

# Enhancement of urinary bladder carcinogenesis by combined treatment with benzyl isothiocyanate and N-butyl-N-(4-hydroxybutyl)nitrosamine in rats after initiation

Kazushi Okazaki,<sup>1,2</sup> Takashi Umemura,<sup>1</sup> Takayoshi Imazawa,<sup>1</sup> Akiyoshi Nishikawa,<sup>1</sup> Toshiaki Masegi<sup>3</sup> and Masao Hirose<sup>1,4</sup>

<sup>1</sup>Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501; <sup>2</sup>The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1193; and <sup>3</sup>Department of Veterinary Pathology, Faculty of Agriculture, Gifu University, 1-1 Yanagido, Gifu City, Gifu 501-1193

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Previously we reported that benzyl isothiocyanate (BITC) strongly enhanced rat urinary bladder carcinogenesis after initiation with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), while potently inhibiting BBN-induction of lesions when given simultaneously with the carcinogen. In the present experiment, the effects of simultaneous treatment with BITC and low-dose BBN on the post-initiation period of rat urinary bladder carcinogenesis were examined. After treatment with 500 ppm BBN for 4 weeks for initiation, groups of 20, 6-week-old, F344 male rats were given 25 ppm BBN alone, basal diet alone, or 100 or 1000 ppm BITC in the diet together with or without 25 ppm BBN in their drinking water for 36 weeks and then killed for autopsy. Further groups consisting of 10 rats each were similarly given BITC or the basal diet together with or without 25 ppm BBN, without initiation treatment. In the initiated groups receiving subsequent BBN exposure, papillary and nodular hyperplasia, dysplasia and carcinoma incidences were significantly increased, and they were further increased by the combined treatment with 100 and 1000 ppm BITC in a dose-dependent manner. In the non-initiation groups, carcinomas were only observed in a single rat in each of the BBN-treated control and BBN/BITC 100 ppm treatment groups. The results indicate that simultaneous treatment with BITC and a low dose of BBN does not inhibit, but rather enhances rat urinary bladder carcinogenesis after appropriate initiation, and further suggest that BITC may be a human risk factor, at least in high-risk populations. (*Cancer Sci* 2003; 94: 948–952)

**E**pidemiologically, risk for bladder cancer is high in cigarette smokers<sup>1,2)</sup> and workers in the rubber and dyeing industries.<sup>3)</sup> Indeed, mutagenic compounds have been demonstrated in human urine,<sup>4,5)</sup> and mutagenicity is higher in smokers than in non-smokers.<sup>6)</sup> It is also well-known that once individuals have suffered from bladder cancer, tumors readily recur after surgery or chemotherapy.<sup>7)</sup> Therefore, such patients are regarded as at high risk for bladder cancers, and are speculated to have undergone multi-focal initiation by genotoxic carcinogens.

Benzyl isothiocyanate (BITC) is one of the many isothiocyanates present in plants as thioglucoside conjugates of glucotropaeolin, a natural constituent that is particularly abundant in cruciferous vegetables such as garden cress.<sup>8,9)</sup> In laboratory animals, several isothiocyanates, including BITC, phenethyl isothiocyanate (PEITC), 3-phenylpropyl isothiocyanate and 4-phenylbutyl isothiocyanate, have been shown to be effective inhibitors of tumorigenesis in a number of organs, such as the colon, esophagus, lung, mammary gland and pancreas of rats, mice or hamsters exposed to genotoxic carcinogens such as azoxymethane, N-nitrosomethylbenzylamine, N'-nitrosocotinine, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK),

benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene or N-nitrosobis(2-oxopropyl)amine.<sup>10–18)</sup> Such chemopreventive activities are particularly strong when the compounds are given during or prior to the carcinogen exposure, and may occur via suppression of metabolic activation<sup>10,11,19–21)</sup> and induction of phase II detoxifying enzymes.<sup>10,11,22)</sup> Therefore, isothiocyanates have been considered as promising chemopreventive agents. In fact, consumption of watercress, which contains abundant isothiocyanates, has been shown to increase urinary excretion of detoxified NNK, a tobacco-related carcinogen, in humans<sup>23)</sup> and to decrease lung cancer risk in current smokers in some epidemiological studies.<sup>24–26)</sup>

We recently reported that dietary 10–1000 ppm BITC administration dramatically inhibited bladder carcinogenesis when it was given simultaneously with a known strong genotoxic bladder carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), at a dose of 50 ppm in drinking water.<sup>27)</sup> On the other hand, dietary administration of 500 or 1000 ppm PEITC or 1000 ppm BITC strongly enhanced rat urinary bladder carcinogenesis in rats pretreated with BBN, and 500 ppm PEITC enhanced bladder carcinogenesis in a multi-organ model.<sup>28–30)</sup> Moreover, continuous dietary administration of 1000 ppm PEITC or BITC for 32 weeks without initiation induced not only hyperplasias, but also papillomas and carcinomas in the urinary bladders of male rats.<sup>28)</sup> These reports suggest that BITC possesses strong promoting activity, weak carcinogenic potential and also, paradoxically, chemopreventive effects on bladder carcinogenesis, depending on the time of exposure.

The present experiment was performed to elucidate whether BITC enhances or inhibits carcinogenesis when given simultaneously with a known strong genotoxic bladder carcinogen, BBN, at a low dose in the post-initiation period.

## Materials and Methods

**Animals and chemicals.** Male 5-week-old F344 rats were obtained from Charles River Japan, Atsugi, and housed five animals per polycarbonate cage under standard laboratory conditions: room temperature, 23±2°C; relative humidity, 60±5%; a 12 h/12 h light-dark cycle. BITC (purity >98%) and BBN were purchased from Kanto Chemical Co., Inc. and Tokyo Kasei Kogyo Co., Ltd. (Tokyo), respectively. BITC was mixed with basal diet at concentrations of 100 and 1000 ppm. The diets were prepared and replaced once a week, and stored at 4°C in the dark before use. BBN was dissolved in distilled water at a concentration of 500 or 25 ppm, and replaced twice a

<sup>4</sup>To whom correspondence should be addressed. E-mail: m-hirose@nihs.go.jp

week. Food (Oriental CRF-1 basal diet, Oriental Yeast, Tokyo) and tap water were available *ad libitum*.

**Treatment.** After a 1-week acclimation period, at the age of 6 weeks, 180 rats were divided into 12 groups, groups 1–6 consisting of 20 animals each and groups 7–12 consisting of 10 animals each. Rats in groups 1–6 were given 500 ppm BBN in their drinking water for 4 weeks as an initiation treatment, then fed a basal diet alone or supplemented with 100 or 1000 ppm BITC, with or without simultaneous 25 ppm BBN in the drinking water for 36 weeks. Rats in groups 7–12 were also fed the same diets with or without BBN as those in groups 1–6, but without prior initiation (Fig. 1). Body weight, food consumption and water intake were recorded at least once every 4 weeks. All surviving animals were killed under ether anesthesia at the end of week 40. The liver, kidneys and urinary bladder of each rat were excised, and the liver and kidneys were weighed and fixed in 10% buffered formalin solution. The urinary bladders were inflated with formalin before being immersed in the

fixative. Then they were weighed, the location and size of urinary bladder tumors were recorded, and 6 slices, including tumors, were prepared and routinely processed for sectioning and staining with hematoxylin and eosin (H&E). Fisher's exact probability test and Dunnett's or Student's *t* test were used for statistical analysis of the data.

## Results

During the experimental period, one animal was found dead in the BITC 1000 ppm with simultaneous BBN treatment group after initiation. Final body weights were significantly decreased in the 1000 ppm BITC groups, irrespective of initiation or simultaneous BBN treatment. On the other hand, the relative kidney and liver weights were slightly or significantly increased in all the same BITC groups (Table 1). There were no clear histopathological changes to explain the increase in organ weights. With BBN initiation, bladder weights of the 1000 ppm BITC

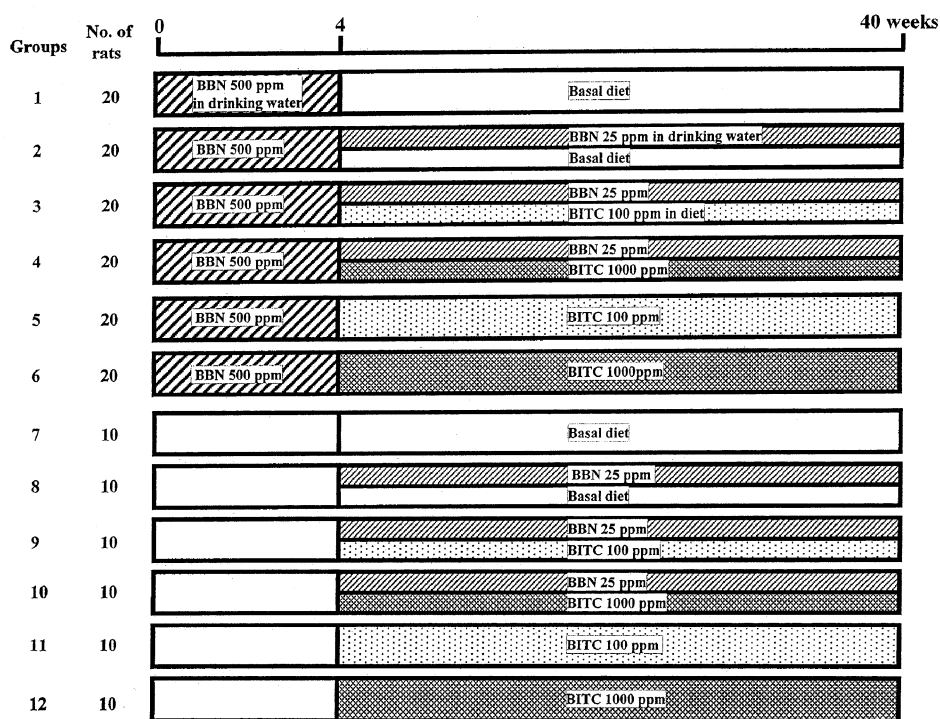


Fig. 1. Experimental design.

Table 1. Final body and organ weights

Treatment		Number of rats	Final body weight (g)	Organ weight		
Initiation	Chemical			Liver (g/100g bw)	Kidneys (g/100g bw)	Bladder (mg/100g bw)
+	Control	20	379±17	2.73±0.15	0.56±0.03	59±13
	BBN 25 ppm	20	386±21	2.76±0.19	0.56±0.04	62±23
	BBN 25 ppm+BITC 100 ppm	20	377±18	2.71±0.09	0.59±0.03**	62±10
	BBN 25 ppm+BITC 1000 ppm	19	352±11**	2.92±0.11	0.63±0.02**	114±34**
	BITC 100 ppm	20	379±16	2.68±0.12	0.56±0.03	57±14
	BITC 1000 ppm	20	353±10**	2.95±0.11**	0.61±0.02**	127±53**
-	Control	10	383±15	2.66±0.11	0.57±0.03	42±15
	BBN 25 ppm	10	382±15	2.79±0.08*	0.57±0.02	46±18
	BBN 25 ppm+BITC 100 ppm	10	369±16	2.64±0.10	0.56±0.03	92±130
	BBN 25 ppm+BITC 1000 ppm	10	357±14**	2.85±0.13**	0.60±0.02*	66±8**
	BITC 100 ppm	10	386±24	2.70±0.15	0.56±0.02	47±11
	BITC 1000 ppm	10	359±20*	2.94±0.11**	0.60±0.03*	59±8**

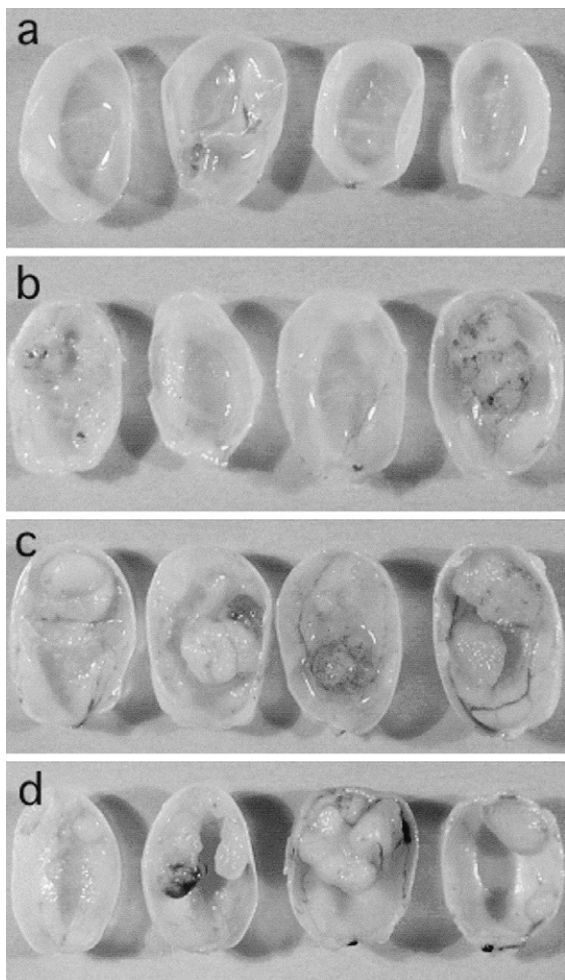
\*  $P < 0.05$ , \*\*  $P < 0.01$  compared to the respective control groups.

group showed a significant 2-fold increase as compared with the control group. In the non-initiation groups, animals receiv-

ing BITC at 1000 ppm demonstrated a similar increase as compared with the control group. Food consumption and water intake were essentially comparable in all groups throughout the experimental period (data not shown).

Grossly, no or only a few nodules were observed in the urinary bladders of BBN-initiated control animals. Their numbers and sizes were clearly increased by additional BBN exposure and further enhancement was observed with simultaneous 100 or 1000 ppm BITC treatment, in a dose-dependent manner. In addition, BITC without simultaneous BBN treatment also increased the development of bladder lesions in a dose-dependent manner (Fig. 2).

Data on the incidences of urinary bladder lesions are summarized in Table 2. The lesions observed were classified as simple hyperplasias, papillary and nodular (PN) hyperplasias, dysplasias, papillomas and transitional cell carcinomas.<sup>27)</sup> In the initiated case, BBN treatment alone significantly increased the incidences of PN hyperplasia, dysplasias, papillomas and papillary carcinomas. Non-papillary carcinomas were not increased by BBN treatment alone. Additional treatment with 100 or 1000 ppm BITC increased the incidences of dysplasias, non-papillary carcinomas and invasive carcinomas in a dose-dependent manner. Treatment with 100 or 1000 ppm BITC alone after initiation significantly increased the incidences of all types of proliferative lesions in a dose-dependent manner. In the non-initiation groups, carcinomas were only observed in single rats of the BBN-treated control and BBN/BITC 100 ppm treatment groups.



**Fig. 2.** Macroscopic findings for urinary bladder lesions. No or only few nodules were observed in rats of the control initiation group (a). Some small nodules are evident in some rats receiving BBN 25 ppm group after the initiation (b). Multiple large nodules are present in the bladders of initiated rats of the BBN 25 ppm+BITC 1000 ppm (c) and BITC 1000 ppm groups (d).

## Discussion

The present study demonstrated that BITC in the diet enhanced the development of BBN-initiated urinary bladder lesions independently or when combined with further BBN exposure. It is of interest that in the group simultaneously given BBN and 100 ppm BITC, the incidence of bladder tumors was additive with respect to BBN-induced papillary carcinomas and BITC-induced non-papillary carcinomas. Previously we reported that 500 or 1000 ppm PEITC or 1000 ppm BITC in the diet strongly enhanced rat bladder carcinogenesis when given in the post-initiation stage.<sup>28-30)</sup> Moreover, continuous dietary administration of 1000 ppm PEITC or BITC for 32 weeks without initiation induced not only hyperplasias, but also papillomas and carcinomas in the urinary bladders of male rats.<sup>28)</sup> Major metabolites of BITC excreted in urine are glutathione conjugates in both rat and human.<sup>31, 32)</sup> This conjugation with thiols is thought to be reversible, and free BITC regenerated in urine may have cyto-

**Table 2.** Incidences of neoplastic lesions in the bladder

Initiation	Treatment Chemical	Number of rats	Hyperplasia		Dysplasia	Papil- oma	Carcinoma			
			Simple	PN			All	Papillary	Non- papillary	Invasive
+	Control	20	20 (100) <sup>1)</sup>	6 (30)	0 (0)	0 (0)	9 (45)	9 (45)	0 (0)	0 (0)
	BBN 25 ppm	20	20 (100)	20 (100)**	8 (40)**	6 (30)*	16 (80)*	15 (75)	1 (5)	1 (5)
	BBN 25 ppm+BITC 100 ppm	20	20 (100)	20 (100)**	8 (40)**	8 (40)**	20 (100)**	18 (90)**	13 (65)****	4 (20)
	BBN 25 ppm+BITC 1000 ppm	19	19 (100)	19 (100)**	19 (100)**##	6 (32)**	19 (100)**	16 (84)*	19 (100)**##	19 (100)**##
	BITC 100 ppm	20	20 (100)	18 (90)**	5 (25)*	1 (5)	10 (50)	5 (25)	10 (50)**	3 (15)
	BITC 1000 ppm	20	20 (100)	20 (100)**	20 (100)**	4 (20)	20 (100)**	16 (80)*	20 (100)**	20 (100)**
-	Control	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)			
	BBN 25 ppm	10	10 (100)**	10 (100)**	0 (0)	0 (0)	1 (10)	1 (10)	0 (0)	0 (0)
	BBN 25 ppm+BITC 100 ppm	10	10 (100)**	9 (90)**	0 (0)	0 (0)	1 (10)	1 (10)	0 (0)	1 (10)
	BBN 25 ppm+BITC 1000 ppm	10	10 (100)**	10 (100)**	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	BITC 100 ppm	10	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	BITC 1000 ppm	10	10 (100)**	10 (100)**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

1) Number (%) of rats with lesions. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared to the respective control groups. ##  $P < 0.01$  compared to the BBN 25 ppm with initiation group.

toxic effects on the bladder epithelium. In fact, allyl isothiocyanate (AITC), BITC and BITC metabolites exert cytotoxic effects after intravesical instillation into the bladders of female F344 rats, and among BITC and its metabolites, BITC itself showed the strongest effects.<sup>33)</sup> We also found that shortly after dietary administration of 1000 ppm BITC to rats, hyperplasia and a prominent increase in BrdU labeling index in the bladder epithelium were observed.<sup>34)</sup> Therefore, regenerative proliferation following cytotoxicity might be involved in promotion of bladder carcinogenesis by isothiocyanates. It is also suggested that such toxicity might be induced in humans after high exposure to isothiocyanates.

On the other hand, we recently reported that continuous 10–1000 ppm BITC treatment in the diet for 40 weeks dramatically inhibited rat urinary bladder carcinogenesis when BITC was given simultaneously with 50 ppm BBN without prior initiation.<sup>27)</sup> Major mechanisms of chemoprevention by isothiocyanates involve selective inhibition of cytochrome P450 enzymes, such as CYP1A2 and CYP2E1, which are responsible for carcinogen metabolic activation, and induction of phase II enzymes, such as glutathione S-transferase, quinone reductase and glucuronosyltransferases, which can detoxify residual electrophilic metabolites generated by P450 enzymes.<sup>10, 11)</sup> BITC has been shown to inhibit CYP1A2 and CYP2E1 in rodents, as well as in humans.<sup>35)</sup> Inhibition of CYP2E1 is partly responsible for the NNK-induced lung carcinogenesis in rodents,<sup>10)</sup> and this may be a factor in the inhibition of tobacco-related lung carcinogenesis by cruciferous vegetables in humans, in addition to the induction of detoxifying enzymes.<sup>23)</sup> Chemopreventive effects of BITC may thus largely be due to inhibition of initiation by the genotoxic carcinogen BBN.

The reason why combined treatment with BBN and BITC inhibits bladder carcinogenesis without prior initiation treatment, while it enhances bladder carcinogenesis after initiation, is currently unknown. It can be speculated that the early phase of lesion development is particularly sensitive, and that when preneoplastic populations have already been induced, then promotion occurs so that suppression of further initiation by BBN is masked. Previously we reported that 10 ppm BITC, a lower dose than the present 100 ppm, also weakly inhibited bladder carcinogenesis induced by simultaneous treatment with 50 ppm BBN, but did not cause any proliferative changes when given alone.<sup>27)</sup> It is of interest to investigate whether a non-toxic dose of 10 ppm or less BITC enhances or inhibits carcinogenesis under the present conditions, as a guide for the risk assessment of human bladder carcinogenesis.

The consumption of average-size portions of vegetables can result in the intake of tens of milligrams of isothiocyanates in man. For example, when 57 g of watercress is consumed, up to 12 mg of PEITC is released.<sup>23, 36)</sup> Human daily intake of total glucosinolates, precursors of isothiocyanates, from cruciferous

vegetables in the United Kingdom is estimated to be 75 mg per person and that of isothiocyanates is a few milligrams.<sup>37)</sup> Exposure levels can therefore be estimated at about 0.2 mg/kg body weight/day in humans. The doses of 5 and 50 mg/kg/day in rats used in this study are about 25–250 times higher than this probable figure.

BITC has been found to be a genotoxicant in *in vitro* experiments, such as the differential DNA repair assay with *Escherichia coli* and the micronucleus assay with human HepG2 cells<sup>38)</sup> and the Ames assay in the presence of S9 mixture,<sup>39)</sup> while both PEITC and BITC induce chromosomal aberrations in an SV40-transformed Indian Muntjac cell line.<sup>40)</sup> Recently it has been shown that the incidence of bladder carcinomas induced in rats by continuous treatment with 0.1% PEITC for 32 weeks and then basal diet for 16 weeks was significantly increased as compared with that induced with PEITC for 32 weeks. Moreover, 58% of the induced tumors had mutations in *p53*.<sup>41)</sup> These results indicate that within a relatively short period, irreversible genetic changes in the DNA of bladder epithelium occur, and that genotoxicity may partly be involved in the induction of bladder carcinomas by PEITC. Moreover, the present study demonstrated that combined treatment with BBN and BITC additively enhanced bladder carcinogenesis, indicating that BITC can act as a risk factor at least in high-risk populations. In a case-control study, protective effects against bladder cancer were shown to be associated with fruit and green-yellow vegetable consumption.<sup>42)</sup> However, with cruciferous vegetables, the situation is less clear, and therefore excess intake of such vegetables should be avoided.

Promoting action of BITC may be limited to the urinary bladder, since early toxic and proliferative lesions were not found in other transitional epithelium such as renal pelvis and urether,<sup>32)</sup> and the urinary bladder was the only target for promotion in rats fed PEITC in a multi-organ carcinogenesis model.<sup>29)</sup> In addition, only the incidence of urinary bladder papillomas was significantly increased in a rat carcinogenicity study of the related compound AITC.<sup>43)</sup> Further accumulation of toxicological data, including underlying mechanisms and possible species variation between the rat and human beings, are very important for assessment of the comparative risk and benefits of isothiocyanates. In addition, since BBN is a synthetic carcinogen that is not present in our environment, the effects of isothiocyanates on urinary bladder carcinogenesis induced by environmental bladder carcinogens, such as aromatic amines, 2-naphthylamine and benzidine, should also be investigated.

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