Jpn. J. Cancer Res. 83, 812–820, August 1992

Modifying Effects of Various Chemicals on Tumor Development in a Rat Wide-spectrum Organ Carcinogenesis Model

Satoshi Uwagawa, Hiroyuki Tsuda,[1] Keisuke Ozaki, Satoru Takahashi, Shuji Yamaguchi, Mamoru Mutai, Toyohiko Aoki and Nobuyuki Ito

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

The efficacy of a wide-spectrum organ carcinogenesis model for detection of modification potential of exogenous agents was investigated in F344 male rats. Groups of animals were sequentially injected with N-bis(2-hydroxypropyl)nitrosamine (1000 mg/kg body weight, i.p., in saline, twice in week 1), N-ethyl-N-hydroxyethylnitrosamine (1500 mg/kg body weight, i.g., in distilled water, twice in week 2) and 3,2′-dimethyl-4-aminobiphenyl (75 mg/kg body weight, s.c., in corn oil, twice in week 3) for wide-spectrum initiation of target organs and then given one of 10 test chemicals, comprising 6 hepatocarcinogens and 4 non-hepatocarcinogens, for 12 weeks. All 10 chemicals exerted modifying effects in their respective target organs. Enhancing influence could be detected in the liver and urinary bladder with 2-acetylaminofluorene, ethionine, and 3′-methyl-4-dimethylaminoazobenzene; in the liver and thyroid with 4,4′-diaminodiphenylmethane and phenobarbital; in the esophagus and urinary bladder with N-butyl-N-(4-hydroxybutyl)nitrosamine; in the forestomach and urinary bladder with butylated hydroxyanisole; in the liver with 7,12-dimethylbenz[*a*]anthracene and in the liver and lung with 3-methylcholanthrene. Inhibitory effects on development of glutathione *S*-transferase placental form-positive liver cell foci were observed with clofibrate. The results indicate that the present model can be reliably utilized as a whole body medium-term bioassay system for assessment of environmental cancer modifiers.

Key words: Wide-spectrum carcinogenesis — Modification — Assay model — Rat

The two-stage concept of neoplasia has been widely used in *in vivo* bioassay systems for assessing carcinogenic and tumor modifying potential. However, modulating effects of chemicals can only be manifested in those organs for which appropriate initiation has been accomplished.[1–13]

For the purpose of developing alternative assay systems for detection of carcinogenicity and modifying effects of chemicals in unknown organs, we have investigated the efficacy of sequential treatment with potent carcinogens possessing wide-spectrum initiating activities. The findings indicate that we can thereby initiate multiple organs in the same animal, allowing assay of modifying potential of test chemicals in various target organs.[14–19] Fukushima *et al.*[17] and Shibata *et al.*[16] used sequential treatment with 3 nitrosocompounds in the initiation stage. Recently we reported a wide-spectrum carcinogenesis model applying N-methyl-N-nitrosourea (MNU[2]).[20] Using this MNU wide-spectrum carcinogenesis model, we could detect carcinogenic modification by test chemicals in as short a period as 20 weeks. However, a major disadvantage with this system was the high rate of hematopoietic tumors induced by MNU.

The present paper concerns a new model for multiple initiation which we investigated for detection of tumor-modifying effects by test chemicals in various target organs. N-Bis(2-hydroxypropyl)nitrosamine (DHPN), N-ethyl-N-hydroxyethylnitrosamine (EHEN) and 3,2′-dimethyl-4-aminobiphenyl (DMAB) were chosen as wide-spectrum initiators, with targets of thyroid, liver, lung, esophagus, kidney and urinary bladder for DHPN[7,21]; liver and kidney for EHEN[10]; and pancreas, intestine, testis, prostate, seminal vesicle, preputial gland, mammary gland, skin and Zymbal gland for DMAB.[22] Ten test chemicals, comprising 6 hepatocarcinogens and 4 non-hepatocarcinogens, were selected to see whether they would enhance induction of neoplastic lesions in their respective organotropic sites. Chemicals, dose levels and known target sites are summarized in Table I.

[1] Present address: Second Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470-11.

[2] The abbreviations used are: MNU, N-methyl-N-nitrosourea; DHPN, N-bis(2-hydroxypropyl)nitrosamine; EHEN, N-ethyl-N-hydroxyethylnitrosamine; DMAB, 3,2′-dimethyl-4-aminobiphenyl; 2-AAF, 2-acetylaminofluorene; DDPM, 4,4′-diaminodiphenylmethane; 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; PB, phenobarbital; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; BHA, butylated hydroxyanisole; DMBA, 7,12-dimethylbenz[*a*]anthracene; 3-MC, 3-methylcholanthrene; GST-P, glutathione *S*-transferase, placental form.

Table I. Dose Level and Target Organs of Orally Administered Test Chemicals

Test chemical	Dose level (%)	Target organs	References
Hepatocarcinogens/promoters			
2-Acetylaminofluorene (2-AAF)	0.01	liver, urinary bladder	23
Clofibrate	1.0	liver	35
4,4′-Diaminodiphenylmethane (DDPM)	0.1	thyroid, liver	24, 25
Ethionine	0.25	liver	26
3′-Methyl-4-dimethylaminoazobenzene (3′-Me-DAB)	0.06	liver	27
Phenobarbital (PB)	0.05	liver	28
Non-hepatocarcinogens			
N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN)	0.1	urinary bladder	29
Butylated hydroxyanisole (BHA)	1.0	forestomach	30, 31
7,12-Dimethylbenz[*a*]anthracene (DMBA)	0.01	mammary gland, ear duct, forestmach	33
3-Methylcholanthrene (3-MC)	0.02	mammary gland	34

MATERIALS AND METHODS

Animals A total of 217 male 6-week-old F344 rats (Charles River Japan Inc., Atsugi) were used. The rats were housed five per plastic cage with hardwood bedding (Charles River Japan Inc.) in an animal room with a 12 h-light, 12 h-dark cycle at 24°C and 60% relative humidity.

Chemicals DHPN was obtained from Nakalai Tesque Co., Kyoto; EHEN from Sakai Research Laboratory, Fukui; DMAB from Matsugaki Pharmaceutical Co., Osaka; ethionine from Sigma Chemical Co., St. Louis; phenobarbital (PB) from Iwaki Seiyaku Co., Tokyo; butylated hydroxyanisole (BHA) from Wako Pure Chemical Industry Ltd., Osaka and 2-acetylaminofluorene (2-AAF), clofibrate, 4,4′-diaminodiphenylmethane (DDPM), 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), 7,12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene (3-MC) from Tokyo Kasei Kogyo Co., Tokyo.

Experimental design After one week for acclimatization, rats were randomly divided into 3 groups (Fig. 1). Group 1: Rats were sequentially treated with DHPN (1000 mg/kg body weight, i.p., in saline, twice on days 1 and 4 of week 1), EHEN (1500 mg/kg body weight, i.g., in distilled water, twice on days 8 and 11 of week 2) and DMAB (75 mg/kg body weight, s.c., in corn oil, twice on days 15 and 18 of week 3) for initiation (DED regimen). Starting one week later, rats were given 2-AAF (0.01%, in diet), clofibrate (1.0%, in diet), DDPM (0.1%, in diet), ethionine (0.25%, in diet), 3′-Me-DAB (0.06%, in diet), PB (0.05%, in diet), BBN (0.1%, in drinking water), BHA (1%, in diet), DMBA (0.01%, in diet) or 3-MC (0.02%, in diet). Group 2: Rats were sequentially treated with the DED regimen as in Group 1 then given basal diet until termination. Group 3: Rats were treated with the carcinogen solvent vehicles for the DED regimen, and then given one of the test chemicals as in Group 1. Food and water were available *ad libitum*. During the experiment, body weights were measured every 4 weeks. Food and water consumption were measured at weeks 8 and 15.

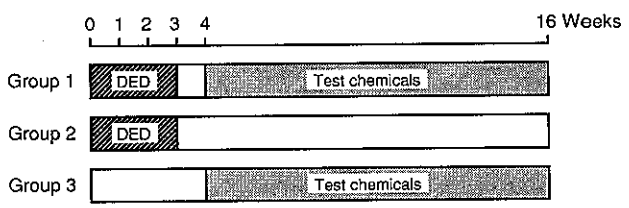

Fig. 1. Experimental design. ▨, sequentially treated with DHPN (1000 mg/kg, i.p., in saline, twice in week 1), EHEN (1500 mg/kg, i.g., in distilled water, twice in week 2) and DMAB (75 mg/kg, s.c., in corn oil, twice in week 3) (DED regimen); ▩, test chemicals; □, basal diet.

Histopathological examination At the end of week 16, all surviving animals were killed and carefully autopsied. The major organs (heart, lymph node, spleen, thyroid, adrenal, trachea, lung, esophagus, stomach, small intestine, large intestine, pancreas, liver, kidney, urinary bladder, prostate, seminal vesicle and brain) were taken and fixed in 10% phosphate-buffered formalin, and routinely prepared paraffin sections were stained with hematoxylin and eosin for histopathological assessment of pre-

neoplastic and neoplastic lesion development. Slices of liver, 2–3 mm in thickness, were also cut with a razor blade from three lobes and fixed in ice-cold acetone for immunohistochemical examination of glutathione *S*-transferase placental form (GST-P) staining. The numbers and areas of GST-P-positive foci larger than 0.2 mm in diameter, were measured using a color video image processor (VIP-21 C, Olympus-Ikegami Tsushin Co., Tokyo). Data on lesion incidences were analyzed for statistical significance using the Fisher exact test and other data were analyzed with Student's *t* test.

RESULTS

Data on the final average body weights are summarized in Table II. The final body weights of rats given test chemicals after the DED regimen (Group 1) all tended to be lower than in the DED regimen alone case (Group 2). However, growth rates for groups 1 (DED regimen) and 3 (chemical alone) were similar throughout the experimental period and no treatment-related deaths occurred in any group. Food and water consumption in the test chemical-treated groups receiving DED (Group 1) were slightly lower than in the DED regimen alone group (Group 2) (Table II).

Liver Immunohistochemically demonstrated GST-P-positive liver cell foci were assessed as preneoplastic endpoint lesions. The hepatocarcinogens, 2-AAF, DDPM, ethionine, 3′-Me-DAB and PB with the DED regimen (Group 1) significantly enhanced the numbers and mean areas per unit area (cm^2) of GST-P-positive foci as compared to the DED regimen alone values (Group 2) as well as the test chemical alone values (Group 3). The other known hepatocarcinogen, clofibrate, significantly reduced both the number and area of GST-P-positive foci. Among the non-hepatocarcinogens tested, DMBA and 3-MC (Group 1) also enhanced the numbers and mean areas of GST-P-positive foci as compared with the DED regimen alone values (Group 2) as well as test chemical alone values (Group 3) (Table III).

Thyroid The incidences of follicular hyperplasias in Group 1 animals treated with DDPM (100%, 15 of 15

Table II. Average Body Weights, and Food and Water Consumption Data

Group	Treatment	No. of rats	Body weight (g) (at week 16)	Food consumption (g/rat/day) (at week 15)	Water consumption (ml/rat/day) (at week 15)
1	DED-2-AAF	16	280.29±16.37[a, c]	12.87±0.57[e]	17.81±1.63[c, e]
	DED-Clofibrate	15	189.76±8.80[c]	8.71±1.53[c]	13.64±1.09[c, e]
	DED-DDPM	15	259.71±9.67[c]	12.51±4.27	16.59±0.51[c, e]
	DED-Ethionine	15	219.73±18.29[c, d]	9.05±0.61[c, e]	12.55±0.48[c, e]
	DED-3′-Me-DAB	14	248.85±10.96[c, e]	10.24±0.62[c, d]	13.79±0.08[c, e]
	DED-PB	15	296.31±19.02[c, d]	13.69±0.69	18.19±1.46[c, e]
	DED-BBN	15	276.73±13.50[c, e]	12.34±0.28[c, e]	18.39±0.53[c]
	DED-BHA	16	283.24±20.76[c, e]	11.49±0.86[c, e]	16.89±2.33[c, e]
	DED-DMBA	16	287.73±16.90[c, e]	11.72±0.87[c, e]	18.47±2.05[b]
	DED-3-MC	16	280.66±14.76[c]	12.20±1.00[c]	18.02±0.96[c, d]
2	DED	15	321.69±11.69	13.37±0.87	20.41±1.96
3	2-AAF	5	288.58±10.18	12.16	16.24
	Clofibrate	5	178.64±18.72	7.94	11.34
	DDPM	5	261.84±20.54	10.29	17.92
	Ethionine	5	244.32±20.87	9.74	12.00
	3′-Me-DAB	5	271.46±7.63	9.82	14.52
	PB	5	317.82±19.70	13.56	21.80
	BBN	5	304.54±8.07	13.27	18.60
	BHA	5	314.44±14.69	12.38	20.34
	DMBA	5	328.50±17.48	13.52	19.16
	3-MC	5	291.42±26.68	12.73	18.63

a) Values are mean±SD.
Significantly different from Group 2 (DED regimen alone) at *b*, $P<0.05$; *c*, $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *d*, $P<0.05$; *e*, $P<0.01$.

Table III. Quantitative Data for GST-P-positive Liver Foci

Group	Treatment	No. of rats	GTS-P-positive foci	
			No./cm^2	Area/cm^2
1	DED-2-AAF	16	24.48±13.19[a, c, d]	91.22±12.60[c, e]
	DED-Clofibrate	15	0.45±0.47[c, e]	0.02±0.02[c, e]
	DED-DDPM	15	14.16±4.88[c, e]	0.82±0.36[c, e]
	DED-Ethionine	15	64.90±10.08[c, e]	27.08±7.25[c, e]
	DED-3′-Me-DAB	14	13.94±5.54[c, e]	78.97±8.35[c, e]
	DED-PB	15	21.78±10.66[c, e]	1.49±0.92[c, e]
	DED-BBN	15	5.53±2.60[e]	0.30±0.16[e]
	DED-BHA	16	3.17±3.72[e]	0.18±0.21[e]
	DED-DMBA	16	6.41±2.43[b, e]	0.41±0.25[b, e]
	DED-3-MC	16	11.56±5.19[c, e]	0.69±0.34[c, e]
2	DED	15	4.36±2.01	0.24±0.14
3	2-AAF	5	38.72±9.89	9.33±5.72
	Clofibrate	5	0	0
	DDPM	5	0	0
	Ethionine	5	1.48±1.14	0.09±0.07
	3′-Me-DAB	5	40.96±8.62	30.01±7.24
	PB	5	0	0
	BBN	5	0	0
	BHA	5	0	0
	DMBA	5	0	0
	3-MC	5	0	0

a) Values are mean±SD.
Significantly different from Group 2 (DED regimen alone) at *b*, $P<0.05$; *c*, $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *d*, $P<0.05$; *e*, $P<0.01$.

rats) and PB (33.3%, 5 of 15 rats) with the DED regimen (Group 1) were significantly higher than that in Group 2 treated with DED alone (0%, 0 of 15 rats). In Group 1 treated with DDPM, the incidences of follicular adenomas (100%, 15 of 15 rats) as well as follicular adenocarcinomas (100%, 15 of 15 rats) were higher than those of Group 2 treated with DED alone or Group 3 treated with DDPM alone (Table IV).

Lung No significant intergroup differences with regard to the incidences of preneoplastic adenomatous hyperplasias were observed. The total incidence of neoplastic lesions, including both adenomas (Fig. 2) and adenocarcinomas, however, was increased in rats treated with 3-MC after DED (Group 1, 43.8%, 7 of 16 rats) as compared to Group 2 treated with DED alone (6.7%, 1 of 15 rats) (Table IV).

Esophagus The incidence of hyperplasia in Group 1 animals treated with BBN (Fig. 3) (26.7%, 4 of 15 rats) was significantly higher than in Group 2 given the DED regimen alone (0%, 0 of 15 rats) (Table V).

Forestomach The incidence of hyperplasia was significantly increased in Group 1 animals treated with BHA (75%, 12 of 16 rats) as compared to the value for Group 2 animals treated with DED alone (0%, 0 of 15 rats) (Table V).

Urinary bladder Two histologically different types of preneoplastic lesion, simple hyperplasia and papillary or nodular hyperplasia (PN hyperplasia) were observed. The incidences of simple hyperplasia were significantly elevated in Group 1 animals treated with 2-AAF (87.5%, 14 of 16 rats), ethionine (86.7%, 13 of 15 rats) or BHA (68.8%, 11 of 16 rats) over the value for Group 2 treated with DED alone (6.7%, 1 of 15 rats) as well as Group 3 treated with test chemical alone. In addition the incidences of simple hyperplasia were significantly increased in rats treated with 3′-Me-DAB (42.9%, 6 of 14 rats) and BBN (93.3%, 14 of 15 rats) after the DED regimen (Group 1) as compared to Group 2, but not to the respective test chemical alone groups (Group 3). The incidence of PN hyperplasia was significantly increased in Group 1 animals treated with BBN (93.3%, 14 of 15 rats) as compared to the Group 2 value (0%, 0 of 15 rats). Total incidence of papillomas plus transitional cell carcinomas was significantly increased in Group 1

Table IV. Data for Lesions in the Thyroid and Lung

Group	Treatment	No. of rats	Thyroid			Lung	
			Hyperplasia	Adenoma	Adenocarcinoma	Adenomatous-hyperplasia	Tumor[a]
1	DED-2-AAF	16	0[b]	0	0	2 (12.5)	2 (12.5)
	DED-Clofibrate	15	0	0	0	0	0
	DED-DDPM	15	15 (100)[d]	15 (100)[d,f]	15 (100)[d,f]	2 (13.3)	1 (6.7)
	DED-Ethionine	15	0	0	0	3 (20.0)	0
	DED-3′-Me-DAB	14	0	0	0	1 (7.1)	2 (14.3)
	DED-PB	15	5 (33.3)[c]	0	0	5 (33.3)	1 (6.7)
	DED-BBN	15	0	0	0	3 (20.0)	1 (6.7)
	DED-BHA	16	0	0	0	0	4 (25.0)
	DED-DMBA	16	2 (12.5)	0	0	1 (6.3)	1 (6.3)
	DED-3-MC	16	0	0	0	6 (37.5)	7 (43.8)[e]
2	DED	15	0	0	0	3 (20.0)	1 (6.7)
3	2-AAF	5	0	0	0	0	0
	Clofibrate	5	0	0	0	0	0
	DDPM	5	5 (100)	1 (20.0)	0	0	0
	Ethionine	5	0	0	0	0	0
	3′-Me-DAB	5	0	0	0	0	0
	PB	5	0	0	0	0	0
	BBN	5	0	0	0	0	0
	BHA	5	0	0	0	0	0
	DMBA	5	0	0	0	0	0
	3-MC	5	0	0	0	0	0

a) Adenoma plus adenocarcinoma.
b) Numbers of rats with lesions (percentage).
Significantly different from Group 2 (DED regimen alone) at *c*, $P<0.05$; *d*, $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *e*, $P<0.05$; *f*, $P<0.01$.

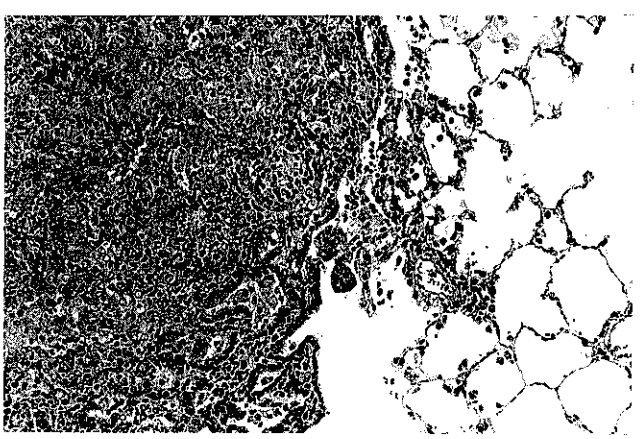

Fig. 2. Adenoma in the lung of a rat treated with 3-MC for 12 weeks after the DED regimen (×140).

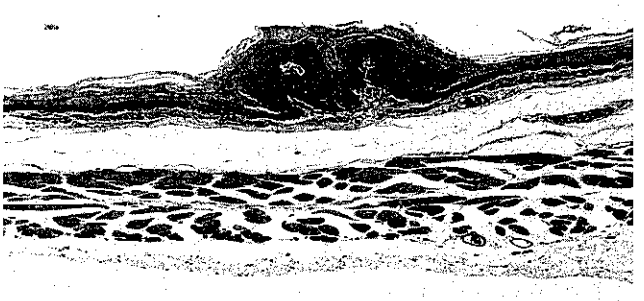

Fig. 3. Hyperplasia in the esophagus of a rat treated with BBN for 12 weeks after the DED regimen (×90).

treated with BBN (93.3%, 14 of 15 rats) as compared to that in Group 2 (0%, 0 of 15 rats) (Table V).

Other tissues Tumors developed in many organs other than those listed in Tables II, III and IV. Included were the pancreas (basophilic foci, acinar cell hyperplasia), glandular stomach (adenomatous hyperplasia), prostate (atypical hyperplasia), seminal vesicle (atypical hyperplasia), and kidney (altered tubules, adenoma, pelvis

Table V. Data for Lesions in the Esophagus, Forestomach and Urinary Bladder

Group	Treatment	No. of rats	Esophagus	Forestomach	Urinary bladder		
			Hyperplasia	Hyperplasia	Simple-hyperplasia	PN-hyperplasia	Tumor[a]
1	DED-2-AAF	16	1 (6.3)[b]	0	14 (87.5)[d,f]	2 (12.5)	0
	DED-Clofibrate	15	0	0	0	0	0
	DED-DDPM	15	0	1 (6.7)	0	0	0
	DED-Ethionine	15	1 (6.7)	0	13 (86.7)[d,f]	2 (13.3)	0
	DED-3′-Me-DAB	14	0	0	6 (42.9)[c]	0	0
	DED-PB	15	0	0	0	1 (6.7)	0
	DED-BBN	15	4 (26.7)[c]	0	14 (93.3)[d]	14 (93.3)[d]	14 (93.3)[d]
	DED-BHA	16	0	12 (75.0)[d]	11 (68.8)[d,e]	3 (18.8)	0
	DED-DMBA	16	0	1 (6.3)	2 (12.5)	0	0
	DED-3-MC	16	0	0	1 (6.3)	0	0
2	DED	15	0	0	1 (6.7)	0	0
3	2-AAF	5	0	0	0	0	0
	Clofibrate	5	0	0	0	0	0
	DDPM	5	0	0	0	0	0
	Ethionine	5	0	0	0	0	0
	3′-Me-DAB	5	0	0	0	0	0
	PB	5	0	0	0	0	0
	BBN	5	0	0	5 (100)	5 (100)	4 (80.0)
	BHA	5	0	5 (100)	0	0	0
	DMBA	5	0	0	0	0	0
	3-MC	5	0	0	0	0	0

a) Papilloma plus transitional cell carcinoma.
b) Numbers of rats with lesions (percentage).
Significantly different from Group 2 (DED regimen alone) at *c*, $P<0.05$; *d*, $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *e*, $P<0.05$; *f*, $P<0.01$.

papilloma and transitional cell carcinoma). These appeared not to be affected by treatment with any of the 10 chemicals tested.

DISCUSSION

The present study confirmed clear modifying influences for all test chemicals on carcinogenesis in their target organs after sequential pretreatment of animals with the 3 different initiators, DHPN, EHEN and DMAB. Enhancing effects on preneoplastic and neoplastic lesions, in agreement with previous reports, were seen in the liver and urinary bladder with 2-AAF,[4,17,23] in the liver and thyroid with DDPM,[17,24,25] in the liver with ethionine,[4,17,26] in the liver with 3′-Me-DAB,[4,17,27] in the liver and thyroid with PB,[4,17,28] in the urinary bladder with BBN,[17,29] in the forestomach and urinary bladder with BHA,[30,31] in the liver with DMBA[17] and in the liver with 3-MC[17] (Table VI). In a previous investigation, DDPM unexpectedly inhibited the development of liver tumors in rats initiated with 2-AAF or 3′-Me-DAB.[32] However, in the present study DDPM promoted liver tumors in line with the results of a long-term carcinogenicity study[24] and another wide-spectrum assay system.[17] This difference in modifying potential of DDPM in the liver is not well understood, but alteration of the drug-metabolizing enzymes by initiating agents may be involved.

In some cases the incidences of lesions in target organs such as the urinary bladder with BBN, and the forestomach with BHA, did not differ between animals given the DED regimen plus test chemicals (Group 1) and those receiving test chemicals alone (Group 3) (Table VI; arrows with asterisks). This was presumably due to strong carcinogenic potential masking the effects of the DED regimen.

It is noteworthy that this system also demonstrated development of preneoplastic and neoplastic lesions in organs for which carcinogenicity had previously not been reported. For example in the urinary bladder with ethionine, in the urinary bladder with 3′-Me-DAB, in the esophagus with BBN and in the lung with 3-MC after

Table VI. Summary of the Experimental Findings for Modification Potential

Treatment	Liver	Thyroid	Lung	Esophagus	Forestomach	Urinary bladder
2-AAF	↑	—	—	—	—	↑
Clofibrate	↓	—	—	—	—	—
DDPM	↑	↑	—	—	—	—
Ethionine	↑	—	—	—	—	↑
3′-Me-DAB	↑	—	—	—	—	Δ
PB	↑	Δ	—	—	—	—
BBN	—	—	—	Δ	—	↑*
BHA	—	—	—	—	↑*	↑
DMBA	↑	—	—	—	—	—
3-MC	↑	—	Δ	—	—	—

↑, strong enhancement; Δ, weak enhancement; ↓, inhibition; —, no modification; *, irrespective of DED initiation treatment.

Table VII. Expected Target Organs Following Initiation Treatment

Initiator	Target organs	References
MNU model	thyroid, lung, liver, pancreas, stomach, intestine, urinary bladder, prostate, nervous system, hematopoietic system	20
DED model	thyroid, lung, liver, pancreas, esophagus, intestine, kidney, urinary bladder, testis, prostate, seminal vesicle, preputial gland, mammary gland, skin, Zymbal gland	7, 10, 21, 22

treatment with DED. This is the first observation of promoting activities of these chemicals in these organs. The DED regimen at the initiation stage played a crucial role in allowing detection of weak carcinogenic potential in a very short time period.

In other cases, however, the limited duration of exposure was not sufficient for induction of measurable lesions in known target organs. For example, DMBA did not induce neoplastic lesions in the ear duct, forestomach and mammary gland[33] and 3-MC did not induce lesions in the mammary gland.[34] This might be a reflection of only weak or no initiation for these agents with the DED regimen. Further investigations are necessary to facilitate optimization of the experimental period and combination of pretreatment carcinogens.

Clofibrate has been demonstrated to induce neoplastic lesions in the liver after long-term application in the rat.[35] While the current finding of an anomalous inhibition using GST-P foci as the preneoplastic marker lesion is not in line with the previous results, it can be explained by suppression of enzyme-altered phenotype of focal liver lesions observed with peroxisome proliferating agents.[4]

Previously, we reported another wide-spectrum carcinogenesis model using MNU, which yielded a high incidence of hematopoietic tumors. However, no treatment-related death occurred in the current DED model. Therefore, the DED regimen is superior to the MNU model because of the satisfactory survival and similar or even wider initiation spectrum (Table VII).

In conclusion, the present results indicate that sequential pretreatment of rats with DHPN, EHEN and DMAB can be effectively used to allow detection of modifying effects of various chemicals at the whole-body level.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to Dr. Kiyomi Sato, Second Department of Biochemistry, Hirosaki University School of Medicine, for generously providing GST-P antibody. This research was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and from the Ministry of Health and Welfare, and for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, as well as by funds from the Society for Promotion of Pathology of Nagoya and from the Experimental Pathology Association.

(Received March 3, 1992/Accepted May 1, 1992)

REFERENCES

1) Masui, T., Shirai, T., Imaida, K., Uwagawa, S. and Fukushima, S. Effects of urinary crystals induced by acetazolamide, uracil, and diethyleneglycol on urinary bladder carcinogenesis in *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine-initiated rats. *Toxicol. Lett.*, **40**, 119–126 (1988).
2) Masui, T., Shirai, T., Takahashi, S., Mutai, M. and Fukushima, S. Summation effect of uracil on the two-stage and multistage models of urinary bladder carcinogenesis in F344 rats initiated by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Carcinogenesis*, **9**, 1981–1985 (1988).
3) Wouterson, R. A., van Garderen-Hoetmer, A. and Longnecker, D. S. Characterization of a 4-month protocol for the quantitation of BOP-induced lesions in hamster pancreas and its application in studying the effect of dietary fat. *Carcinogenesis*, **8**, 833–837 (1987).
4) 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).
5) 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).
6) Fukushima, S., Sakata, T., Tagawa, Y., Shibata, A., Hirose, M. and Ito, N. Different modifying response of butylated hydroxyanisole, butylated hydroxytoluene, and other antioxidants in *N,N*-dibutylnitrosamine esophagus and forestomach carcinogenesis in rats. *Cancer Res.*, **47**, 2113–2116 (1987).
7) Moore, M. A., Thamavit, W., Tsuda, H., Sato, K., Ichihara, A. and Ito, N. Modifying influence of dehydroepiandrosterone on the development of dihydroxy-di-*n*-propylnitrosamine-initiated lesions in the thyroid, lung and liver of F344 rats. *Carcinogenesis*, **7**, 311–316 (1986).
8) Hirose, M., Masuda, A., Inoue, T., Fukushima, S. and Ito, N. Modification by antioxidants and p,p′-diaminodiphenylmethane of 7,12-dimethylbenz[a]anthracene-induced carcinogenesis of the mammary gland and ear duct in CD rats. *Carcinogenesis*, **7**, 1155–1159 (1986).
9) Shirai, T., Ikawa, E., Hirose, M., Thamavit, W. and Ito, N. Modification by five antioxidants of 1,2-dimethylhydrazine-initiated colon carcinogenesis in F344 rats. *Carcinogenesis*, **6**, 637–639 (1985).
10) Tsuda, H., Sakata, T., Masui, T., Imaida, K. and Ito, N. Modifying effects of butylated hydroxyanisole, ethoxyquin and acetaminophen on induction of neoplastic lesions in rat liver and kidney initiated by N-ethyl-N-hydroxyethylnitrosamine. *Carcinogenesis*, **5**, 525–531 (1984).
11) Shirai, T., Masuda, A., Fukushima, S., Hosoda, K. and Ito, N. Effects of sodium L-ascorbate and related compounds on rat stomach carcinogenesis initiated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. *Cancer Lett.*, **29**, 283–288 (1985).
12) 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*-nitrosomethylurea. *Cancer Res.*, **43**, 3292–3296 (1983).
13) 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).
14) 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).
15) 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).
16) 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).
17) Fukushima, S., Hagiwara, A., Hirose, M., Yamaguchi, S., Tiwawech, D. and Ito, N. Modifying effects of various chemicals on preneoplastic and neoplastic lesion development in a wide-spectrum organ carcinogenesis model using F344 rats. *Jpn. J. Cancer Res.*, **82**, 642–649 (1991).
18) Ikawa, E., Tagawa, Y., Shirai, T., Hirose, M. and Fukushima, S. Induction of preneoplastic lesions by sequential treatment of rats with three carcinogens: N-butyl-N-(4-hydroxybutyl)nitrosamine, 7,12-dimethylbenz(a)anthracene, and N-methyl-N′-nitro-N-nitrosoguanidine. *J. Toxicol. Pathol.*, **4**, 63–66 (1991).
19) Fukushima, S., Shibata, M-A., Hirose, M., Kato, T., Tatematsu, M. and Ito, N. Organ-specific modification of tumor development by low-dose combinations of agents in a rat wide-spectrum carcinogenesis model. *Jpn. J. Cancer Res.*, **82**, 784–792 (1991).
20) Uwagawa, S., Tsuda, H., Inoue, T., Tagawa, Y., Aoki, T., Kagawa, M., Ogiso, T. and Ito, N. Enhancing potential of 6 different carcinogens on multi-organ tumorigenesis after initial treatment with N-methyl-N-nitrosourea in rats. *Jpn. J. Cancer Res.*, **82**, 1397–1405 (1991).
21) Shirai, T., Kurata, Y., Fukushima, S. and Ito, N. Dose-related induction of lung, thyroid and kidney tumors by N-bis(2-hydroxypropyl)nitrosamine given orally to F344 rats. *Gann*, **75**, 502–507 (1984).

22) Shirai, T., Nakamura, A., Fukushima, S., Takahashi, S., Ogawa, K. and Ito, N. Effects of age on multiple organ carcinogenesis induced by 3,2′-dimethyl-4-aminobiphenyl in rats, with particular reference to the prostate. *Jpn. J. Cancer Res.*, **80**, 312–316 (1989).
23) 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).
24) IARC monographs on the evaluation of the 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.
25) Hiasa, Y., Kitahori, Y., Enoki, N., Konishi, N. and Shimoyama, T. 4,4′-Diaminodiphenylmethane: promoting effect on the development of thyroid tumors in rats treated with N-bis(2-hydroxypropyl)nitrosamine. *J. Natl. Cancer Inst.*, **72**, 471–476 (1984).
26) 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).
27) Kitagawa, T. and Sugano, H. Timetable for hepatocarcinogenesis in rats. *In* "Analytical and Experimental Epidemiology of Cancer," ed. W. Nakahara, T. Hirayama, T. Nishioka and H. Sugano, pp. 91–105 (1973). Japan Scientific Societies Press, Tokyo.
28) Shirai, T., Masuda, A., Imaida, K., Ogiso, T. and Ito, N. Effects of phenobarbital and carbazole on carcinogenesis of the lung, thyroid, kidney, and bladder of rats pretreated with N-bis(2-hydroxypropyl)nitrosamine. *Jpn. J. Cancer Res.*, **79**, 460–465 (1988).
29) 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).
30) 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).
31) Ito, N. and Hirose, M. The role of antioxidants in chemical carcinogenesis. *Jpn. J. Cancer Res.*, **78**, 1011–1026 (1987).
32) Tsuda, H., Ogiso, T., Hasegawa, R., Imaida, K., Masui, T. and Ito, N. Inhibition of neoplastic development in rat liver, kidney, oesophagus and forestomach by 4,4′-diaminodiphenylmethane administration. *Carcinogenesis*, **8**, 719–722 (1987).
33) 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).
34) Shay, H., Gruenstein, 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).
35) IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, Vol. 24. "Some Pharmaceutical Drugs," pp. 39–58 (1980). International Agency for Research on Cancer, Lyon.