Jpn. J. Cancer Res. 81, 754–759, August 1990

Structure-Activity Relations in Promotion of Rat Urinary Bladder Carcinogenesis by Phenolic Antioxidants

Yasushi Kurata,[1] Shoji Fukushima, Ryohei Hasegawa, Masao Hirose, Masa-Aki Shibata, Tomoyuki Shirai and Nobuyuki Ito

First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467

The urinary bladder tumor-promoting potentials of the phenolic antioxidants, 2-*tert*-butyl-4-methylphenol (TBMP), propylparaben, catechol, resorcinol and hydroquinone, which are structurally related to butylated hydroxyanisole (BHA), were investigated in 170 male F344 rats. The animals were initially given 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) as an initiator in their drinking water for 4 weeks. Three days later, groups of 20 rats received diet containing 1.0% TBMP, 3% propylparaben, 0.8% catechol, 0.8% resorcinol, 0.8% hydroquinone or basal diet alone until the end of week 36. Significant increases in the incidences and average numbers of the putative preneoplastic lesions, papillary or nodular (PN) hyperplasia, and papillomas of the urinary bladder were only observed in the group given TBMP after BBN. Development of these lesions was not enhanced by diet containing the other test compounds and no induction was associated with any of the test chemicals alone. The results thus clearly showed that TBMP, which most closely resembles BHA, promoted urinary bladder carcinogenesis. The similar effects of TBMP and BHA on urinary bladder carcinogenesis suggest a direct link between chemical structure and biological potency.

Key words: Tumor promotion — Antioxidant — Urinary bladder

Since phenolic antioxidants, not only synthetic but also naturally occurring species, are ubiquitous in our environment, investigation of their carcinogenicity and/or capacity for modification of carcinogenesis has been regarded as important in the clarification of the effects of environmental factors on human cancer. There is now substantial evidence that butylated hydroxyanisole (BHA[2]), an antioxidant commonly used in food, is in fact caricinogenic for the forestomach of the rat[1)] and hamster.[2,3)] In addition a number of investigations have shown that BHA and related compounds, such as butylated hydroxytoluene (BHT), which are widely used as preservatives in foods and cosmetics, can modify tumor development, exerting an inhibitory effect in the liver,[4–7)] but conversely promoting 2-stage urinary bladder carcinogenesis.[8–10)] Catechol and TBMP have also been reported to possess promoting activity for forestomach carcinogenesis.[11)]

To better understand BHA carcinogenesis, a number of studies have been conducted on isomers and/or structurally related phenolic compounds to analyze the relationship between structure and carcinogenic potential. 2-*tert*-Butyl-4-methylphenol (TBMP), *p*-*tert*-butylphenol, catechol, *p*-methylphenol, *p*-methoxyphenol, caffeic acid, methylhydroquinone and pyrogallol were found to induce epithelial hyperplasia in the forestomach of the hamster, whereas resorcinol, hydroquinone, propylparaben and *tert*-butylhydroquinone (TBHQ) did not[12)] thus suggesting a link between ability to cause proliferation and promotion potential. In the light of this finding, the present study was designed to evaluate the effect of five dietary antioxidants, catechol, TBMP, propylparaben, resorcinol and hydroquinone, on both promotion of rat urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and the level of DNA synthesis in the epithelial cells using 5-bromo-2′-deoxyuridine (BrdU) incorporation as a marker.

MATERIALS AND METHODS

Animals A total of 170 male F344 rats (Charles River Japan Inc., Kanagawa), 6 weeks old at the beginning of the experiments, were used. The animals were housed 5 to a plastic cage with wood chips for bedding, under conditions of controlled temperature (22±2°C) and humidity (60±10%), on a 12-h light-dark cycle, and fed Oriental MF basal diet (Oriental Yeast Co., Tokyo) and given tap water *ad libitum*.

Chemicals BBN (CAS No. 3817-11-6, purity >98%) was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. TBMP (CAS No. 2409-55-4, purity >99%) was

[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: BHA, butylated hydroxyanisole; TBMP, 2-*tert*-butyl-4-methylphenol; PN, papillary or nodular; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; BHT, butylated hydroxytoluene; TBHQ, *tert*-butylhydroquinone; BrdU, 5-bromo-2′-deoxyuridine; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine.

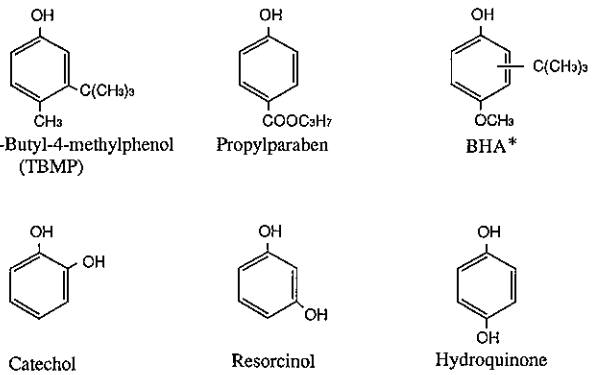

Fig. 1. Chemical formulae of the phenolic antioxidants tested.
* BHA is shown for reference.

obtained from Aldrich Chemical Co., Milwaukee, WI. Propylparaben (purity>99%), catechol (CAS No. 120-80-9, purity>98%), resorcinol (CAS No. 108-46-3, purity>99.5%) and hydroquinone (CAS No. 123-31-9, purity>99%) were from Wako Pure Chemical Industries, Osaka. The chemical structures of these compounds are shown in Fig. 1.

Experiment 1 Rats were divided into 3 groups. Group 1 consisted of 100 rats given BBN in their drinking water for 4 weeks, and then tap water until they were killed. The BBN was dissolved in a solution of Tween 80 in distilled water (0.5%) at a concentration of 0.5% and refrigerated (4°C). Every other day an aliquot of this stock solution was diluted with tap water to a concentration of 0.05%. At three days after the end of the treatment with BBN, the rats were further divided into 5 subgroups of 20 rats each and given Oriental MF powdered diet containing 1.0% TBMP, 3.0% propylparaben, 0.8% catechol, 0.8% resorcinol or 0.8% hydroquinone, until the end of week 36. These dietary levels of chemicals were chosen on the basis of previous studies.[12] Group 2 consisted of 20 rats treated with BBN in the same manner as group 1 and given tap water thereafter. They were given basal diet throughout the experiment. Group 3 contained 50 rats without BBN treatment divided into 5 subgroups, each given one of the test chemicals alone after 4 weeks and 3 days in the same manner as for group 1. All rats were killed for histological examination at the end of week 36.

The urinary bladders were inflated by intraluminal injection of 10% buffered formalin solution, and were cut in half, weighed and sliced into eight strips for histological analysis. Tissues were embedded in paraffin and sections were stained with hematoxylin and eosin. Urinary bladder lesions were classified histologically into three types, papillary or nodular (PN) hyperplasia, a putative preneoplastic lesion, papilloma and carcinoma as described previously.[13] For quantitative analysis, the numbers of these lesions were counted microscopically, the total length of the basement membrane analyzed was measured with the aid of a color video image processor (VIP-21CH, Olympus-Ikegami, Tokyo), and lesion frequency per 10 cm of basement membrane was calculated.

Experiment 2 A total of 30 male 6-week-old Fischer 344 rats were divided into 6 groups of 5 rats each and then were given one of the antioxidants at the same doses as in Experiment 1. The control group was given basal diet for 4 weeks. At the end of week 4, all animals were given an intraperitoneal injection of 100 mg/kg of BrdU (Sigma Chemical Co., St. Lois, MO) dissolved in 0.9% NaCl and dimethylsulfoxide (3:1) solution. The animals were killed 1 h after injection of BrdU. At the time of killing, the urinary bladder was excised in the same manner as in Experiment 1 and processed routinely for immunohistochemical demonstration of BrdU incorporation in the epithelial cells using the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, PK-6102, Vector Laboratories, Inc., Burlingame, CA) with anti-BrdU monoclonal antibody (Dako Patts, Dako Japan Co., Ltd.). The labeling indices of the epithelial cells were calculated as the ratio (expressed as a percentage) of the number of BrdU-labeled cells to the total number of counted cells (1000 cells per animal).

Statistical analysis Data, expressed as mean±SD of measurements, were analyzed when appropriate by using F- and *t*-tests. The significance of differences in the incidences of neoplastic lesions among different groups was evaluated by using Fisher's exact test. The criterion of significance was $P<0.05$.

RESULTS

Experiment 1 Table I shows the final body and urinary bladder absolute weights and organ-to-body weight ratios. Significantly decreased final body weights were seen in rats treated with BBN followed by TBMP, propylparaben, catechol or hydroquinone when compared to control values (group 2). The body weights of the groups given TBMP, propylparaben or catechol alone without BBN were also decreased. Although no treatment-related effects on absolute urinary bladder weight were evident in any of the groups, the relative bladder weights of animals given BBN followed by TBMP were significantly higher than those of animals treated with BBN alone. Grossly, small numbers of nodules were observed in urinary bladders in all groups pretreated with BBN. No urinary bladder lesions were found in rats of group 3, treated with test chemicals alone.

The microscopically determined incidences and quantitative values for epithelial lesions in the urinary bladder

are summarized in Table II. Incidences of transitional cell carcinoma in groups treated with BBN varied from 5 to 25%. Although not statistically significant, increasing trends were noted in those groups given TBMP and propylparaben (group 1). A significantly increased incidence of papillomas was observed in the BBN-followed-by-TBMP group compared to that for BBN alone (group 2). The numbers of papilloma per 10 cm of basement membrane were significantly increased in the group given TBMP. The incidence and quantitative values of PN hyperplasia were also significantly increased by TBMP. However, there were no differences in the incidences or numbers of papillomas or PN hyperplasias in the groups treated with BBN followed by propylparaben, catechol, resorcinol or hydroquinone. In groups given the test chemical alone without BBN, TBMP induced simple hyperplasia, which consisted of diffuse or focal thickening of the epithelium with four to eight layers of transitional cells.

Experiment 2 The labeling indices in the urinary bladder epithelium from control and antioxidant-treated rats are summarized in Table III. TBMP-treated rats demon-

Table I. Mean Final Body Weights and Urinary Bladder Weights

Group	Treatment		Dose	No. of	Final body wt.	Bladder weight	
	BBN	Test compound	(%)	rats	(g)	Abs.[d] (g)	% of body wt.
1	+	TBMP	1.0	20	314±15[a, b]	0.16±0.04[a]	0.050±0.014[a, c]
	+	Propylparaben	3.0	20	382±16[b]	0.14±0.04	0.037±0.010
	+	Catechol	0.8	19	366±19[b]	0.14±0.04	0.038±0.010
	+	Resorcinol	0.8	20	420±18	0.14±0.03	0.035±0.009
	+	Hydroquinone	0.8	20	406±18[c]	0.13±0.03	0.031±0.073
2	+	—	—	19	420±18	0.14±0.04	0.034±0.010
3	−	TBMP	1.0	10	307±8	0.12±0.03	0.038±0.010
	−	Propylparaben	3.0	10	377±13	0.15±0.05	0.041±0.013
	−	Catechol	0.8	10	362±18	0.11±0.02	0.030±0.006
	−	Resorcinol	0.8	10	432±15	0.14±0.04	0.031±0.008
	−	Hydroquinone	0.8	10	411±21	0.13±0.01	0.031±0.040

a) Mean±SD.
b) Significantly different from group 2 at $P<0.01$.
c) Significantly different from group 2 at $P<0.05$.
d) Abs, absolute.

Table II. Incidence and Quantitative Values of Bladder Epithelial Lesions in Rats Treated with BBN Followed by Antioxidants

Group	Treatment		No. of	PN[a] hyperplasia		Papilloma		Carcinoma	
	BBN	Test compound	rats	Incidence (%)	No./10 cm of BM[b]	Incidence (%)	No./10 cm of BM	Incidence (%)	No./10 cm of BM
1	+	TBMP	20	20 (100)[c]	10.1±10.1[c, d]	16 (80)[c]	1.6±1.2[c, d]	5 (25)	0.20±0.40[d]
	+	Propylparaben	20	9 (45)	0.6±0.7	4 (20)	0.2±0.4	4 (20)	0.20±0.40
	+	Catechol	19	7 (36)	0.4±0.6	4 (21)	0.3±0.7	2 (10)	0.20±0.70
	+	Resorcinol	20	3 (15)	0.2±0.4	5 (25)	0.2±0.4	1 (5)	0.05±0.20
	+	Hydroquinone	20	3 (15)	0.2±0.4	2 (10)	0.1±0.3	1 (5)	0.05±0.20
2	+	—	19	6 (32)	0.3±0.6	2 (11)	0.1±0.3	1 (5)	0.05±0.20
3	−	TBMP	10	0	0	0	0	0	0
	−	Propylparaben	10	0	0	0	0	0	0
	−	Catechol	10	0	0	0	0	0	0
	−	Resorcinol	10	0	0	0	0	0	0
	−	Hydroquinone	10	0	0	0	0	0	0

a) PN, papillary or nodular.
b) BM, basement membrane.
c) Significantly different from group 2 at $P<0.01$.
d) Mean±SD.

Table III. DNA Synthesis in Bladder Epithelium Assessed by Incorporation of BrdU

Test compound	Dose (%)	No. of rats	Labeling index[a] (%)
TBMP	1.0	5	1.52±0.92[b, c]
Propylparaben	3.0	5	0.24±0.11
Catechol	0.8	5	0.12±0.13
Resorcinol	0.8	5	0.28±0.19
Hydroquinone	0.8	5	0.38±0.38
—	—	5	0.13±0.05

a) Values are percentages of cells labeled with BrdU.
b) Mean±SD.
c) Significantly different from the control at $P<0.05$.

strated significantly elevated labeling indices as compared to control rats (non-treated) and simple hyperplasia. No increases in labeling indices were evident in propylparaben-, catechol-, resorcinol- or hydroquinone-treated rats.

DISCUSSION

The results clearly demonstrated that TBMP, which chemically closely resembles BHA, can promote urinary bladder carcinogenesis, whereas the other antioxidants tested were without effect. In addition, only TBMP induced an elevation of DNA synthesis in rat urinary bladder epithelial cells.

In a previous study,[12] TBMP and BHA showed strong activity and catechol weak activity for inducing hyperplasia and papillomatous lesions of the hamster forestomach, all three chemicals causing increased cell proliferation in the epithelium. BHT, propylparaben, resorcinol and hydroquinone, in contrast, were without effect. Thus, the results indicated that one hydroxy and one *tert*-butyl substituent may be associated with strong forestomach lesion induction potential in hamsters. In the rat, TBMP, BHA and catechol clearly enhanced forestomach carcinogenesis after treatment with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), BHA strongly and both TBMP and catechol weakly inducing forestomach hyperplasia.[11] With respect to urinary bladder carcinogenesis, all phenolic antioxidant compounds with the *tert*-butyl substituent (BHA, BHT and TBHQ) were found to enhance the development of neoplastic lesions induced by BBN.[8-10] The present results provided further evidence for the postulated association between possession of this substituent and promotion in the urinary bladder while demonstrating that phenolic species with hydroxy substituents alone, such as catechol, resorcinol and hydroquinone, lack the activity. In view of these observations, the similar effects of TBMP, BHA, BHT and TBHQ on urinary bladder carcinogenesis suggest a direct link between chemical structure and biological potency.

Since catechol is the most abundant phenol in cigarette smoke condensate[14-17] and is a major industrial chemical,[18] many tests have been conducted to analyze its carcinogenic potential, e.g., catechol was established to be a cocarcinogen when it was applied with benzo[*a*]-pyrene to the skin of mice[19, 20] or administered together with methyl-*n*-amylnitrosamine in rat esophagus.[21] Recently, catechol was shown to promote MNNG-induced rat forestomach and glandular stomach carcinogenesis, and additionally, treatment with catechol alone induced adenomatous hyperplasia and adenocarcinoma of the glandular stomach.[11] Catechol did not act as a cocarcinogen in rat urinary bladder carcinogenesis when administered together with BBN in the drinking water to rats[22] and did not show any promoting activity in our medium-term bioassay system using BBN.[23] The results of the present study were thus consistent with our previous observations regarding the influence of catechol on urinary bladder carcinogenesis. It was found that structural isomers of catechol, such as resorcinol and hydroquinone, also did not promote the development of preneoplastic urinary bladder changes induced by BBN.

A clear correlation between ability to promote lesion development and capacity for induction of DNA synthesis was found in the present work. The fact that propylparaben neither enhanced DNA synthesis nor promoted BBN-initiated rat urinary bladder carcinogenesis indicates possible inter-species difference since our previous study showed that this chemical increased the labeling index of hamster urinary bladder epithelium.[12]

The present investigation is a continuation of a large series of investigations utilizing BBN as the initiator.[13, 24-27] This experimental model of urinary bladder carcinogenesis has revealed promoting activity of many environmental chemicals, particularly sodium salts,[28-31] in association with increased urinary pH. While the antioxidants, BHA, BHT and TBHQ did not influence urinary components, they did exert promoting potential in urinary bladder carcinogenesis.[32] The nature of promotion by TBMP is presumably similar to that exerted by these other related compounds, although the exact urinary metabolite derived from antioxidants with a *tert*-butyl substituent which could be responsible for their promoting activity remains unidentified. Although the strength of TBMP as an antioxidant is unknown, BHA, TBHQ, hydroquinone and catechol all have strong antioxidant activity as evaluated by the active oxygen method.[33] Therefore, in view of the differing effects of these compounds in the urinary bladder, promotion by TBMP seems more likely to be attributable to its structural characteristics than directly to its antioxidant properties.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, and Grants-in-Aid from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare.

(Received April 13, 1990/Accepted May 15, 1990)

REFERENCES

1) Ito, N., Fukushima, S., Hagiwara, A., Shibata, M. and Ogiso, T. Carcinogenicity of butylated hydroxyanisole in F344 rats. *J. Natl. Cancer Inst.*, **70**, 343–352 (1983).
2) Ito, N., Fukushima, S., Imaida, K., Sakata, T. and Masui, T. Induction of papilloma in the forestomach of hamsters by butylated hydroxyanisole. *Gann*, **74**, 459–461 (1983).
3) Ito, N., Hirose, M., Kurata, Y., Ikawa, E., Mera, Y. and Fukushima, S. Induction of forestomach hyperplasia by crude butylated hydroxyanisole, a mixture of 3-*tert* and 2-*tert* isomers, in Syrian golden hamsters is due to 3-*tert*-butylated hydroxyanisole. *Gann*, **75**, 471–474 (1984).
4) Wattenberg, L. W. Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic antioxidants and ethoxyquin. *J. Natl. Cancer Inst.*, **48**, 1425–1430 (1972).
5) Wattenberg, L. W. Inhibition of chemical carcinogen-induced pulmonary neoplasia by butylated hydroxyanisole. *J. Natl. Cancer Inst.*, **50**, 1541–1544 (1973).
6) Wattenberg, L. W. Inhibition of chemical carcinogenesis. *J. Natl. Cancer Inst.*, **60**, 11–18 (1978).
7) Wattenberg, L. W. Inhibitory effects of butylated hydroxyanisole on methylazoxymethanol acetate-induced neoplasia of the large intestine and on nicotinamide adenine dinucleotide-dependent alcohol dehydrogenase activity in mice. *J. Natl. Cancer Inst.*, **63**, 219–222 (1979).
8) Imaida, K., Fukushima, S., Shirai, T., Ohtani, M., Nakanishi, K. and Ito, N. Promoting activities of butylated hydroxyanisole and butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of γ-glutamyl transpeptidase-positive foci development in the liver of rats. *Carcinogenesis*, **4**, 895–899 (1983).
9) Imaida, K., Fukushima, S., Shirai, T., Masui, T., Ogiso, T. and Ito, N. Promoting activities of butylated hydroxyanisole, butylated hydroxytoluene and sodium L-ascorbate on forestomach and urinary bladder carcinogenesis initiated with methylnitrosourea in F344 male rats. *Gann*, **75**, 769–775 (1984).
10) Tamano, S., Fukushima, S., Shirai, T., Hirose, M. and Ito, N. Modification by α-tocopherol, propylgallate and tertiary butylhydroquinone of urinary bladder carcinogenesis in Fischer 344 rats pretreated with N-butyl-N-(4-hydroxybutyl)nitrosamine. *Cancer Lett.*, **35**, 39–46 (1987).
11) Hirose, M., Fukushima, S., Kurata, Y., Tsuda, H., Tatematsu, M. and Ito, N. Modification of N-methyl-N′-nitro- N-nitrosoguanidine-induced forestomach and glandular stomach carcinogenesis by phenolic antioxidants in rats. *Cancer Res.*, **48**, 5310–5315 (1988).
12) Hirose, M., Inoue, T., Asamoto, M., Tagawa, Y. and Ito, N. Comparison of the effect of 13 phenolic compounds in induction of proliferative lesions of the forestomach and increase in the labelling indices of the glandular stomach and urinary bladder epithelium of Syrian golden hamsters. *Carcinogenesis*, **7**, 1285–1289 (1986).
13) Fukushima, S., Murasaki, G., Hirose, M., Nakanishi, K., Hasegawa, R. and Ito, N. Histopathological analysis of preneoplastic changes during N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced urinary bladder carcinogenesis in rats. *Acta Pathol. Jpn.*, **32**, 243–250 (1982).
14) Clemo, G. R. Some aspects of chemistry of cigarette smoke. *Tetrahedron*, **3**, 168–174 (1958).
15) Brunnenmann, K. D., Lee, H-C. and Hoffmann, D. Chemical studies on tobacco smoke. XLVII. On the quantitative analysis of catechols and their reduction. *Anal. Lett.*, **9**, 939–955 (1976).
16) Hoffmann, D., Schmeltz, I., Hecht, S. S. and Wynder, E. L. Chemical studies on tobacco smoke. XXXIX. On the identification of carcinogens, tumor promoters, and cocarcinogens in tobacco smoke. *In* "Smoking and Health. Vol. 1 Modifying Risk for the Smoker," ed. E. L. Wynder, D. Hoffmann and G. B. Gori, DHEW publication No. (NIH) 76-1221, pp. 125–146 (1976). Department of Health, Education, and Welfare, Washington, D.C.
17) Schlotzhauer, W. S., Walters, D. B., Snook, M. E. and Higman, H. C. Characterization of catechols, resorcinols, and hydroquinones in an acidic fraction of cigarette smoke condensate. *J. Agric. Food Chem.*, **26**, 1277–1281 (1978).
18) IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Vol. 15, pp. 115–175 (1977). IARC, Lyon.
19) Van Duuren, B. L. Tumor-promoting and cocarcinogenic agents in chemical carcinogenesis. *In* "Chemical Carcinogens," ed. C. E. Searle, American Chemical Society Monograph 173, pp. 24–51 (1976). Am. Chem. Soc., Washington, D.C.
20) Hecht, S. S., Carmella, S., Mori, H. and Hoffmann, D. A study of tobacco carcinogenesis. XX. Role of catechol as a major cocarcinogen in the weakly acidic fraction of smoke condensate. *J. Natl. Cancer Inst.*, **66**, 163–169 (1981).
21) Mirvish, S. S., Salmasi, S., Lawson, T. A., Pour, P. and Sutherland, D. Test of catechol, tannic acid, *bidens pilosa*, croton oil, and phorbol for cocarcinogenesis of esophageal tumors induced in rats by methyl-n-amylnitrosamine. *J. Natl. Cancer Inst.*, **74**, 1283–1290 (1985).
22) LaVoie, E. J., Shigematsu, A., Mu, B., Rivenson, A. and Hoffmann, D. The effects of catechol on the urinary

bladder of rats treated with N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Jpn. J. Cancer Res.*, **76**, 266–271 (1985).
23) Miyata, Y., Fukushima, S., Hirose, M., Masui, T. and Ito, N. Short-term screening of promoters of bladder carcinogenesis in N-butyl-N-(4-hydroxybutyl)nitrosamine-initiated, unilaterally ureter-ligated rats. *Jpn. J. Cancer Res.*, **76**, 828–834 (1985).
24) Ito, N., Matayoshi, K., Arai, M., Yoshioka, Y., Kamamoto, Y., Makiura, S. and Sugihara, S. Effect of various factors on induction of urinary bladder tumors in animals by N-butyl-N-(4-hydroxybutyl)nitrosamine. *Gann*, **64**, 151–159 (1973).
25) Ito, N., Arai, M., Sugihara, S., Hirao, K., Makiura, S., Matayoshi, K. and Denda, A. Experimental urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nirosamine. *Gann Monogr. Cancer Res.*, **17**, 367–381 (1975).
26) Nakanishi, K., Fukushima, S., Shibata, M., Shirai, T., Ogiso, T. and Ito, N. Effect of phenacetin and caffeine on the urinary bladder of rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamine. *Gann*, **69**, 395–400 (1978).
27) Cohen, S. M., Arai, M., Jacobs, J. B. and Friedell, G. H. Promoting effect of saccharin and DL-tryptophan in urinary bladder carcinogenesis. *Cancer Res.*, **39**, 1207–1217 (1979).
28) Fukushima, S., Friedell, G. H., Jacobs, J. B. and Cohen, S. M. Effect of DL-tryptophan and sodium saccharin on urinary tract carcinogenesis initiated by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide. *Cancer Res.*, **41**, 3100–3103 (1981).
29) Fukushima, S., Imaida, K., Sakata, T., Okamura, T., Shibata, M-A. and Ito, N. Promoting effects of sodium L-ascorbate on two-stage urinary bladder carcinogenesis in rats. *Cancer Res.*, **43**, 4454–4457 (1983).
30) Fukushima, S., Kurata, Y., Shibata, M-A., Ikawa, E. and Ito, N. Promotion by ascorbic acid, sodium erythorbate and ethoxyquin of neoplastic lesions in rats initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine. *Cancer Lett.*, **23**, 29–37 (1984).
31) Fukushima, S., Thamavit, W., Kurata, Y. and Ito, N. Sodium citrate: a promoter of bladder carcinogenesis. *Jpn. J. Cancer Res.*, **77**, 1–4 (1986).
32) Fukushima, S., Shibata, M-A., Kurata, Y., Tamano, S. and Masui, T. Changes in the urine and scanning electron microscopically observed appearance of the rat bladder following treatment with tumor promoters. *Jpn. J. Cancer Res.*, **77**, 1074–1082 (1986).
33) Rosenwald, R. H. and Chenicek, J. A. Alkyl hydroxyanisole as antioxidants. *J. Am. Oil Chem. Soc.*, **28**, 185–188 (1951).