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Local and Systemic Carcinogenic Effects of Alkylating Carcinogens in Rats Treated by Intravesicular Administration

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Several nitrosamines and an azoxyalkane have been administered intravesically to groups of 12 female F344 rats, twice a week for 20 or 30 weeks. Many of the nitrosamines were as efficacious in giving rise to the same tumors of internal organs as when similar doses were administered orally, showing that absorption from the bladder was as rapid as from other sites. The tumors produced included lung and kidney tumors by nitrosodimethylamine, colon and Zymbal gland tumors by azoxymethane, liver tumors by methylnitrosoethylamine (but not by nitrosodimethylamine), liver and esophagus tumors by nitrosodiethylamine, liver and lung tumors by methylnitrosamino-3-pyridylbutanone, liver tumors by nitrosomorpholine, and tumors of the esophagus by methylnitroso-*n*-butylamine, 2,6-dimethylnitrosomorpholine and methylnitrosamino-N,N-dimethylethylamine. Bladder tumors were induced by intravesicular administration of only low doses of nitrosobis-(2-oxopropyl)amine and to a lesser extent by methylnitroso-*n*-hexylamine and nitroso-(2-hydroxypropyl)(2-oxopropyl)amine, which all induced tumors systemically in addition. The bladder mucosa seemed to lack enzymes necessary to activate most nitrosamines to locally acting proximate carcinogens, but was quite transparent to the passage of carcinogenic nitrosamines present in the urine into the body to induce tumors in distant organs.

Key words: Nitrosamine — Alkylating agent — Rat — Intravesicular — Tumor

N-Nitroso compounds induce bladder tumors, the most notable being nitrosodi-*n*-butylamine (NDBA),[1] methylnitrosourea[2] and several methylnitroso-*n*-alkylamines having even-numbered carbon chains.[3] With the exception of the alkylnitrosourea, these compounds must be metabolized extensively until a derivative that induces bladder tumors is formed; the initial metabolite formed from NDBA, for example, is butylnitroso-4-hydroxybutylamine formed in the liver and itself a bladder carcinogen when given by mouth.[1] Further studies of the metabolites of these nitrosamines revealed that a more proximate bladder carcinogen from NDBA is butylnitroso-3-carboxypropylamine, especially when the latter is introduced directly into the bladder of rats.[4]

This last finding was complemented by the report of Thomas *et al.*,[5] that intravesicular administration of methylnitroso-3-carboxypropylamine (MNCPA), a urinary metabolite of methylnitroso-*n*-alkylamines,[6] also led to induction of bladder tumors, as nitrosamino acid did when given to rats by mouth.[7] In the same study, methylnitroso-2-oxopropylamine (MNOPA) and methylnitroso-2-hydroxypropylamine (MNHPA) administered intravesically also gave rise to bladder tumors, the ketone being much more effective than the alcohol; both nitrosamines also induced some tumors of other organs in the same study, suggesting that absorption of the nitrosamine from the urine through the bladder wall took place readily. These results indicated that the cells of the bladder mucosa had the capacity to metabolize the non-directly acting nitrosamines to proximate carcinogenic forms. Furthermore, the much lesser potency of MNCPA compared with MNOPA suggested that the former was metabolized to the latter by the bladder epithelium.

As has been suggested,[8] nitrosation of amino compounds could take place in the bladder through reduction of urinary nitrate to nitrite by bacteria present in infected bladders. The N-nitroso compounds so formed could have carcinogenic effects, both local and systemic. This could contribute to the risk of cancer in people with infected bladders.

Having established the efficacy of intravesicular administration of nitrosamines as a means of delivering carcinogenic nitrosamines to rats, we undertook an investigation of the tumor-inducing effects of a variety of alkylating compounds by this route. It was hoped to gain insight into the possible consequences of exposure to members of this group of carcinogens when present in the urine. The compounds chosen for study were nitrosamines and azoxyalkanes that require metabolic activation.

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MATERIALS AND METHODS

The carcinogenic compounds used (structures in Fig. 1) have all been examined previously by other routes of administration to rats (mainly orally in drinking water or by gavage), and were prepared and purified in this laboratory from the amines as has been described.[1, 7, 9-12] Methylnitrosamino-3-pyridylbutanone (NNK) was purchased from Chemsyn Science Laboratories, Lenexa, Kansas, and azoxymethane from Chemical Dynamics Corp., S. Plainfield, New Jersey. The purity of all compounds was above 98%.

The animals were 156 female F344 rats of the colony of the Frederick Cancer Research Facility, born and raised within a barrier facility. They were housed four to a plastic cage and were fed Purina Autoclavable Laboratory Chow *ad libitum*. There were 12 animals in each treatment group. Treatment began at approximately 10 weeks of age, at which time it was determined that they would tolerate repeated intravesicular injections of solutions of such compounds as were to be used. As previously described,[5] under light metophane anesthesia, urine was expressed as completely as possible from the bladder of each rat, following which the needle of a syringe was inserted and 0.2 ml of solution of the test compound was injected. Solutions were prepared to appropriate concentration by dissolving the compound in ethanol (to ensure sterility) and diluting the solution to the desired concentration by adding three times the volume of deionized water that had been sterilized by boiling and then cooled. That this procedure was effective is attested by the rarity of bladder infection in rats so treated. It was determined initially that a 25% solution of ethanol in water was tolerated by the rats with no observable adverse effects and rats given this treatment served as controls. The solutions were administered twice a week for up to 30 weeks. At the end of the treatment the animals were maintained until death or until they became moribund, when they were killed. Tissues and organs examined included brain, pituitary gland, spleen, lymph nodes, thyroid gland, parathyroids, salivary glands, lungs, trachea, heart, esophagus, tongue, stomach, duodenum, jejunum-ileum, large intestine, pancreas, kidneys, adrenals, liver, skin, ovaries, urinary bladder, uterus, mammary gland and any lesions or masses. Tissues were fixed in buffered formalin, embedded in paraffin, sectioned and stained for histologic examination.

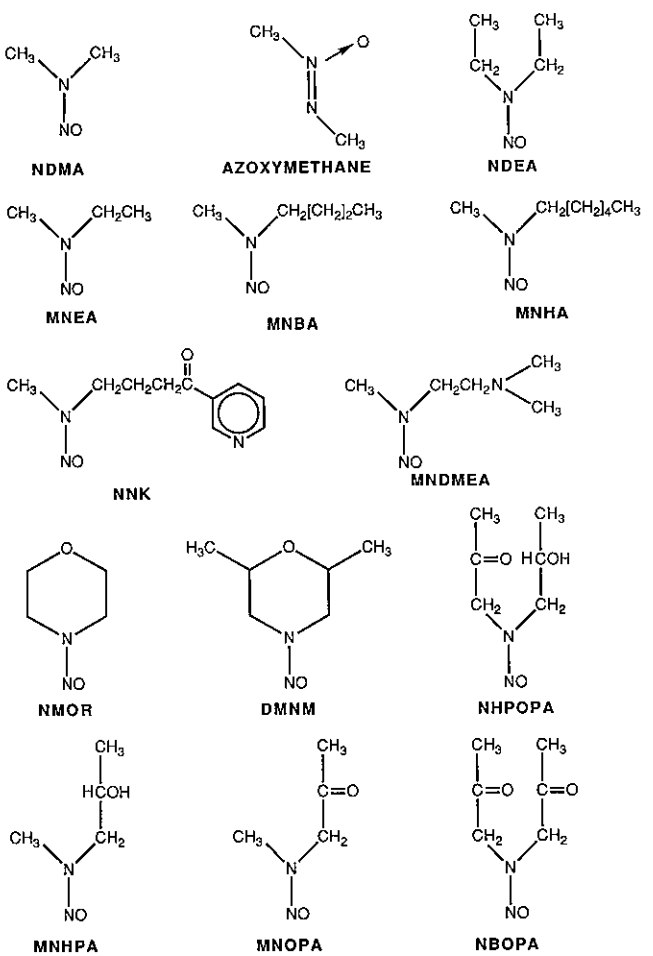

Fig. 1. Structures of compounds given intravesically to rats.

RESULTS

The compounds studied, their concentrations, the length of treatment and the tumors observed in the animals are shown in Table I. The tumors diagnosed included transitional cell papillomas and carcinomas of the urinary bladder, mesenchymal neoplasms of the kidney (NDMA and NBOPA), tubular cell neoplasms of the kidney (MNEA), hepatocellular adenomas and carcinomas, liver hemangiosarcomas (NDEA, MNEA and MNBA), nasal mucosa adenomas and carcinomas, lung alveolar/bronchiolar adenomas and carcinomas, squamous cell carcinomas (MNHA), papillomas and carcinomas of the esophagus, adenomas and adenocarcinomas of the colon, and carcinomas of the Zymbal gland. All of these neoplasms in F344 rats have been described elsewhere and those seen in this study conform to the established criteria. Only in the few treated animals that survived beyond week 70 and in the controls were the several tumors considered "spontaneous" in this strain (pituitary, adrenal pheochromocytoma, mononuclear cell leukemia and mammary gland fibroadenoma) observed. For comparison, the tumors induced by similar doses of the same compounds given to rats by gavage are

Table I. Tumors in Female Rats Induced by Twice-weekly Administration of Alkylating Carcinogens

Compound	Weekly dose (mg) and weeks of treatment	Intravesicular				Gavage Treatment			Ref.
		Total dose (mmol)	Median wk of death	% Rats with tumors of bladder	% Rats with other tumors	Total dose (mmol)	Median wk of death	% Rats with tumors of:	
Nitrosodimethylamine (NDMA)	4 mg×30	1.6	59	0	Kidney 100 Lung 92	0.8	59	Kidney 83 Lung 75 Liver 42	27
Azoxymethane	4 mg×22	1.2	28	0	Colon 82 Zymbal gland 67	0.8	42	Kidney 100 Colon 25 Zymbal gland 25	27
Methylnitrosoethylamine (MNEA)	4.6 mg×30	1.6	54	0	Liver 92 Lung 17 Kidney 25	1.4	30	Liver 100 Lung 25	27
Nitrosodiethylamine (NDEA)	5 mg×25	1.2	32	0	Liver 92 Esophagus 67	1.0	29	Liver 100 Esophagus 25	27
Methylnitroso-*n*-butylamine (MNBA)	6 mg×17	0.9	22	0	Esophagus 100 Liver 25	0.8	20	Esophagus 60	9
Methylnitroso-*n*-hexylamine (MNHA)	7.5 mg×30	1.6	65	22	Lung 100 Uterus 67	1.2	63	Lung 90 Liver 80 Esophagus 50	9
Methylnitrosamino-N,N-dimethylethylamine (MNDMEA)	14 mg×23	2.5	36	0	Esophagus 100 Nasal 33	2.2	26	Esophagus 100 Nasal 42	12
Methylnitrosamino-3-pyridylbutanone (NNK)	11 mg×30	1.5	70	0	Lung 42 Liver 33	1.3	43	Liver 83 Lung 25	12
Nitrosomorpholine (NMOR)	10 mg×30	2.6	35	0	Liver 58 Nasal 100 Esophagus 17	1.9	26	Liver 92 Esophagus 67	12
Dimethyl-2,6-nitrosomorpholine (DMNM)	12 mg×18	1.5	21	0	Esophagus 100	1.7	26	Esophagus 100	10
Hydroxypropylnitroso-2-oxopropylamine (NHPOPA)	5 mg×22	0.7	48	17	Liver 17	1.1	45	Esophagus 100 Lung 100 Nasal 90 Liver 30 Thyroid 30 Colon 20	11
Nitrosobis-(2-oxopropyl)amine (NBOPA)	1.6 mg×30	0.3	87	83	Lung 50 Kidney 50 Liver 17	1.1	59	Liver 92 Lung 83	22
*Methylnitroso-2-hydroxypropylamine (MNHPA)	4 mg×30	1.0	83	67	Esophagus 25 Nasal 67	0.6	50	Esophagus 100 Nasal 95 Lung 30 Liver 25	12
*Methylnitroso-2-oxopropylamine (MNOPA)	4 mg×30	1.0	43	75	Nasal 58	0.6	22	Esophagus 100	12
*Methylnitroso-3-carboxypropylamine (MNCPA)	30 mg×30	6	55	75	—	12	66	Bladder 100	
Control		—	102	0	—	—	96	—	

* The results of intravesicular administration of these compounds to rats were reported by Thomas *et al.*[5]

shown; the latter results have been published in other reports.

Other than the compounds previously reported, only one nitrosamine given intravesically gave rise to a high incidence of bladder tumors, nitrosobis-(2-oxopropyl)-amine (NBOPA). Methylnitroso - *n* - hexylamine (MNHA) and hydroxypropylnitroso-2-oxopropylamine (NHPOPA) induced bladder tumors in a small number of rats, but the main effect of the former was induction of tumors systemically, mostly in the lung and uterus. Nitrosodimethylamine (NDMA), nitrosodiethylamine (NDEA), methylnitroso-*n*-butylamine (MNBA), methylnitrosoethylamine (MNEA), methylnitrosamino-dimethylethylamine (MNDMEA), azoxymethane, NNK, nitrosomorpholine and 2,6-dimethylnitrosomorpholine (DMNM) were all carcinogenic, but did not induce bladder tumors. Controls given 25% ethanol intravesically survived 100 weeks or more and had no bladder tumors or treatment-related tumors.

Most of the methylnitrosoalkylamines had similar effectiveness when given by intravesicular administration or by gavage, although the animals treated with the same dose by gavage tended to succumb earlier. However, the organ in which tumors appeared, and the incidence, tended to be the same by both routes. Thus, MNBA produced esophageal tumors in high incidence by gavage,[9] and intravesically, although a few animals also had liver tumors (hemangiosarcomas) following intravesicular administration. This nitrosamine under a variety of conditions has failed to induce liver tumors in F344 rats by the oral route, although liver tumors were reported in another strain of rat following oral administration.[13] MNDMEA induced tumors of the esophagus and nasal mucosa with similar effectiveness by intravesicular or oral administration; there were no bladder tumors. The tobacco-specific nitrosamine NNK, another basic nitrosamine, also did not induce tumors of the bladder by intravesicular administration, but it induced tumors of the lung and liver in rats, as it did when given by gavage; the effectiveness, as measured by the rate of mortality from tumors, was less when injected into the bladder than when given by gavage.

MNHA was not highly soluble in water and had to be administered intravesically as a solution containing 9 mg/ml in water/ethanol 3:1, which was cloudy; 0.4 ml was given twice a week, instead of the usual 0.2 ml. Most of the animals had lung tumors, as they did when a similar dose was given to rats by gavage. Other tumors were induced in the uterus and bladder by intravesicular administration, but in the liver and esophagus by gavage.[9] The mortality rate of the rats treated by the two routes was similar.

The cyclic nitrosamine, nitrosomorpholine, induced tumors of the nasal mucosa and liver by intravesicular administration and few tumors of the esophagus, whereas by gavage, in addition to liver tumors there were only tumors of the esophagus. The derivative, DMNM, was more effective intravesically than by gavage and in both cases induced only tumors of the esophagus. Although it is well-known that this nitrosamine is metabolized to a number of acyclic oxygen-bearing propylnitrosamines,[14] none of the tumors that have been induced in rats by these metabolites was seen. On the other hand, intravesicular treatment with two of these metabolites, NBOPA and NHPOPA, produced very different results. Quite small doses of NBOPA gave rise, after a long interval (due to the low dose), to tumors of the bladder in high incidence, which were not induced by gavage in female rats, but were in male rats.[15] The intravesicular treatment also induced lung tumors and mesenchymal tumors of the kidney, whereas by gavage there were lung tumors and liver tumors in high incidence, but no kidney tumors. A dose of NBOPA administered intravesically (2.5 mg twice a week) caused toxic changes in the bladder and kidneys (pyelonephritis) and the rats eventually succumbed without evidence of tumors; all of the animals died between week 21 and week 38. The related DMNM metabolite NHPOPA was given intravesically at 2.5 mg twice a week, and also proved quite toxic to the bladder and kidneys, causing death of almost half of the rats by week 34. Of the animals that survived longer, a small number had bladder tumors and others had liver tumors (hepatocellular adenomas), but most of these animals died without tumors. On the other hand, by gavage NHPOPA gave rise to high incidences of tumors of the esophagus, lung and nasal mucosa, as well as smaller numbers of tumors of the liver, thyroid and colon[11]; most animals had more than one type of tumor related to the treatment and the last died at week 58.

DISCUSSION

The main objective of the study was achieved, namely the demonstration that N-nitroso compounds placed in the bladder were absorbed into the circulation of rats and gave rise to tumors in a compound-related manner, as effectively as when given by mouth. This suggests that if N-nitroso compounds are formed in the bladder by reaction of amines excreted in the urine with excreted nitrites or with nitrite formed by bacterial reduction of nitrate which is derived from vegetables or formed endogenously,[16] and always present in urine, they can easily enter the circulation and contribute to increased risk of cancer. This could be of considerable importance since a substantial part of the human population suffers from occasional or chronic bladder infections, and the etiology of many human cancers is imperfectly understood.

In most cases, death of the rats from the tumors induced by the treatment occurred at very similar times following either intravesicular or oral gavage treatments, indicating that absorption and distribution of the compounds from the urine through the bladder epithelium was rapid, and the potency of the carcinogens was similar by the two routes of administration. In most cases, the pattern of tumors induced by a compound was similar by either route, suggesting that the pharmacokinetics were similar in both instances.

The nitrosamines that have been tested by intravesicular administration can be divided into those that induce tumors in the bladder in addition to tumors in other organs, and those that induce only tumors in other organs, a majority. The differences can suggest explanations based on the chemical structures of the compounds. MNOPA is the product of metabolism of methylnitroso-*n*-alkylamines having a carbon chain with even number, that is excreted in the urine[17] and is activated by the bladder mucosa to the proximate carcinogen. The more weakly carcinogenic MNHPA and methylnitroso-3-carboxypropylamine, which also induce bladder tumors when given intravesically, seem likely to act through conversion to MNOPA. It is possible that MNHA, which induces many lung tumors when given intravesically, but few bladder tumors, is absorbed and oxidized in part in the liver to MNOPA, which is excreted in the urine, as was demonstrated by Singer *et al.*[17] in the case of oral administration of the *n*-hexylnitrosamine. The mechanism of action of MNOPA in inducing bladder tumors is not known, but it does give rise to methylation of DNA *in vivo*, derived entirely from the N-methyl group.[18]

MNBA, on the other hand, did not form more than traces of MNOPA excreted in the urine when given orally to rats,[17] which partially explains why intravesicular administration did not give rise to bladder tumors. Instead there was a high incidence of tumors of the esophagus and a few liver tumors. The liver tumors are something of an enigma, because methylnitroso-*n*-butylamine has not induced liver tumors in F344 rats by oral administration, whereas methylnitroso-*n*-hexylamine induced many liver tumors orally, but none by intravesicular administration. Such differences illustrate the complexity of the pharmacokinetics of nitrosamines in rodents, which has much to do with organ-specific carcinogenesis. These pharmacokinetics appear to be less complicated in the case of MNDMEA, which induced only tumors of the esophagus and nasal mucosa with similar potency, whether given intravesically or orally, and whether by gavage or in drinking water.

The tobacco-specific methylating nitrosamine NNK[19] was apparently not metabolized to a bladder carcinogen, whether given intravesically or orally to rats. On the other hand, it seemed to be readily absorbed through the bladder wall and induced tumors of the lung and liver, as it did when given by gavage, although with greater potency by the latter route. This result suggests, but does not prove, that NNK is not likely to be the carcinogenic agent in tobacco smoke related to the bladder cancer found in smokers.

Nitrosomorpholine and DMNM induced liver tumors and esophageal tumors, respectively, by intravesicular or oral administration. Nitrosomorpholine also induced a high incidence of tumors of the nasal mucosa and some esophageal tumors when injected into the bladder, but no nasal tumors by gavage.

Metabolites of DMNM include NHPOPA, which is excreted in the urine.[20] The ease of excretion of this asymmetric nitrosamine possibly is related to its hydrophilicity and to the very weak carcinogenic effect when it is administered intravesically. It appears not to be very readily activated or absorbed, and so gave rise to only a small number of tumors of the bladder and liver (although there was extensive toxicity to the bladder and kidneys), in contrast with its broad effectiveness when given by gavage or in drinking water to rats.[15] The related nitrosamine NBOPA was very toxic to the bladder and kidneys when given intravesically to rats at 30 μmol per week, so that none of the animals developed tumors before dying. At one-third of that dose rate, the animals survived well and developed tumors rather late, but most of them had bladder tumors and many had tumors of the lungs and kidneys (mesenchymal); the latter have been almost exclusively induced in rats by compounds that give rise metabolically to a methyldiazonium ion, and it can be assumed that formation of a methylating agent (for DNA) is part of the process of tumor-induction by NBOPA.[21] However, although NBOPA is carcinogenic when given orally it induced lung and bladder tumors in male rats (but no liver tumors), while inducing liver tumors, but no bladder tumors in female rats.[22] It appears that NBOPA acts locally on the bladder mucosa when given intravesically, and systemically in the lung and kidneys, whereas by oral administration to females it is extensively metabolized so that the metabolites excreted in the urine (including NHPOPA)[23] are weak bladder carcinogens or do not act on the bladder mucosa. MNOPA and MNHPA both induced high incidences of bladder tumors when given intravesically, but none when given by mouth, showing that local action is important.

The remaining compounds considered here are the simple methylating and ethylating nitrosamines which are presumed to act as carcinogens by forming methyl- or ethyl-diazonium ions, which act directly on DNA in the tissues in which they are formed by metabolism. Enzymes capable of activating these compounds are apparently absent from the bladder mucosa, so that they are

absorbed and have their carcinogenic effects in their respective target organs and tissues. This is straightforward for MNEA and NDEA, which produce essentially the same spectrum of tumors whether given intravesically or by gavage, although the potency was considerably lower intravesically in the case of MNEA. However, the powerful role of pharmacokinetics is shown in the difference of effect of NDMA and azoxymethane between the intravesical and oral routes of administration. Thus, lung and kidney tumors are induced by both routes in high incidence with NDMA, but no liver tumors intravesically. Azoxymethane induced no liver tumors by either route, but induced Zymbal gland tumors and colon tumors, the latter in high incidence when azoxymethane was injected into the bladder. However, in contrast with NDMA, azoxymethane induced no mesenchymal tumors of the kidney when given intravesically, whereas the nitrosamine induced 100% of these tumors by the same route. Yet both compounds are believed to be activated to a methylating agent in a very similar way and similar levels of DNA methylation have been shown with both compounds.[24] Clearly, these results indicate that DNA alkylation explains only partly, or not at all, the organ-specific tumor induction by these compounds following different routes of administration.

Methylation of DNA in the various organs, target and nontarget, seems to be little different whatever the route of administration (and whatever the species.[24,25] The similarities in DNA alkylation by the compounds, in contrast to the sharp differences in target organ specificity of tumor-induction, favor the hypothesis that DNA-adduct formation is not the main action of these carcinogens precipitating tumor formation.

Several of the compounds in this study could be methylating agents, and several of them have been shown to methylate DNA in liver and other organs at mutagenic sites. However, the correlation of the extent of such methylation with the induction of tumors in particular organs of the rat was not convincing of a relationship since there was methylation in organs in which tumors did not appear and no detectable methylation by some compounds in organs in which tumors were induced. The compounds studied included NDMA, MNEA, MNBA and MNHA.[26] The present results indicate that the pharmacokinetics of conversion of these carcinogens to metabolites other than simple methylating agents plays a dominant role in organ-specific tumor induction.[27]

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