—RAPID COMMUNICATION—

Jpn. J. Cancer Res. (Gann)
79, 413–417; April, 1988

WIDE-SPECTRUM INITIATION MODELS: POSSIBLE APPLICATIONS TO MEDIUM-TERM MULTIPLE ORGAN BIOASSAYS FOR CARCINOGENESIS MODIFIERS

Nobuyuki ITO, Katsumi IMAIDA, Hiroyuki TSUDA, Masa-aki SHIBATA, Toyohiko AOKI, Joao Lauro V. de CAMARGO and Shoji FUKUSHIMA

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

Two wide-spectrum initiation models were investigated in F344 male rats. Model I: After sequential treatment with diethylnitrosamine (DEN), N-methylnitrosourea (MNU) and dihydroxy-di-N-propylnitrosamine (DHPN), animals were given phenobarbital (PB) or N, N-dibutylnitrosamine (DBN) as a test compound for 14 weeks and sacrificed at week 18. Model II: Animals were treated with DHPN, followed by N-ethyl-N-hydroxyethylnitrosamine (EHEN), and then 3,2′-dimethyl-4-aminobiphenyl (DMAB) as initiators and were subsequently given 3-methylcholanthrene (MCA) or PB as a test compound for 11 weeks. Animals were sacrificed 16 weeks after the commencement. In both models, assessment of lesion yield revealed significant enhancement of carcinogenesis by the test compounds in their respective target organs. Since, in many cases, treatment with PB, DBN and MCA subsequent to the combined initiation procedures brought about a marked increase in lesion development, far greater than a simple sum of the yields given by initiators and test compounds alone, the presently described approach appears promising for development of medium-term bioassay systems for detection of environmental carcinogens.

Key words: Multiple organs — Bioassay system — Carcinogens — Promoters — Neoplastic change — Rat

In vivo assays, based on the two-stage concept of carcinogenesis,[1,2] have been developed to assess the promoting potential of short-term treatment with carcinogenic and non-carcinogenic chemicals on carcinogenesis of several organs, including the urinary bladder,[3] stomach[4] and liver. However, the majority of these assays concern only one organ. In the liver, the altered cell foci which are believed to be preneoplastic changes are easily recognizable because of their strongly increased expression of the placental form of glutathione S-transferase (GST-P). Although GST-P-positive foci have been widely used in short-term assay systems[5–7] to investigate the modifying effects of test chemicals on hepatocarcinogenesis, the fact that not all compounds which exert carcinogenic or promoting effects act on the liver limits the efficacy of this approach. The present paper reports on two different models for multiple organ initiation which were investigated for detection of modification of lesion development by test chemicals in various target organs. Sequential treatment with potent carcinogens having different wide-spectrum initiating activities was used as the initiation step. DEN is well known to be a strong initiator of hepatocarcinogenesis, whereas DHPN has thyroid, lung, kidney, and urinary bladder as target organs in rats. MNU also initiates thyroid and urinary bladder carcinogenesis and in addition causes tumor development in the hematopoietic system. PB and DBN were chosen as test compounds in model I and PB and MCA in model II, since their modification potential has been the subject of a number of earlier reports.[8–13]

Model I: A total of 65 6-week-old male F344 rats (Charles River Japan Inc., Atsugi) were used. Animals were divided into 3 groups. Groups 1 and 2 were treated sequentially with DEN (100 mg/kg, ip, single dose at commencement), MNU (20 mg/kg, ip, in citrate-buffered solution adjusted to pH 6.0, 4 doses at days 2, 5, 8 and 11), and DHPN (0.1% in drinking water, during weeks 3 and 4). After this initiating procedure, Groups 1 and 3 were fed the test compound, PB at 0.05% in the diet or DBN at

0.005% in the drinking water. Group 2 was given basal diet and tap water after the initiation treatment and served as a control. Group 3 received vehicles without carcinogens during the initiation period. All animals were sacrificed at week 18, and the main organs were excised and fixed in buffered formalin, then hematoxylin and eosin-stained sections

Table I. The Numbers and Areas of GST-P$^+$ Foci in the Livers of F344 Male Rats in Model I (Initiation by DEN, MNU and DHPN) and Model II (Initiation by DHPN, EHEN and DMAB)

Group	Initiation	Test chemical	No. of rats	GST-P$^+$ foci[a]	
				No./cm^2	Area (mm^2/cm^2)
Model I					
1	DEN+MNU+DHPN	PB	15	5.26±1.76**	0.29±0.12**
1	DEN+MNU+DHPN	DBN	15	0.19±0.52	0.02±0.02
2	DEN+MNU+DHPN	Control	15	0.47±0.46	0.05±0.09
3	—	PB	10	0	0
3	—	DBN	10	0	0
Model II					
1	DHPN+EHEN+DMAB	PB	15	21.78±10.67***	1.49±0.92***
1	DHPN+EHEN+DMAB	MCA	16	11.57±5.12***	0.69±0.34***
2	DHPN+EHEN+DMAB	Control	15	4.36±2.01	0.24±0.14
3	—	PB	5	0	0
3	—	MCA	5	0	0

a) Mean±SD.
** Statistically different from the respective control group at $P<0.01$.
*** Statistically different from the respective control group at $P<0.001$.

Table II. Incidences of Neoplastic Lesions in Lung, Thyroid, Esophagus, Forestomach and Large Intestines of Rats in Model I

Group	Initiation	Test chemical	Effective no. of rats	Lung (%)	
				Hyperplasia	Adenoma
1	DEN+MNU+DHPN	PB	15	14 (93)	4 (27)
1	DEN+MNU+DHPN	DBN	15	15 (100)	7 (47)*
2	DEN+MNU+DHPN	Control	15	15 (100)	1 (7)
3	—	PB	10	1 (10)	0 (0)
3	—	DBN	10	1 (10)	0 (0)

* Statistically different from the control group at $P<0.05$.
** Statistically different from the control group at $P<0.01$.
*** Statistically different from the control group at $P<0.001$.

Table III. Incidences of Neoplastic Lesions in Lung, Thyroid, Kidney, Pancreas and Urinary Bladder Tissues of Rats in Model II

Group	Initiation	Test chemical	Effective no. of rats	Lung (%)	
				Hyperplasia	Adenoma
1	DHPN+EHEN+DMAB	MCA	16	16 (100)	7 (44)*
1	DHPN+EHEN+DMAB	PB	14	14 (100)	1 (7)
2	DHPN+EHEN+DMAB	Control	15	15 (100)	1 (7)
3	—	MCA	5	0 (0)	0 (0)
3	—	PB	5	0 (0)	0 (0)

* Statistically different from the control group at $P<0.05$.

were examined for histopathological lesion development. Liver fixed in ice-cold acetone was also used for the quantitative assessment of immunohistochemically stained GST-P-positive foci (number and area/cm^2) using an image analyzer (Olympus VIP-21C).[6] Student's t test and Fischer's exact probability test were used in the statistical evaluation of frequency and incidence data, respectively.

Model II: A total of 55 F344 male rats were divided into 3 groups. Groups 1 and 2 were consecutively treated with 3 different carcinogens, DHPN (1000 mg/kg, ip, 2 doses in week 1), EHEN (1500 mg/kg, ig, 2 doses in week 2) and DMAB (75 mg/kg, sc, 2 doses in week 3) for initiation. Starting one week later, Group 1 rats were given 0.05% PB or 0.02% MCA in the diet. Group 2 rats were given carcinogens as in Group 1 but did not receive PB or MCA. Group 3 rats were given vehicles only then fed the respective test chemicals. At the end of week 16 of the experiment, surviving animals were sacrificed and all organs were carefully examined as for Model I.

In Model I, the number and the area of the GST-P-positive foci in the liver were significantly increased by the PB treatment as compared to Group 2 values (Table I). The incidences of thyroid hyperplasia and adenoma in PB-treated animals were also significantly higher than those of controls [87% ($P<0.01$) and 47% ($P<0.01$), respectively, Table II]. In Group 1, the incidence of lung adenoma was significantly increased by DBN treatment (47%, $P<0.05$), as were those of hyperplasia and papilloma of the esophagus [47% ($P<0.01$) and 87% ($P<0.001$), respectively], and forestomach hyperplasia (27%, $P<0.05$, Table II).

In Model II, the number and area of GST-P-positive liver foci were significantly increased by both MCA and PB treatments (see Table I). The incidence of lung tumors (adenomas) in the MCA-treated group was 44%, this being significantly higher than the control group value (7%, $P<0.05$, Table III). PB treatment also significantly increased the incidence of thyroid hyperplasia (33%, $P<0.05$).

The results of the present experiments clearly demonstrated enhancing effects of PB in the liver and thyroid gland, of DBN in the esophagus and forestomach and of MCA in

Thyroid (%)		Esophagus (%)		Forestomach (%)		Intestines (%)	
Hyperplasia	Adenoma	Hyperplasia	Papilloma	Hyperplasia	Papilloma	Hyperplasia	Adenoma
13 (87)**	7 (47)**	0 (0)	1 (7)	0 (0)	0 (0)	0 (0)	1 (7)
4 (27)	2 (13)	7 (47)**	12 (80)***	4 (27)*	3 (20)	1 (7)	0 (0)
4 (27)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7)	1 (7)
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Thyroid (%)	Kidney (%)		Pancreas (%)		Urinary bladder (%)
Hyperplasia	Altered tubules	Adenoma	Basophilic foci	Hyperplasia	PN hyperplasia
0 (0)	7 (44)	0 (0)	2 (13)	0 (0)	0 (0)
5 (33)*	5 (33)	2 (13)	1 (7)	1 (7)	1 (7)
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

the lung and liver. These findings are in clear agreement with earlier reports[8-13] and, although more experiments are necessary to optimize the observation period and dose of initiating carcinogens, they do suggest that the models used are very sensitive for detection of modifying effects of the test chemicals investigated. While DBN itself is carcinogenic, no neoplastic changes were observed in animals receiving this test compound without initiation (Group 3 in Model II). Thus, the initiation procedures that induced lesions in multiple organs were necessary to sensitize the animals for detection of enhancing and/or modifying effects. The present data after administration of different wide-spectrum carcinogens indicate the potential of their use for development of medium-term bioassay systems for environmental carcinogens to supplement those already established for the liver.[6,7] The probability that medium-term multiple organ bioassay systems are capable of detecting not only hepatocarcinogens but also carcinogens acting in other organs warrants further investigation.

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan, a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan, and a grant from the Society for Promotion of Pathology of Nagoya, Japan.

(Received Dec. 28, 1987/Accepted Feb. 26, 1988)

REFERENCES

1) Ito, N., Tsuda, H., Hasegawa, R. and Imaida, K. Comparison of the promoting effects of various agents in induction of preneoplastic lesions in rat liver. *Environ. Health Perspect.*, **50**, 131–138 (1983).
2) Bannasch, P., Griesemer, R. A., Anders, F., Becker, R., Cabral, J. R., Della Porta, G., Feron, V. J., Henschler, D., Ito, N., Kroes, R., Magee, P. N., McKnight, B., Mohr, U., Montesano, R., Napalkov, N. P., Nesnow, S., Roberfroid, M., Slaga, T., Turusov, Wilbourn, J. and Williams, G. M. Assays for initiating and promoting activities. *In* "Long-term and Short-term Assays for Carcinogens: A Critical Appraisal," ed. R. Montesano, H. Bartsch, H. Vainio, J. Wilbourn and H. Yamasaki, IARC Scientific Publications No. 83, pp. 103–126 (1986). Oxford University Press, London.
3) 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).
4) Tatematsu, M., Furihata, C., Katsuyama, T., Mera, Y., Inoue, T., Matsushima, T. and Ito, N. Immunohistochemical demonstration of pyloric gland-type cells with low-pepsinogen isozyme 1 in preneoplastic and neoplastic tissues of rat stomachs treated with N-methyl-N′-nitro-N-nitrosoguanidine. *J. Natl. Cancer Inst.*, **78**, 771–777 (1987).
5) Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, N. The placental form of glutathione *S*-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gann*, **75**, 199–202 (1984).
6) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **82**, 3964–3968 (1985).
7) Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase-P form positive foci in rats — an approach for a new medium-term bioassay system. *Carcinogenesis*, **9**, 387–394 (1988).
8) Kitagawa, T. and Sugano, H. Enhancing effect of phenobarbital on the development of enzyme-altered islands and hepatocellular carcinomas initiated by 3′-methyl-4-(dimethylamino)azobenzene or diethylnitrosamine. *Gann*, **69**, 679–687 (1978).
9) 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-methylnitrosourea. *Cancer Res.*, **43**, 3292–3296 (1983).

10) Imaida, K. and Wang, C. Y. Effect of sodium phenobarbital and sodium saccharin in AIN-76A diet on carcinogenesis initiated with *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide and N,N-dibutylnitrosamine in male F344 rats. *Cancer Res.*, **46**, 6160–6164 (1986).
11) Druckrey, H., Preussmann, R., Ivankovic, S., Schmidt, C. H., Mennel, H. D. and Stahl, K. W. Selektive Erzeugung von Blasenkrebs an Ratten durch Dibutyl- und N-Butyl-N-butanol(4)-nitrosamine. *Z. Krebsforsch.*, **66**, 280–290 (1964).
12) Pitot, H., Barsness, L., Goldsworthy, T. and Kitagawa, T. Biochemical characterization of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine. *Nature*, **271**, 456–458 (1978).
13) Tatematsu, M., Nakanishi, K., Murasaki, G., Miyata, Y., Hirose, M. and Ito, N. Enhancing effect of inducers of liver microsomal enzymes on induction of hyperplastic liver nodules by N-2-fluorenylacetamide in rats. *J. Natl. Cancer Inst.*, **63**, 1411–1416 (1979).