%. In this study, tumor incidences were reduced to 9% and 57% in the DEN+BITC group and DEN+BTC group compared to the DEN-alone group, respectively. On the other hand, only an 8–9% body weight reduction was present in the group treated with DEN and BITC or BTC. Therefore, the reduction of tumor incidence was not due to body weight loss. BITC and BTC also decreased the enzyme-altered foci in this experiment. Glauert and Pitot examined the effect of high fat on DEN-induced GGT-positive foci in rats, and reported that GGT-positive foci were not enhanced by high fat diet, which was given in the postinitiation phase without phenobarbital promotion for 4 and 10 months.[16] Thus, changes of body weight and liver weight were not related with number and volume of GGT-positive foci. In this experiment, GGT-positive foci and GST-P-positive foci were reduced in the BITC+DEN and BTC+DEN groups, and these changes appear to be owing mainly to the direct effect of BITC or BTC. Among the groups treated with DEN in this study, BITC and BTC reduced the liver weights and relative liver weights. One of the causes was the development of the liver tumors. The relative liver weight of BTC-fed rats was also significantly lower than that of rats fed the basal diet, but BITC diet had no effect. This may be related to the suppressive effect of BTC in RDS, because such an effect of BTC is stronger than that of BITC.

Chemopreventive agents have been found among both synthetic and natural products.[1–5] Some have been proved to decrease liver tumorigenesis. BHA and BHT are typical synthetic antioxidants and act as chemopreventors of hepatocarcinogenesis.[17–19] Thamavit *et al.* analyzed GGT- and GST-P-positive foci to examine the modifying effects of BHA and BHT in a medium-term bioassay system using 200 ppm DEN as an initiator.[19] The suppressive effect was stronger with BHA than BHT, and the effect was more marked for GGT than GST-P. Both chemicals, however, had to be given at a higher dose than BITC to cause a significant decrease in both GGT- and GST-P-positive foci. In this study, the GST-P-positive foci were better correlated than GGT-positive foci with tumorigenesis.

Glucosinolates and indoles are typical natural chemopreventive agents in cruciferous vegetables. Sinigrin and indole-3-carbinol, which are constituents of cruciferous vegetables, have been administered in the diet in the initiation phase (1200 and 100 ppm, respectively), and shown to inhibit DEN-induced hepatocarcinogenesis in ACI/N rats.[20] Some isothiocyanates have inhibitory actions against carcinogenesis in other organs. Consumption of diet containing phenethyl isothiocyanate (30 mmol/kg), another cruciferous vegetable component, in the initiation phase has been reported to reduce 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone(NNK)-induced lung tumors in rats.[21] BITC has been shown to inhibit DMBA-induced mammary carcinogenesis in rats[7, 8] and to suppress the development of forestomach or lung tumors induced by DMBA, DEN or B[a]P in mice.[7, 9] BTC has also been reported to reduce DMBA-induced mammary carcinogenesis in rats.[7] In these studies, indoles and glucosinolates were basically given by oral intubation prior to carcinogen treatment or diet in the initiation phase. Sinigrin and phenethyl isothiocyanate decreased α-hydroxylation and DNA methylation induced by NNK and DEN in hepatic DNA.[22, 23] Indole-3-carbinol induced GST and epoxide hydrolase activities.[24–26]

The mechanism by which BITC or BTC affects DEN-induced hepatocacinogenesis is still not clear. Thiono-sulfur-containing compounds, including BITC and BTC, have been shown to inhibit hepatic microsomal mixed function oxidase reaction, hepatic α-hydroxylase and DNA methylation reactions of nitrosamines.[9, 22, 27, 28] Wattenberg *et al.* reported that BITC enhanced GST activity in the cytosol of mouse liver.[24] Concerning BTC, low-dose diets (25 and 50 ppm) did not inhibit DNA methylation by azoxymethane (AOM) in the colon and liver,[29] but a higher-dose BTC diet (30 mmol/kg) inhibited formation of α-hydroxylation products of

nitrosamines in the liver.[27] Moreover, 100 ppm BTC suppressed ornithine decarboxylase activity induced by AOM in colonic mucosa.[30] The protective effect of BITC or BTC against DEN-induced carcinogenesis in the present study, thus, may also be related to inhibition of activation of the hepatocarcinogen. In our recent study, BITC and BTC inhibited not only UDS and but also RDS in rat hepatocytes in response to DEN exposure,[11] suggesting that both glucosinolates suppress the DEN-induced genotoxicity and cell proliferation.

In conclusion, the results of the present investigation suggest that BITC and BTC are possible chemopreventive agents against liver neoplasms.

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