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Organ-specific Modification of Tumor Development by Low-dose Combinations of Agents in a Rat Wide-spectrum Carcinogenesis Model

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The combined effects of low doses of various carcinogens and carcinogenesis modifiers on tumor development were investigated by using a wide-spectrum organ carcinogenesis model in F344 rats. These agents were administered as three groups: (1) a group of known hepatocarcinogens; (2) a group of nitroso compounds having various target organ specificities; and (3) a group of antioxidants having various inhibiting or enhancing activities depending on the target organ. Doses were used which were generally below the known effective level for the individual chemical. These groups of chemicals were administered with or without prior administration of N-diethylnitrosamine (100 mg/kg body wt., i.p.), N-methylnitrosourea (4 × 20 mg/kg body wt., i.p.) and dihydroxy-di-N-propylnitrosamine (0.1% in drinking water for 2 weeks). The hepatocarcinogen group in combination with various nitroso compounds increased the incidences of liver hyperplastic nodules and hepatocellular carcinomas. In contrast, incidences were clearly reduced when the hepatocarcinogens and/or the nitroso compounds were administered in combination with the antioxidants. For the urinary bladder, the combination with nitroso compounds and antioxidants enhanced cancer development, and the addition of hepatocarcinogens further increased tumorigenesis. For the glandular stomach, additive effects on the numbers of pepsinogen isozyme 1-altered pyloric glands, a putative preneoplastic lesion, were produced by the combination treatment of antioxidants and the nitroso compounds. No synergistic effects on tumor development were seen in other organs. The results of the present study demonstrated that combinations of various compounds at low doses can additively or synergistically exert either enhancing or inhibitory effects on the development of preneoplastic and neoplastic lesions in different organs in a single model having a wide spectrum of organ effects.

Key words: Combination effect — Low dose — Multiple organ — Wide-spectrum carcinogenesis — Rat

A high proportion of human cancers has been attributed to environmental factors. Among these factors, in particular, are numerous chemical carcinogens and chemicals which either enhance or inhibit their effects. When administered in combination, modifying effects can be observed, as has been reported for various experimental carcinogenesis systems. For instance, both synergistic and additive effects on tumor development for two or more carcinogens have been clearly demonstrated.[1-4] Also, the combined administration of various hepatocarcinogens after initiation increases the numbers of preneoplastic glutathione *S*-transferase P-form (GST-P[4])-positive foci in rat liver.[5] However, antagonistic effects have also been reported; for example, combined antioxidant and carcinogen treatments result in a reduction of tumor yield.[5-8]

Many of these chemicals require quite high doses in experimental models to produce a detectable effect. The human environment, however, contains an enormous number of chemicals, usually present at low concentrations. It remains uncertain what the effects of these low levels of exposure might be, particularly with respect to their interactions, which could often be complex.

The majority of animal experimentation performed in the past has concentrated on analysis of the influence of

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[4] The abbreviations used are: A, antioxidants; 2-AAF, 2-acetylaminofluorene; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DBN, dibutylnitrosamine; DEN, N-diethylnitrosamine; DHPN, dihydroxy-di-N-propylnitrosamine; DMD, sequential combination of DEN, MNU and DHPN; DMN, dimethylnitrosamine; EHEN, N-ethyl-N-hydroxyethylnitrosamine; GST-P, glutathione *S*-transferase P-form; H, hepatocarcinogens; HCC, hepatocellular carcinoma; 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MNU, N-methylnitrosourea; N, nitroso compounds; PAPG, pepsinogen isozyme 1-altered pyloric glands; PB, sodium phenobarbital; PG, propyl gallate; Pg1, pepsinogen isozyme 1; TBHQ, *tert*-butylhydroquinone.

single compounds. For example, sodium phenobarbital (PB) was reported to have promoting potential for liver and thyroid carcinogenesis in rats initiated with methylnitrosourea (MNU).[9] Butylated hydroxyanisole (BHA), a phenolic antioxidant, was found to be a forestomach carcinogen in rats,[10] and, in addition, it promotes tumor development in rat urinary bladder carcinogenesis.[11, 12] However, BHA can also significantly reduce tumor yield in the liver, lung and large intestine of mice or rats.[13–16] Thus, the situation where chemicals can exert various actions in a multitude of organs requires *in vivo* experimental models which can detect effects in a wide spectrum of organs.[17–19] The present investigation was therefore designed to assess the combined effects of various compounds administered at low doses on the development of tumors after sequential application of multiple carcinogens in a rat wide-spectrum organ model.

MATERIALS AND METHODS

Chemicals The compounds administered at low doses in order to evaluate the combined effects on tumor development in various organs are shown in Table I. The chemicals purchased from Tokyo Kasei Kogyo Co., Tokyo were: 2-acetylaminofluorene (2-AAF), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), N,N-dibutylnitrosamine (DBN), N-diethylnitrosamine (DEN), N,N-dimethylnitrosamine (DMN), 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), propyl gallate (PG), *tert*-butylhydroquinone (TBHQ) and thioacetamide. BHA and catechol were obtained from Wako Pure Chemical Industries, Osaka; N-ethyl-N-hydroxyethylnitrosamine (EHEN) and MNU from Iwai Chemical Co., Tokyo; dihydroxy-di-N-propylnitrosamine (DHPN) from Nakalai Tesque Inc., Kyoto; and PB from Iwaki Pharmaceutical Co., Tokyo.

Animals and housing A total of 190 male F344 rats (from Charles River Japan, Inc., Atsugi), aged 6 weeks at the commencement of the experiment, were used. The animals were housed five to a plastic cage on hardwood chip bedding in an environmentally controlled room, maintained at 22±2°C and 60±10% relative humidity with artificial illumination for 12 h each day.

Experimental procedure Rats were divided into 15 groups of 10 or 15 rats each. As shown in Fig. 1, groups 1 to 8 were sequentially treated with DEN (100 mg/kg body wt., i.p., in saline, at the commencement of the study), MNU (20 mg/kg body wt., i.p., in citrate-buffered solution, pH 6.0, four injections at days 2, 5, 8 and 11) and DHPN (0.1% in drinking water for 2 weeks, during weeks 3 and 4). The doses and treatment periods

Table I. Administration Levels of Various Compounds and Their Target Organs in Rats

Compounds	Levels (ppm)	Target organs	References
Antioxidant group (A) in diet			
BHA	1,000	forestomach, bladder	10, 11, 20, 35, 39
Catechol	800	forestomach, glandular stomach	21, 45
Propyl gallate	100	forestomach	20
TBHQ	1,000	bladder	22, 38
Hepatocarcinogen group (H) in diet			
2-AAF	20	liver, bladder	2, 3, 8, 23–25, 40
DMN	10	liver	23–25
3′-Me-DAB	60	liver	23–25
Phenobarbital	50	liver, thyroid	9, 18, 23–25
Thioacetamide	60	liver	23–25
Nitroso compound group (N) in drinking water			
BBN	100	bladder	11, 29, 30
DBN	100	bladder, esophagus, tongue, liver	31, 39
EHEN	50	liver, kidney	12, 26
MNNG	5	forestomach, glandular stomach	21, 42–44
PNU	60	intestine, tongue, thymus forestomach, glandular stomach	27, 28

of these three carcinogens were chosen based on their ability to cause epithelial cell proliferation in various organs as determined in a preliminary study (unpublished data). After administration of these three carcinogens (DMD treatment), group 1 was fed basal diet (Oriental MF, Oriental Yeast Co., Tokyo) without any further chemical supplement (to serve as the control group), and groups 2 to 8 were given the various combination treatments as listed in Table II. Groups 9 to 15 did not receive DMD treatment but were given the same combination treatments as for groups 2 to 8, respectively (Table II). The level administered of each compound and the respective target organs are shown in Table I. Most dosage levels were one-tenth of those used in earlier studies.[8, 10, 19-28] However, since bladder cancer development requires a particularly long period, the levels of BBN and DBN used were one-fifth of the usual dosage levels.[19, 29-31]

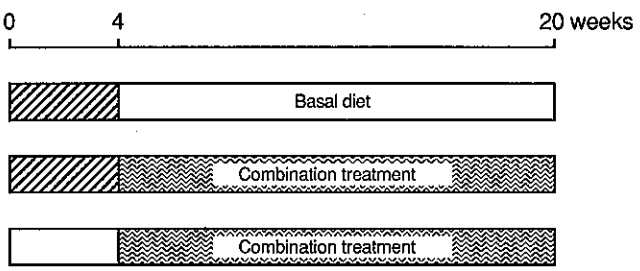

Fig. 1. Experimental protocol for evaluation of the combined effects of various compounds on rat carcinogenesis. ▨: DMD treatment. 1st, DEN (100 mg/kg/body wt., i.p., ×1); 2nd, MNU (20 mg/kg/body wt., i.p., at days 2, 5, 8 and 11); 3rd, DHPN (0.1% in drinking water from weeks 3 to 4). ▩: Combination treatment with various chemicals. For details of chemical treatments, see Table II.

All rats were killed by exsanguination under ether anesthesia at week 20. The organs (nasal cavity, lungs, trachea, tongue, esophagus, salivary glands, liver, pancreas, small intestine, large intestine, spleen, thymus, thyroid, kidneys, urinary bladder, testes, epididymis, seminal vesicles, prostate and brain) were removed and fixed in 10% phosphate-buffered formalin, routinely embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological examination. The stomachs were removed, fixed in sublimated formaldehyde[32, 33] and cut into 6 strips (forestomach) and 4 strips (glandular stomach) for histopathology and for immunohistochemical pepsinogen isozyme 1 (Pg1) staining (Vectastain Elite ABC Kit, Vector Laboratories, Inc., CA, USA) as previously described.[34] Pyloric glands which otherwise appeared normal but stained weakly or were negative for Pg1 immunohistochemically were defined as Pg1-altered pyloric glands (PAPG).[32, 33] The numbers of PAPG per 100 pyloric glands were counted.

Table II. Mean Body Weights, and Food and Water Consumptions of Rats Sequentially Treated with Three Different Carcinogens Followed by Various Chemicals

Group	DMD treatment	Combination of compounds[a]	No. of rats	Body weight (g) at week:					Food consumption (g/rat/day)	Water consumption (g/rat/day)
				0	4	8	16	20		
1	+	—	15	121	171	245	300	313	12.4	18.9
2	+	A	15	120	172	246	299	311	12.2	17.8
3	+	H	15	121	177	243	295	304	12.2	18.2
4	+	N	15	120	171	243	290	305	11.7	17.0
5	+	A+H	15	120	176	248	295	308	12.0	16.8
6	+	A+N	15	120	175	241	286*	288**	11.5	16.9
7	+	H+N	15	118	174	222**	264**	275**	11.4	14.2
8	+	A+H+N	15	121	179	235*	272**	279**	11.6	14.9
9	−	A	10	121	231	300	368	387	15.0	20.9
10	−	H	10	119	230	300	357	370	14.2	20.0
11	−	N	10	119	231	295	347	363	14.3	19.6
12	−	A+H	10	121	232	295	350	365	13.6	19.9
13	−	A+N	10	117	227	296	359	374	14.7	19.1
14	−	H+N	10	120	229	276	318	334	13.4	16.4
15	−	A+H+N	10	120	229	276	324	336	13.6	15.3

a) A, antioxidants; H, hepatocarcinogens; N, nitroso compounds.
* Significantly different from control (group 1), $P<0.05$.
** Significantly different from control (group 1), $P<0.01$.

Statistical analyses The significance of differences between the means of treated and control groups in body weights and quantitative data regarding numbers of PAPG were analyzed by using Student's t test. The significance of differences in lesion incidences between groups was assessed by using the chi-square test.

RESULTS

Two rats in group 5 died from malignant lymphomas in week 20. One rat in group 7 and one in 8 were found dead at week 18; the rat in group 7 was diagnosed as having malignant lymphoma and the cause of death in the rat in group 8 could not be determined. Body weight and food and water consumption data are summarized in Table II. Final mean body weights in groups 6 to 8 were significantly reduced compared to group 1 (DMD treatment only). Food consumption of groups 1 to 8 given the DMD treatment were similar, whereas consumption in the non-DMD treated groups was slightly higher than in the respective DMD-treated groups. Water consumption showed a reduction in groups 7, 8, 14 and 15 compared to the other groups.

Histopathology Synergistic or additive enhancing or inhibitory effects of the combination treatments on lesion development were noted for the liver, urinary bladder and glandular stomach.

Liver: DMD-treated groups As shown in Fig. 2, although the incidences of hyperplastic nodules were increased in groups 4 (N), 7 (H+N) and 8 (A+H+N) compared to group 1, groups 5 (A+H) and 6 (A+N) had decreased incidences compared to groups 3 (H) and 4 (N), respectively. Treatments in groups 7 and 8 significantly increased the incidences of hyperplastic nodules to 100% compared to the corresponding groups 2 to 6, respectively. No HCCs were observed in groups 1 to 5 (this is a particularly noteworthy outcome in groups 3 and 4), and only a few HCCs were found in group 6. In groups 7 and 8, there were significant incidences of HCC, which were greater than the sums for the relevant singly treated groups. However, HCC development in group 8 was significantly reduced by the addition of antioxidants.

Non-DMD-treated groups As shown in Fig. 3, the combined effects on liver tumor development in the non-DMD-treated groups (groups 9 to 15) were similar to those of the DMD-treated groups, but the incidences of the lesions, especially HCC, were lower than in the respective DMD-treated groups. Although there were striking differences in the incidences of hyperplastic nodules between the two N-treated groups with or without DMD treatment, there were no equivalent differences in the H-treated groups.

Urinary bladder: DMD-treated groups As shown in Fig. 4, the occurrence of urinary bladder tumors was greatly increased in groups 4, 6, 7 and 8, associated with nitroso compound treatment. In particular, nitroso compounds in combination with antioxidants (groups 6 and 8) markedly increased the incidences of papillomas and carcinomas. Furthermore, animals receiving the nitroso com-

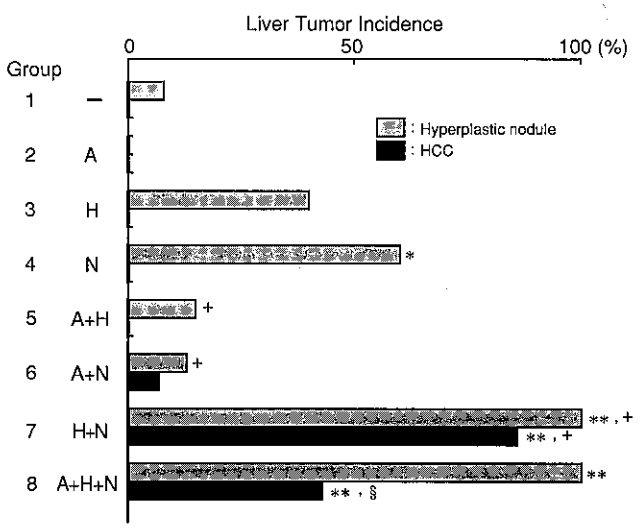

Fig. 2. The combined effects of various compounds on liver tumor development in rats given the initial DMD treatment. Data presented are incidences (%). Significantly different from the control value (* $P<0.05$, ** $P<0.01$) or from the corresponding value for the H or N groups (+ $P<0.05$, § $P<0.01$) or from the corresponding value for the H+N group (§ $P<0.05$).

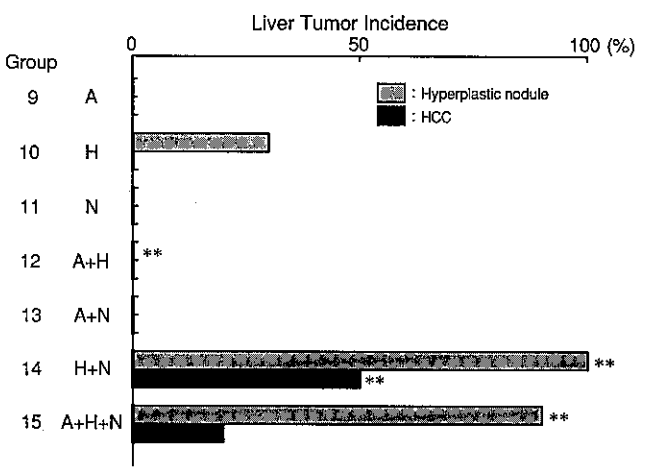

Fig. 3. The combined effects of various compounds on liver tumor development in rats without the initial DMD treatment. Data presented are incidences (%). Significantly different from the corresponding value for the H or N groups, ** $P<0.01$.

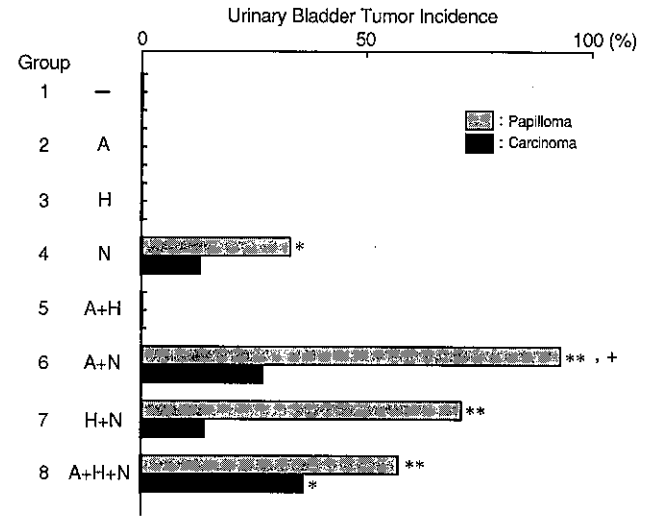

Fig. 4. The combined effects of various compounds on bladder tumor development in rats given the initial DMD treatment. Data presented are incidences (%). Significantly different from the control value ($*$ $P<0.05$, $**$ $P<0.01$) or from the corresponding value for the N group ($+$ $P<0.05$).

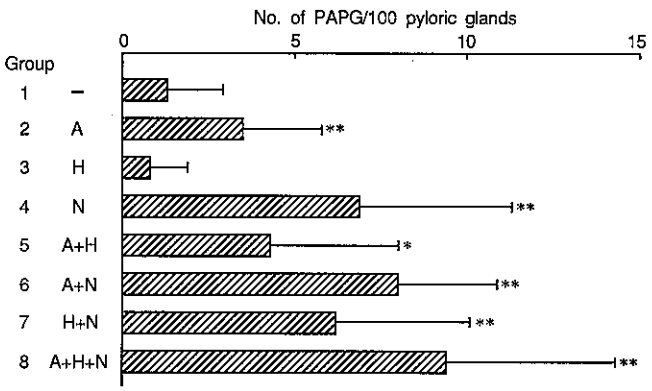

Fig. 5. The combined effects of various compounds on PAPG development in the stomach of rats given the initial DMD treatment. Values are mean±SD data for numbers of PAPG per 100 pyloric glands. Significantly different from the control value, $*$ $P<0.05$ or $**$ $P<0.01$.

pounds in combination with the hepatocarcinogens (group 7) showed a tendency for increased tumor development. The increased tumor incidences in groups 6 to 8 were synergistic.

Non-DMD-treated groups The combined effects on urinary bladder tumor development in the non-DMD-treated groups were similar to those of the DMD-treated groups, but the incidences of tumors, particularly carcinomas, were lower than in the respective DMD-treated groups.

PAPG expression in the glandular stomach: DMD-treated groups As shown in Fig. 5, significant increases in numbers of PAPG were observed in groups receiving the nitroso compounds and/or antioxidants (groups 2, and 4 to 8) as compared to group 1, the increase being additive in nature.

Non-DMD-treated groups The combined effects on PAPG expression in the non-DMD-treated groups were similar to those in the DMD-treated groups, but the numbers of PAPG were lower.

Other organs: DMD-treated groups (Table III) The incidences of esophageal papillomas were significantly increased in groups receiving the nitroso compounds (groups 4, and 6 to 8) compared to group 1. The occurrence of forestomach papillomas also tended to be increased in the antioxidant-treated groups (groups 2, 5, 6 and 8). However, no synergistic effects of the different groups of compounds were observed for tumor development in these organs. Although preneoplastic or neoplastic lesions were found in various other organs, their incidences were similar to those in the DMD alone-treated rats.

Non-DMD-treated groups Esophageal hyperplasia in groups receiving the nitroso compounds (groups 11, and 13 to 15) was frequently seen, and forestomach hyperplasia was sporadically observed in groups 9, 13 and 15. Hyperplasia of the lung was moderate in groups given the nitroso compounds. Thus, the extent of lesion development and the frequency were much lower in the respective non-DMD-treated groups, the results indicating the efficacy of prior DMD treatment.

DISCUSSION

In the present study, synergistic or additive enhancing and inhibitory effects of various compounds administered at low doses were clearly shown for preneoplastic and neoplastic lesion development in the rat liver, urinary bladder and glandular stomach.

Thus, hepatocarcinogens in combination with various nitroso compounds synergistically increased the development of both hyperplastic nodules and HCCs in the liver as compared to the control, and H- and N-treated groups. It is particularly noteworthy that no HCCs were seen in the H or N groups, but the incidence was almost 100% when they were administered in combination. The reason for the enhancing effects may be due to the inclusion of DBN and EHEN in the N group, since both of these nitroso-compounds are also hepatocarcinogens. The addition of antioxidants to the combination treatment with hepatocarcinogens and nitroso compounds (A+H+N group) reduced the occurrence of HCCs.

Table III. Incidences of Proliferative or Neoplastic Lesions in Various Organs of Rats Given DMD Treatment

Site/Finding	Group	1	2	3	4	5	6	7	8
	Treatment	—	A	H	N	A+H	A+N	H+N	A+H+N
	No. of rats	15	15	15	15	13	15	14	14
		Incidences of lesions (%)							
Esophagus									
Papilloma		0	1 (7)	2 (13)	15 (100)**	0	14 (93)**	12 (86)**	14 (100)**
SCC		0	0	0	3 (20)	0	0	1 (7)	1 (7)
Forestomach									
Papilloma		1 (7)	10 (67)**	1 (7)	1 (7)	4 (31)	6 (40)	2 (14)	10 (71)**
Tongue									
Papilloma		0	0	0	1 (7)	0	2 (13)	1 (7)	3 (21)
Small Intestine									
Adenoma		2 (13)	1 (7)	4 (27)	0	0	0	0	0
Adenocarcinoma		0	0	2 (13)	3 (20)	0	1 (7)	0	1 (7)
Large Intestine									
Adenoma		1 (7)	0	0	0	1 (8)	1 (7)	0	1 (7)
Nasal cavity									
Hyperplasia		6 (40)	4 (27)	6 (40)	4 (27)	1 (8)	3 (20)	4 (29)	2 (14)
Papilloma		0	0	0	4 (27)	1 (8)	1 (7)	1 (7)	0
Lung									
Adenoma		3 (20)	11 (73)	4 (27)	6 (40)	3 (23)	7 (47)	5 (36)	8 (57)
Adenocarcinoma		1 (7)	0	1 (7)	0	1 (8)	0	1 (7)	0
Thyroid									
Hyperplasia		2 (13)	5 (33)	6 (40)	4 (27)	1 (8)	1 (7)	1 (7)	2 (14)
Adenoma		1 (7)	3 (20)	3 (20)	0	2 (15)	1 (7)	0	1 (7)
Carcinoma		1 (7)	1 (7)	0	2 (13)	3 (23)	0	0	0
Thymus									
Hyperplasia		2 (13)	5 (33)	3 (20)	2 (13)	1 (8)	0	1 (7)	3 (21)

Significantly different from the DMD treatment alone (group 1), * $P<0.05$ or ** $P<0.01$, respectively.

This was expected, since there have been several reports of hepatocarcinogenesis inhibition by phenolic antioxidants, such as BHA.[6-8, 11-16, 24, 25] Recently, our laboratory demonstrated that BHA, even at the very low dose of 400 ppm (1/50 of the forestomach carcinogenic dose), significantly reduced the incidence of 7,12-dimethylbenz-[*a*]anthracene-induced liver neoplastic lesions.[35] The inhibitory effects are presumably related to its ability to prevent *in vivo* metabolic activation of carcinogens to proximate or ultimate forms or to increase detoxication of reactive intermediates. This could be caused by elevation in activity of epoxide hydrase or glutathione-*S*-transferase by BHA treatment.[36, 37] Since TBHQ is an analog of BHA, it is possible that TBHQ inhibits liver tumor development by the same mechanism as BHA. Therefore, in the present study, the mechanism of reduction of the liver tumor development by the antioxidant combination was probably similar. There are no data regarding the effect of catechol or propyl gallate on liver carcinogenesis. The reason for the striking differences in incidences of hyperplastic nodules between the two N-treated groups with or without DMD treatment, which were not apparent in the H-treated groups, remains unclear.

Synergistic enhancement of urinary bladder carcinogenicity by nitroso compounds was likely to be due to the presence of BBN and DBN, known urinary bladder carcinogens, given simultaneously with antioxidants (A+N group), some of which are known to promote rat bladder carcinogenesis.[11, 12] Also, treatment with BHA or TBHQ for 4 weeks induces an increase in DNA synthesis in the urinary bladder epithelium.[38] Whether this is the basis for the observed synergism with bladder carcinogens at low doses remains unclear. Since BBN and DBN are well known to require metabolic activation to exert their carcinogenic potential, it is also possible that antioxidants may modify their metabolism, and indeed, excretion of ultimate carcinogens is increased in urine as a feature of synergistic enhancement of tumor development.[39] Furthermore, the present study showed that the addition of hepatocarcinogens to the nitroso compounds, with or without antioxidants (H+N and

A+H+N groups), can amplify urinary bladder tumorigenesis. Since the hepatocarcinogen, 2-AAF, is also a bladder carcinogen,[3, 8] it is probable that it may have played an important role in this effect. Previously it was reported that simultaneous treatment with BHT and 2-AAF enhances cancer development in rats.[8, 40] N-Hydroxylation is a key element in the induction of urinary bladder cancer by aromatic amines and amides,[41] and the previous demonstration of increased excretion of glucuronic acid conjugates of N-OH-AAF resulting from alteration of liver metabolism by BHT further indicates an important role for antioxidants in the enhancement of urinary bladder carcinogenesis.[8, 40] In the present study, a similar mechanism involving alteration of 2-AAF metabolism by BHA, resulting in increased excretion of N-OH-AAF in the urine, might have occurred.

Pg1 is a promising marker for stomach preneoplasia, and PAPG are likely to be directly involved in gastric carcinogenesis. For example, Pg1-negative preneoplastic lesions detectable on the basis of altered histopathology are preceded by development of PAPG in the pyloric mucosa during the early stages of MNNG-induced gastric carcinogenesis.[42, 43] This biochemical alteration has also been consistently found in gastric tumors in rats.[44] In the present study, the numbers of PAPG were increased in rats treated with nitroso compounds or with the antioxidants. In addition, combination treatment with the nitroso and antioxidant chemicals exerted additive effects on PAPG expression. The fact that the groups of antioxidants or nitroso compounds included glandular stomach carcinogens, such as catechol or MNNG,[20, 42–45] probably forms the basis for these effects.

It has been recently recognized that activated oncogenes and loss of tumor suppressor genes may be of direct relevance to human neoplasia.[46, 47] Successive stages of promotion and progression from altered epithelium through epithelial hyperplasia and benign epithelial tumors to carcinomas are well accepted for a number of organs in chemical carcinogenesis. The possibility that gene alteration evoked in altered epithelia by the DMD treatment might lead, under the influence of the combination treatments at low doses, to activation of various oncogenes and/or inactivation of tumor suppressor genes in one of the stages of promotion or progression in the organs affected synergistically or additively in the present study therefore deserves further attention.

There are very large numbers of chemicals in the human environment. The present study has demonstrated that combination treatment with various compounds, even at low doses, exerts organ-dependent effects in the whole body. Therefore, the results are of direct relevance to risk assessment of human cancer development, and the present model, which can detect modifying effects of agents in multiple organs, offers a useful new approach to this endeavor.

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