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Modifying Effects of Various Chemicals on Preneoplastic and Neoplastic Lesion Development in a Wide-spectrum Organ Carcinogenesis Model Using F344 Rats

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Modifying potentials of various chemicals on tumor development were investigated in a wide-spectrum organ carcinogenesis model using male F344/DuCrj rats. The animals were treated with N-nitrosodiethylamine (100 mg/kg body weight, ip, single injection at the commencement of the study), N-methyl-N-nitrosourea (20 mg/kg body weight, ip, 4 times during weeks 1 and 2) and N-bis(2-hydroxypropyl)nitrosamine (0.1% in drinking water, during weeks 3 and 4) for multi-organ initiation and then were given one of 14 test chemicals including 6 hepatocarcinogens, 7 non-hepatocarcinogens and 1 non-carcinogen, or basal diet for 16 weeks. All rats were killed at the end of week 20, and the major organs were carefully examined for preneoplastic and neoplastic lesions. Immunohistochemical demonstration of glutathione *S*-transferase-positive foci was also used for quantitative assessment of liver preneoplastic lesion development. Modifying effects were shown for 11 out of 14 test agents in the liver, forestomach, glandular stomach, lung, urinary bladder or thyroid, 7 of them targeting more than two organs. This was the first demonstration to our knowledge that clofibrate possesses enhancing potential for urinary bladder carcinogenesis and an inhibiting effect on thyroid carcinogenesis. Caprolactam showed no effect in any organ, in agreement with its established inactivity. The results indicated that the system could be reliably applied as a medium-term multiple organ bioassay for assessment of the modification potential of test agents in unknown target sites.

Key words: Wide-spectrum organ carcinogenesis model — Modifying effect — Neoplastic lesion development

In vivo single organ bioassay systems for assessing carcinogenic and tumor-promoting potential after relatively short-term treatment with test agents have been developed for several organs, including the liver, stomach, urinary bladder and thyroid. The medium-term liver bioassay system developed in our laboratory has proved a very useful model for detecting risk of unknown compounds,[1–3] because more than half of the chemicals which have been evaluated as carcinogenic, target the liver.[4,5] However, 40 (45%) out of 88 carcinogens detected by NCI/NTP carcinogenesis bioassay were found to have multiple sites of action.[5] Moreover, numerous investigations utilizing initiation-promotion protocols have revealed that many nongenotoxic chemicals modify carcinogenesis in sites which are not targetted in oncogenicity studies.[6–8]

For the purpose of developing alternative assay systems for detection of carcinogenicity and modification activity of compounds in unknown target organs we have investigated several multi-organ wide-spectrum initiation models.[9–12] They have proved to have advantages, as whole-body surveys of carcinogenic potential in a relatively short experimental period and at low cost, for rapid screening of large numbers of chemicals. It was considered that the DEN-MNU-DHPN (DMD) model was the best among the several systems investigated.[11]

The objective of the present study was to determine the modifying effects of various chemicals in our wide-spectrum carcinogenesis model using multi-organ initiation by sequential treatment with the three potent carcinogens, N-nitrosodiethylamine (DEN), N-methyl-N-nitrosourea (MNU) and N-bis(2-hydroxypropyl)nitrosamine (DHPN), each possessing a different organ spectrum of carcinogenicity. Effects were judged by comparing the incidences of preneoplastic and/or neoplastic lesions between control and treated groups.

MATERIALS AND METHODS

Animals Male F344/DuCrj rats were obtained at 5 weeks of age from Charles River Japan, Inc., Kanagawa. They were housed five to a plastic cage with hard wood chips for bedding, and fed powdered diet MF (Oriental Yeast, Co., Ltd., Tokyo) and water *ad libitum*. Animals were kept in an environmentally controlled room main-

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Table I. Dosage Levels and Target Sites (Oral Exposure) of the Test Chemicals Used in the Study

Test chemicals No.	Chemical name	Level (ppm)	Target sites	References
Hepatocarcinogens				
1	2-Acetylaminofluorene (2-AAF)	100	liver, bladder	13, 14
2	Clofibrate	10,000	liver, pancreas	15, 16
3	4,4′-Diaminodiphenylmethane (DDPM)	1,000	thyroid, liver	17
4	Ethionine	2,500	liver	18
5	3′-Methyl-4-dimethylaminoazobenzene (3′-Me-DAB)	600	liver	19
6	Sodium phenobarbital (PB)	500	liver	20
Non-hepatocarcinogens				
7	Benzo[*a*]pyrene (B[a]P)	200	no adequate data available	21
8	N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN)	1,000	bladder	22, 23
9	Butylated hydroxyanisole (BHA)	10,000	forestomach	24
10	Catechol	8,000	glandular stomach, forestomach	25
11	7,12-Dimethylbenz[*a*]anthracene (DMBA)	100	mammary gland, ear duct, forestomach	26
12	3-Methylcholanthrene (3-MC)	200	forestomach, mammary gland	27
13	N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG)	50	glandular stomach, forestomach, esophagus, small intestine	28, 29
Noncarcinogen				
14	Caprolactam	10,000	—	30

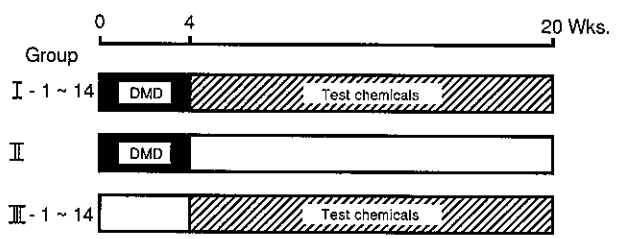

Fig. 1. Experimental design. DMD treatment: Animals were sequentially given DEN (100 mg/kg, ip, single dose at the commencement), MNU (20 mg/kg, ip, 4 times during weeks 1 and 2) and DHPN (0.1%, in the drinking water during weeks 3 and 4). For test chemical details, see Table I.

tained at a temperature of 22±2°C, a relative humidity of 55±10% and a 12-h light/dark cycle. After a one week acclimation period, they were used in this study.

Chemicals DEN (Tokyo Kasei Kogyo Co., Ltd., Tokyo), MNU (Iwai Chemical Co., Tokyo) and DHPN (Nakalai Tesque Inc., Kyoto) were used in the initiation treatment. Fourteen chemicals, comprising 6 hepatocarcinogens, 7 non-hepatocarcinogens and one non-carcinogen, were selected for the investigation of modification potential. Chemical names, dose levels and known target sites are summarized in Table I.[13-30] They were obtained from the following suppliers (chemical nos. 1, 4–8, 11–13, Tokyo Kasei Kogyo Co. Ltd., Tokyo; nos. 2, 9, 10, 14, Wako Pure Chemical Industries, Osaka; no. 3, Aldrich Chemical Co., Milwaukee, Wis.).

Experimental design A total of 310 rats were divided randomly into 3 groups, groups I and III then being subdivided into subgroups (15 rats each for groups I 1–14, 5 rats each for groups III 1–14) for treatment with test compounds as shown in Fig. 1 and Table I. Animals in groups I and II (30 rats) were treated sequentially with DEN (100 mg/kg, ip, single dose at commencement), MNU (20 mg/kg, ip, 4 times during weeks 1 and 2) and DHPN (0.1%, in drinking water, during weeks 3 and 4), as previously reported.[9, 11, 12] Starting 4 weeks later, the rats in groups I and III were administered the test compounds. Group II animals were given basal diet and tap water after the first step procedure and served as controls. Group III received vehicles without carcinogens during the first step treatment period. All animals were killed under ether anesthesia for examination of lesion development at week 20.

Histopathological examination and quantitation of glutathione *S*-transferase (GST-P)-positive foci At autopsy, the liver, kidneys, spleen and thyroid were immediately excised and weighed, and organ-to-body weight ratios calculated. The livers were cut into about 3

mm thick sections with a razor blade and fixed in ice-chilled acetone for subsequent immunohistochemical staining of GST-P. The numbers and areas of GST-P-positive foci greater than 0.2 mm in diameter were measured using a color video image processor (SPICCA-II, Nippon Avionics Co., Tokyo). The results were assessed by comparing the values between group I and group II. The following organs of each rat were also examined histopathologically: heart, lymph node, spleen, bone marrow, thymus, thyroid, adrenal, nasal cavity, trachea, lung, tongue, esophagus, stomach, small intestine, large intestine, pancreas, liver, kidney, urinary bladder, testis, prostate, seminal vesicle, brain, spinal cord and other tissues of abnormal appearance.

Statistical analysis The significances of intergroup differences in numerical data obtained for body weight, organ weight, and enzyme-altered foci were assessed using the two-sided Student's *t* test. Insufficient homogeneity of variance was corrected with respect to the degree of freedom according to the method of Welch.

RESULTS

Growth rates among the groups I-1–14 and II were essentially similar during the DMD treatment period of 4 weeks. Marked body weight retardation was found in rats given ethionine, DDPM and clofibrate as the second-step treatment. However, no treatment-related deaths occurred in any group.

Organ weights Prominent increases of liver weight were found in rats which received the strong hepatocarcinogens, 2-AAF and 3′-Me-DAB, and moderate increases were seen in rats fed phenobarbital, ethionine and clofibrate (data not shown). Statistically significant increases of liver weight were also observed in animals treated with non-hepatocarcinogens, BBN, BHA, catechol, DMBA and 3-MC. Marked thyroid weight increase was noted in rats fed DDPM, and a slight increase was seen in rats given PB.

Quantitative evaluation of GST-P-positive foci As shown in Fig. 2, relative values of the numbers and areas

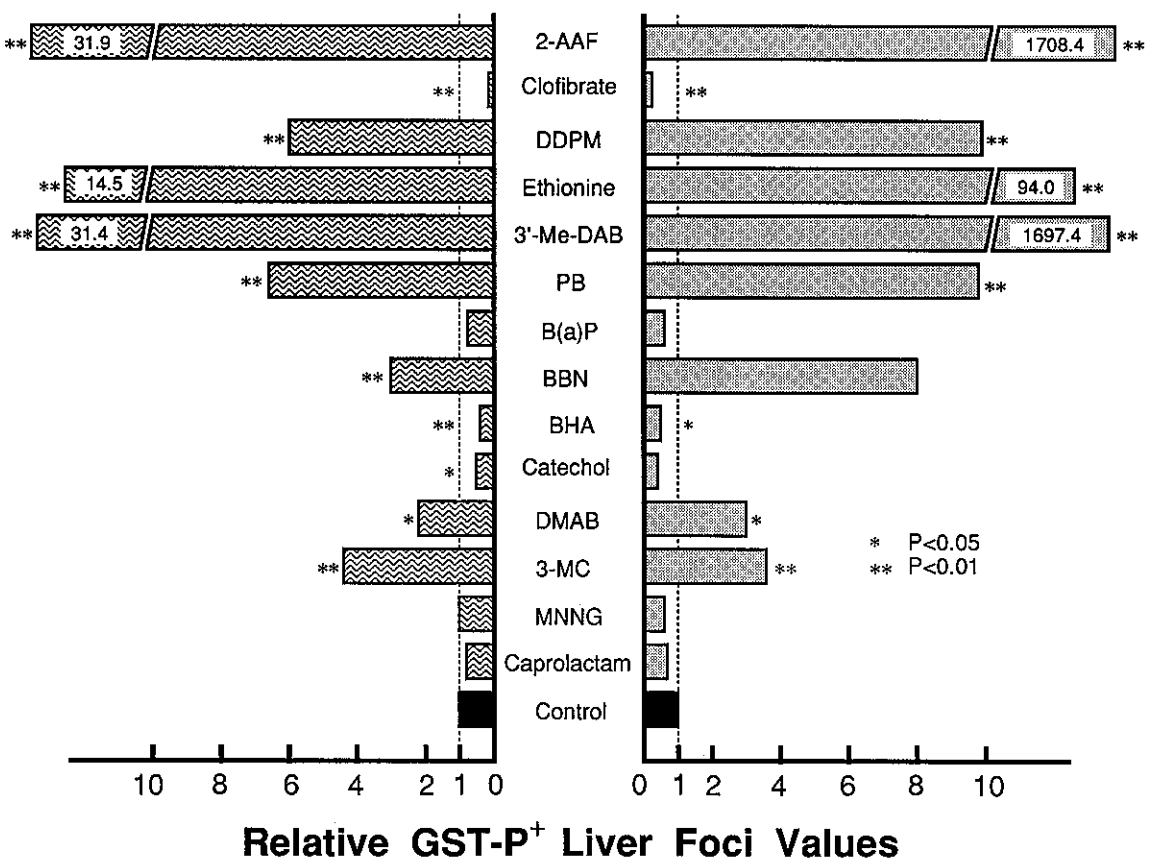

Fig. 2. Relative values for the numbers (▨) and areas (▭) of GST-P-positive hepatocyte foci.

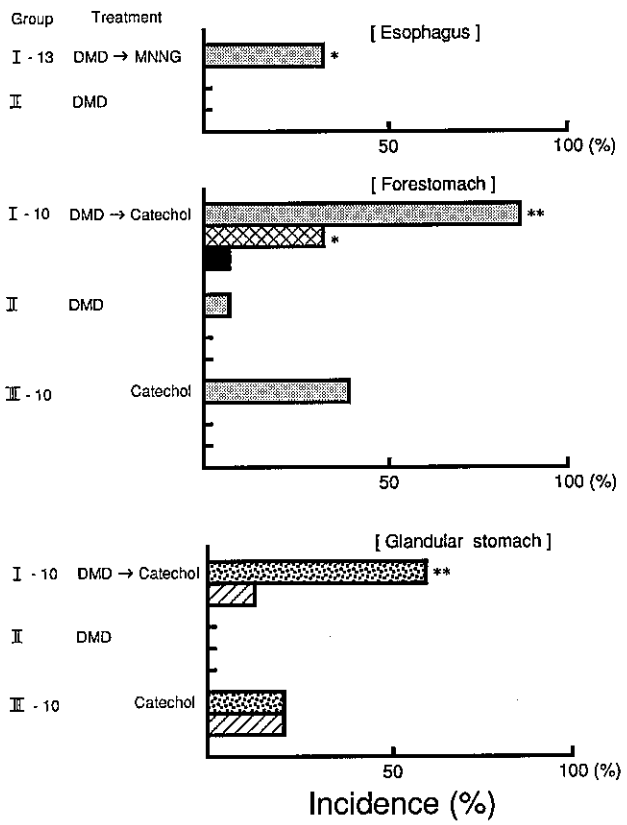

Fig. 3. Development of squamous cell hyperplasia (▒), papillomas (▒) and carcinomas (■) in the esophagus and forestomach. Induction of submucosal hyperplasia (▒) and adenoma (▒) in the glandular stomach.

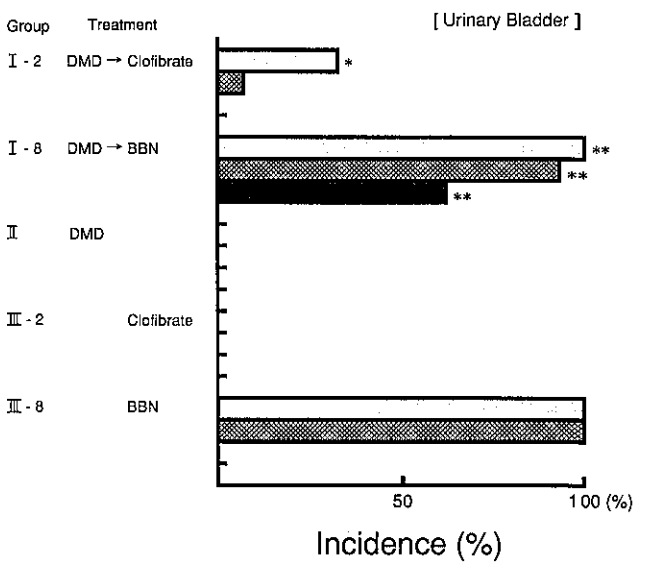

Fig. 4. Induction of PN hyperplasia (▒), papilloma(▒), and carcinoma (■) in the urinary bladder.

of GST-P-positive foci per square centimeter in animals exposed to strong hepatocarcinogens, namely 2-AAF and 3′-Me DAB, after DMD treatment were markedly increased as compared to the values for rats treated DMD alone. The other hepatocarcinogens, ethionine, DDPM and PB also clearly enhanced the development of GST-P-positive foci, while clofibrate inhibited their induction. Among the non-hepatocarcinogens tested, BBN, 3-MC and DMBA also significantly enhanced the development of GST-P-positive foci, while BHA and catechol exerted inhibitory effects. MNNG, B[a]P and caprolactam did not quantitatively influence the GST-P-positive foci.

Esophagus, forestomach and glandular stomach Fig. 3 summarizes values only for the chemicals which significantly increased the induction of preneoplastic and neoplastic lesions. MNNG treatment was associated with a statistically significant increase in the incidence of squamous cell hyperplasia of the esophagus. Catechol significantly increased the incidences of squamous cell hyperplasia and papilloma of the forestomach, as compared to the DMD-pretreated control values. A squamous cell carcinoma was found in this catechol group. Catechol also significantly increased the incidence of hyperplasias (submucosal hyperplasia) and adenomas of the glandular stomach. In the non DMD-treatment group, catechol itself induced hyperplasia of the forestomach and hyperplasia and adenoma of the glandular stomach at low incidences.

Urinary bladder All rats exposed to BBN, a strong and specific urinary bladder carcinogen, demonstrated papillary or nodular (PN) hyperplasia and papillomas, with or without DMD treatment. A high incidence of transitional cell carcinomas was also found in rats given DMD and then BBN (Fig. 4). Clofibrate post-treatment significantly increased the incidence of PN hyperplasia (5 out of 15, 33%) and was associated with papilloma development (1 out of 15, 7%). No proliferative urinary bladder lesions were found in either carcinogen-pretreated controls (group II) or the clofibrate alone group (group III-2). BHA, an established urinary bladder tumor promoter, as well as ethionine caused simple hyperplasia (data not shown), but not PN hyperplasia or papilloma, in the present study.

Thyroid DDPM, a thyroid carcinogen and goitrogen, significantly enhanced the development of follicular cell hyperplasias, adenomas, and follicular cell carcinomas (100%), as shown in Fig. 5. Eight out of 15 rats (53%) in this group had metastasis to the lung. Although DDPM alone also induced diffuse follicular hyperplasia (goiter), no preneoplastic or neoplastic lesions were found.

PB, a known thyroid tumor promoter, also clearly enhanced the development of follicular cell hyperplasias and adenomas. In contrast, clofibrate and ethionine exerted clear inhibitory effects on follicular cell hyperplasia and adenomas. PB, clofibrate or ethionine alone did not cause any change in thyroid follicular tissue.

Lung 3-MC and 2-AAF showed a tendency for increasing the incidence of alveolar type II cell adenomas, but this was not statistically significant (data not shown). Although all rats which received the DMD treatment developed alveolar type II cell hyperplasia, there were no significant differences in the incidences of this lesion between the DMD alone and the DMD plus test chemical groups. In the non DMD-treatment group, proliferative lesions were not observed in the lung.

Other organs While DMD treatment induced preneoplastic and/or neoplastic lesions in the nasal cavity, kidney and seminal vesicle, no second-stage modification was found in any group. A case of hyperplasia of the tongue was found in the DMD+catechol group. A case of basophilic foci development in the pancreas was noted in the DMD+DMBA group. Medullary hyperplasia of the adrenals was seen in one DMD+2-AAF group animal. Adenomas of the Zymbal gland were found in single DMD+BBN and DMD+DMBA group rats. One case of neurofibroma of the spinal cord was observed in the DMD+DMBA group.

The site-specific modification effects of the different agents investigated are summarized in Table II.

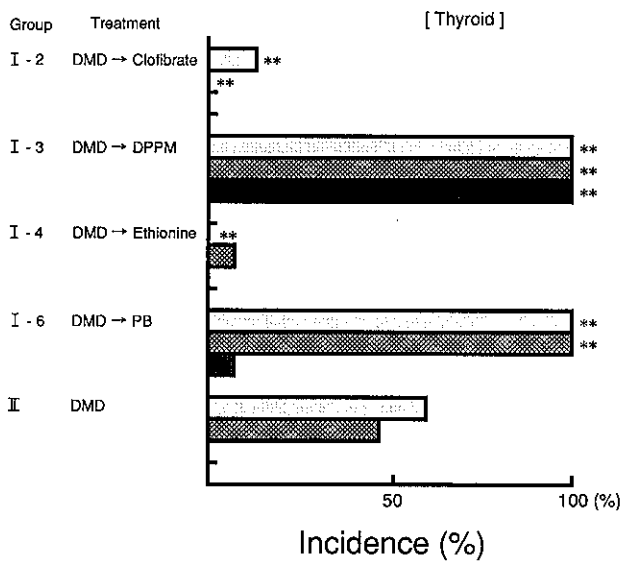

Fig. 5. Development of follicular cell hyperplasia (▨), follicular cell adenoma (▩) and follicular cell carcinoma (■) in the thyroid.

DISCUSSION

The results of the present investigation clearly demonstrated that modification potential of test agents can be assessed at the whole-body level, a great advantage as compared to previous initiation-promotion protocols for single organs. Eleven out of 14 test chemicals possessed modifying effects, 7 agents exerting enhancing and/or inhibiting effects on more than two types of neoplastic lesions.

Table II. Summary of Modification of Carcinogenic Response by Test Chemicals

Test chemicals	Liver	Esophagus	Forestomach	Glandular stomach	Thyroid	Bladder
2-AAF	⬆	→	→	→	→	→
Clofibrate	⬇	→	→	→	↓	↑
DDPM	⬆	→	→	→	⬆	→
Ethionine	⬆	→	→	→	↓	→
3′-Me-DAB	⬆	→	→	→	→	→
PB	⬆	→	→	→	↑	→
B[a]P	→	→	→	→	→	→
BBN	↑	→	→	→	→	⬆
BHA	↓	→	→	→	→	→
Catechol	↓	→	↑	↑	→	→
DMBA	↑	→	→	→	→	→
3-MC	⬆	→	→	→	→	→
MNNG	→	↑	→	→	→	→
Caprolactam	→	→	→	→	→	→

⬆, strong enhancement; ↑, weak enhancement; ⬇, strong inhibition; ↓, weak inhibition; →, no modification.

The most striking finding in the present study was that clofibrate, a hypolipidemic medicine and peroxisome proliferator, exerted a weak enhancing effect on urinary bladder carcinogenesis, and an inhibiting influence on thyroid carcinogenesis. It was previously reported that clofibrate, a known non-genotoxic hepatocarcinogen,[15,16] did not possess tumor-promoting activity in any organ other than the liver.[16] This illustrates the great advantage of the present approach.

All of the hepatocarcinogens tested, with the exception of clofibrate, clearly enhanced the development of enzyme-altered hepatocellular foci, in agreement with the results of a medium-term bioassay system based on the liver.[1-3] The non-hepatocarcinogen, 3-MC showed strong tumor-promoting activity on liver carcinogenesis, while BBN and DMBA also possessed weak enhancing effects in confirmation of the positive results gained using a modified liver medium-term bioassay system.[31] This provides further support for the conclusion that the present wide-spectrum carcinogenesis model is useful for the detection of liver tumor promoters and carcinogens.

Catechol, a natural antioxidant and stomach carcinogen,[25] enhanced the incidence of hyperplasia in both the forestomach and glandular stomach. In contrast, BHA[24] and MNNG,[28,29] which are known to target the stomach, did not induce any preneoplastic or neoplastic stomach lesions, presumably due to the low exposure level to these chemicals. MNNG induced hyperplasia in the esophagus, but not the small intestine in the present study. This suggests that the critical dose for appearance of modifying activity on tumor development may be organ-specific.

Among the 14 chemicals tested, DDPM[17] and PB,[6,7] which are known to target the thyroid, obviously enhanced follicular cell tumor development after pretreatment with the three carcinogens in the present investigation. Furthermore, clofibrate[15,16] and ethionine,[18] not hitherto reported to act as inhibitors of thyroid tumorigenesis, clearly reduced the induction of follicular cell tumors, the other chemicals being inactive. The results thus indicate that the wide-spectrum initiation (DMD) model may be particularly useful as a tool for the detection of thyroid carcinogens or modifiers.

Other sites initiated by DEN,[1-3,11] MNU[6-8,32] and DHPN[33] are reported to include the kidney, nervous system, and hematopoietic system. Putative preneoplastic renal tubular cell lesions were observed in the DMD treatment control group at very low incidence. However, none of the test chemicals showed enhancement of these changes, in line with their reported lack of toxicity or carcinogenicity for the kidney. Preneoplastic and/or neoplastic lesions developing in the nasal cavity and seminal vesicle were similarly not affected by test chemicals. Neither nervous nor hematopoietic system neoplastic lesions were observed in the present study, presumably because the initiation dose or duration of the experiment was insufficient for tumor development in these organs.

In the present study, caprolactam did not demonstrate modification of carcinogenesis in any organ, consistent with the previously reported results indicating non-genotoxic and noncarcinogenic character.[30] B[a]P also proved inactive, as in our previous study (oral exposure).[1]

In conclusion, with the aim of detecting carcinogenic agents at the whole-organ level, as an alternative to the medium-term bioassay system for liver carcinogens, we have developed two different types of multi-organ carcinogenesis model using one wide-spectrum initiator,[6-8] or a combination of potent carcinogens having different organ spectra of carcinogenicity.[9-12] Continuing research is being directed at improvement of the model by increasing the number of carcinogens used in the initiation stage.

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REFERENCES

1) Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Kagawa, M., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats — an approach for a new medium-term bioassay system. *Carcinogenesis*, **9**, 387–394 (1988).

2) Ito, N., Imaida, K., Hasegawa, R. and Tsuda, H. Rapid bioassay methods for carcinogens and modifiers of hepatocarcinogenesis. *CRC Crit. Rev. Toxicol.*, **19**, 385–415 (1989).

3) Ito, N., Tatematsu, M., Hasegawa, R. and Tsuda, H. Medium-term bioassay system for detection of carcinogens and modifiers of hepatocarcinogenesis utilizing the GST-P positive liver cell focus as an endpoint marker. *Toxicol. Pathol.*, **17**, 630–641 (1989).

4) Chu, K. C., Cueto, C., Jr. and Ward, J. M. Factors in the evaluation of 200 National Cancer Institute carcinogen bioassays. *J. Toxicol. Environ. Health,* **8,** 251–280 (1981).
5) Gold, L. S., Ward, J. M., Bernstein, L. and Stern, B. Association between carcinogenic potency and tumor pathology in rodent carcinogenesis bioassays. *Fundam. Appl. Toxicol.,* **6,** 677–690 (1986).
6) Tsuda, H., Fukushima, S., Imaida, K., Kurata, Y. and Ito, N. Organ-specific promoting effect of phenobarbital and saccharin in induction of thyroid, liver, and urinary bladder tumors in rats after initiation with N-nitrosourea. *Cancer Res.,* **43,** 3292–3296 (1983).
7) Tsuda, H., Sakata, T., Shirai, T., Kurata, Y., Tamano, S. and Ito, N. Modification of N-methyl-N-nitrosourea initiated carcinogenesis in the rat by subsequent treatment with antioxidants, phenobarbital and ethinyl estradiol. *Cancer Lett.,* **24,** 19–27 (1984).
8) 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).
9) Ito, N., Imaida, K., Tsuda, H., Shibata, M-A., Aoki, T., de Camargo, J. L. V. and Fukushima, S. Wide-spectrum initiation models: possible applications to medium-term multiple organ bioassays for carcinogenesis modifiers. *Jpn. J. Cancer Res.,* **79,** 413–417 (1988).
10) Thamavit, W., Fukushima, S., Kurata, Y., Asamoto, M. and Ito, N. Modification by sodium L-ascorbate, butylated hydroxytoluene, phenobarbital and pepleomycin of lesion development in a wide-spectrum initiation rat model. *Cancer Lett.,* **45,** 93–101 (1989).
11) Ito, N., Tsuda, H., Hasegawa, R., Tatematsu, M., Imaida, K. and Asamoto, M. Medium-term bioassay models for environmental carcinogens — two-step liver and multi-organ carcinogenesis protocols. *In* "Biologically-based Methods for Cancer Risk Assessment," ed. C. C. Travis, pp. 209–230 (1989). Plenum Publishing Co., New York.
12) Shibata, M-A., Fukushima, S., Takahashi, S., Hasegawa, R. and Ito, N. Enhancing effects of sodium phenobarbital and N,N-dibutylnitrosamine on tumor development in a rat wide-spectrum organ carcinogenesis model. *Carcinogenesis,* **11,** 1027–1031 (1990).
13) Oyasu, R., Battifora, H. A., Eisenstein, R., McDonald, J. H. and Hass, G. M. Enhancement of tumorigenesis in the urinary bladder of rats by neonatal administration of 2-acetylaminofluorene. *J. Natl. Cancer Inst.,* **40,** 377–388 (1968).
14) Maeura, Y., Weisburger, J. H. and Williams, G. M. Dose-dependent reduction of N-2-fluorenylacetamide-induced liver cancer and enhancement of bladder cancer in rats by butylated hydroxytoluene. *Cancer Res.,* **44,** 1604–1610 (1984).
15) IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol. 24. "Some Pharmaceutical Drugs," pp. 39–58 (1980). International Agency for Research on Cancer, Lyon.
16) IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, suppl. 7. "Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42," pp. 171–172 (1987). International Agency for Research on Cancer, Lyon.
17) IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol. 39. "Some Chemicals Used in Plastics and Elastomers," pp. 347–364 (1985). International Agency for Research on Cancer, Lyon.
18) Leopold, W. L., Miller, J. A. and Miller, E. C. Comparison of some carcinogenic, mutagenic, and biochemical properties of S-vinylhomocysteine and ethionine. *Cancer Res.,* **42,** 4364–4374 (1982).
19) Kitagawa, T. and Sugano, H. Timetable for hepatocarcinogenesis in rats. *In* "Analytical and Experimental Epidemiology of Cancer," ed. W. Nakahara, T. Hirayama, K. Nishioka and H. Sugano, pp. 91–105 (1973). Japan Scientific Societies Press, Tokyo.
20) Butler, J. G. Long-term effects of phenobarbitone-Na on male Fischer rats. *Br. J. Cancer,* **37,** 418–423 (1978).
21) IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol. 32. "Polynuclear Aromatic Compounds, Part 1, Chemical, Environmental and Experimental Data," pp. 211–224 (1983). International Agency for Research on Cancer, Lyon.
22) 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).
23) Fukushima, S., Hagiwara, A., Ogiso, T., Shibata, M. and Ito, N. Promoting effects of various chemicals in rat urinary bladder carcinogenesis initiated by N-nitroso-n-butyl-(4-hydroxybutyl)amine. *Food Chem. Toxicol.,* **21,** 59–68 (1983).
24) 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).
25) Hirose, M., Kurata, Y., Tsuda, H., Fukushima, S. and Ito, N. Catechol strongly enhances rat stomach carcinogenesis: a possible new environmental stomach carcinogen. *Jpn. J. Cancer Res.,* **78,** 1144–1149 (1987).
26) Hirose, M., Masuda, A., Fukushima, S. and Ito, N. Effects of subsequent antioxidant treatment on 7,12-dimethylbenz-[a]anthracene-initiated carcinogenesis of the mammary gland, ear duct and forestomach in Sprague-Dawley rats. *Carcinogenesis,* **9,** 101–104 (1988).
27) Shay, H., Grunstein, M. and Kessler, W. B. Experimental mammary adenocarcinoma of rats. Some consideration of methylcholanthrene dosage and hormonal treatment. *J. Natl. Cancer Inst.,* **27,** 503–513 (1961).
28) Sugimura, T. and Fujimura, S. Tumor production in glandular stomach of rat by N-methyl-N′-nitro-N-nitrosoguanidine. *Nature,* **216,** 943–944 (1967).

29) IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol. 4. "Some Aromatic Amines, Hydrazine and Related Substances, N-Nitroso Compounds and Miscellaneous Alkylating Agents," pp. 183–195 (1974). International Agency for Research on Cancer, Lyon.
30) IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, vol. 39. "Some Chemicals Used in Plastics and Elastomers," pp. 247–276 (1985). International Agency for Research on Cancer, Lyon.
31) Ito, N., Imaida, K., de Camargo, J. L. V., Takahashi, S., Asamoto, M. and Tsuda, H. A new medium-term bioassay system for detection of environmental carcinogens using diethylnitrosamine-initiated rat liver followed by D-galactosamine treatment and partial hepatectomy. *Jpn. J. Cancer Res.*, **79**, 573–575 (1988).
32) Maekawa, A., Matsuoka, C., Onodera, H., Tanigawa, H., Furuta, K., Ogiu, T., Mitsumori, K. and Hayashi, Y. Organo-specific carcinogenicity of N-methyl-N-nitrosourea in F344 and ACI/N rats. *J. Cancer Res. Clin. Oncol.*, **109**, 178–182 (1985).
33) Konishi, Y., Kondo, H., Ikeda, I., Kawabata, A., Shoji, Y. and Denda, A. Effect of dose on the carcinogenic activity of orally administered N-bis(2-hydroxypropyl)nitrosamine to rats. *Gann*, **69**, 573–577 (1978).