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Effects of Antioxidant 1-*O*-Hexyl-2,3,5-trimethylhydroquinone or Ascorbic Acid on Carcinogenesis Induced by Administration of Aminopyrine and Sodium Nitrite in a Rat Multi-organ Carcinogenesis Model

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The effect of antioxidant, 0.25% 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) or 0.25% ascorbic acid (AsA), on carcinogenesis induced by administration of 0.05% aminopyrine (AP) and 0.05% sodium nitrite ($NaNO_2$), was examined using a rat multi-organ carcinogenesis model. Groups of twenty F344 male rats were treated sequentially with an initiation regimen of *N*-diethylnitrosamine, *N*-methyl-*N*-nitrosourea, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, *N*,*N*′-dimethylhydrazine and 2,2′-dihydroxy-di-*n*-propylnitrosamine during the first 4 weeks, followed by AP+$NaNO_2$, AP+$NaNO_2$+HTHQ, AP+$NaNO_2$+AsA, $NaNO_2$+HTHQ, $NaNO_2$+AsA, each of the individual chemicals alone or basal diet and tap water as a control. All surviving animals were killed at week 28, and major organs were examined histopathologically for development of preneoplastic and neoplastic lesions. In the AP+$NaNO_2$ group, the incidences of hepatocelluar adenomas and hemangiosarcomas were 95% and 35%, respectively. When HTHQ or AsA was simultaneously administered, the incidences decreased to 58% and 11%, or to 80% and 15%, respectively. On the other hand, in the AP+$NaNO_2$ group and the $NaNO_2$-alone group, when HTHQ, but not AsA, was simultaneously administered, the incidence of carcinomas in the forestomach significantly increased. The results suggest that HTHQ can prevent tumor production induced by AP and $NaNO_2$ more effectively than AsA. On the other hand, an enhancing or possible carcinogenic effect of simultaneous administration of HTHQ and $NaNO_2$ only on the forestomach is suggested, while simultaneous treatment with the same dose of AsA and $NaNO_2$ may not be carcinogenic to the forestomach or other organs.

Key words: Antioxidants — Aminopyrine — Sodium nitrite — Carcinogenesis — Rat

Many nitrosoamines are known to induce tumors in various organs.[1)] It was reported that some drugs with secondary or tertiary amino groups reacted with nitrite under acidic conditions to form nitrosamines *in vitro* and *in vivo*.[2–5)] In particular, aminopyrine (AP) is known to react with sodium nitrite ($NaNO_2$) to form dimethylnitrosamine (DMN), which is a potent liver, lung and renal carcinogen.[2, 3, 6)] Feeding of AP and $NaNO_2$ to rats has been shown to cause acute liver damage due to formation of DMN and eventually to cause liver tumors upon chronic treatment.[2, 7, 8)]

It is also known that ascorbic acid (AsA), and phenolic antioxidants such as caffeic acid, ferulic acid, butylated hydroxyanisole (BHA) and propyl gallate, inhibit the formation of DMN in rats receiving AP and $NaNO_2$.[4, 5)] An inhibitory effect of caffeic acid on the endogenous formation of *N*-nitrosoproline was demonstrated in man.[9)] Kuenzig *et al.* suggest that dietary phenols may play an important role in the prevention of carcinogenesis by inhibiting the formation of nitrosamines.[5)]

On the other hand, it has been reported that mutagenic compounds were formed by the interaction of polyphenols with nitrite *in vitro*.[10, 11)] It was also reported that when several phenolic antioxidants such as catechol, hydroquinone, gallic acid, *t*-butylhydroquinone (TBHQ) or AsA and 0.2% $NaNO_2$ are simultaneously administered to rats, strong toxicity and cell proliferation are induced in the forestomach epithelium. Furthermore, continuous administration of AsA, sodium ascorbate, catechol or 3-methoxycatechol and 0.2–0.3% $NaNO_2$ for 24–51 weeks induced forestomach tumors, and also enhanced rat forestomach carcinogenesis initiated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG).[12–15)] Such synergistic enhancing

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effects of carcinogenesis were limited to the forestomach epithelium in the case of catechol and 3-methoxycatechol.[12, 13] However, the combined effects of AsA and $NaNO_2$ on other organs have not yet been evaluated.

In recent years, attention has been focused on a strong lipophilic phenolic antioxidant, 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ). This antioxidant possesses the strongest potential for inhibiting lipid peroxidation in liver microsomes among known antioxidants, including BHA, butylated hydroxytoluene (BHT), propyl gallate, TBHQ, and α-tocopherol.[16] Its anti-mutagenic activity against 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), as evaluated in the Ames assay, was also found to be stronger than those of other antioxidants.[17] In addition to these *in vitro* effects, HTHQ potently inhibits Glu-P-1 or 2-amino-3,8-dimethyl-imidazo[4,5-*f*]quinoxaline (MeIQx)-induced hepatocarcinogenesis,[18] 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary carcinogenesis by simultaneous treatment,[19] and 7,12-dimethylbenz(*a*)anthracene (DMBA)-initiated mammary carcinogenesis in the post-initiation period[20] in rats. On the other hand, HTHQ was shown to enhance tongue and forestomach carcinogenesis in rats pretreated with *N*-ethylnitrosourethane.[21] It is possible that this antioxidant also inhibits carcinogenesis by blocking nitrosamine formation, and strongly enhances forestomach carcinogenesis in the presence of $NaNO_2$, like other phenolic antioxidants.

In the present experiment, we evaluated the inhibitory effects of HTHQ or AsA on nitrosamine-induced carcinogenesis by simultaneous administration of $NaNO_2$ and AP, and also assessed the possibility of modification of carcinogenesis in major organs by simultaneous administration of HTHQ or AsA and $NaNO_2$, using a rat medium-term multi-organ carcinogenesis model.

MATERIALS AND METHODS

Animals and chemicals Two hundred and fifty 5-week-old male F344 rats were obtained from Charles River Japan, Inc., Atsugi. They were randomly divided into groups of five animals per cage with hard wood chips as bedding in an air-conditioned room at 24±2°C and 55±5% humidity with a 12 h light/dark cycle. Food (Oriental MF, Oriental Yeast Co., Tokyo) and tap water were available *ad libitum*. *N*-Diethylnitrosamine (DEN), *N*,*N*′-dimethylhydrazine (DMH) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo), *N*-methyl-*N*-nitrosourea (MNU) from Sigma-Aldrich (Tokyo) and 2,2′-dihydroxy-di-*n*-propylnitrosamine (DHPN) from Nakalai Tesque, Inc. (Osaka). HTHQ was provided from Nippon Hypox Laboratories, Inc. (Yamanashi), $NaNO_2$, AP and AsA from Wako Pure Chemical Industries, Ltd. (Osaka).

Experimental design As shown in Fig. 1, at the age of 6 weeks, a total of 250 rats were divided into 20 groups. Twenty animals each in groups 1–10 received the DMBDD initiation treatment, composed of DEN (100 mg/kg b.w., i.p., single dose at commencement), MNU (20 mg/kg b.w., i.p., on day 2, 5, 8 and 11), and DMH (40 mg/kg b.w., s.c. on day 14, 17, 20 and 23). Animals were simultaneously given BBN (0.05% in drinking water during weeks 1 and 2) and DHPN (0.1% in drinking water during weeks 3 and 4). Starting 1 week after this DMBDD treatment, the animals were treated as follows: group 1, AP+$NaNO_2$; group 2, AP+$NaNO_2$+HTHQ; group 3, AP+$NaNO_2$+AsA; group 4, $NaNO_2$+HTHQ; group 5, $NaNO_2$+AsA; group 6, AP; group 7, $NaNO_2$; group 8, HTHQ; group 9, AsA; group 10, basal diet. Five animals each in groups 11 to 20 were treated in the same way as groups 1 to 10 without DMBDD treatment. AP and $NaNO_2$ were dissolved in tap water at a dose of 0.05%, and HTHQ and AsA were mixed in powdered basal diet at a dose of 0.25%. Diet and water were prepared once every 1 to 2 weeks and stored in the dark until use. Body weight and food consumption were measured once every 2 to 4 weeks during the administration periods. All survivors were killed under ether anesthesia at the end of week 28 and subjected to complete autopsy. The liver, kidneys and spleen were weighed. Ten-percent buffered formalin solution-fixed and paraffin-embedded sections of the liver, kidneys, spleen, heart, thyroid, lungs, tongue, esophagus, stomach, intestines, testes, urinary bladder and other tissues of abnormal appearance were prepared routinely and stained with hematoxylin-eosin (H&E) for histopathological examination. In addition, 3 slices were cut from each liver and fixed in cold acetone for immunohistochemical staining for liver glutathione *S*-transferase placental form (GST-P). Numbers and area of GST-P-positive lesions

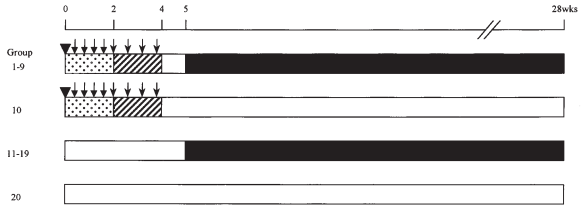

Fig. 1. Experimental protocol. Six-week-old male Fischer 344 rats were used. ▼, DEN (100 mg/kg b.w., i.p.); ↓, MNU (20 mg/kg b.w., i.p.); ⊡, BBN (0.05% in drinking water); ⇓, DMH (40 mg/kg b.w., s.c.); ▨, DHPN (0.1% in drinking water); ■, test compounds; □, basal diet. Test compounds: 1, AP+$NaNO_2$; 2, AP+$NaNO_2$+HTHQ; 3, AP+$NaNO_2$+AsA; 4, $NaNO_2$+HTHQ; 5, $NaNO_2$+AsA; 6, AP; 7, $NaNO_2$; 8, HTHQ; 9, AsA. Aminopyrine (AP), 0.05% in drinking water; $NaNO_2$, 0.05% in drinking water; HTHQ, 0.25% in diet; ascorbic acid (AsA), 0.25% in diet.

larger than 0.2 mm in diameter, including adenomas and carcinomas were measured with the aid of an image analyzer (VIP-21, Olympus Co., Tokyo).

Statistical analysis Student's *t* test and Fisher's exact probability test were used for statistical analysis of the data.

RESULTS

Body and organ weights The final body weights and relative organ weights of liver and kidneys are summarized in Table I. Regardless of DMBDD, HTHQ or AsA treatment, the final body weight of rats significantly decreased in all the AP+$NaNO_2$-treated groups as compared with the control group. Relative liver weights significantly increased in all test compound groups given DMBDD as compared with the control group. On the other hand, in the AP+$NaNO_2$, $NaNO_2$ and AsA groups without DMBDD treatment, a significant decrease was noted. In the AP+$NaNO_2$+HTHQ group, the value significantly increased. Relative kidney weights significantly increased in the AP+$NaNO_2$, AP+$NaNO_2$+HTHQ and HTHQ groups as compared with the control group. A significant increase was also found in the AP+$NaNO_2$ group without DMBDD treatment. The AP-alone group showed a significant decrease. Further, regardless of DMBDD, HTHQ or AsA treatment, relative spleen weights showed a significant increase in all the AP+$NaNO_2$-treated groups compared with the control group.

GST-P-positive lesions Quantitative data for numbers and areas of GST-P-positive lesions in the liver are summarized in Table II. In the AP+$NaNO_2$ group with DMBDD treatment, the number and area of GST-P-positive lesions significantly increased as compared with the control group, regardless of HTHQ or AsA administration. However, when HTHQ or AsA was administered simultaneously, the area of GST-P-positive lesions in the AP+$NaNO_2$+HTHQ group (9.64 mm^2/cm^2, $P<0.01$) and that in the AP+$NaNO_2$+AsA group (11.35 mm^2/cm^2, $P<0.01$) were significantly decreased in comparison with the AP+$NaNO_2$ group (33.6 mm^2/cm^2). In the AP-alone group, the number and area of GST-P-positive lesions significantly increased as compared with the control group. In the AP+$NaNO_2$ group without DMBDD treatment, both the number and area of GST-P-positive lesions significantly increased as compared with the control group. However, when HTHQ or AsA was administered simultaneously, both the number and area of GST-P-positive lesions decreased, though not with statistical significance.

Histopathology Quantitative data for neoplastic and preneoplastic lesions in major organs of the DMBDD-treated groups are summarized in Table III.

In the liver, the incidences of hepatocellular adenomas, carcinomas and hemangiosarcomas in the AP+$NaNO_2$

Table I. Final Body and Relative Organ Weights

Group	Treatment		No. of rats survived	Final body wt.[a)] (g)	Relative organ wt. (g/100 g body wt.)[a)]		
	DMBDD	Test compound			Liver	Kidneys	Spleen
1	+	AP+$NaNO_2$	14	274±17**	2.95±0.42**	0.75±0.08**	0.29±0.10**
2	+	AP+$NaNO_2$+HTHQ	19	290±17**	2.45±0.16**	0.70±0.08**	0.22±0.03**
3	+	AP+$NaNO_2$+AsA	20	294±14**	2.32±0.09**	0.71±0.07**	0.22±0.02**
4	+	$NaNO_2$+HTHQ	20	309±13	2.19±0.09**	0.68±0.19*	0.18±0.02
5	+	$NaNO_2$+AsA	20	306±14*	2.05±0.06**	0.65±0.05**	0.18±0.02
6	+	AP	19	311±18	2.19±0.14**	0.99±0.99	0.28±0.39
7	+	$NaNO_2$	20	321±13	2.07±0.07**	0.63±0.09	0.18±0.01
8	+	HTHQ	20	314±20	2.17±0.08**	0.65±0.08**	0.18±0.02
9	+	AsA	20	313±13	2.07±0.08**	0.63±0.05*	0.18±0.01
10	+	Control	19	318±17	1.99±0.06	0.59±0.03	0.18±0.01
11	−	AP+$NaNO_2$	5	313±6**	1.98±0.03**	0.60±0.01*	0.19±0.02**
12	−	AP+$NaNO_2$+HTHQ	5	325±18**	2.23±0.07*	0.59±0.02	0.18±0.01**
13	−	AP+$NaNO_2$+AsA	5	328±11**	2.09±0.07	0.57±0.02	0.17±0.01**
14	−	$NaNO_2$+HTHQ	5	345±10*	2.22±0.08	0.56±0.03	0.15±0.01
15	−	$NaNO_2$+AsA	5	373±8	2.05±0.05	0.58±0.01	0.15±0.01
16	−	AP	5	350±21	2.04±0.06	0.55±0.02*	0.15±0.01
17	−	$NaNO_2$	5	345±22	1.94±0.03**	0.57±0.02	0.14±0.01
18	−	HTHQ	5	354±15	2.19±0.08	0.56±0.02	0.14±0.00
19	−	AsA	5	345±10	1.90±0.16*	0.56±0.03	0.15±0.02
20	−	Control	5	363±12	2.11±0.06	0.58±0.02	0.15±0.01

a) Mean±SD.

*, ** Significantly different from group 10 or 20 at $P<0.05$, 0.01.

Table II. Numbers and Areas of GST-P-positive Liver Lesions

Group	Treatment		No. of rats examined	GST-P-positive lesions[a)]	
	DMBDD	Test compounds		No./cm^2	mm^2/cm^2
1	+	AP+$NaNO_2$	10	66.22±14.83**	33.62±13.61**
2	+	AP+$NaNO_2$+HTHQ	10	64.99±10.58**	9.64±5.17**§§
3	+	AP+$NaNO_2$+AsA	10	69.03±13.83**	11.35±6.59**§§
4	+	$NaNO_2$+HTHQ	10	2.52±2.36	0.12±0.10
5	+	$NaNO_2$+AsA	10	2.73±2.49	0.13±0.11
6	+	AP	10	9.79±6.18**	0.55±0.36**
7	+	$NaNO_2$	10	2.66±1.70	0.13±0.08
8	+	HTHQ	10	2.47±1.35	0.13±0.08
9	+	AsA	10	3.05±2.82	0.16±0.17
10	+	Control	9	2.01±2.03	0.13±0.12
11	–	AP+$NaNO_2$	5	7.17±0.87**	0.40±0.09**
12	–	AP+$NaNO_2$+HTHQ	5	4.28±3.28	0.25±0.21
13	–	AP+$NaNO_2$+AsA	5	3.60±2.29	0.22±0.21
14	–	$NaNO_2$+HTHQ	5	0.00±0.00	0.00±0.00
15	–	$NaNO_2$+AsA	5	0.00±0.00	0.00±0.00
16	–	AP	5	0.00±0.00	0.00±0.00
17	–	$NaNO_2$	5	0.00±0.00	0.00±0.00
18	–	HTHQ	5	0.00±0.00	0.00±0.00
19	–	AsA	5	0.00±0.00	0.00±0.00
20	–	Control	5	0.00±0.00	0.00±0.00

a) Mean±SD.
** Significantly different from group 10 or 20 at $P<0.01$.
§§ Significantly different from group 1 at $P<0.01$.

group were 95%, 20% and 35%, respectively, while the incidences in the AP+$NaNO_2$+HTHQ group decreased to 58% ($P<0.01$), 5% and 11%, respectively. On the other hand, in the AP+$NaNO_2$+AsA group, the incidences decreased slightly to 80%, 0% and 15%, respectively.

In the forestomach, the incidences of hyperplasias and papillomas were significantly increased in the HTHQ group. Moreover, the incidence of squamous cell carcinomas tended to be increased by the addition of $NaNO_2$ (40% vs. 10% in HTHQ-alone group). A similar tendency was observed in other HTHQ+$NaNO_2$ groups. On the other hand, the incidences of forestomach lesions in all the AsA+$NaNO_2$-treated groups were not increased as compared with the control group.

In the kidney, the incidences of renal cell tumors in all test compound groups were higher than those of the control group, and those of renal cell tumors in the AP+$NaNO_2$+AsA and AsA-alone groups were significantly increased. However, when the incidences of those in the AP+$NaNO_2$+AsA or the AP+$NaNO_2$+HTHQ groups were compared with those in the AP+$NaNO_2$ group, there was no significant difference.

In the lung, the incidences of carcinomas in all test compound groups were lower than those of the control group. Those in the AP+$NaNO_2$+HTHQ and AP-alone groups were significantly decreased as compared with the control group. However, a significant difference was not found when the incidences in the AP+$NaNO_2$+HTHQ group were compared with those in the AP+$NaNO_2$ group.

In the tongue, the incidence of hyperplasias in the HTHQ-treated group tended to increase (25%) as compared with the control group value (5%). The incidence was statistically significantly increased by the addition of $NaNO_2$ (45%). However, the incidence of this lesion was not statistically significantly different between the HTHQ and $NaNO_2$+HTHQ groups.

In other organs, the incidences of mesotheliomas in the abdominal cavity in all the HTHQ-treated groups and the AP+$NaNO_2$+AsA group were lower than those of the control group.

Among the groups treated with the same combination or with the individual chemicals alone without DMBDD treatment, incidences of forestomach hyperplasias were significantly increased in all HTHQ-treated groups. Forestomach tumors were not found in these groups. The incidence of lung alveolar hyperplasias significantly increased in the AP+$NaNO_2$ group (80% vs. 0% in control), and the incidence was not decreased by the addition of HTHQ or AsA (Table IV).

Table III. Incidences of Preneoplastic and Neoplastic Lesions in Initiated Groups

Group	1	2	3	4	5	6	7	8	9	10
DMBDD treatment	+	+	+	+	+	+	+	+	+	+
Test compounds	AP +$NaNO_2$	AP +$NaNO_2$ +HTHQ	AP +$NaNO_2$ +AsA	$NaNO_2$ +HTHQ	$NaNO_2$ +AsA	AP	$NaNO_2$	HTHQ	AsA	Control
No. of rats examined	20	19	20	20	20	20	20	20	20	20
Liver										
Hepatocellular adenoma	19 (95)**	11 (58)**§§	16 (80)**	4 (20)	2 (10)	2 (10)	1 (5)	1 (5)	1 (5)	1 (5)
Hepatocellular carcinoma	4 (20)	1 (5)	0	1 (5)	0	0	0	0	0	1 (5)
Hepatocholangiocellular adenoma	1 (5)	0	0	0	0	0	0	0	0	0
Hepatocholangiocellular carcinoma	1 (5)	0	0	0	0	0	0	0	0	0
Hemangioma	0	1 (5)	1 (5)	0	1 (5)	0	0	0	1 (5)	1 (5)
Hemangiosarcoma	7 (35)**	2 (11)	3 (15)	0	0	0	0	0	0	0
Forestomach										
Hyperplasia	4 (20)	19 (100)**	5 (25)	20 (100)**	5 (25)	6 (30)	2 (10)	20 (100)**	4 (20)	4 (20)
Papilloma	2 (10)	8 (42)*	0	10 (50)**	1 (5)	2 (10)	4 (20)	13 (65)*	1 (5)	1 (5)
Squamous cell carcinoma	0	5 (26)*	0	8 (40)**	0	0	0	2 (10)	0	0
Kidney										
Renal cell tumor	12 (60)	11 (58)	16 (80)**	9 (45)	11 (55)	10 (50)	11 (55)	8 (40)	13 (65)*	5 (25)
Nephroblastoma	15 (75)	13 (68)	16 (80)	9 (45)	10 (50)	13 (65)	10 (50)	9 (45)	9 (45)	12 (60)
Transitional cell hyperplasia	2 (10)	3 (16)	0	1 (5)	0	2 (10)	1 (5)	0	0	1 (5)
Transitional cell carcinoma	0	1 (5)	0	0	0	4 (20)	3 (15)	0	1 (5)	0
Thyroids										
Follicular adenoma	6 (30)	10 (53)	11 (55)	7 (35)	6 (30)	12 (60)	8 (40)	9 (45)	7 (35)	7 (35)
Follicular carcinoma	4 (20)	3 (16)	5 (25)	8 (40)	6 (30)	9 (45)	7 (35)	11 (55)	3 (15)	6 (30)
Lung										
Adenoma	8 (40)	8 (42)	12 (60)	9 (45)	6 (30)	9 (45)	9 (45)	7 (35)	9 (45)	8 (40)
Carcinoma	9 (45)	5 (25)*	8 (40)	10 (50)	7 (35)	5 (25)*	9 (45)	8 (40)	9 (45)	13 (65)
Tongue										
Hyperplasia	0	4 (21)	0	9 (45)**	1 (5)	0	0	5 (25)	0	1 (5)
Papilloma	0	3 (16)	1 (5)	1 (5)	0	0	1 (5)	3 (15)	0	1 (5)
Esophagus										
Papilloma	0	2 (11)	1 (5)	0	1 (5)	1 (5)	0	0	1 (5)	1 (5)
Carcinoma	0	0	0	1 (5)	0	1 (5)	1 (5)	0	0	0
Small intestine										
Adenoma	0	0	0	0	1 (5)	3 (15)	6 (30)	0	0	4 (20)
Adenocarcinoma	0	0	0	0	1 (5)	1 (5)	0	0	2 (10)	0
Colon/Rectum										
Adenoma	0	1 (5)	0	0	0	0	0	0	2 (10)	2 (10)
Adenocarcinoma	0	1 (5)	1 (5)	0	0	0	2 (10)	1 (5)	3 (15)	1 (5)
Urinary bladder										
Papilloma	0	1 (5)	0	0	1 (5)	0	0	2 (10)	0	0
Carcinoma	1 (5)	1 (5)	0	1 (5)	1 (5)	0	2 (10)	0	2 (10)	1 (5)
Nasal cavity										
Papilloma	1 (5)	0	0	3 (15)	5 (25)	1 (5)	1 (5)	3 (15)	1 (5)	1 (5)
Carcinoma	1 (5)	0	3 (15)	1 (5)	3 (15)	1 (5)	4 (20)	0	3 (15)	1 (5)
Abdominal cavity										
Schwannoma	0	0	0	4 (20)	0	0	1 (5)	1 (5)	0	3 (15)
Mesothelioma	2 (10)	0*	0*	0*	6 (30)	1 (5)	5 (25)	0*	3 (15)	5 (25)

*, ** Significantly different from group 10 at $P<0.05$, 0.01.
§§ Significantly different from group 1 at $P<0.01$.

Table IV. Incidences of Preneoplastic and Neoplastic Lesions in Non-initiated Groups

Group	11	12	13	14	15	16	17	18	19	20
DMBDD treatment	–	–	–	–	–	–	–	–	–	–
Test compounds	AP +$NaNO_2$	AP +$NaNO_2$ +HTHQ	AP +$NaNO_2$ +AsA	$NaNO_2$ +HTHQ	$NaNO_2$ +AsA	AP	$NaNO_2$	HTHQ	AsA	Control
No. of rats examined	5	5	5	5	5	5	5	5	5	5
Liver										
Foci	3 (60)	3 (60)	3 (60)	0	0	0	0	0	0	0
Hemangiosarcoma	0	0	1 (20)	0	0	0	0	0	0	0
Forestomach										
Hyperplasia	0	5 (100)**	0	5 (100)**	0	0	0	5 (100)**	0	0
Lung										
Alveolar hyperplasia	4 (80)*	3 (60)	5 (100)**	0	0	0	0	0	0	0
Adenocarcinoma	0	1 (20)	0	0	0	0	0	0	0	0

*, ** Significantly different from group 20 at $P<0.05$, 0.01.

DISCUSSION

In our present experiment, we evaluated the inhibitory effect of HTHQ or AsA on nitrosamine-induced carcinogenesis by simultaneous administration of $NaNO_2$ and AP, and also assessed the enhancing effects of combined treatment with HTHQ or AsA and $NaNO_2$ on major organ carcinogenesis including the forestomach, using a rat medium term multi-organ carcinogenesis model.

As expected, simultaneous administration of $NaNO_2$ and AP induced hepatocellular adenomas and hemangiosarcomas in the liver. When HTHQ was simultaneously given, these incidences remarkably decreased. Furthermore, the area of GST-P-positive lesions was also reduced. These results indicate that HTHQ inhibits nitrosamine-induced hepatocarcinogenesis induced by $NaNO_2$ and AP. On the other hand, when AsA was given with $NaNO_2$ and AP, the inhibitory effect was less potent than that of HTHQ. It was reported that AsA and sodium ascorbate (NaAsA) protected rats against liver tumor production or hepatotoxicity by simultaneous treatment with $NaNO_2$ and secondary amines such as AP[3, 22] or morpholine.[23] AsA has been shown to inhibit the genotoxicity of nitrosoamines derived from $NaNO_2$ and secondary amines such as morpholine or proline in the Comet assay,[24] and is suggested to inhibit nitrosamine formation by reducing nitrous anhydride to nitric oxide, a non-nitrosating species, in the absence of catalysts.[25] Kuenzig *et al.*[5] reported that caffeic acid and ferulic acid blocked the elevation of serum DMN level in rats receiving AP and $NaNO_2$. Caffeic acid and chlorogenic acid were able to scavenge the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and inhibit nitrosamine formation by scavenging a nitrosating agent, nitrogen sesquinixide.[26] HTHQ has potent radical-scavenging activity, in addition to reducing activity, as evaluated using the stable free radical, DPPH[27] like caffeic acid and chlorogenic acid.[26] Such an effect may be partly responsible for the observed inhibition of hepatocarcinogenesis by HTHQ.

The number and area of GST-P-positive lesions significantly increased when AP alone was given after DMBDD treatment. It was reported that AP does not possess mutagenic or carcinogenic activity in mice.[28] Previously, we demonstrated that the number and area of GST-P-positive foci were increased by treatment with AP after initiation with DEN in the rat medium-term liver bioassay system.[29] The present results are in line with our previous results and suggest that AP is a weak promoter of hepatocarcinogenesis in rats.

It was shown that kidney and lung tumors were induced by combined oral treatment with AP and $NaNO_2$ at dose levels 0.1–0.2% AP and 0.1–0.2% $NaNO_2$.[3, 6] In the present study, however, tumor incidences were not significantly increased in the kidney and lung by the combined treatment with AP and $NaNO_2$. The reasons for the lack of enhancing effect may be the lower dose levels or strong initiation for these organs in view of the high incidences of tumors in groups without AP and $NaNO_2$. The incidence of lung carcinomas, but not adenomas, in the AP+$NaNO_2$+HTHQ group and the AP-alone group significantly decreased as compared with the control group. This was considered to be false-positive due to the high incidence in the control group.

In the upper digestive tracts, incidences of tongue hyperplasias, and forestomach hyperplasias and papillomas were increased in all the HTHQ-treated groups. The results are in line with our previous observations that 0.25% HTHQ weakly enhanced carcinogenesis in the

tongue and forestomach, but not in the esophagus, in a rat medium term multi-organ model[30] and in an *N*-ethylnitrosourethane-initiated rat two-stage carcinogenesis model.[21] Furthermore, incidence of forestomach carcinomas was further increased by additional treatment with $NaNO_2$. Although the data are not shown, the grade of hyperplasias in the HTHQ+$NaNO_2$ group was greater than that in the HTHQ-alone group in the present experiment. Although the carcinogenicity of these combination treatments was not demonstrated in long-term experiments, the present experiment confirms forestomach carcinogenicity of this combination treatment, because potential for promotion and cell proliferation activities in the forestomach parallels the potential for forestomach carcinogenicity.[31] However, the strong enhancing or possible carcinogenic effect may be limited in the forestomach epithelium, because enhancement of proliferative lesions by the combination was not observed in any other organ, including the esophagus and other squamous cell epithelium.

Previously, we demonstrated that cell proliferation of forestomach mucosa was markedly increased when phenolic antioxidants such as TBHQ, gallic acid, catechol and hydroquinone, and $NaNO_2$ were simultaneously given to F344 male rat for 4 weeks.[12] Continuous oral administration of catechol or 3-methoxycatechol and $NaNO_2$ for 24 weeks was demonstrated to increase the incidences of hyperplasias and/or papillomas in the forestomach.[13, 14] When catechol and $NaNO_2$ are given simultaneously to rats, DNA adducts were detected by ^{32}P-postlabeling methods in the forestomach epithelium.[32] Furthermore, reaction of catechol or phenol and nitrite produced *o*-benzoquinone and *p*-nitrosocatechol, and mutagenic diazonium compounds were produced after nitrosation under acidic conditions.[10, 33] Therefore, in simultaneous administration of phenolic compounds, including HTHQ, and $NaNO_2$, the production of these substances might have played a role in induction of cell proliferation and carcinogenesis.

AsA and NaAsA, at a dose of 1% in diet, have already been shown to promote forestomach carcinogenesis on simultaneous administration with 0.3% $NaNO_2$ in rats pretreated with MNNG,[15] and they also induced forestomach tumors without prior MNNG treatment. In the present experiment, AsA did not show tumor promotion in any organ, including the forestomach, when administered simultaneously with 0.05% $NaNO_2$. Although the preventive effect of AsA against liver tumor production by $NaNO_2$ and AP was weaker than that of HTHQ, 0.25% AsA did not exert any harmful effect in any organ, even in the presence of 0.05% $NaNO_2$.

Humans ingest large amounts of NaAsA or AsA in foodstuffs such as vegetables and fruits, and the average daily intake is estimated to be 100 mg per person.[34] The daily intake of nitrite is 6 to 10 mg from saliva and 1.5 mg from exogenous sources such as water, vegetables and meats.[35] The intake increases with stimulation of salivary flow-rate,[36] and in vegetarians, intake is several times higher than in non-vegetarians. Furthermore, intake of nitrate is much higher than that of nitrite, and the latter can be formed by reduction of nitrate *in vivo*.[37] Therefore considerable amounts of nitrite can be formed in the human stomach. However, the amounts of NaAsA and AsA applied in the present experiment are more than 25 times the average exposure in human, and the figure for the amount of $NaNO_2$ may be more than 50 times. Therefore, low levels of exposure to AsA might not exert harmful effects in any human organs in the presence of low-dose $NaNO_2$.

In conclusion, HTHQ prevents tumor production induced by AP and $NaNO_2$ more effectively than AsA. A strong enhancing or possible carcinogenic effect only in the forestomach became clear when HTHQ and $NaNO_2$ were administered simultaneously. However, considering that humans do not possess a forestomach, HTHQ may be expected to be useful as a chemopreventive agent to inhibit nitrosamine-induced carcinogenesis caused by the administration of $NaNO_2$ and AP. Low-dose AsA weakly protected against carcinogenesis induced by AP and $NaNO_2$ without harmful effects in any other organs in the presence of low-dose $NaNO_2$.

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