Jpn. J. Cancer Res. 85, 226–230, March 1994

Effects of Inhibition of O^6-Alkylguanine-DNA Alkyltransferase in Rats on Carcinogenesis by Methylnitrosourea and Ethylnitrosourea

William Lijinsky,[1,4] Anthony E. Pegg,[2] Miriam R. Anver[3] and Robert C. Moschel[1]

[1]Chemistry of Carcinogenesis Laboratory, ABL-Basic Research Program, and [3]Pathology/Histotechnology Laboratory, Program Resources, Inc./DynCorp., NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702, [2]Department of Cellular and Molecular Physiology, Penn State University College of Medicine, Hershey Medical Center, 500 University Drive, Hershey, PA 17033

Many alkylating agents are potent carcinogens and there is considerable evidence that the formation of O^6-alkylguanine in DNA can lead to mutations and the initiation of neoplastic growth. The repair of O^6-methyl- or O^6-ethylguanine in DNA is known to be brought about by the action of a protein termed O^6-alkylguanine-DNA alkyltransferase. In order to investigate the role of this activity in the carcinogenic action of methylnitrosourea and ethylnitrosourea, O^6-benzylguanine, a potent inhibitor of the alkyltransferase, was used. Groups of 20 female F344 rats were treated with the nitrosourea (0.2 mmol) by gavage in 10 weekly doses and a parallel group was also treated with 4 mg of O^6-benzylguanine, 2 h prior to each dose of the nitrosourea. This dose of O^6-benzylguanine was sufficient to reduce the alkyltransferase activity to zero in the liver for at least 8 h but activity had returned to about 60% of normal within 24 h. Animals were maintained until they became moribund, when they were killed, or until death related to tumors. The median week of death in the animals receiving methylnitrosourea was reduced from 60 wk to 52 wk by co-treatment with O^6-benzylguanine. There was a smaller reduction from 55 to 50 wk in the rats receiving ethylnitrosourea. The treatment with O^6-benzylguanine caused no significant change in the incidence of the principal tumors induced by the alkylnitrosoureas and there were no liver tumors produced by the combined treatments. These results show that the level of inactivation of alkyltransferase produced by this dose of O^6-benzylguanine was not sufficient to greatly alter the potent carcinogenic effect of these doses of alkylnitrosoureas in this system.

Key words: O^6-Alkylguanine-DNA alkyltransferase — Carcinogenesis — Methylnitrosourea — Ethylnitrosourea

A corollary of the hypothesis that cancer comes about through mutations in somatic cells caused by alkylation of DNA, which initiates the process, is that repair of the alkylated damage can obviate the initiation. The importance of DNA repair to the health of organisms exposed to radiation and other mutagens has been investigated for many years since the work of Hart and Setlow[1] and others. The effectiveness of DNA repair was related to longevity of species[1] and to organ-specific induction of cancer,[2] and both have become well-established concepts.

The experiments of Goth and Rajewsky[2] showed that the repair of DNA ethylated by exposure of rats to ethylnitrosourea was appreciably faster in the liver than in the brain, a result that correlated with the induction by ethylnitrosourea of brain tumors in rats, but not of liver tumors. The equating of this correlation with a cause/effect relation was questioned[3,4] and experiments in other species including hamsters[5] and gerbils[6] showed the same biochemical differential, but no susceptibility to tumors of the nervous system. Nevertheless, extensive studies of the nature and action of the O^6-alkylguanine-DNA alkyltransferase (AGT) repair protein[7] indicated the importance of DNA repair in carcinogenesis. The hypothesis could be further verified experimentally following discovery of the readiness with which the alkyltransferase protein could be inactivated by O^6-benzylguanine (BzlG).[8] Therefore, we have examined the effect of a series of small doses of BzlG on depletion of AGT and on tumorigenesis by multiple doses of two directly acting alkylating carcinogens, methylnitrosourea (MNU) and ethylnitrosourea (ENU). These were chosen because of the extensive background information we have accumulated from rats treated with them.[9,10] Groups of rats were treated with BzlG to inactivate the repair protein, followed 2 h later by treatment with one of the alkylnitrosoureas. This was repeated once a week for 10 weeks, after which the animals were observed until they died or were moribund because of tumors induced. Control groups of rats received parallel treatment with each of the alkylnitrosoureas and with BzlG alone. Additional rats treated with BzlG only were killed after 1 week, 5 weeks and 10 weeks and the level of alkyltransferase activity in the liver was determined to ensure that

[4] Present address: 5521 Woodlyn Road, Frederick, MD 21702, USA.

adaptive synthesis of the enzyme had not been induced by the treatments. Levels of alkyltransferase activity were measured at 4, 8 and 24 h after treatment with BzlG.

If AGT repair of O^6-substituted guanine residues in DNA can inhibit carcinogenesis by these alkylating carcinogens then rats treated with BzlG would be expected to show an increased susceptibility to tumorigenesis by the alkylnitrosourea treatments compared with the parallel groups not treated with BzlG, as measured by time-to-death with tumors or by incidence of tumors, or both. The present study was designed to test this proposition.

MATERIALS AND METHODS

Chemicals MNU and ENU were prepared as described previously[11] and were dissolved in ethyl acetate/corn oil (1:1) at concentrations of 10.5 and 12 mg/ml, respectively. BzlG was prepared as reported[8] and was dissolved in dimethylsulfoxide (DMSO) (1 part) and Cremophor (Sigma Chemical Company) (1 part) to which was added 8 parts of sterile water, forming a fine suspension containing 20 mg BzlG/ml.

Animal treatments Animals were female F344 rats of the colony of the Frederick Cancer Research and Development Center, born, bred and maintained within a barrier facility. They were 8 weeks old at the beginning of the experiment and were housed 4 to a polycarbonate cage. They were fed Ziegler NIH31 Rodent Chow and were given tap water *ad libitum*. Each experimental group for long-term studies consisted of 20 animals. Animal care was provided in accordance with procedures in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication number 86-23, 1985).

One group was given an intraperitoneal (i.p.) injection of 0.2 ml of BzlG suspension by syringe under light metophane anesthesia at approximately 8:00 a.m. A second group was given the same BzlG treatment followed 2 h later (approximately 10:00 a.m.) with 0.2 ml of MNU solution by gavage; this was repeated once a week for 10 weeks. Similarly a third group was treated with BzlG followed 2 h later with 0.2 ml of ENU solution by gavage, a protocol also repeated for ten weeks. The fourth and fifth groups of 20 rats were given 0.2 ml of MNU and ENU solutions, respectively, by gavage once a week for 10 weeks. A group of 20 controls were given 0.2 ml of DMSO/Cremophor/water (1:1:8) by i.p. injection followed by 0.2 ml of ethyl acetate/corn oil (1:1) by gavage once a week for 40 weeks. The group of rats given BzlG was also treated for 40 weeks to assess the possible adverse effects of prolonged treatment with BzlG.

Additional groups of solvent controls and BzlG controls were treated for one, 5 and 10 weeks for examination of AGT activity in liver at those times. Assays were performed as described by Domoradzki *et al.*[12] on three or more solvent controls and on two or more BzlG controls in animals killed 4 h after treatment and in two instances 24 h after treatment. Results are shown in Table I.

Few animals treated with ENU or MNU died during the 10 weeks of treatment. Rats that died during the 10-week treatment period were excluded from Tables II and III. The remaining rats in the 4 groups treated with the alkylnitrosoureas were allowed to die naturally or were killed when moribund. Rats of the BzlG control group and the solvent-treated controls were killed at week 85. Animals of all six groups were necropsied. Lesions and major organs and tissues were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histologic examination.

RESULTS

As shown in Table I the single i.p. treatment with 4 mg of BzlG was sufficient to inhibit almost completely alkyltransferase activity measured in the rat liver. This inhibition was reported to occur rapidly[8] and at 4 h in the present study, the activity was close to zero. At 8 h it was still almost immeasurable, but at 24 h approximately 60% of the original activity had regenerated. Measurement of alkyltransferase activity in liver of rats that had

Table I. Alkyltransferase Activities in Rat Liver Samples

Week	Treatment	Alkyltransferase activity (fmol/mg protein)	Mean
0	Control	48.4, 53.1, 70.4, 86.2	64.5±8.6
	+BzlG, 4 h	0.1, 0.1	0.1
	+BzlG, 8 h	0, 0.1	0.1
	+BzlG, 24 h	33.3, 47.6	40.4
1	Control	38.8, 45.6, 53.5	45.9
	+BzlG, 4 h	0, 7.2, 1.0	2.7
5	Control	46.0, 51.8, 55.4	51.1
	+BzlG, 4 h	0, 5.6, 0	1.9
10	Control	71.5, 61.8, 60.2, 58.9	63.1±5.7
	+BzlG, 4 h	0.5, 0, 0.1	0.2
	+BzlG, 24 h	42.2, 38.4, 36.4	39.0

Table II. Survival of Female Rats Treated with Alkylnitrosoureas and O^6-Benzylguanine

Treatment	Number of animals alive at week								
	10	20	30	40	50	60	70	80	90
MNU	18	18	18	16	14	9	3	0	
MNU+BzlG	20	20	19	16	12	4	2	1	0
ENU	18	18	18	15	13	5	2	0	
ENU+BzlG	18	18	16	12	9	4	1	0	
BzlG	20	20	20	20	20	20	20	18	0
Solvent control	20	20	20	20	20	20	20	19	0

been treated with BzlG for 1, 5 or 10 weeks gave the same result as that following a single BzlG treatment, so there seemed to be no adaptation to the inhibition of alkyltransferase by BzlG.

One of the criteria by which the potency of a carcinogen treatment can be judged is the rate at which animals die of tumors induced by the treatment; in a dose-response study the time to death is inversely related to the size of the dose of a carcinogen. Table II shows the number of animals surviving as a function of time in the groups treated with MNU or ENU, alone and combined with BzlG treatment. The six animals that died during the ten week treatment (two in three groups, because of technological problems) are omitted from the tables. Almost none of the BzlG-treated or solvent-treated controls died before termination at week 85.

In Table III are listed the tumors diagnosed in the groups of rats treated with MNU and ENU; in each case

Table III. Number and Types of Tumors in Female Rats Following Treatment with Alkylnitrosoureas and O^6-Benzylguanine

Tumors	Treatment					
	MNU	MNU/BzlG	ENU	ENU/BzlG	BzlG	Solvent
Nonglandular stomach						
Squamous cell papilloma	6/18[a](33)[b]	2/20 (10)	3/18 (17)	2/18 (11)	0	0
Squamous cell carcinoma	14/18 (78)	19/20 (95)	3/18 (17)	3/18 (17)	0	0
Mammary gland						
Fibroadenoma	7/18 (39)	4/20 (20)	9/18 (50)	4/18 (22)	4/20 (13)	3/20 (15)
Adenocarcinoma	0	4/20 (20)	8/18 (44)	11/18 (61)	0	1/20 (5)
Ovary						
Sertoli cell tumor	0	0	1/17 (6)	5/18 (28)	0	0
Other tumors	1/18 (5)	2/20 (10)	1/17 (6)	1/18 (6)	0	0
Uterus						
Stromal polyp/adenoma	2/18 (11)	1/20 (5)	6/17 (35)	4/16 (25)	6/20 (30)	4/20 (20)
Stromal sarcoma/adenocarcinoma	1/18 (5)	1/20 (5)	2/17 (12)	2/16 (13)	0	0
Hemangioma/hemangiosarcoma	0	0	2/17 (12)	4/16 (25)	1/20 (5)	0
Kidney						
Malignant mesenchymal tumor	3/17 (18)	0	0	0	0	0
Brain — glial tumors	2/18 (11)	4/20 (20)	5/18 (28)	4/18 (22)	1/20 (5)	0
Thyroid						
Follicular cell adenoma	1/16 (6)	1/16 (6)	0	0	0	0
Follicular cell carcinoma	0	1/16 (6)	2/15 (13)	0	0	0
C cell adenoma	0	0	4/15 (27)	1/14 (7)	0	2/19 (11)
C cell carcinoma	0	0	4/15 (27)	2/14 (14)	0	0
Lung						
Alveolar cell adenoma	0	1/20 (5)	2/18 (11)	3/18 (17)	0	0
Alveolar cell carcinoma	0	0	1/18 (6)	0	0	0
Liver						
Hepatocellular adenoma	0	1/20 (5)	0	1/18 (6)	0	2/20 (10)
Tongue/oral mucosa						
Squamous cell papilloma	3/18 (17)	2/20 (10)	4/18 (22)	2/18 (11)	1/20 (5)	0
Squamous cell carcinoma	1/18 (6)	1/20 (5)	0	0	0	0
Intestine						
Adenoma	0	0	4/18 (22)	1/18 (6)	0	0
Adenocarcinoma	0	0	1/18 (6)	0	0	0
Pituitary						
Adenoma/carcinoma	3/17 (18)	8/19 (42)	4/17 (24)	2/14 (14)	6/20 (30)	9/19 (47)
LGL leukemia	3/18 (17)	2/20 (10)	9/18 (50)	9/18 (50)	3/20 (15)	6/20 (30)
Schwannoma — various sites	2/18 (11)	3/20 (15)	0	2/18 (11)	0	0
Other	[a]	[b]	[c]	[d]	[e]	[f]

a) Number with tumor/number tissues examined. *b*) Percentage.

Other tumors: [a] Zymbal gland carcinoma 1, glandular stomach carcinosarcoma 1, fibrosarcoma 1, adrenal cortex adenoma 1; [b] Zymbal gland carcinoma 1, clitoral gland carcinoma 1, meningeal sarcoma 1, spleen hemangioma 1, esophagus carcinoma 1, adrenal pheochromocytoma 1, thymoma 1; [c] kidney lipoma 1, clitoral gland carcinoma 1, parathyroid adenoma 1, histiocytic sarcoma 1, adrenal cortex adenoma 1, adrenal medulla pheochromocytoma 1; [d] spleen hemangiosarcoma 1, histiocytoma 1, urinary bladder transitional cell papilloma 1; [e] pancreas islet cell carcinoma 1; [f] clitoral gland carcinoma 1, adrenal pheochromocytoma 1.

the group pretreated with BzlG is compared with the group without pretreatment. In the 4 groups all of the animals surviving the 10-week treatment had tumors induced by the alkylnitrosourea treatment (i.e., not usually seen in untreated control rats of this strain[13,14]). Many animals bore more than one such tumor.

The pattern of treatment-related tumors in this experiment was similar to those observed with MNU and ENU administered in somewhat different dose regimens in previous studies.[10] The incidences of the so-called "spontaneous" tumors, including large granular lymphocyte leukemia, anterior pituitary tumors, adrenal medulla pheochromocytomas, mammary gland fibroadenomas and thyroid C-cell tumors varied considerably from one group to another, as reported elsewhere to be true among untreated F344 rats, for reasons not understood.[14]

The small differences that were seen in tumor incidence included mammary adenocarcinomas in 4 rats treated with BzlG and MNU compared with none in the MNU group, and zero kidney mesenchymal tumors in the BzlG/MNU group compared with 3 in rats treated with MNU alone; 5 rats treated with ENU had tumors of the intestine compared with 1 in rats treated also with BzlG and 5 rats treated with ENU and BzlG had Sertoli cell tumors of the ovary compared with 1 in rats receiving ENU alone.

DISCUSSION

Each rat treated with an alkylnitrosourea received 0.2 mmol during the 10 weeks of treatment. The effect of BzlG pretreatment in each case was to reduce the median time to death with tumors induced by the alkylnitrosourea, from week 60 to week 52 in the case of MNU ($P=0.04$) and from week 55 to week 50 in the case of ENU (not significant). This reduction in the median week of death suggests a modest increase in the carcinogenic effectiveness of the treatment. That is, pretreatment with BzlG increased the tendency for lethal tumors to be induced. The finding that BzlG pretreatment had a larger effect on carcinogenesis by MNU than ENU is consistent with a greater role for AGT in repair of O^6-methylguanine residues in DNA than O^6-ethylguanine residues.[7] However, these effects were small compared with the results of previous similar experiments, in which a two-fold higher dose (0.4 mmol) of MNU reduced the median week of death of female F344 rats to 33, and in the case of ENU to 40.[10] These larger doses caused a considerably greater reduction in median week of death than did BzlG pretreatment in the present experiment.

A comparison of the number and distribution of tumors in the rats given MNU or ENU with those also treated with BzlG does not show large differences. Most of the alkylnitrosourea-induced tumors occurred at similar incidences in the groups with and without BzlG treatment, although there were large differences in the tumor patterns between rats treated with MNU and ENU, respectively, which has been discussed elsewhere.[15] Only in the case of the five benign Sertoli cell tumors of the ovary in the group treated with ENU and BzlG and the four mammary adenocarcinomas in the group treated with MNU and BzlG was there a possibly significant increase in incidence related to BzlG treatment. Notably lacking was any increase in liver tumors, although the vigor of DNA repair in the liver was originally proposed by Goth and Rajewsky[2] as the reason for the failure of ENU to induce liver tumors in rats. That explanation is not supported by our results, and it seems that other factors are responsible for the insusceptibility of the adult rat liver to carcinogenesis by many alkylnitrosoureas.[16]

Our results contrast with the report from Gerson's laboratory[17] that transgenic mice in which human AGT is expressed in the thymus are much less susceptible to thymic lymphomas induced by a single dose of MNU than their non-transgenic counterparts, which clearly supports a major role for the strongly expressed AGT activity in the mouse thymus in inhibiting carcinogenesis by MNU. More closely relevant is the report by Nakatsuru *et al.*[18] that transgenic mice in which expression of AGT activity in the liver is greatly enhanced develop fewer liver tumors following a single treatment of very young mice with nitrosodimethylamine (NDMA) or nitrosodiethylamine (NDEA). These results are much less convincing support for a role of the alkyltransferase in modulating carcinogenesis by these nitrosamines. In the males treated with the higher dose of NDMA and with NDEA there was no difference between the transgenic and normal mice in the incidence of liver tumors; in the other groups the differences might be fortuitous, because the mice were killed at an arbitrary time. Single dose treatments of adults with high doses of these nitrosamines does not induce liver tumors, whereas multiple treatment with very low doses (when repair of DNA alkylation would be expected to have a relatively greater influence) are very effective in inducing liver tumors.[19]

The modest acceleration of the appearance of alkylnitrosourea-induced tumors by pretreatment of F344 rats with BzlG suggests either that AGT plays only a limited role in modulating carcinogenesis by these agents, or that the dose of BzlG administered was not sufficient to alter significantly the persistence of O^6-substituted guanine residues in DNA produced by these carcinogens. Table I shows that after 24 h about 60% of the AGT activity was restored in the liver of animals treated with BzlG. Thus, it may be that AGT activity was not suppressed for long enough by this dose of BzlG to affect significantly the

impact of the delay in O^6-alkylguanine repair on carcinogenesis. Experiments to test this latter possibility are in progress.

ACKNOWLEDGMENTS

This research was sponsored in part by the National Cancer Institute, DHHS, under contracts NO1-CO-74101 with ABL (W.L. and R.C.M.) and NO1-CO-72102 with PRI/DynCorp. (M.R.A.), and grant CA19137 (A.E.P.). We are indebted to Barbara Thomas and Kristin Swenn for expert technical assistance. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. Government and its agents and contractors to retain a nonexclusive, royalty-free license in and to any copyright covering the article.

(Received August 9, 1993/Accepted November 15, 1993)

REFERENCES

1) Hart, R. W. and Setlow, R. B. Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species. *Proc. Natl. Acad. Sci. USA,* **71,** 2169–2173 (1974).
2) Goth, R. and Rajewsky, M. J. Molecular and cellular mechanisms associated with pulse-carcinogenesis in the rat nervous system by ethylnitrosourea: ethylation of nucleic acids and elimination rates of ethylated bases from the DNA of different tissues. *Z. Krebsforsch.,* **82,** 37–64 (1974).
3) Singer, B. The chemical effects of nucleic acid alkylation and their relation to mutagenesis and carcinogenesis. *Prog. Nucleic Acid Res. Mol. Biol.,* **15,** 219–284 (1975).
4) Lijinsky, W. Interaction with nucleic acids of carcinogenic and mutagenic N-nitroso compounds. *Prog. Nucleic Acid Res. Mol. Biol.,* **17,** 247–269 (1976).
5) Lickhachev, A. F., Ivanov, M. N., Bresil, H., Planche-Martel, G., Montesano, R. and Margison, G. P. Carcinogenicity of single doses of N-nitroso-N-methylurea and N-nitroso-N-ethylurea in Syrian golden hamsters and the persistence of alkylated purines in the DNA of various tissues. *Cancer Res.,* **43,** 829–833 (1983).
6) Kleihues, P., Hodgson, R. M., Veit, C., Schweinsberg, F. and Wiessler, M. DNA modification and repair *in vivo*: towards a biochemical basis of organ-specific carcinogenesis by methylating agents. *In* "Organ and Species Specificity in Chemical Carcinogenesis," ed. R. Langenbach, S. Nesnow and J. M. Rice, pp. 509–529 (1983). Plenum Press, New York.
7) Pegg, A. E. Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.,* **50,** 6119–6129 (1990).
8) Dolan, M. E., Moschel, R. C. and Pegg, A. E. Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc. Natl. Acad. Sci. USA,* **87,** 5368–5372 (1990).
9) Lijinsky, W., Saavedra, J. E. and Kovatch, R. M. Carcinogenesis in rats by nitrosodialkylureas containing methyl and ethyl groups given by gavage and in drinking water. *J. Toxicol. Environ. Health,* **28,** 27–38 (1989).
10) Lijinsky, W. and Kovatch, R. M. Similar carcinogenic actions of nitrosoalkylureas of varying structure given to rats by gavage. *J. Toxicol. Ind. Health,* **5,** 925–935 (1989).
11) Lijinsky, W., Garcia, H., Keefer, L. and Loo, J. Carcinogenesis and alkylation of rat liver nucleic acids by nitrosomethylurea and nitrosoethylurea administered by intraportal injection. *Cancer Res.,* **32,** 893–897 (1972).
12) Domoradzki, J., Pegg, A. E., Dolan, M. E., Maher, V. M. and McCormick, J. J. Correlation between O^6-methylguanine-DNA methyltransferase activity and resistance of human cells to the cytotoxic and mutagenic effect of N-methyl-N′-nitro-N-nitrosoguanidine. *Carcinogenesis,* **5,** 1641–1647 (1984).
13) Solleveld, H. A., Haseman, J. K. and McConnell, E. E. Natural history of body weight gain, survival, and neoplasia in the F344 rat. *J. Natl. Cancer Inst.,* **72,** 929–940 (1984).
14) Lijinsky, W., Riggs, C. W. and Walters, P. T. Lack of effect of carcinogen treatment on development of tumors arising spontaneously in Fischer 344 rats. *J. Toxicol. Environ. Health,* **39,** 527–538 (1993).
15) Lijinsky, W. A view of the relation between carcinogenesis and mutagenesis. *Environ. Mol. Mutagen.,* **14** (Suppl. 16), 78–84 (1989).
16) Ludeke, B., Schubert, M., Yamada, Y., Lijinsky, W. and Kleihues, P. DNA hydroxyethylation by hydroxyethylnitrosoureas in relation to their organ specific carcinogenicity in rats. *Chem.-Biol. Interact.,* **79,** 207–216 (1991).
17) Dumenco, L. L., Allay, E., Norton, K. and Gerson, S. L. The prevention of thymic lymphomas in transgenic mice by human O^6-alkylguanine-DNA alkyltransferase. *Science,* **259,** 219–222 (1993).
18) Nakatsuru, Y., Matsukuma, S., Nemoto, N., Sugano, H., Sekiguchi, M. and Ishikawa, T. O^6-Methylguanine-DNA methyltransferase protects against nitrosamine-induced hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA,* **90,** 6468–6472 (1993).
19) Peto, R., Gray, R., Brantom, P. and Grasso, P. Effects on 4080 rats of chronic ingestion of N-nitrosodiethylamine or N-nitrosodimethylamine: a detailed dose-response study. *Cancer Res.,* **51,** 6415–6451 (1991).