Jpn. J. Cancer Res. 80, 632–636, July 1989

Dose-Response Carcinogenicity in Rats on Low-dose Levels of *N*-Ethyl-*N*-nitrosourethane

Akihiko Maekawa,[1] Hiroshi Onodera,[1] Yuko Matsushima,[1] Takaharu Nagaoka,[1] Asahi Todate,[1] Makoto Shibutani,[1] Yukio Kodama[2] and Yuzo Hayashi[1]

[1]*Division of Pathology and* [2]*Division of Toxicology, Biological Safety Research Center, National Institute of Hygienic Sciences, Kamiyooga 1-18-1, Setagaya-ku, Tokyo 158*

A dose-response study on the carcinogenicity of *N*-ethyl-*N*-nitrosourethane (ENUR) was undertaken to examine its effect at low doses. Six-week-old female F344 rats were divided into 5 groups, each consisting of 40 animals. ENUR was dissolved in distilled water at dose levels of 0 (control), 0.15, 0.6, 2.5 and 10 ppm, and rats were given these solutions *ad libitum* for 2 years. Significant increase of the total tumor incidences and shortening of the mean survival times were observed in groups given 2.5 and 10 ppm ENUR. In groups given 0.6 ppm or more ENUR, digestive tract tumors were induced dose-dependently. They were restricted to the upper digestive tract from the oral cavity to the forestomach, and were histologically squamous cell papillomas or carcinomas. Dose-related differences in the location and incidence of these tumors were found. The virtually safe doses (VSDs) calculated by using the Weibull, Logit and Probit models were 0.365×10^{-2}, 0.110×10^{-1} and 0.779×10^{-1} ppm, respectively. The VSDs estimated in the present study are discussed in comparison with those of other carcinogens.

Key words: *N*-Ethyl-*N*-nitrosourethane — Dose-response carcinogenicity — F344 rat — Upper digestive tract carcinoma — Virtually safe dose

Environmental chemicals have been suspected of being the most important cause of human cancers. For assessment of the risk of chemical carcinogens, it seems important to carry out quantitative estimation of the carcinogenicity of chemicals. As one method for this purpose, estimation of a relative potency index such as virtually safe dose (VSD) based on dose-response data in experimental animals has been proposed.[1-5] VSD is defined as a value corresponding to the dose level or range which can induce tumors at extremely low rates, such as 10^{-6} or 10^{-8}. This value can be obtained by downward extrapolation (low-dose extrapolation) of animal dose-response data by use of proper mathematical models.

Previously, we reported a dose-response carcinogenicity study of *N*-ethyl-*N*-nitrosourea (ENU) to estimate its VSD.[6] In the present study, *N*-ethyl-*N*-nitrosourethane (ENUR) which has a chemical structure analogous to ENU but a different organ specificity from ENU, was selected as a model compound, and a dose-response carcinogenicity study was conducted to estimate its VSD and compare it with that of ENU.

MATERIALS AND METHODS

Animals and diet Five-week-old female F344/DuCrj rats purchased from Charles River Japan Inc. (Kanagawa) were maintained on basal diet (CRF-1, Oriental Yeast Ind. Co., Tokyo) and tap water until they were 6 weeks old when the study was started. Previously, we reported carcinogenicity studies of *N*-propyl-[7] and *N*-butyl-*N*-nitrosourethane.[8] In these studies, no sex-difference was found in the location and incidence of induced tumors. Therefore, we used only female rats in the previous carcinogenicity study of *N*-ethyl-*N*-nitrosourethane[9,10] and also in the present study. Rats were housed five animals to a plastic cage and maintained in an air-conditioned animal room (25±2°C, 55±5% relative humidity). The basal diet was analyzed and was found to be free from contaminants such as pesticides, benzopyrene and aflatoxin. *N*-Nitroso compounds in the diet were also examined and a small amount of *N*-nitrosodimethylamine (mean amount; 3 ppb) was detected.

N-Ethyl-N-nitrosourethane *N*-Ethyl-*N*-nitrosourethane (ENUR) was purchased from Nakarai Chemical Ltd. (Kyoto) and kept in a refrigerator (4°C) before use.

Experimental design Rats were divided into 5 groups, each consisting of 40 animals. ENUR was dissolved in distilled water at levels of 0 (control), 0.15, 0.6, 2.5 and 10 ppm. ENUR solution was freshly prepared 3 times a week. Rats were given these solutions *ad libitum* from a light-proof bottle in place of drinking water for 2 years, and thereafter tap water was given to all groups and observation was continued until experimental week 112, when all survivors were killed.

During the experimental period, all animals were examined daily for abnormalities and mortality was recorded. Body weights were recorded once a week

during the first 4 weeks and then every 4 weeks. Moribund or dead animals were autopsied completely to determine the development of tumors in all organs and/or tissues. Tumor masses, and all organs and/or tissues were fixed in 10% buffered formalin and sections were stained routinely with hematoxylin and eosin.

RESULTS

The first rat with tumors was autopsied in week 43. Therefore, all rats that survived beyond this week were included in effective numbers, except for a few in which autolysis was too far advanced.

Tumor incidence and mean survival time Table I shows the incidences of all tumors and mean survival times of rats in each group. As shown in the table, the incidence of tumors was about 100% in the 2.5 and 10 ppm groups and significantly higher than that (77–83%) in the other 3 groups including the control group. An inverse dose-effect relation with the mean survival time was observed in treated groups given 0.6 ppm or more ENUR: that is, all animals died before the end of the study in the 10 ppm group, and the mean survival time in the 2.5 ppm group was also shorter than that in the other 3 groups. There was significant difference in the mean survival times between these two groups and the other 3 groups.

Table I. Tumor Incidence and Mean Survival Time of Female F344 Rats Given Low Doses of ENUR in the Drinking Water for 2 Years

Dose (ppm)	No. of rats			Mean survival time and range
	initial	effective	with tumors (%)	
0	40	40	33 (82.5)	109 (56–112)
0.15	40	39	30 (76.9)	105 (73–112)
0.6	40	38	31 (81.6)	109 (78–112)
2.5	40	39	39 (100.0)**	97*** (66–112)
10.0	40	38	37 (97.4)*	57*** (43–77)

Significantly different from the control group: * $P<0.05$, ** $P<0.01$ (chi-square test); *** $P<0.001$ (Student's t-test).

Organ distribution and histological type of tumors The organ distribution and histological types of the main tumors observed in each group are summarized in Table II. Tumors were observed mainly in the uterus, hematopoietic organs, mammary gland and endocrine organs in the control and treated groups except for the 10 ppm group, although their incidences differed in each group. In the 10 ppm group, the incidences of these tumors were very low. On the contrary, upper digestive tract tumors were observed with high incidence in treated groups given 0.6 ppm or more ENUR.

Histologically, the types of tumors observed in all organs, except for the upper digestive tract, were quite similar to those of spontaneous ones observed commonly in female F344 rats.[11] Upper digestive tract tumors were found in the region from the oral cavity to the forestomach, and all of them were histologically squamous cell papillomas or carcinomas.

Table III summarizes the location and type of upper digestive tract tumors in each group. In the 0.6 ppm group, almost all tumors were found in the forestomach. In the 2.5 and 10 ppm groups, however, esophageal tumors were the most frequent and the incidence of forestomach tumors decreased slightly. On the other hand, tumors of the oral cavity increased dose-dependently. In the oral cavity and esophagus, many of the tumors were carcinomas. In the forestomach, however, papillomas were more frequent than carcinomas.

Table II. Main Tumors Observed in Female F344 Rats Given Low Doses of ENUR in the Drinking Water for 2 Years

Location and type of tumors	Incidence of tumors (%)				
	0	0.15	0.6	2.5	10.0 ppm
Pituitary gl: adenoma/carcinoma	30	13	8*	0***	3**
Hematopoietic organs: leukemia	15	13	18	8	0*
Uterus: endometrial stromal polyp	18	21	24	23	0**
Mammary gl: fibroadenoma	15	15	11	3	0*
Thyroid gl: C-cell adenoma/carcinoma	5	3	8	5	0
Adrenal gl: pheochromocytoma	3	3	0	3	0
Pancreas: islet cell adenoma	5	3	3	3	0
Clitoral gl: adenoma	8	5	3	3	3
Lung: adenoma/carcinoma	3	5	0	0	0
Upper digestive tract: papilloma/carcinoma	3	0	42***	97***	97***

Significantly different from the control group (Fisher's exact probability test): * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table III. Localization and Histological Types of Tumors in the Upper Digestive Tract

Dose (ppm)	No. of rats examined	No. of rats with tumors								
		Oral cavity			Esophagus			Forestomach		
		H	P	C	H	P	C	H	P	C
0	40	0	0	0	0	1	0	0	0	0
0.15	39	0	0	0	0	0	0	0	0	0
0.6	38	1	0	0	1	1	1	12	13	2
2.5	39	1	1	6	1	9	26	7	14	18
10.0	38	6	6	17	5	6	23	9	19	9

H: hyperplasia, P: papilloma, C: carcinoma.

Table IV. Estimated Virtually Safe Doses (VSDs) for Upper Digestive Tract Carcinomas in F344 Rats at a Risk Level of 10^{-6}

	Weibull model	Logit model	Probit model
ENUR			
Chi-square value	0.166	0.070	0.005
P-value	0.920	0.966	0.998
VSD[a)]	0.365×10^{-2}	0.110×10^{-1}	0.779×10^{-1}
ENU			
VSD[a, b)]	0.343×10^{-1}	0.376×10^{-1}	
VSD[a, c)]	0.214×10^{-2}	0.332×10^{-2}	

a) Dose (ppm) in the drinking water.
b) VSD for digestive tract tumors in female F344 rats.[6)]
c) VSD for digestive tract tumors in male F344 rats.[6)]

In addition to these tumors, squamous cell hyperplasia was also commonly observed in these organs.

Estimation of virtually safe dose (VSD) To calculate the dose level of ENUR expected to induce squamous cell carcinomas of the upper digestive tract at a risk level of 10^{-6}, we adopted the Weibull model, Logit model and Probit model. Estimated VSDs of ENUR are shown in Table IV. The VSDs calculated by using these models were 0.365×10^{-2}, 0.110×10^{-1} and 0.779×10^{-1} ppm, respectively.

DISCUSSION

In female F344 rats, endometrial stromal polyp of the uterus, mononuclear cell leukemia, fibroadenoma of the mammary gland and adenoma/carcinoma of the endocrine organs are the most common spontaneous tumors, while tumor of the upper digestive tract is very rare. The organ distribution and incidence of tumors observed in the control group were similar to those in our historical data.[11)] In the control and 0.15 ppm groups, no marked differences in the organ distribution and incidences of tumors were found. On the contrary, in groups given 0.6 ppm or more ENUR, the incidence of upper digestive tract tumors increased dose-dependently. These results indicate that ENUR induced selectively upper digestive tract tumors, and administration of 10 ppm ENUR might mask the development of spontaneous tumors in many organs by eliminating animals early in the study because of selective induction of upper digestive tract tumors.

Previously, we reported carcinogenicity of ENUR in rats.[9, 10)] ENUR induced not only upper digestive tract tumors but also duodenal tumors in female Donryu rats, when high doses (25–100 ppm) of ENUR were given in the drinking water.[10)] In the present study, however, ENUR caused only development of upper digestive tract tumors and no duodenal tumor was found. The reason for the difference between the previous and present studies is considered to depend on the dose levels used, although the difference in the strain of rats used may also be a factor. In the previous study, the incidence of duodenal tumors was found to be dose-related. Dose levels used in the present study might be low for induction of duodenal tumors, because ENUR is a direct-acting carcinogen, and most of the ENUR presumably reacted with biomolecules of the upper digestive tract, leaving insufficient ENUR for induction of tumors in the duodenum. ENUR is oily and remains in the forestomach for the longest period when given in the drinking water. This is presumably the reason why tumors of the forestomach were most common in the 0.6 ppm group. On the other hand, tumors of the oral cavity were most frequently found in the 10 ppm group. In the 2.5 and 10 ppm groups, the incidences of esophageal tumors increased and those of forestomach tumors decreased slightly. These findings may support the view that higher doses of ENUR solution remain longer in the oral cavity and esophagus than low-dose solutions because of their viscosity. These dose-related differences in the location and incidence of the tumors were also observed in the previous study.[10)]

In the low-doses carcinogenicity study of ENU, the organ distribution and incidences of tumors observed were different from those in the high-doses study.[6] In the present low-doses carcinogenicity study of ENUR, the main target organ was restricted in the upper digestive tract, similar to that in the previous high-doses study.[10] It is well known that the chemical reactivity of ENUR *in vitro* with amines or amino acids is greater than that of ENU. This may be one of the reasons why the main target organ was restricted to the upper digestive tract in both studies, when ENUR was given orally in the drinking water.

In all models used for estimation of VSDs, the *P*-values were large (near 1.0), which indicated a good fit, although the calculated VSDs were slightly different in the three models. Estimated VSDs were compared with those of ENU reported previously.[6] As shown in Table IV, VSD of ENUR was almost the same as that of ENU for digestive tract tumors in females when the Logit model was used, although VSD of ENUR calculated by using the Weibull model was about 10 times lower than that of ENU. As mentioned above, no sex-difference was found in the incidence of upper digestive tract tumors, when *N*-alkyl-*N*-nitrosourethanes were given to both sexes of rats in the drinking water.[7,8] Therefore, we may speculate that VSD for upper digestive tract tumors in males is similar to that in females, although only female rats were used in the present study. VSD of ENUR was also close to that for *N*-nitrosodimethylamine (Weibull model, 1.9×10^{-2} ppm), but very higher than that for aflatoxin B_1 (Weibull model, 40×10^{-3} ppb), as reviewed by Oser.[12] These results indicate that these three alkylating *N*-nitroso compounds have almost the same carcinogenic potency *in vivo*, though their organ specificities are quite different.

It is well known that many mathematical models have been proposed for low-dose extrapolation, and VSDs differ greatly depending on the mathematical models used. At present, however, no single model is recognized as the most appropriate and no definite criteria have been established for selection of mathematical models.[13] Thus, further investigations on this problem are needed.

On the other hand, due to the increasing demand for safety evaluation of environmental chemicals, a number of chemicals have been tested for carcinogenicity during the last two decades. However, only a few selected carcinogens have been tested for estimation of VSD for many reasons, such as the limitations in manpower, budget, animal facilities, time, etc. Further hazard or dose-response assessment including estimation of VSD not only for genotoxic carcinogens but also for epigenetic carcinogens is needed, although fortunately ENUR used in the present study is not present in our environment.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan. Part of this work was presented at the 47th Annual Meeting of the Japanese Cancer Association in Tokyo, September, 1988.

(Received April 6, 1989/Accepted May 27, 1989)

REFERENCES

1) Mantel, N. and Schneiderman, M. A. Estimating "safe" levels, a hazardous undertaking. *Cancer Res.*, **35**, 1379–1386 (1975).
2) Fishbein, L. Overview of some aspects of quantitative risk assessment. *J. Toxicol. Environ. Health*, **6**, 1275–1296 (1980).
3) Munro, I. C. and Krewski, D. R. Risk assessment and regulatory decision making. *Food Cosmet. Toxicol.*, **19**, 549–560 (1981).
4) Carlborg, F. W. The threshold and the virtually safe dose. *Food Chem. Toxicol.*, **20**, 219–221 (1982).
5) Hayashi, Y., Kurokawa, Y., Maekawa, A. and Takahashi, M. Strategy of long-term animal testing for quantitative evaluation of chemical carcinogenicity. *In* "New Concepts and Developments in Toxicology," ed. P. L. Chambers, P. Gehring and F. Sakai, pp. 383–391 (1986). Elsevier Sci. Pub., Amsterdam.
6) Maekawa, A., Ogiu, T., Matsuoka, C., Onodera, H., Furuta, K., Kurokawa, Y., Takahashi, M., Kokubo, T., Tanigawa, H., Hayashi, Y., Nakadate, M. and Tanimura, A. Carcinogenicity of low doses of N-ethyl-N-nitrosourea in F344 rats; a dose-response study. *Gann*, **75**, 117–125 (1984).
7) Maekawa, A., Kamiya, S. and Odashima, S. Tumors of the upper digestive tract of ACI/N rats given N-propyl-N-nitrosourethan in the drinking water. *Gann*, **67**, 549–559 (1976).
8) Takeuchi, M., Kamiya, S. and Odashima, S. Induction of tumors of the forestomach, esophagus, pharynx, and oral cavity of the Donryu rat given N-butyl-N-nitrosourethan in the drinking water. *Gann*, **65**, 227–236 (1974).
9) Hirose, M., Maekawa, A., Kamiya, S. and Odashima, S. Carcinogenic effect of N-ethyl- and N-amyl-N-nitrosourethans on female Donryu rats. *Gann*, **70**, 653–662 (1979).
10) Onodera, H., Furuta, K., Matsuoka, C., Kamiya, S. and Maekawa, A. Carcinogenicities of l-ethyl- and l-amyl-l-nitrosourethans in female Donryu rats: dose-effect relations. *Gann*, **73**, 48–54 (1982).
11) Maekawa, A., Kurokawa, Y., Takahashi, M., Kokubo, T.,

Ogiu, T., Onodera, H., Tanigawa, H., Ohno, Y., Furukawa, F. and Hayashi, Y. Spontaneous tumors in F-344/DuCrj rats. *Gann*, **74**, 365–372 (1983).
12) Oser, B. L. The rat as a model for human toxicological evaluation. *J. Toxicol. Environ. Health*, **8**, 521–542 (1981).
13) U.S. Interagency Staff Group on Carcinogens. Chemical carcinogens: a review of the science and its associated principles. *Environ. Health Perspect.*, **67**, 201–282 (1986).