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Enhancing Potential of 6 Different Carcinogens on Multi-organ Tumorigenesis after Initial Treatment with N-Methyl-N-nitrosourea in Rats

Satoshi Uwagawa, Hiroyuki Tsuda,[1] Tadashi Inoue, Yoshiaki Tagawa, Toyohiko Aoki, Masataka Kagawa, Tadashi Ogiso and Nobuyuki Ito

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

The advantages of applying a whole-body concept to the assessment of carcinogenic potential of compounds in a two-stage model after initiation by N-methyl-N-nitrosourea (MNU) were investigated. Male, 6-week-old F344 rats were injected with MNU (20 mg/kg, i.p.) twice a week for 4 weeks and they then received 3,2′-dimethyl-4-aminobiphenyl (DMAB) (50 mg/kg, s.c., once a week), N,N′-dibutylnitrosamine (DBN) (0.05%, in drinking water), N-bis(2-hydroxypropyl)nitrosamine (DHPN) (0.1%, in drinking water), diethylstilbestrol (DES) (2.5 ppm, in diet), sodium *o*-phenylphenate (S.OPP) (2%, in diet) or captafol (0.15%, in diet) for 20 weeks. All six carcinogens enhanced the incidences of preneoplastic and neoplastic lesions in their respective target organs: liver, pancreas, small intestine and urinary bladder with DMAB; liver, esophagus, forestomach and urinary bladder with DBN; thyroid, lung, liver, esophagus, forestomach, small intestine and urinary bladder with DHPN; liver and forestomach with DES; and thyroid, forestomach, kidney and urinary bladder with S.OPP; liver and forestomach with captafol. The results suggested that prior treatment with MNU sensitized the tissues to the organotropic carcinogenic potential of chemicals given thereafter for as short a period as 20 weeks. Thus, this system could be utilized as a whole-body medium-term bioassay system for the screening of environmental carcinogens, bridging the gap between *in vitro* mutagenicity and long-term carcinogenicity tests.

Key words: Multi-organ tumorigenesis — Rat — N-Methyl-N-nitrosourea — Neoplastic lesion development

Many experimental systems have been developed for the detection of carcinogenic potential of environmental chemicals. In particular, *in vivo* assay systems based on the two-stage concept of carcinogenesis have been utilized for the development of short-term systems for assaying the modifying potential of chemicals. The majority of these assays predict carcinogenicity of test chemicals to only a few organs, because modulating effects of chemicals can be manifested only in those organs for which appropriate initiation has been accomplished.[1–7] In our laboratory, we have shown that separate or sequential treatment with potent carcinogens possessing wide-spectrum initiating activities can initiate multiple organs in the same animal, allowing the assay of modifying potential of test chemicals in various target organs.[8–17] N-Methyl-N-nitrosourea (MNU), which is known to induce tumors in many different organs, including the thyroid, lung, liver, pancreas, stomach, intestine, urinary bladder, prostate, nervous system and hematopoietic system,[15–24] is therefore convenient for use as a single carcinogen in such assay systems.

In the present study, 6 different established carcinogens, 3,2′-dimethyl-4-aminobiphenyl (DMAB), N,N′-dibutylnitrosamine (DBN), N-bis(2-hydroxypropyl)-nitrosamine (DHPN), diethylstilbestrol (DES), sodium *o*-phenylphenate (S.OPP) and captafol, were used as "known" test chemicals, to examine the efficacy of our MNU-initiation, medium-term assay for modification potential in their respective target organs in rats.

MATERIALS AND METHODS

Animals A total of 315 male, 6-week-old F344 rats (Charles River Japan Inc., Atsugi) were used. The rats were housed five per plastic cage with wood chip bedding (Charles River Japan Inc.) in an animal room with a 12h-light, 12h-dark cycle at 24°C and 60% relative humidity.

Chemicals The chemicals used in the experiment were MNU (Sigma Chemical Co., St. Louis, MO), DMAB (Matsugaki Pharmaceutical Co., Osaka), DBN (Tokyo Chemical Industry Co., Tokyo), DHPN (Nakarai Chemical Industry Co., Kyoto), DES (Sigma Chemical Co.), S.OPP (Dow Chemical Co., Midland, MI) and captafol (Nissan Chemical Co., Tokyo). MNU was dissolved at a concentration of 4 mg/ml in ice-cold citrate buffer adjusted to pH 6.0 shortly before each treatment. DMAB was dissolved at 10 mg/ml in dimethylsulfoxide. DBN

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

and DHPN were dissolved in tap water, and DES, S.OPP and captafol were added to the basal diet.

Experimental design (Fig. 1) After one week for acclimatization, rats were randomly divided into 3 groups. Group 1: Rats were treated with MNU (20 mg/kg body weight, i.p.) twice a week for 4 weeks, and then given DMAB (50 mg/kg, s.c., once a week), DBN (0.05%, in drinking water), DHPN (0.1%, in drinking water), DES (2.5 ppm, in basal diet, Oriental MF, Oriental Yeast Co., Tokyo), S.OPP (2%, in basal diet) or captafol (0.15%, in basal diet). Group 2: Rats were treated with MNU, and then given basal diet. Group 3: Rats were treated with the citrate buffer vehicle adjusted to pH 6.0, and then given one of the test chemicals. Food and water were available *ad libitum*. During the experiment, body weights were measured every 4 weeks. Food and water consumption were measured at week 15.

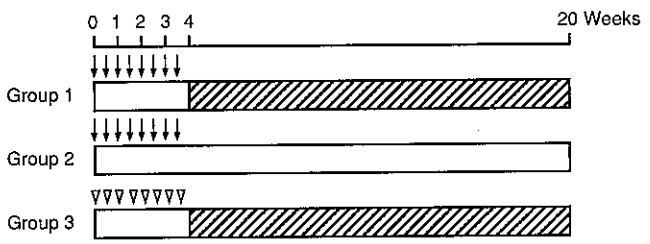

Fig. 1. Experimental design. ↓, Groups 1, 2: MNU (20 mg/kg i.p. 2 times weekly); ▽, Group 3: citrate buffer (i.p. 2 times weekly); ▨, DMAB (50 mg/kg s.c. once a week), DBN (0.05% in water), DHPN (0.1% in water), DES (2.5 ppm in diet), S.OPP (2% in diet) or captafol (0.15% in diet); □, basal diet.

Histopathological examination At the end of week 20, all surviving animals were killed, and carefully autopsied. Sections of all tissues were stained with hematoxylin and eosin, and examined histopathologically for any preneoplastic and neoplastic lesion development. In addition, sections of liver 2–3 mm thick were 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 or areas, larger than 0.1 mm in diameter, were measured using a color video image processor (VIP-21 C, Olympus-Ikegami Tsushin Co., Tokyo). Induced lesions were counted independently even when they were found within the same animal. Data on lesion incidences were analyzed for statistical significance using the Fisher exact test and other data were analyzed by the use of Student's *t* test.

RESULTS

Many rats died from hematopoietic or lymphatic system tumors (leukemia, malignant lymphoma) and nervous system tumors (neurofibrosarcoma, ganglioneuroma) before the termination of the experiment, these being excluded from the effective numbers.

Data on the final average body weights, as well as food and water consumption are summarized in Table I. The final body weights of rats given all test chemicals after

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

Group	Treatment	Body weight (g) (at week 20)	Food consumption (g/rat/day) (at week 15)	Water consumption (ml/rat/day) (at week 15)
1	MNU-DMAB	219.7±28.7[b, c]	12.6±2.3	19.8±5.4
	MNU-DBN	245.3±30.9[b]	10.8±1.7	20.1±4.1
	MNU-DHPN	216.7±22.1[b, d]	9.5±1.6	14.1±2.3[a]
	MNU-DES	170.1±8.0[b, d]	12.7±1.8	11.8±0.8[b]
	MNU-S.OPP	248.9±37.7[a, d]	14.8±1.4[b]	22.1±2.9
	MNU-Captafol	260.5±22.3[d]	14.9±1.7[a]	20.3±1.1
2	MNU	271.5±24.0	11.5±0.4	19.5±0.7
3	DMAB	258.9±12.5	12.1±0.3	17.4±1.4
	DBN	268.8±32.4	12.6±0.7	17.1±1.2
	DHPN	260.8±9.8	11.3±1.2	16.5±1.3
	DES	190.7±15.5	9.9±1.5	15.9±5.0
	S.OPP	296.6±16.4	13.8±0.7	23.3±0.8
	Captafol	289.2±11.8	13.7±1.4	21.6±0.5

Significantly different from Group 2 (MNU alone) at *a*) $P<0.05$, *b*) $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *c*) $P<0.05$, *d*) $P<0.01$.

MNU (Group 1) tended to be lower than those of the controls without MNU (Group 3). While significant differences in food and water consumption were noted among several treatments, no differences between Groups 1 and 3 given the same test chemical were evident. Thus, food and water consumption did not directly correlate with body weight change.

Histopathological examination revealed multiple preneoplastic and neoplastic lesions in the tongue, ear duct, thyroid, lung, liver, pancreas, esophagus, forestomach, small and large intestine, kidney, urinary bladder, urethra, prostate, seminal vesicle, coagulating gland, preputial gland, mammary gland, nervous system and hematopoietic system.

Thyroid The incidence of follicular hyperplasia in Group 1 treated with S.OPP (25%, 5 of 20 rats) was significantly higher than that in Group 2 treated with MNU alone (0%, 0 of 23 rats). The incidences of adenoma and

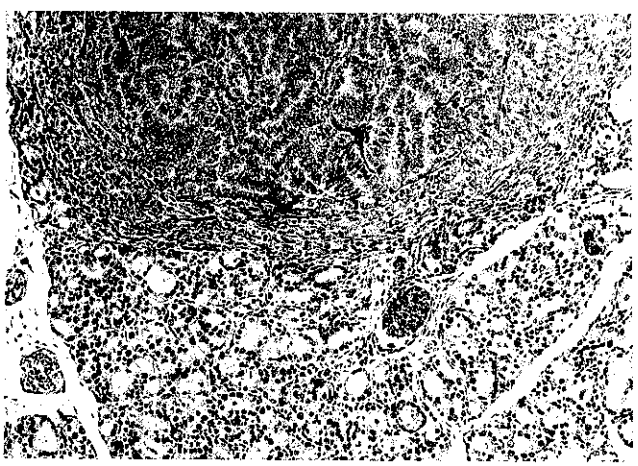

Fig. 2. Adenocarcinoma in the thyroid of a rat treated with DHPN after MNU at week 20 (×100).

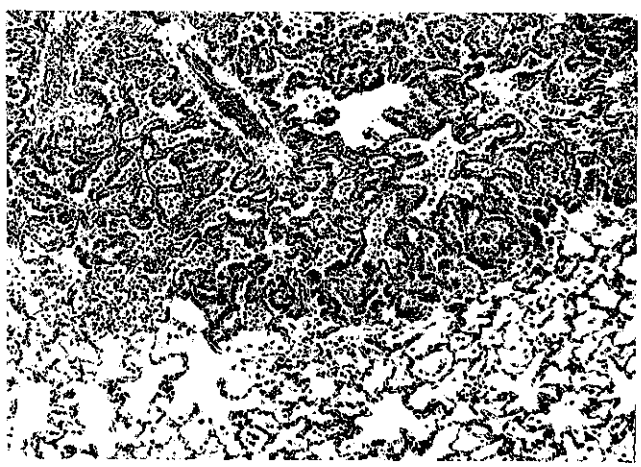

Fig. 3. Adenomatous hyperplasia in the lung of a rat treated with DHPN after MNU at week 20 (×75).

Table II. Lesions in the Thyroid and Lung

Group	Treatment	Effective No. of rats	Thyroid			Lung		
			Hyperplasia	Adenoma	Adeno-carcinoma	Adenomatous hyperplasia	Adenoma	Cancer[a]
1	MNU-DMAB	5	0[b]	0	0	0	0	0
	MNU-DBN	15	1 (6.7)	0	0	3 (20)	2 (13.3)	0
	MNU-DHPN	11	2 (18.2)	2 (18.2)	2 (18.2)	6 (54.5)[d]	2 (18.2)	3 (27.3)[b]
	MNU-DES	20	0	0	0	0	1 (5)	0
	MNU-S.OPP	20	5 (25)[c]	2 (10)	0	2 (10)	0	0
	MNU-Captafol	25	1 (4)	0	0	3 (12)	0	0
2	MNU	23	0	1 (4.3)	0	0	0	0
3	DMAB	14	0	0	0	0	0	0
	DBN	10	0	0	0	0	0	0
	DHPN	11	5 (45.5)	3 (27.3)	1 (9.1)	2 (18.2)	3 (27.3)	0
	DES	15	0	0	0	0	0	0
	S.OPP	14	0	0	0	0	0	0
	Captafol	15	0	0	0	0	0	0

a) Adenocarcinoma and squamous cell carcinoma.
b) Numbers of rats with lesions (percentage).
Significantly different from Group 2 (MNU alone) at *c*) $P<0.05$, *d*) $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *e*) $P<0.05$, *f*) $P<0.01$.

Table III. Lesions in the Liver and Pancreas

Group	Treatment	Effective No. of rats	Liver		Pancreas	
			GST-P-positive foci		Basophilic	Acidophilic
			(No./cm^2)	(mm^2/cm^2)	foci	foci
1	MNU-DMAB	5	12.000±4.959[c, e]	0.214±0.121[b]	4 (80)[a, c]	0
	MNU-DBN	15	35.617±16.903[c]	1.726±1.854[c]	0	0
	MNU-DHPN	11	14.803±7.074[c, d]	0.276±0.158[c, d]	1 (9.1)	0
	MNU-DES	20	4.634±3.560[c, e]	0.116±0.129[c, e]	0	0
	MNU-S.OPP	20	0.260±0.385[e]	0.014±0.048	0	1 (5)
	MNU-Captafol	25	0.357±0.416[b]	0.004±0.005[b]	1 (4)	0
2	MNU	23	0.115±0.284	0.001±0.004	0	0
3	DMAB	14	3.140±4.722	0.075±0.171	14 (100)	0
	DBN	10	47.892±25.619	2.701±1.801	0	0
	DHPN	11	7.672±5.630	0.124±0.111	0	0
	DES	15	0.077±0.203	0.001±0.003	0	0
	S.OPP	14	0.000±0.000	0.000±0.000	0	0
	Captafol	15	0.190±0.294	0.002±0.004	0	0

a) Numbers of rats with lesions (percentage).
Significantly different from Group 2 (MNU alone) at *b*) $P<0.05$, *c*) $P<0.01$.
Significantly different from Group 3 (test chemical alone) at *d*) $P<0.05$, *e*) $P<0.01$.

adenocarcinoma did not significantly differ among the groups. However, the total tumor incidence, consisting of adenomas plus adenocarcinomas (Fig. 2) was significantly increased in Group 1 treated with DHPN (36.4%, 4 of 11 rats) as compared with Group 2 treated with MNU alone (4.3%, 1 of 23 rats) (Table II).

Lung The incidence of preneoplastic adenomatous hyperplasias (Fig. 3) was significantly higher in Group 1 treated with DHPN (54.5%, 6 of 11 rats) than in Group 2 treated with MNU alone (0%, 0 of 23 rats). Three histologically different types of neoplastic lesions were observed, adenoma, adenocarcinoma and squamous cell carcinoma. The incidence of carcinomas including both adenocarcinoma and squamous cell carcinoma varieties in Group 1 treated with DHPN (27.3%, 3 of 11 rats) was also significantly higher than that in Group 2 given MNU alone (0%, 0 of 23 rats) (Table II).

Liver Immunohistochemically demonstrated GST-P-positive liver cell foci were used as preneoplastic endpoint lesions. The numbers per unit area (cm^2) were significantly increased in rats treated with MNU plus DMAB, DBN, DHPN, DES or captafol (Group 1) as compared to the MNU-alone value (Group 2). Similarly the mean areas of GST-P-positive foci were significantly increased in rats treated with MNU plus DMAB, DBN, DHPN, DES or captafol (Table III). When Group 1 animals were compared with Group 3 given test chemicals alone, the numbers as well as areas of GST-P-positive foci in Group 1 were significantly higher than those in Group 3 treated with test chemical alone in the DHPN and DES cases. Similarly the number of GST-P-positive foci in Group 1 given MNU+DMAB was significantly higher than in Group 3 treated with DMAB alone.

Fig. 4. Hyperplasia in the forestomach of a rat treated with DBN after MNU at week 20 (×100).

Pancreas Two histologically different focal preneoplastic lesions, one basophilic foci and the other acidophilic, were observed in pancreatic tissue.[25] The incidence of basophilic foci was significantly higher in Group 1 treated with MNU+DMAB (80%, 4 of 5 rats) than in

Table IV. Lesions in the Esophagus, Forestomach and Small Intestine

Group	Treatment	Effective No. of rats	Esophagus			Forestomach			Small intestine	
			Hyperplasia	Papilloma	SCC[a]	Hyperplasia	Papilloma	SCC[a]	Adenoma	Adeno-carcinoma
1	MNU-DMAB	5	0[b]	0	0	5 (100)[f]	2 (40)	0	5 (100)[d,f]	1 (20)
	MNU-DBN	15	15 (100)[d]	5 (33.3)[c]	0	15 (100)[d,f]	3 (20)	2 (13.3)	8 (53.3)[f]	1 (6.6)
	MNU-DHPN	11	11 (100)[d]	4 (36.4)[c]	1 (9.1)	11 (100)[c,f]	2 (18.2)	0	9 (81.8)[d,f]	2 (18.2)
	MNU-DES	20	0	0	0	19 (95)[d,f]	2 (10)	1 (5)	0	1 (5)
	MNU-S.OPP	20	2 (10)	0	0	20 (100)[d,f]	5 (25)	0	5 (25)	2 (10)
	MNU-Captafol	25	4 (16)	0	0	25 (100)[d]	4 (16)	1 (4)	10 (40)[f]	2 (8)
2	MNU	23	2 (8.7)	1 (4.3)	0	13 (56.5)	2 (8.7)	0	6 (26.1)	1 (4.3)
3	DMAB	14	0	0	0	0	0	0	0	1 (7.1)
	DBN	10	8 (80)	2 (20)	0	5 (50)	0	0	0	0
	DHPN	11	11 (100)	2 (18.2)	1 (9.1)	1 (9.1)	0	0	0	0
	DES	15	0	0	0	0	0	0	0	0
	S.OPP	14	0	0	0	0	0	0	0	0
	Captafol	15	0	0	0	14 (93.3)	0	0	0	0

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

Table V. Lesions in the Kidney and Urinary Bladder

Group	Treatment	Effective No. of rats	Kidney (pelvis)			Urinary bladder		
			PN hyperplasia	Papilloma	TCC[a]	PN hyperplasia	Papilloma	TCC[a]
1	MNU-DMAB	5	0[b]	0	0	4 (80)[d,f]	0	0
	MNU-DBN	15	1 (6.7)	0	0	10 (66.7)[d,e]	1 (6.7)	0
	MNU-DHPN	11	1 (9.1)	0	1 (9.1)	3 (27.3)[c]	0	0
	MNU-DES	20	0	0	0	0	0	0
	MNU-S.OPP	20	8 (40)[d,e]	2 (10)	0	16 (80)[d,f]	6 (30)[d,e]	4 (20)[c]
	MNU-Captafol	25	0	0	0	0	0	0
2	MNU	23	0	0	0	0	0	0
3	DMAB	14	0	0	0	0	0	0
	DBN	10	0	0	0	2 (20)	0	0
	DHPN	11	0	0	0	1 (9.1)	0	0
	DES	15	0	0	0	0	0	0
	S.OPP	14	0	0	0	4 (28.6)	0	0
	Captafol	15	0	0	0	0	0	0

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

Group 2 treated with MNU alone (0%, 0 of 23 rats) (Table III).

Esophagus The incidences of hyperplasia were significantly higher in Group 1 animals treated with DBN (100%, 15 of 15 rats) and DHPN (100%, 11 of 11 rats) than in Group 2 given MNU alone (8.7%, 2 of 23 rats). The incidences of papilloma were also significantly increased in Group 1 treated with DBN (33.3%, 5 of 15 rats) and DHPN (36.4%, 4 of 11 rats) as compared to the Group 2 value (4.3%, 1 of 23 rats) (Table IV).

Forestomach The incidences of hyperplasia (Fig. 4) were significantly increased in Group 1 (DBN, DHPN,

DES, S.OPP) as compared with Group 2, and also with the respective Group 3 rats. The incidence of hyperplasia in Group 1 animals treated with captafol was significantly higher than that in Group 2, though there was no significant difference between Groups 1 and 3. No significant intergroup differences with regard to papilloma and squamous cell carcinoma incidences were observed (Table IV).

Small intestine The incidences of adenomas were significantly increased in Group 1 animals treated with DMAB (100%, 5 of 5 rats) and DHPN (81.8%, 9 of 11 rats) as compared with that in Group 2 (26.1%, 6 of 23 rats), and also with the respective Group 3, test chemical-alone values (Table IV).

Kidney (renal pelvis) The incidence of papillary or nodular hyperplasia (PN hyperplasia) in Group 1 treated with S.OPP (40%, 8 of 20 rats) was significantly higher than that in Group 2 (0%, 0 of 23 rats), and also that in Group 3 (0%, 0 of 14 rats). The incidences of papillomas and transitional cell carcinomas did not significantly differ among the groups (Table V).

Urinary bladder The incidences of papillary or nodular hyperplasia (PN hyperplasia) were significantly increased in Group 1 animals treated with DMAB (80%, 4 of 5 rats), DBN (66.7%, 10 of 15 rats), DHPN (27.3%, 3 of 11 rats) or S.OPP (80%, 16 of 20 rats) compared to the Group 2 value (0%, 0 of 23 rats). The incidences of this preneoplastic lesion in the cases of DMAB, DBN and S.OPP after MNU were also higher than in the respective Group 3 animals. The incidence of papillomas was significantly increased in Group 1 treated with S.OPP (30%, 6 of 20 rats) compared to that in Group 2 (0%, 0 of 23 rats), and also that in Group 3 treated with S.OPP alone (0%, 0 of 14 rats). The incidence of transitional cell carcinoma (Fig. 5) in Group 1 treated with S.OPP was significantly higher than that in Group 2 (Table V).

Other tissues Tumors developed in many organs other than those listed in Tables II to V. Included were the tongue (papilloma, squamous cell carcinoma), ear duct (sebaceous carcinoma), large intestine (adenoma, adenocarcinoma), kidney (adenoma), prostate (carcinoma), preputial gland (adenocarcinoma), mammary gland (adenocarcinoma), nervous system (neurofibrosarcoma, ganglioneuroma) and hematopoietic system (leukemia, malignant lymphoma). These appeared not to be affected by treatment with any of the 6 chemicals tested.

DISCUSSION

The results of the present study clearly demonstrated the usefulness of *in vivo* medium-term multiple organ initiation systems for assessing modifying effects of chemicals on carcinogenesis in unknown target organs. After multi-organ initiation by MNU, all 6 chemicals tested induced preneoplastic and neoplastic lesions in their own target organs by 20 weeks. Thus, a positive influence was exerted in the liver, pancreas, small intestine and urinary bladder with DMAB; liver, esophagus, forestomach and urinary bladder with DBN; thyroid, lung, liver, esophagus, forestomach, small intestine and urinary bladder with DHPN; liver and forestomach with DES; and thyroid, forestomach, kidney and urinary bladder with S.OPP; and liver and forestomach with captafol (Table VI). The findings were essentially in line with those reported previously, as indicated by the arrows in Table VI with or without asterisks. Thus DMAB induced tumors in the liver, pancreas, small intestine and urinary bladder,[26-28] DBN in the liver, esophagus, forestomach

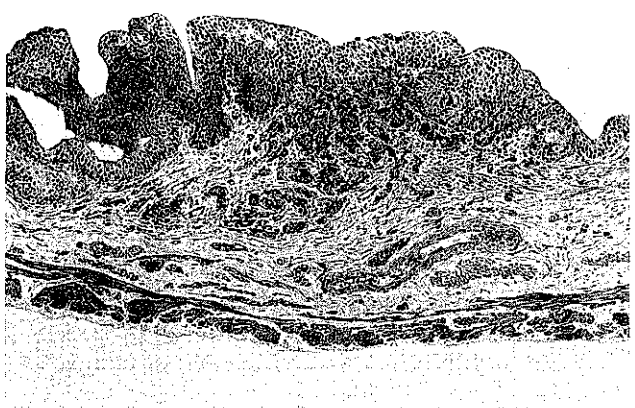

Fig. 5. Transitional cell carcinoma in the urinary bladder of a rat treated with S.OPP after MNU at week 20 (×75).

Table VI. Summary of the Experimental Findings

Organ	Test chemicals					
	DMAB	DBN	DHPN	DES	S.OPP	Captafol
Thyroid	—	—	↑*	—	Δ*	—
Lung	—	—	↑*	—	—	—
Liver	↑	↑*	↑	↑	—	↑*
Pancreas	↑*	—	—	—	—	—
Esophagus	—	↑*	↑*	—	—	—
Forestomach	—	↑	Δ	Δ	Δ	Δ*
Small intestine	↑	—	Δ	—	—	—
Kidney (pelvis)	—	—	—	—	Δ	—
Urinary bladder	↑	↑	↑*	—	↑	—

—, No effect.
↑, Enhancement.
Δ, Enhancement (not previously reported).
*, Independent of MNU treatment.

and urinary bladder,[11, 29] DHPN in the thyroid, lung, liver, esophagus and urinary bladder,[10, 30, 31] DES in the liver,[5] S.OPP in the urinary bladder[32, 33] and captafol in the liver.[34] The incidences of lesions in target organs such as the pancreas with DMAB, the liver and esophagus with DBN, the thyroid, lung and urinary bladder with DHPN, and the liver with captafol, unexpectedly did not differ between animals given MNU plus test chemicals (Group 1) and these receiving test chemicals alone (Group 3) (Table VI; arrows with asterisks). This is presumably due to strong carcinogenic potential masking the effects of MNU.

It is noteworthy that this system demonstrated development of preneoplastic and neoplastic lesions in organs for which carcinogenicity has previously been unknown. For example in the forestomach and small intestine with DHPN, in the forestomach with DES and in the forestomach and kindney (renal pelvis) with S.OPP after treatment with MNU (Table VI; open arrowheads). To the best of our knowledge, this is the first observation of promoting activities in these organs with these chemicals. In some organs (Table VI; open arrowheads with asterisks), such as the thyroid with S.OPP and the forestomach with captafol, the incidences of such preneoplastic and neoplastic lesions did not significantly differ between Groups 1 and 3. Captafol induced tumors in the forestomach of mice after 96 weeks of treatment[35] as well as forestomach preneoplastic lesions such as basal cell hyperplasia and squamous cell hyperplasia in rats after 104 weeks.[34] Our results also suggest that captafol exerts modifying potential in the forestomach. The MNU step strongly enhanced this effect and allowed detection of such weak carcinogenic potential in a much shorter time period.

Induction of measurable lesions in known target organs was, however, not complete for some test chemicals. For example, DMAB did not induce neoplastic lesions in the large intestine, prostate, preputial gland, ear duct, skin or lung[26, 27] and captafol did not induce neoplastic lesions in the kidney,[34] this presumably being due to the short duration of the experiment. Shirai *et al.*[10] found that 0.2% DHPN caused neoplastic lesions to develop in the kidney after 52 weeks, but in addition to the shorter time span, the concentration of DHPN was also lower (0.1%) in the present study. Similarly, while DES did not induce mammary gland and pituitary gland neoplastic lesions in contrast to the findings from a 2-year long-term carcinogenicity test,[36] this might have been associated with the difference in strain used: F344 rats in the present study as compared to Sprague-Dawley rats in the long-term carcinogenicity test.

In a recent study Ito *et al.*[5] reported on the validity of using immunohistochemically demonstrated liver GST-P-positive foci, showing good conformity with gamma-glutamyl transpeptidase positivity, as the end point in a two-stage carcinogenesis model. Accordingly, the results confirmed possible carcinogenicity of MNU for the liver. Recent studies indicated that by using enzyme histochemical or immunohistochemical techniques, for example pepsinogen isozyme 1 in the glandular stomach[37] and adenosine triphosphatase in the pancreas,[38] preneoplastic lesions in other organs could also be utilized for early detection of hazard. Establishment of the minimum time period necessary for generation of reliable results is clearly a high priority.

In conclusion, the present results indicate that pretreatment of rats with MNU could be effectively used to allow detection of the modifying effects of various chemicals, because it enhances neoplastic development in a wide range of organs such as the thyroid, lung, liver, pancreas, esophagus, forestomach, small intestine, kidney and urinary bladder.

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