Inhibition of Urinary Bladder Carcinogenesis by Broccoli Sprouts

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Abstract

Isothiocyanates are a well-known class of cancer chemopreventive agents, and broccoli sprouts are a rich source of several isothiocyanates. We report herein that dietary administration to rats of a freeze-dried aqueous extract of broccoli sprouts significantly and dose-dependently inhibited bladder cancer development induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. The incidence, multiplicity, size, and progression of bladder cancer were all inhibited by the extract, while the extract itself caused no histologic changes in the bladder. Moreover, inhibition of bladder carcinogenesis by the extract was associated with significant induction of glutathione *S*-transferase and NAD(P)H:quinone oxidoreductase 1 in the bladder, enzymes that are important protectants against oxidants and carcinogens. Isothiocyanates are metabolized to dithiocarbamates *in vivo*, but dithiocarbamates readily dissociate to isothiocyanates. We found that >70% of the isothiocyanates present in the extract were excreted in the urine as isothiocyanate equivalents (isothiocyanates + dithiocarbamates) in 12 h after a single p.o. dose, indicating high bioavailability and rapid urinary excretion. In addition, the concentrations of isothiocyanate equivalents in the urine of extract-treated rats were 2 to 3 orders of magnitude higher than those in plasma, indicating that the bladder epithelium, the major site of bladder cancer development, is most exposed to p.o. dosed isothiocyanate. Indeed, tissue levels of isothiocyanate equivalents in the bladder were significantly higher than in the liver. In conclusion, broccoli sprout extract is a highly promising substance for bladder cancer prevention and the isothiocyanates in the extract are selectively delivered to the bladder epithelium through urinary excretion. [Cancer Res 2008;68(5):1593–600]

Introduction

The risk of urothelial and bladder cancers increases significantly in individuals who carry genetic variants of glutathione *S*-transferase (GST) or NAD(P)H:quinone oxidoreductase 1 (NQO1) that yield either a null or a suboptimal phenotype (1–4). This is not surprising, because both these phase 2 enzymes are important cellular protectants against carcinogens and oxidants (5, 6). On the other hand, high consumption of fruit and vegetables, cruciferous vegetables in particular, is associated with reduced risk of bladder cancer (7, 8). Cruciferous vegetables are the major source of human exposure to organic isothiocyanates, many of which are known to induce both GST and NQO1 in the bladder *in vivo* (9). A recent case-control study directly linked higher isothiocyanate consumption to lower bladder cancer risk (10).

Broccoli sprouts are a very rich source of isothiocyanates, the majority of which is sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane], a highly promising cancer chemopreventive agent (11), with smaller amounts of two close sulforaphane analogues, iberin and erucin (12, 13). These compounds are stored in plants in a stable precursor form (glucosinolates) and are generated through enzymatic hydrolysis of the latter substances by endogenous myrosinase, which is released when plant cells are disrupted. We recently showed that a freeze-dried aqueous extract of broccoli sprouts strongly induced both GST and NQO1 in cultured bladder cells and in rat bladder *in vivo* and that the inducer activity of the extract was comparable with that of sulforaphane on the basis of total isothiocyanate concentrations (9). These findings suggest that broccoli sprout extract is not only potentially useful for bladder cancer prevention but is also an excellent substitute for sulforaphane. Moreover, this study revealed that the bladder was one of the most responsive organs to induction of the phase 2 enzymes by the extract.

In the present report, we show the strong inhibitory effect of broccoli sprout extract on bladder cancer development in an *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)–induced rat bladder cancer model. We also show that such inhibition by the extract is associated with significant and persistent induction of GST and NQO1 in the bladder. BBN is a specific bladder carcinogen and represents nitrosamines that are an important class of human carcinogens. BBN-induced bladder tumorigenesis in rodents also resembles the development of the majority of human bladder cancers with respect to histopathology (14, 15) and the role of phase 2 enzymes (16). Previous studies have shown that sulforaphane and other isothiocyanates are primarily metabolized through the mercapturic acid pathway, and their metabolites are bioactive and almost exclusively excreted in the urine (17). Further examination of the pharmacokinetics and disposition of isothiocyanates in rats after p.o. administration of broccoli sprout extract in the present study indicates that the cancer chemopreventive activity of broccoli sprout extract is efficiently and selectively delivered to the bladder target tissue.

Materials and Methods

Chemicals. 1,2-Benzenedithiol was purchased from Aldrich, purified by vacuum distillation, and stored in small aliquots at −20°C. Sulforaphane and BBN were purchased from LKT Laboratories and TCI America,

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respectively. *N*-acetylcysteine conjugate of sulforaphane was synthesized as previously described (18). Lyophilized broccoli sprout extract was prepared from 3-day-old broccoli sprouts, analyzed for isothiocyanate content, and stored at −70°C before use as previously described (13). Each gram of extract contained 140 μmol isothiocyanates, of which 70%, 25%, and 5% were sulforaphane, iberin, and erucin, respectively, with no glucosinolate remaining.

Animals. Female Sprague-Dawley rats were used in animal experiments. The animals were bred and housed at the Ruakura Agricultural Research Center (Hamilton, New Zealand). The animals were maintained in solid bottom cages, containing bedding of softwood shavings, and allowed free access to food and water. The temperature of the animal room was maintained at 21°C to 23°C with a 12-h light/dark cycle. All experimental protocols were approved by the Institutional Animal Ethics Committee.

Assay of GST and NQO1 induction in the bladder *in vivo*. Rats (11–12 weeks of age) were randomly assigned to two groups of 11 rats and acclimatized to AIN-76A powder diet (Crop & Food Research Ltd.) for 2 weeks. In the 3rd week, rats in one group remained on the control diet, whereas those in the second group were switched to a diet supplemented with broccoli sprout extract to provide isothiocyanates at 160 μmol/kg body weight/d (see next paragraphs for details of diet preparation). Five rats in each group were killed by carbon dioxide inhalation after 6 weeks and the remaining six rats in each group were killed after 12 weeks. Their bladders were promptly removed, washed in saline, and weighed. Each bladder was cut longitudinally into two portions. One half was preserved in 10% buffered formalin for histology, and the other half was frozen for determination of activities of NQO1 and GST as previously described (9). During the experiment, the rats were checked thrice each week for signs of ill health, and their body weights were recorded each week.

Bladder cancer prevention trial. Rats (11–12 weeks of age) were randomly assigned to five groups (groups 1–5; see Fig. 2*A*) and acclimatized to AIN-76A powder diet for 2 weeks. Rats then either remained on the same diet (groups 1 and 2) or were switched to diets supplemented with different amounts of broccoli sprout extract (groups 3–5). After a further 2 weeks, rats in groups 2, 3, and 4 received 0.05% BBN in their drinking water continuously for 8 weeks to induce bladder cancer, based on a published protocol (19). Administration of broccoli sprout extract continued for 2 weeks after cessation of BBN exposure, after which all rats were switched to control AIN-76A diet for 1 week and then to pelletized Teklad Global 2016 rodent diet (Harlan Teklad) for the remainder of the experimental period (pelletized AIN-76A diet was not available at the time). AIN-76A diet was used during treatment with broccoli sprout extract in view of its low content of antioxidants and inducers of phase 2 enzymes, but the subsequent switch to pelletized diet was designed to decrease the incidence of dental problems and to reduce wastage of diet. During the experiment, the rats were checked thrice each week for signs of ill health, and their body weights were recorded weekly.

During the period of broccoli sprout administration, the food consumption of the rats was measured thrice weekly. The amount of broccoli sprout extract to be incorporated into the diet was calculated on the basis of the food intake and body weights of the animals, to give isothiocyanate doses of 40 and 160 μmol/kg body weight/d. The concentration of the extract in the diet varied somewhat throughout the feeding period due to animal growth. For example, at the dose of 160 μmol/kg/d, the concentration of the extract in the diet was ~18 g/kg (1.8%) at the beginning of the experiment but was ~23 g/kg (2.3%) at the end. The extract-containing diets were made up thrice per week to minimize degradation of the extract in the diet at room temperature. BBN solutions were also made up thrice per week and given to the rats in polycarbonate drinking bottles covered with aluminum foil to avoid photodegradation.

Thirty-six weeks after the start of the experiment, the rats were killed by carbon dioxide inhalation and their bladders were removed. Bladder weights were recorded, and, after opening, they were examined under a hand lens and macroscopic lesions were recorded. Tumors were graded as small (<0.1 cm in diameter), medium (0.1–0.3 cm in diameter), and large (>0.3 cm in diameter). After photographing, the bladders were preserved in formalin for subsequent histologic examination.

Histologic examination of bladder and bladder cancer. After gross examination, rat bladder tissues were fixed in formalin, cut, and embedded on edge in cassettes. Paraffin-embedded tissue blocks were cut at 3 μm and stained with H&E. The slides were examined on an Olympus microscope (Microscoptics), and tumor staging was recorded using the 2004 WHO classification (20). Tumors were classified as pT_a (noninvasive superficial tumors), pT_1 (tumors invading the lamina propria), and pT_2 (tumors invading the muscularis propria; Fig. 3*A*). When more than one tumor was present in a bladder, the diagnosis was given based on the most advanced tumor. The histologic diagnosis was performed twice by the same pathologist.

Measurement of isothiocyanate levels in plasma, urine, and tissue specimens. Seven groups of four rats (11–12 weeks of age) were given a single p.o. dose of broccoli sprout extract to provide an isothiocyanate dose of 160 μmol/kg and were then immediately placed in metabolism cages, with food (Teklad Global 2016 rodent diet) and water available *ad libitum*. The extract was suspended in water and was administered by gavage in a volume (calculated on the basis of body weight) of ~0.5 mL. Urine was collected from the rats at 1, 2, 3, 4, 8, 12, and/or 24 h after dosing (Fig. 4*A*). One group of rats was killed at each time point by carbon dioxide inhalation for immediate collection of blood using heparin as anticoagulant. Plasma was obtained from the blood by low-speed centrifugation. The eighth group of four rats was given 0.5 mL of water by gavage, placed in metabolism cages for 24-h urine collection, and killed at this time for blood collection. Both urine and plasma specimens were stored at −70°C before use. In addition, bladder and liver were collected at the time of death from all rats, washed thoroughly in ice-cold PBS, and stored at −70°C.

Total levels of isothiocyanate equivalents (isothiocyanates plus their metabolites) in bladder, liver, plasma, and urine were measured using the high-performance liquid chromatography–based cyclocondensation assay (reaction with 1,2-benzenedithiol; refs. 21, 22). Isothiocyanates are metabolized to dithiocarbamates *in vivo*, and the cyclocondensation assay detects both isothiocyanates and dithiocarbamates. Plasma and urine specimens were analyzed without further manipulation, whereas the bladders and livers were homogenized before analysis. In the latter case, each tissue specimen was homogenized in ~3 volumes (for the liver) and 16 volumes (for the bladder) of ice-cold 10 mmol/L Tris-HCl (pH 7.4) with 0.25 mol/L sucrose using either a Polytron homogenizer or a glass tissue grinder, and the crude homogenates were analyzed. Briefly, each 1-mL reaction mixture in a 4-mL glass vial contained 0.25 mL of 100 mmol/L potassium phosphate buffer (pH 8.5), 0.5 mL of 10 mmol/L 1,2-benzenedithiol in acetonitrile, and up to 0.25 mL sample (adjusted with water). For urine samples, the phosphate buffer was replaced with 500 mmol/L sodium borate buffer (pH 9.25), to maintain the required pH of the reaction solution. The reaction mixture was incubated for 2 h at 65°C and, after cooling to room temperature, was centrifuged at low speed to sediment insoluble materials. A 0.1-mL aliquot of the supernatant was analyzed by high-performance liquid chromatography for content of isothiocyanate equivalents. Control samples of bladder, liver, plasma, and urine were added to sulforaphane and *N*-acetylcysteine conjugate of sulforaphane standards to determine recovery of isothiocyanate equivalents. Full recovery of the standards was achieved in urine samples, whereas 85% to 95% of the standards were detected in other samples (detailed data not shown). Concentrations of isothiocyanate equivalents measured in samples were adjusted accordingly.

Statistical analysis. Statistical significance was tested by ANOVA, followed by the Student-Newman-Keuls multiple comparisons test, using Instat software (GraphPad).

Results

Induction of GST and NQO1 in rat bladder by broccoli sprout extract. We recently showed that dietary supplementation of freeze-dried aqueous broccoli sprout extract, at a level that provided isothiocyanate at 160 μmol/kg body weight/d, elevated the activities of GST and NQO1 1.3- and 2.5-fold in the bladders of female Sprague-Dawley rats after 2 weeks of feeding (9). Such

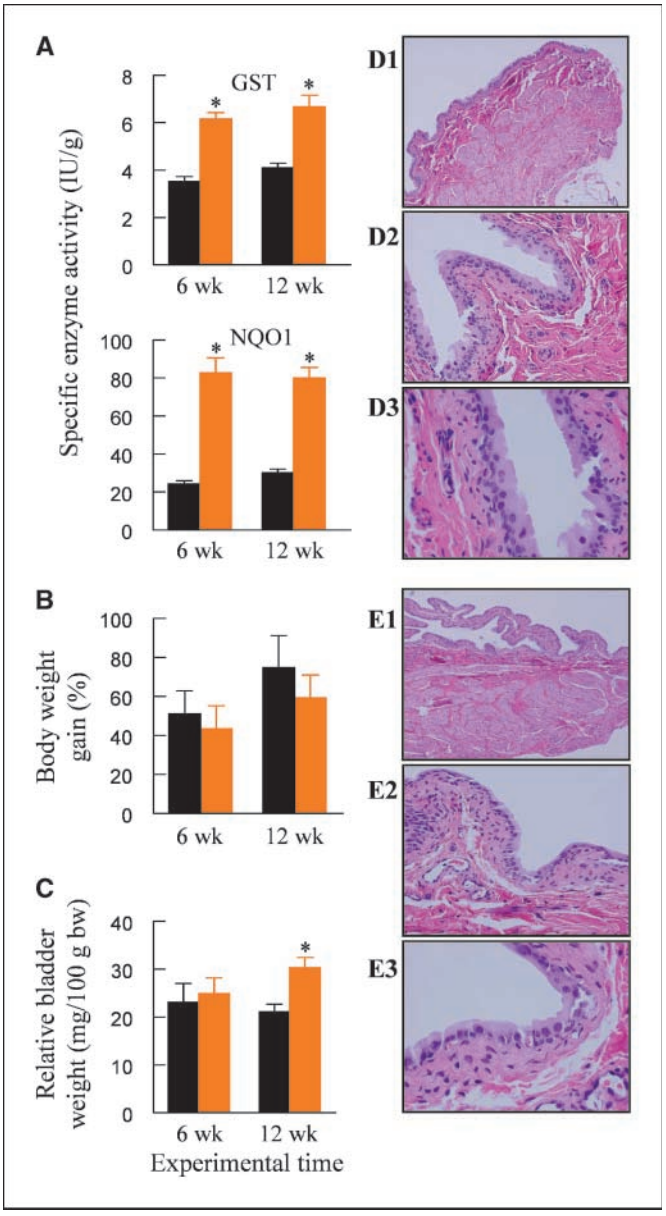


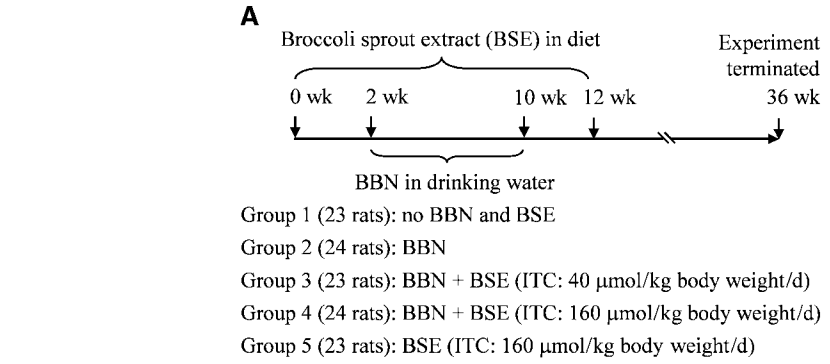
Figure 1. Effect of dietary broccoli sprout extract on phase 2 enzymes, body weight, and bladder weight in rats *in vivo*. Adult female Sprague-Dawley rats were fed a control diet (*black columns*) or a diet containing broccoli sprout extract at an isothiocyanate dose of 160 μmol/kg body weight/d (*orange columns*) for 6 and 12 wk, during which body weight was measured weekly. The animals were killed at the end of extract feeding, and each bladder was cut longitudinally into two equal portions. One half of each bladder was homogenized and measured for specific activity of GST and NQO1, whereas the other half was fixed, sectioned, and stained with H&E for histologic examination. *A,* specific enzyme activity. *B,* body weight gain. *C,* relative bladder weight. *Columns,* mean (n = 5 or 6); *bars,* SD. *D1* to *D3,* microscopic views at increasing magnification of a representative bladder of a control rat. *E1* to *E3,* microscopic views at increasing magnification of a representative bladder of an extract-treated rat. *, significantly different from the corresponding control value ($P < 0.001$).

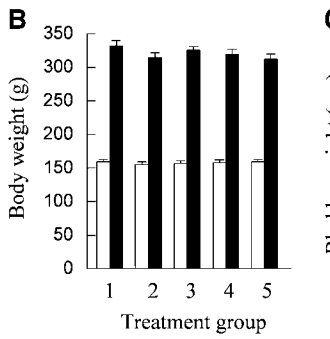
enzyme induction was not associated with any indication of toxicity to the urinary bladder. However, it was not clear if a longer-term treatment with broccoli sprout extract would alter enzyme induction or if it would cause toxic effects. This was an important issue, because it was necessary to feed the rats with broccoli sprout extract for a longer time in the cancer prevention trial as described in this report. Thus, groups of five to six adult female Sprague-Dawley rats were fed with either the control diet or the diet supplemented with broccoli sprout extract at 160 μmol isothiocyanate/kg body weight/d. The activities of GST and NQO1 in the bladder of rats treated with broccoli sprout extract were elevated 1.8- and 3.4-fold at 6 weeks and 1.6- and 2.7-fold at 12 weeks, respectively (Fig. 1*A*). These values not only show significant induction of the enzymes in the bladder but also are higher than those obtained from rats treated with the same dose of extract for 2 weeks as mentioned above. This indicates that induction of GST and NQO1 in the bladder is sustained under prolonged treatment with broccoli sprout extract.

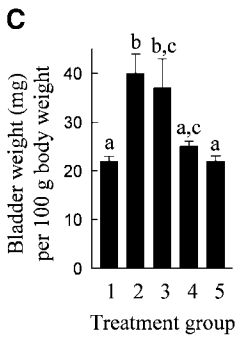
The animals appeared healthy and their behavior was normal during the experiment, but the body weight gains of the extract-fed rats were 7.9% and 15.6% lower than that of the control animals at 6 and 12 weeks, respectively (Fig. 1*B*). Moreover, there was a time-dependent increase of 7.4% and 43.1% in relative bladder weight in extract-fed rats (Fig. 1*C*), although no histologic abnormalities were detected in the bladders of control and extract-fed rats (Fig. 1*D* and *E*). The reason for these changes remains unknown, but the changes were reversible. No increase in bladder weight and only a 6% decrease in body weight (not statistically significant) were seen 24 weeks after the removal of the extract from the diet (Fig. 2*B* and *C*).

Inhibition of bladder carcinogenesis by broccoli sprout extract. Having shown that feeding rats continuously with broccoli sprout extract in the diet at the isothiocyanate dose of 160 μmol/kg body weight/d leads to significant and sustained induction of GST and NQO1 in the bladder, we next asked if dietary broccoli sprout extract would inhibit bladder carcinogenesis. The extract was evaluated in a BBN-induced rat bladder cancer model, and the dosing schedules are shown in Fig. 2*A*. Briefly, rats were randomly assigned to five groups (24 rats per group), consisting of a control group (group 1), a BBN group (group 2), two groups of combination treatment with BBN and broccoli sprout extract (groups 3 and 4), and one group of broccoli sprout extract alone (group 5). Rats in the control group did not receive any specific treatment during the entire experimental period. For induction of bladder cancer, BBN was administered to rats in their drinking water for 8 weeks beginning in the 3rd week. Broccoli sprout extract was added to the diet during the first 12 weeks at dose levels that provided 40 and 160 μmol isothiocyanate/kg body weight/d. The low dose of extract was included to permit assessment of dose-response. Only the high dose of extract (160 μmol/kg/d) was evaluated for potential adverse effects. All rats were switched to regular diets and water after completion of treatment with BBN and broccoli sprout extract and were killed 24 weeks thereafter. Three rats had to be removed before the experiment was terminated, including a rat in group 1 with severe and intractable dental malocclusion, a rat in group 3 with renal failure (marked calcification at the level of the corticomedullary junction, tubular dilation and loss within the cortex, and areas of fibrosis), and a rat in group 5 with severe respiratory distress, associated with acute pulmonary congestion and edema of unknown etiology.

The average body weight gain of rats in groups 2 to 5 over the experimental period was slightly lower than that in group 1, but the difference was not statistically significant (Fig. 2*B*). The increases in bladder weight in groups 2 to 4 seem to reflect tumor burden (Fig. 2*C*). The average bladder weight after adjustment by body weight (mg bladder weight per 100 g body weight) was identical between group 1 and group 5, indicating that the acute bladder







D

Treatment group	% Rats with tumor	No. tumors per rat	% Tumors with listed size (cm length)		
			<0.1	0.1-0.3	>0.3
Group 1	0	0	0	0	0
Group 2	95.8	1.96	46.8	42.6	10.6
Group 3	73.9	1.39	37.5	34.4	21.9
Group 4	37.5	0.46	63.6	36.4	0
Group 5	0	0	0	0	0

Figure 2. Effect of broccoli sprout extract on bladder carcinogenesis in rats. *A,* adult female Sprague-Dawley rats were treated with BBN and/or broccoli sprout extract (*BSE*) or vehicle. Body weights were recorded weekly during the experimental period, and all rats were killed 36 wk after the experiment was started. Bladder was removed from each rat, weighed, opened, examined under a hand lens for macroscopic lesions, and the size of each tumor was measured with a ruler. *B,* initial (*white columns*) and final (*black columns*) body weights. *Columns,* mean (n = 23 or 24); *bars,* SE. The differences among groups are not statistically significant. *C,* relative bladder weights (mg per 100 g body weight). *Columns,* mean (n = 23 or 24); *bars,* SE. Values marked with different alphabetical letters are significantly different at $P < 0.01$. *D,* bladder cancer incidence, multiplicities, and size.

weight increase due to extract feeding observed in the short-term experiment (Fig. 1*C*) was completely reversible.

There was no evidence of bladder cancer in rats in either group 1 (control) or in group 5 (extract only), and no histologic abnormalities were detected in bladders chosen randomly from these groups. In contrast, 4.2% and 95.8% of rats in the BBN group (group 2) developed dysplasia and carcinoma in the bladder, respectively (Figs. 2*D* and 3*B*), and the average cancer multiplicity was 1.96 (Fig. 2*D*). The majority of the bladders (70.8%) presented with noninvasive cancer (pT_a), whereas 16.7% and 8.3% of the bladders bore cancers invading either the lamina propria (pT_1) or the muscularis propria (pT_2), respectively (Fig. 3*B*). Feeding broccoli sprout extract to rats resulted in marked and dose-dependent inhibition of bladder tumorigenesis. Cancer incidence and multiplicity were 37.5% (60.9% inhibition) and 0.46 (76.5% inhibition) in group 4 (BBN plus the high dose extract) and 73.9% (22.9% inhibition) and 1.39 (29.1% inhibition) in group 3 (BBN plus the low-dose extracts; Fig. 2*D*). Moreover, there was a marked and dose-dependent decrease in tumor size and cancer progression in extract-treated rats (Figs. 2*D* and 3*B*). These results clearly show that dietary consumption of broccoli sprout extract is highly effective in inhibiting BBN-induced bladder cancer development in these animals.

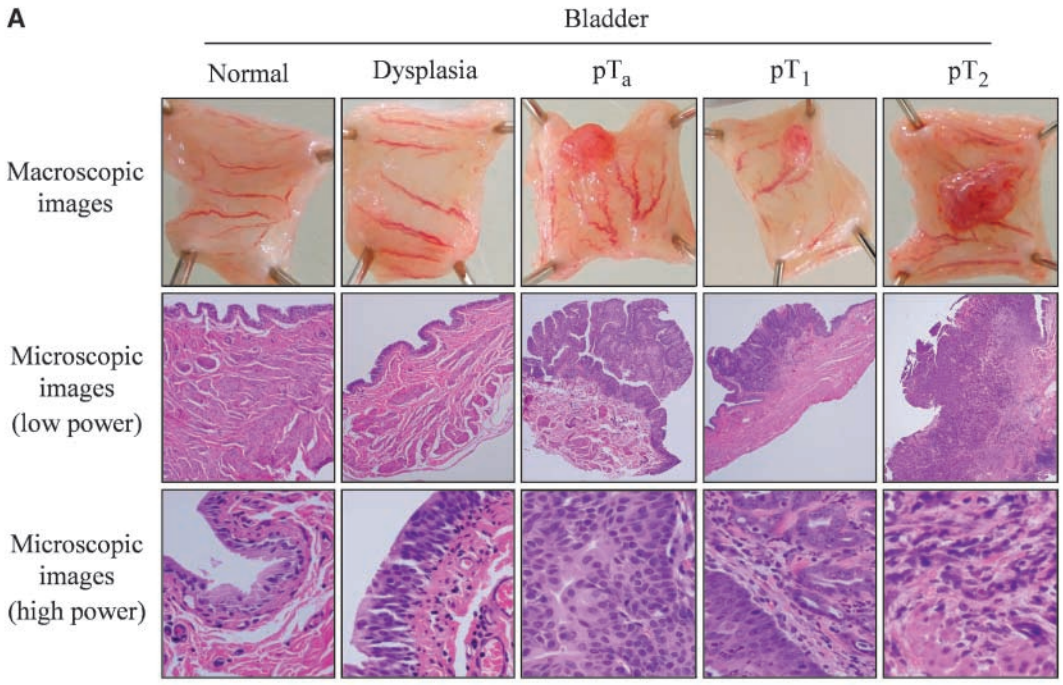
Isothiocyanates are selectively delivered to the bladder epithelium after p.o. dosing of broccoli sprout extract. Our previous studies have shown that isothiocyanates, sulforaphane in particular, are the principal if not the only cancer chemopreventive agents in broccoli sprout extract (see Introduction). Broccoli sprout isothiocyanates and other isothiocyanates are mainly metabolized through the mercapturic acid pathway *in vivo*. An initial conjugation of isothiocyanate with glutathione gives rise to the corresponding conjugates, which are further metabolized to form sequentially the cysteinylglycine, cysteine, and *N*-acetylcysteine conjugates, which are excreted in urine (23, 24). The conjugates, which are collectively known as dithiocarbamates, can dissociate to free isothiocyanates (25) and therefore act as isothiocyanate carriers. The cyclocondensation assay, which we had previously developed for measuring the total amount of both isothiocyanates and their conjugates (dithiocarbamates; ref. 21), was used to measure isothiocyanate equivalents in bladder, liver, plasma, and urine after a single p.o. dose of broccoli sprout extract. Seven groups of four rats were dosed by gavage with broccoli sprout extract at an isothiocyanate dose of 160 μmol/kg and sacrificed at 1, 2, 3, 4, 8, 12, and 24 h for collection of bladder, blood, and liver. Urine was collected from each rat at these time points until the animals were killed (Fig. 4*A*). An eighth group of four rats was used as control and was given vehicle (water) by gavage, and 24-h urine was collected from each animal in addition to blood and organ collection after 24 h (Fig. 4*A*).

Low levels of isothiocyanate equivalents were present in the plasma (0.14 ± 0.07 μmol/L) and urine (14 ± 9 μmol/L) of rats in the control group (Fig. 4*B*), likely resulting from the presence of a small amount of isothiocyanates in the diet. Oral administration of

broccoli sprout extract, however, led to a rapid increase in the levels of isothiocyanate equivalents in both plasma and urine. The isothiocyanates were absorbed rapidly; plasma concentrations of isothiocyanate equivalents were highest at 1 h after dosing (16.3 ± 0.4 μmol/L) and declined thereafter with first-order kinetics (half-life of 8.4 h). Urinary concentrations of isothiocyanate equivalents reached their peak 4 h after dosing (6.7 ± 0.8 mmol/L) and declined thereafter with first-order kinetics (half-life of 10.7 h). The cumulative urinary excretion of isothiocyanate equivalents was almost linear within 12 h after dosing, and 70.3 ± 4.4% of the isothiocyanate dose was recovered during this period (Fig. 4*C*). Only 3.4% of the dose was excreted in the urine in the next 12 h (from 12 to 24 h after dosing), indicating that urinary excretion of isothiocyanates is largely completed within 12 h after dosing. The most remarkable finding, however, was that urinary concentrations of isothiocyanate equivalents were 2 to 3 orders of magnitude higher than those in plasma. This indicates that the bladder epithelium, which is directly exposed to urine, was exposed to isothiocyanates and dithiocarbamates at profoundly higher concentrations than other organs when the animals were fed with broccoli sprout extract.

Levels of total isothiocyanate equivalents in both bladder and liver tissues were highest at 1 h after dosing, reaching 142.8 and 39.7 pmol/mg tissue, respectively, and decreased thereafter (Fig. 4*D*). Total isothiocyanate equivalent levels in the bladder were 2.7 to 3.6 times higher than in the liver at 1 to 12 h after dosing and 19.7 times higher than in the liver at 24 h (Fig. 4*D*). Notably, the relatively modest higher levels of isothiocyanate

A



B

	Incidence (% of bladders)				
BBN in drinking water (0.05%)	–	–	+	+	+
BSE in diet (μmol ITC/kg bw)	–	160	–	40	160
Normal	n/a*	n/a	0	0	20.8
Dysplasia	n/a	n/a	4.2	26.1	41.7
pT$_a$	0	0	70.8	56.5	37.5
pT$_1$	0	0	16.7	8.7	0
pT$_2$	0	0	8.3	8.7	0

Figure 3. *A,* morphology of stage of BBN-induced bladder carcinogenesis in rats and the blocking effect of broccoli sprout extract on bladder carcinogenesis. Treatment detail of female Sprague-Dawley rats with BBN and/or broccoli sprout extract, including the number of rats in each group, is shown in Fig. 2*A* and *B*. The macroscopic images were taken after opening and extending the bladders. The microscopic images were obtained after H&E staining of sections of paraffin-embedded bladder tissues. *B,* pT$_a$, pT$_1$, and pT$_2$ refer to tumors that are noninvasive (superficial tumors); invade the lamina propria; and invade the muscularis propria, respectively. *, no histologic abnormalities were detected in bladders chosen randomly from these groups. *ITC,* isothiocyanate.

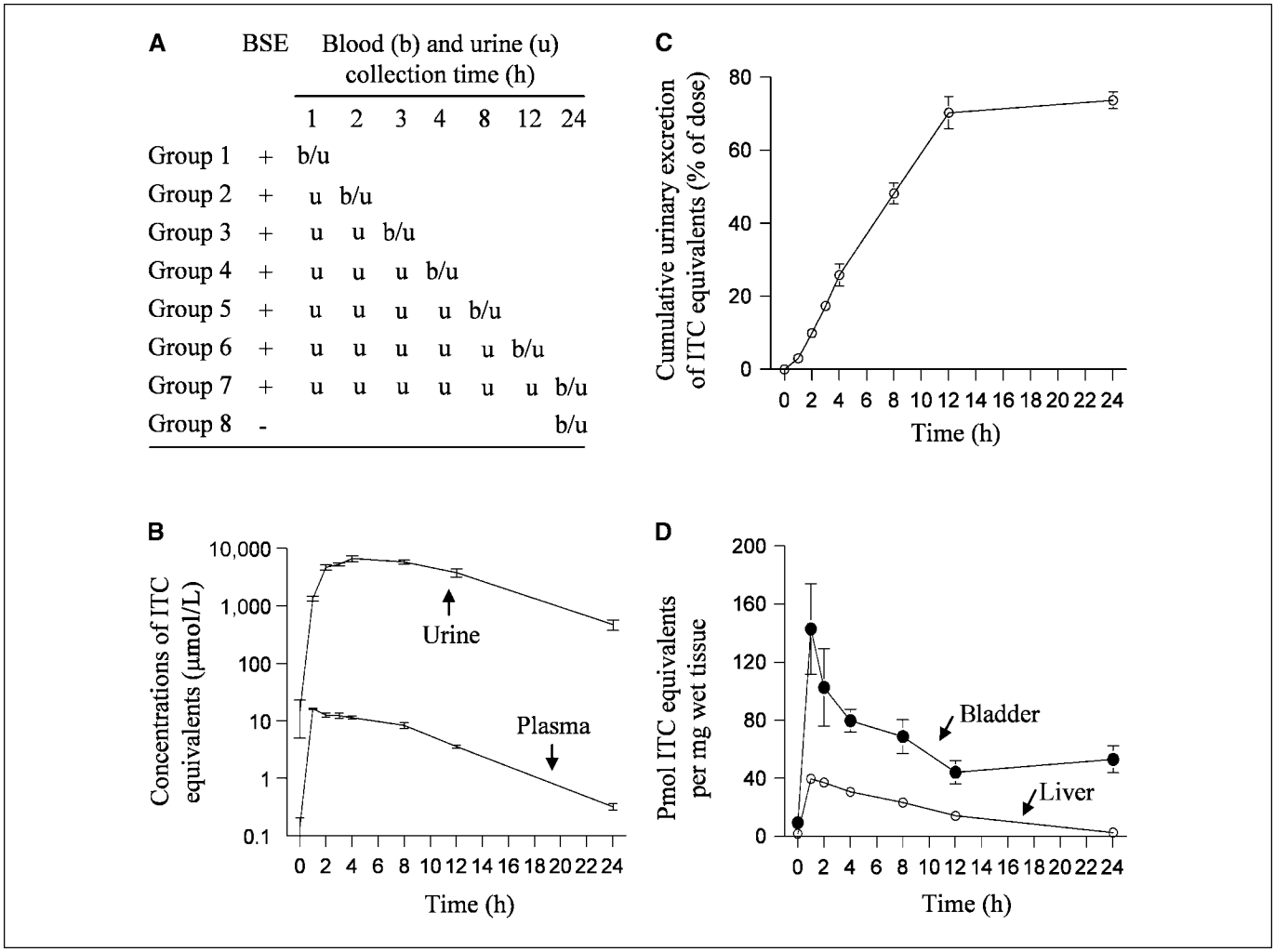


Figure 4. Pharmacokinetics of broccoli sprout isothiocyanate in rats. *A,* eight groups of adult female Sprague-Dawley rats were given a single p.o. dose of vehicle or broccoli sprout extract at an isothiocyanate level of 160 μmol isothiocyanate/kg and then individually housed in a metabolism cage for urine and blood collection at 1, 2, 3, 4, 8, 12, or 24 h after dosing. Rats were killed at various time intervals for blood collection. Total isothiocyanate equivalents in bladder, liver, plasma, and urine samples were measured with the cyclocondensation assay (see Materials and Methods). *B,* the urinary and plasma concentrations of isothiocyanate equivalents were plotted against time. *C,* cumulative urinary excretion of isothiocyanate equivalents was calculated. *D,* tissue levels of isothiocyanate equivalents in the bladder and liver were also plotted against time. *Points,* mean ($n \geq 4$); *bars,* SE.

equivalents in the bladder compared with those in the liver contrast sharply to the dramatically higher concentrations of isothiocyanate equivalents in the urine compared with those in the plasma. However, considering that the bladder epithelium probably comprises <1% of the total bladder weight but is directly exposed to isothiocyanate in urine, the isothiocyanate levels measured in the entire bladder may not fully reflect the levels in the epithelium. It is likely that the levels of isothiocyanate equivalents in the epithelium are markedly higher than those in other areas of the bladder. In view of the fact that bladder cancer occurs almost exclusively in the epithelium, the present finding is significant in that, after ingestion, broccoli sprout isothiocyanates are selectively delivered to the bladder target tissue through urinary excretion.

Discussion

BBN is a well known and widely used experimental bladder carcinogen. The dosing regimen used in the present study (0.05% BBN in drinking water for 8 weeks) was highly effective in inducing bladder cancer in the animals (Figs. 2 and 3). Whereas no rats in the control group developed bladder cancer, 23 of 24 BBN-treated rats (95.8%) had bladder cancer 26 weeks after BBN treatment, and the remaining rat (4.2%) had dysplasia in the bladder. The majority of the rats (70.8%) had bladder cancer localized within the epithelium (pT_a), whereas 16.7% and 8.3% rats presented with submucosal (pT_1) and muscular (pT_2) invasion. The superficial nature of the majority of bladder cancers in these rats closely resembles that of human bladder cancer as 70% to 80% of bladder cancers in humans are pT_a and pT_1 (26). Feeding rats with broccoli sprout extract in the diet at isothiocyanate doses of 40 and 160 μmol/kg body weight/d for 12 weeks, starting 2 weeks before carcinogen exposure, caused significant and dose-dependent inhibition of bladder cancer development, including lower cancer incidence and multiplicity, smaller tumor size, and reduced cancer invasiveness (Figs. 2*D* and 3*B*). Broccoli sprout extract alone was not carcinogenic to the bladder. These findings show for the first time that

broccoli sprout extract strongly inhibits carcinogenesis in the bladder. The results are consistent with epidemiologic studies showing that increased consumption of broccoli and other cruciferous vegetables is associated with reduced bladder cancer risk (7, 8, 10).

The exact mechanism by which broccoli sprout extract inhibits BBN-induced bladder carcinogenesis is unknown at the present time. Our study showed that feeding rats with the extract in the diet at an isothiocyanate dose of 160 μmol/kg/d for 6 and 12 weeks resulted in significant and sustained induction of both GST and NQO1. Both enzymes protect cells against carcinogens and have previously been reported to play an important role in bladder cancer prevention in humans (1–4). We previously showed that broccoli sprout extract giving an isothiocyanate dose of 40 μmol/kg body weight/d in the diet induced GST and NQO1 in the rat bladder, but the degree of enzyme induction was lower than that caused by the higher dose of extract used in this study (9). Thus, induction of both enzymes by the extract in the bladder seems to correlate with inhibition of bladder carcinogenesis, suggesting that induction of these enzymes may play a role and/or act as a reliable biomarker in the inhibition of bladder carcinogenesis by broccoli sprout extract. Broccoli sprout extract and sulforaphane have also been shown to induce apoptosis and cell cycle arrest in cultured bladder cancer cells (13, 27, 28), and were also shown to inhibit angiogenesis and cancer metastasis in nonbladder systems (29–32). Whether any of these mechanisms contribute significantly to the chemopreventive effect of broccoli sprout extract in the present model is uncertain, because the extract was administered only in the initiation phase. In light of the multiple anticancer mechanisms possessed by broccoli sprout extract and sulforaphane, it will be interesting to determine if the extract inhibits bladder cancer development when given in the postinitiation phase. It is of note that both broccoli sprout extract and sulforaphane were previously shown to inhibit tumorigenesis when given in the postinitiation phase and inhibited the growth of tumor xenograft in several nonbladder cancer models (33–36).

We have shown that 70.3% and 4.3% of the isothiocyanate dose were excreted in the urine in the first and second 12 h, respectively. Our recent study in rats showed that ~75% of the isothiocyanate contained in a single p.o. dose of broccoli sprout extract was excreted in the urine within 24 h after dosing and only 1% of the dose was excreted in the second 24 h time period (9). These results indicate that urinary excretion of isothiocyanate equivalents is largely completed within 12 h after dosing. In the present study, we measured the total levels of isothiocyanate and its dithiocarbamate metabolites and expressed the results as isothiocyanate equivalents. Previous studies in both rats and humans have indicated that broccoli sprout isothiocyanates are present in several chemical forms in both blood and urine, including free isothiocyanates and their metabolites formed in the mercapturic acid pathway (mainly cysteine conjugate in blood, and *N*-acetylcysteine and cysteine conjugates in urine; refs. 23, 24, 37). As mentioned before, these conjugates act as carriers of isothiocyanates and are similar to the cognate isothiocyanates in biological activity. Perhaps the most striking finding in the present study was that urinary concentrations of isothiocyanate equivalents were 2 to 3 orders of magnitude higher than those in the plasma within 24 h after dosing. Isothiocyanate levels in the bladder were 2.7- to 19.7-fold higher than that in the liver when compared at the same tissue weight. The actual isothiocyanate levels in bladder epithelium (the target tissue) may be much higher than that measured in the whole bladder tissue, because the bladder epithelium most likely comprises <1% of the whole bladder weight and the epithelium is directly exposed to isothiocyanate in urine. In the light of these findings and considerations, we conclude that p.o. dosed broccoli sprout isothiocyanates are selectively delivered to the bladder tissue through urinary excretion. Given that nearly all bladder cancers occur in the epithelium, isothiocyanate-enriched broccoli sprout extract and other cruciferous vegetables rich in isothiocyanates may be particularly useful for prevention of bladder cancer.

The average 24-h urinary concentrations of isothiocyanate equivalents in rats dosed with broccoli sprout isothiocyanates at 40 and 160 μmol/kg were ~0.32 and 2.1 mmol/L in our previous study (9). The present data show that urinary concentrations are as high as 6.7 mmol/L at shorter time intervals after dosing rats with the isothiocyanates at 160 μmol/kg (Fig. 4*B*). These concentrations are tens- to hundreds-fold higher than that required for isothiocyanates and their dithiocarbamates metabolites to exert chemopreventive activity in cultured bladder cells, as shown in our previous studies (13, 18, 27). It remains to be determined if lower urinary concentrations of isothiocyanate equivalents *in vivo* are effective for bladder cancer prevention. Interestingly, the daily intake of total isothiocyanate in humans through consumption of cruciferous vegetables is estimated to be 10 to 100 μmol per person (38–40), equivalent to 0.14 to 1.43 μmol/kg for a 70-kg individual. This range is markedly lower than the doses given to the animals in the present study. Because epidemiologic studies have shown that dietary isothiocyanates and cruciferous vegetable intake are inversely associated with bladder cancer risk in humans (see Introduction), it is possible that isothiocyanate doses much lower than those given to the rats in the present study may be adequate for bladder cancer prevention.

In conclusion, dietary broccoli sprout isothiocyanate extract is highly effective in inhibiting bladder carcinogenesis in rats. Inhibition of bladder carcinogenesis by the extract is associated with induction of GST and NQO1 in the bladder, which is significant because deficiency of either of these enzymes was previously reported to increase bladder cancer risk in humans. Moreover, the active ingredients in broccoli sprout extract, namely isothiocyanates, are selectively delivered to bladder epithelium (the site of bladder cancer development) through urinary excretion.

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Conflict of interest: Dr. Jed W. Fahey is a founder of Brassica Protection Products, LLC (BPP), a company that sells broccoli sprouts. He is an unpaid consultant to BPP, and he, as well as Johns Hopkins University, owns stock in BPP, which is subject to certain restrictions under University policy. The terms of this arrangement are being managed by Johns Hopkins University in accordance with its conflict of interest policies. Neither the preparation of broccoli sprout extract used in the present study nor the study itself involved BPP.

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