Jpn. J. Cancer Res. 80, 952–957, October 1989

# Inhibition by Dietary Benzylselenocyanate of Hepatocarcinogenesis Induced by Azoxymethane in Fischer 344 Rats[1]

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**The effect of dietary benzylselenocyanate (BSC), a novel organoselenium compound and its sulfur analog, benzylthiocyanate (BTC), on hepatocarcinogenesis induced by azoxymethane (AOM) was investigated in male F344 rats. Eighty-one weanling rats were divided into 3 groups and were raised on a semipurified diet (control diet). Starting from 5 weeks of age, groups of animals consuming the control diet were fed one of the experimental diets containing 25 ppm BSC or BTC. An additional group was continued on the control diet. At 7 weeks of age, animals were given weekly sc injections of AOM (15 mg/kg body weight once weekly for 2 weeks). One week after the second AOM injection, those groups receiving BSC and BTC diets were transferred to the control diet and continued on this diet until termination of the experiment at 34 weeks after the last AOM injection. For quantitative analysis of enzyme-altered liver cell foci, glutathione *S*-transferase placental form was stained by an immunohistochemical technique. The results indicate that the incidence and the density of the enzyme-altered foci were significantly lower in AOM-treated rats fed the diet containing 25 ppm BSC (foci incidence 56%, foci density 2.43/cm$^2$) than in AOM-treated animals fed the control diet (foci incidence 92%, foci density 4.79/cm$^2$). The incidence of small altered foci was significantly inhibited in rats fed the BTC diet (35%) as compared to those fed the control diet (68%), but the degree of inhibition was more pronounced in animals fed the BSC diet than in those fed the BTC diet.**

Key words: Benzylselenocyanate — Benzylthiocyanate — Chemoprevention — Azoxymethane — Rat hepatocarcinogenesis

Chemoprevention of cancer is based on the concept that certain chemical agents, either synthetic or naturally occurring substances, can inhibit tumor development. A large body of evidence in animals indicates that supplementation of diet or drinking water with inorganic selenium protects against cancer induced by a variety of chemical carcinogens, inducing cancer of the colon, mammary gland, pancreas and liver, to cite a few.[1-21] These data have resulted in considerable interest in the potential of selenium and its derivatives as chemopreventive agents.

In the case of liver carcinogenesis, the first experimental evidence that selenium could modify 3′-methyl-4-dimethylaminobenzene-induced hepatocarcinogenesis was reported by Clayton and Baumann in 1949.[9] Similar reductions in hepatocarcinogenesis by inorganic selenium were reported in studies using N-2-fluorenylacetamide,[10,13] diethylnitrosamine[14] and aflatoxin $B_1$.[12]

Although inorganic selenium has been shown to inhibit carcinogenesis, there is concern about its toxicity. Toxic reproductive and teratogenic effects of selenium have been reported for both animals and humans.[22] Generally, chronic feeding of 5 to 10 ppm of selenium is toxic in animals.

Since selenium occurs in foodstuffs predominantly as an organic form, such as selenomethionine, attention has been focused on the effect of organic forms of selenium in carcinogenesis. In the 7,12-dimethylbenz[*a*]anthracene and methylnitrosourea-induced mammary carcinogenesis, inorganic selenium at 4 to 6 ppm in the diet provided greater inhibition of mammary carcinogenesis in female rats than did an equivalent amount of selenomethionine.[23] Dietary selenium at 6 ppm in the form of selenomethionine induced liver damage.[23] These observations pointed to the need to develop novel forms of organoselenium compounds that are less toxic but more effective than inorganic forms. Synthetic organoselenium compounds offer greater promise as chemopreventive agents because their chemical structures can be altered to provide maximum efficacy with minimal toxicity.

Recently, we synthesized two organoselenium compounds, namely methoxybenzeneselenol (MBS)[6] and

[1] This paper is No. 6 in the series "Selenium in Chemoprevention of Carcinogenesis."

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[6] Abbreviations: MBS, *p*-methoxybenzeneselenol; AOM, azoxymethane; BSC, benzylselenocyanate; BTC, benzyl thiocyanate; GST-P, glutathione *S*-transferase placental form.

benzylselenocyanate (BSC), which were found to be effective inhibitors of benzo[*a*]pyrene-induced forestomach tumors in mice.[24] Diet containing 50 ppm MBS fed during the initiation phase of carcinogenesis inhibited azoxymethane (AOM)-induced colon, kidney and liver carcinogenesis in rats.[20, 21] In addition, feeding of 25 ppm BSC but not its sulfur analog, benzylthiocyanate (BTC), during the initiation phase inhibited AOM-induced colon carcinogenesis in rats.[25] In the present study, we have investigated the potential inhibitory effect of dietary BSC and its sulfur analog BTC in AOM-induced hepatocarcinogenesis in male F344 rats.

## MATERIALS AND METHODS

**Animals, diets and carcinogen** A total of 81 weanling male F344 rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. AOM (CAS: 25843-42-2) was obtained from Ash Stevens, Detroit, MI, and BTC from Aldrich Chemicals, Milwaukee, WI. BSC was synthesized by a previously described procedure and added to the diet.[24] AIN-76A semipurified diet was used throughout the study.[26, 27] BSC or BTC was added to the semipurified diet at a level of 25 ppm. The AIN-76A (control) diet contained 0.1 ppm selenium as sodium selenite. The experimental BSC and BTC diets contained, in addition to the 0.1 ppm inorganic selenium introduced with the AIN-76A diet base, 10 ppm and 0 ppm selenium, respectively.

**Experimental procedure** Male F344 rats received at weanling were quarantined for 10 days and had access to AIN-76A semipurified diet (control diet). They were then randomly allocated by weight to one of three dietary groups (control diet, 25 ppm BSC diet and 25 ppm BTC diet).

Beginning at 5 weeks of age, groups of rats fed the control diet were transferred to diets containing 25 ppm BSC or 25 ppm BTC. At 7 weeks of age, the animals in each group, received two weekly sc injections of AOM at a dose level of 15 mg/kg body weight/week. One week after AOM, animals receiving the BSC and BTC diets were transferred to the control diet and continued on this diet until the termination of the experiment. The animals receiving the control diet were continued on the same diet. The experiment was terminated at 34 weeks. As scheduled, AOM-treated animals were killed by $CO_2$ asphixiation. Following laparotomy, the liver was examined grossly for tumors. Tissues were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by routine histological methods with the use of hematoxylin and eosin (H-E) stains and immunohistochemical demonstration of glutathione *S*-transferase placental form (GST-P).

Anti-GST-P antibody was kindly provided by Dr. K. Sato, Hirosaki University School of Medicine, Hirosaki. For immunohistochemical study of GST-P, the indirect immunoperoxidase method [avidin-biotin-peroxidase complex (ABC) method] was performed. After deparaffinization and hydration in the usual manner, the sections were washed in phosphate-buffered saline (PBS) at pH 7.6 for 5 min. Endogenous peroxidase activity was blocked with hydrogen peroxidase (0.5% in methanol) for 5 min and the sections were rinsed with PBS. Then they were preincubated with normal swine serum (1 ml/100 ml PBS) for 20 min for blocking non-specific reaction and incubated with rabbit anti-GST-P antibody (dilute 1:2000) in a cold room at 4°C overnight. After rinsing, they were incubated with biotinylated anti-rabbit antibody (Dako Corp., Santa Barbara, CA) for 20 min, rinsed and incubated with avidin and biotinylated horseradish peroxidase complex (Dako ABC kit) for 20 min at room temperature. Color reactions were developed with 0.4 mg/ml of 3-amino-9-ethylcarbazole and one drop of 3% hydrogen peroxidase in 0.1 *M* sodium acetate buffer at pH 5.2. The sections were counterstained with hematoxylin and mounted in gelatin.

The area of GST-P-positive foci and the number of foci/cm$^2$ were measured by means of an image analyzer with a microscope (Videoplan, Carl Zeiss, Inc., NY). GST-P-positive lesions composed of more than 5 cells were recognized as foci, measured and classified into two categories according to the number of cells composing the foci as follows: 6–10 cells, small foci and more than 10 cells, large foci.

Differences of incidence or density of pathological lesions in the liver between groups were analyzed by the $\chi^2$ test, Fisher's exact probability test or Student's $t$ test.

## RESULTS

**General observations** There was no evidence of toxicity in animals fed the BSC and BTC diets. At termination, the body weights of animals fed the control, BSC and BTC diets were $454 \pm 24$ g, $449 \pm 22$ g and $459 \pm 19$ g, respectively. Experimental diets caused no growth retardation of animals as compared with control diet.

**Incidence of hepatocellular lesions** Macroscopically, liver tumors were detected in few rats. The diameter of tumors ranged from 0.1 to 0.2 cm in all groups, and the tumors were neoplastic nodules, microscopically. Administration of AOM resulted in small hepatocellular lesions. Animals fed the control diet had 203 lesions whereas the animals fed the BSC diet had 61 lesions and those fed the BTC diet had 99 lesions in the liver. Microscopically, three types of hepatocellular foci (clear, eosinophilic and basophilic) were found in all groups. GST-P-positive foci (small or large foci) were observed in all groups (Fig. 1).

The results of a quantitative examination of GST-P-positive foci are summarized in Table I. The incidence (% animals) of GST-P-positive foci in the liver was significantly lower in animals fed the diets containing BSC ($P<0.005$) than in those fed the control diet (Table I). The incidence of GST-P-positive foci was lower in BSC- or BTC-fed animals compared to that in animals fed the control diet, but the difference between the BTC diet and control diet groups lacked statistical significance. The incidence of small foci was significantly lower in rats fed the BSC or BTC diets than in those fed the control diet. Large and total foci incidences were significantly inhibited only in animals fed the BSC diet compared to those fed the control diet. There were no differences in large and total foci incidences between the control and BTC groups. Thus, the degree of inhibition is more pronounced in the BSC group than in the BTC group.

With regard to the density of GST-P-positive foci (Table I), total foci were significantly inhibited in animals fed the BSC diet ($P<0.05$) compared to those fed the control diet. Although the density of total foci was lower in animals fed the BTC diet than in those fed the control diet, the difference lacked statistical significance. A significant inhibition in the density of small foci was observed in the animals fed the BSC ($P<0.001$) and BTC ($P<0.02$) diets compared to those fed the control diet. There was no significant difference in the average area of foci between the animals fed the BTC and the control diets (Table I); however, the unit area of foci was significantly inhibited in the group fed the BSC diet. The average area of total foci was significantly higher in the rats fed the BTC diet than in those fed the control diet, although there was no significant difference in the average areas of small and large foci.

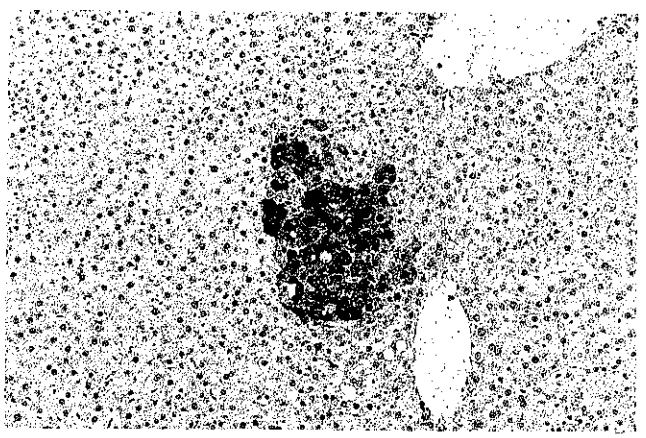

Fig. 1. GST-P-positive focus composed of more than 10 hepatocytes.

## DISCUSSION

The results of the present study are of considerable interest because the synthetic organoselenium compound, BSC, when fed during the initiation phase inhibited AOM-induced hepatocarcinogenesis in F344 rats. We are not aware of any previous study of a potential hepatocarcinogenesis inhibition by BSC. These results are consistent with our previous finding that another

Table I. Results of the Quantitative Analysis of GST-P-positive Foci in Rats of Each Group

| Treatment | No. of rats (effective /initial) | No. of rats with foci | | | Density (No. of foci/cm² area of liver section) | | | Average area of foci ($\times 10^{-3}$ mm²) | | | Unit area of foci (mm²/cm²) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Small foci[a] | Large foci[b] | Total | Small foci | Large foci | Total | Small foci | Large foci | Total | |
| AOM+ Basal diet | 25/27 | 17 (68%) | 22 (88%) | 23 (92%) | 1.36[c] ±1.27 | 3.43 ±2.07 | 4.79 ±2.90 | 8.91 ±2.66 | 48.18 ±47.78 | 37.26 ±44.37 | 0.185 ±0.124 |
| AOM+ BSC | 27/27 | 5[d] (19%) | 14[e] (52%) | 15[f] (56%) | 0.29[g] ±0.69 | 2.14 ±3.89 | 2.43[h] ±4.12 | 8.44 ±2.34 | 46.72 ±29.38 | 40.95 ±30.32 | 0.100[i] ±0.151 |
| AOM+ BTC | 26/27 | 9[j] (35%) | 19 (73%) | 21 (81%) | 0.56[k] ±0.87 | 2.65 ±2.55 | 3.21 ±2.77 | 8.91 ±1.56 | 65.63 ±64.06 | 57.69[l] ±62.99 | 0.163 ±0.178 |

*a*) Small foci: Composed of 6–10 GST-P-positive cells.
*b*) Large foci: Composed of more than 10 GST-P-positive cells.
*c*) Mean±SD.
*d–f*) Significantly different from AOM alone by Fisher's exact probability test (*d*, $P<0.0005$; *e*, *f*, $P<0.005$).
*g–i*, *k*, *l*) Significantly different from AOM alone by Student's *t* test (*g*, $P<0.01$; *h*, *i*, $P<0.05$; *k*, *l*, $P<0.02$).
*j*) Significantly different from AOM alone by the $\chi^2$ test ($P<0.025$).

form of organoselenium, MBS, inhibited AOM-induced hepatocarcinogenesis.

The effect of BSC and BTC on the development of liver altered foci by AOM was observed by measuring both the number and area of GST-P-positive foci. The liver cell foci are generally recognized as preneoplastic lesions in the lineage of hepatocellular carcinoma development and reflect carcinogenic potential due to the consistent manner in which liver cell foci appear during the post-initiation stage of hepatocarcinogenesis.[28] GST-P is a sensitive marker, and is also useful to detect early altered lesions (single GST-P-positive cells and mini foci).[29] The result of the present study demonstrated that both the incidence (% of rats with lesions) and the density (no. of foci/cm$^2$ area of liver section) of GST-P-positive lesions of liver were inhibited in animals fed the diets containing BSC during the inhibition phase of carcinogenesis.

It was also demonstrated that feeding of BSC and BTC had no effect on the incidence of neoplastic nodules in the liver. It should be noted that in the present study, the incidence of AOM-induced neoplastic nodules in the liver was small, which might be due to the lower dose of AOM and shorter experimental period than in our previous study.[21]

In the present study, the average area of foci was not significantly affected by feeding BSC, although the unit area of foci (mm$^2$/cm$^2$) was inhibited in animals fed the BSC diet. Moreover, a significant difference was found in the average area of total foci between BTC and control diets. These results can be explained on the basis of the occurrence of GST-P-positive small and large foci in animals fed the experimental and control diets. The densities of large foci in the BSC and BTC groups were less than that of the control group; nevertheless, the proportions of the large foci in the BSC and BTC groups were larger than that of the control group. In animals fed the control diet, there was an increase in the proportion of small foci. The inhibition of small foci was more pronounced than the inhibition of large foci in BSC- and BTC-fed animals. Therefore when the areas of foci were calculated for the various groups, the significant differences observed in the number of foci among the experimental and control groups disappeared, although the unit area of foci was inhibited in the BSC group (Table I). The reason for the insignificant differences in the density of large foci between the animals fed the control diet and those fed the BSC and BTC diets may be explained on the basis that the BSC and BTC diets were fed to the animals during the initiation phase of carcinogenesis. It is possible that initiated hepatocytes were inhibited by BSC and BTC feeding, but the initiated cells of BSC- and BTC-fed animals developed in the same way as those in animals fed the control diet. Studies by Fishbein,[2] and Medina[30] also indicated that selenium supplementation produced an insignificant effect in terms of tumor incidence after selenium was withdrawn from the diet.

The mechanism by which BSC affects AOM-induced carcinogenesis when fed during the initiation phase remains to be elucidated. There is some evidence that selenium may act by altering the metabolism of chemical carcinogens, as observed by Marshall.[31] It is possible that, in the present study, the inhibitory effect of BSC may be related to some action of this compound on the metabolic activation and detoxification of AOM. The metabolic activation of AOM to a reactive species capable of alkylating DNA occurs in two steps: (a) the hydroxylation of AOM to methylazoxymethanol, and (b) the oxidation of methylazoxymethanol to methylazoxyformaldehyde.[32-34] The hydroxylation of AOM to methylazoxymethanol was found mainly to occur in rat liver, probably by a cytochrome P-450-dependent pathway.[35] The oxidation of methylazoxymethanol to methylazoxyformaldehyde is affected by microsomes from both liver and colon[33] as well as by alcohol dehydrogenase from the cytosol of both of these organs.[34] Whether the inhibition of AOM-induced liver carcinogenesis by BSC is due to alteration of metabolic activation and detoxification of AOM remains to be investigated. The results of this study support those of our earlier study in which another synthetic organoselenium compound, namely MBS, inhibited AOM-induced hepatocarcinogenesis in rats[21] fed a high fat diet. Our recent studies have indicated that dietary MBS and BSC inhibited the development of benz[*a*]pyrene-induced forestomach tumors in mice[24] and AOM-induced colon carcinogenesis in rats.[20, 27] These studies together suggest that the introduction of the element selenium into an organic molecule is a feasible approach to the chemoprevention of certain types of cancer. The results of this and our previous studies[20, 21, 24, 27] may open new approaches to the development of effective and less toxic selenium-containing chemopreventive agents.

ACKNOWLEDGMENTS

We thank Yves-Marie Louis and Jeffery Rigotty for expert technical assistance and Edwigh Beauvoir for preparation of the manuscript.

*(Received April 27, 1989/Accepted August 14, 1989)*

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