Mechanism of Esophageal Tumor Induction in Rats by N-Nitrosomethylbenzylamine and Its Ring-methylated Analog N-Nitrosomethyl(4-methylbenzyl)amine

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ABSTRACT

The metabolism of the esophageal carcinogen N-nitrosomethylbenzylamine (MBN) and its ring-methylated analog N-nitrosomethyl(4-methylbenzyl)amine (4-MeMBN) was investigated in male Wistar rats. When given in the drinking water, both compounds have been shown to induce a high incidence of esophageal carcinomas but, after systemic administration of equimolar doses, 4-MeMBN is considerably less toxic and carcinogenic than is MBN. Following a single i.v. injection, 4-MeMBN disappeared from serum faster than did MBN. After 5 hr, neither compound was detectable in serum. Within 12 hr after a single i.v. injection (0.017 mmol/kg) of [methyl-14C]MBN, 49% of the radioactivity was exhaled as 14CO2, and less than 5% was in the urine, compared with only 13% as 14CO2 and 65% in the urine after an equimolar dose of 4-Me[methyl-14C]MBN. The urinary metabolite of 4-MeMBN was identified as its benzoic acid derivative. Methylation of DNA purines 4 hr after a single i.v. injection (0.017 mmol/kg) of [methyl-14C]MBN was highest in the esophagus (344 μmol 7-methylguanine per mol guanine), followed by liver, lung, and stomach. Considerably less DNA methylation was produced by an equimolar dose of 4-MeMBN, with highest values in liver, followed by esophagus (22 μmol 7-methylguanine per mol guanine) and lung. However, s.c. injections of equitoxic doses of MBN (18 mg/kg) and 4-MeMBN (394 mg/kg) produced similar amounts of 7-methylguanine in esophageal nucleic acids. These data indicate that the lower toxicity and carcinogenicity of 4-MeMBN after systemic administration are due to the rapid formation (mainly in the liver) and excretion via the urine of its benzoic acid derivative. The strong carcinogenic effect of orally administered 4-MeMBN can be explained by direct uptake from the drinking water into the esophageal mucosa.

INTRODUCTION

Structure-activity studies have shown that nonsymmetrical aliphatic dialkynitrosamines, particularly those with one methyl group, preferentially induce esophageal carcinomas in rats (1). For MBN, this effect is independent of the route of administration, esophageal neoplasms being induced in all experimental animals after both p.o. (1, 11) and s.c. (13) administration. Previous in vitro (2, 10) and in vivo (4, 6) studies suggest that this organ-specific effect is due to an extraordinarily high capacity of the esophageal mucosa of rats to enzymatically convert the parent carcinogen into a methylating intermediate. The present work extends these studies to 4-MeMBN which differs from MBN only in having a methyl group in the para position of the benzyl moiety (Chart 1). Schweinsberg et al. (9, 11) have shown that 4-MeMBN is considerably less toxic than MBN but equipotent in its capacity to induce esophageal carcinomas after p.o. administration to rats. To elucidate the biochemical basis of this finding, we have investigated the metabolism of both compounds and their reaction with cellular DNA in vivo. Experiments were carried out using the parent compounds labeled with 14C in the methyl group or the methylene bridge (Chart 1), since hydroxylation may occur at either site (14), leading to a benzylating or methylating intermediate as the ultimate carcinogen. Some preliminary data have been reported elsewhere (3, 5).

MATERIALS AND METHODS

Animals. Adult male Wistar rats (100 to 130 g body weight) were obtained from Han Versuchstiere, Hannover, West Germany. A standard laboratory diet and water were available ad libitum.

Chemicals. [methyl-14C]MBN and [methylene-14C]MBN were synthesized as described by Skipper (12) at specific radioactivities of 21 and 11.3 mCi/mmol, respectively. 4-Me[methyl-14C]MBN (specific activity, 6.19 mCi/mmol) and 4-Me[methylene-14C]MBN (specific activity, 3.23 mCi/mmol) were obtained from New England Nuclear, Boston, Mass. Prior to use, the radiochemical purity was checked by high-pressure liquid chromatography using a Lichrosorb RP 18 column (4.6 x 250 mm; Merck, Darmstadt, West Germany) eluted with 62% (v/v) aqueous methanol and was found to be greater than 98%. Sephasorb HP ultratrace was purchased from Deutsche Pharmacia, Freiburg, West Germany. Lumagel was from LKB, Karlsruhe, West Germany.

Animal Experiments. 14C-labeled MBN and 4-MeMBN were injected i.v. or s.c. at dose levels given in Table 1. Details on the doses used, the route of administration, and the survival time are given in the chart legends and in Table 2.

Determination of Serum Concentrations. Carcinogens were given as a single i.v. dose of 0.017 and 0.12 mmol/kg, at specific radioactivities of 0.49 and 0.094 mCi/mmol for [methyl-14C]MBN, and 0.568

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4 The abbreviations used are: MBN, N-nitrosomethylbenzylamine; 4-MeMBN, N-nitrosomethyl(4-methylbenzyl)amine; [methyl-14C]MBN, N-nitroso[methyl-14C]methylbenzylamine; [methylene-14C]MBN, N-nitroso[methylene-14C]benzylamine; 4-Me[methyl-14C]MBN, N-nitroso[4-methylbenzyl]amine; 4-Me[methylene-14C]MBN, N-nitroso[4-methylbenzyl]amine; TCA, trichloroacetic acid; i.g., intragastric.

2836 CANCER RESEARCH VOL. 42
Metabolism of MBN in Vivo

Chart 1. Routes of metabolism potentially involved in the bioactivation of MBN and 4-MeMBN. Metabolic pathways indicated by question marks may occur, but benzylated DNA bases were not detectable in vivo.

Table 1

| Experimental doses of [14C]-labeled MBN and 4-MeMBN |  
|-----------------|-----------------|-----------------|
| Molar dose (mmol/kg) | MBN (mg/kg) | 4-MeMBN (mg/kg) |
| 0.0067 | 1.0 | |
| 0.017 | 2.5 | 2.74 |
| 0.04 | 6.0 | |
| 0.08 | 12.0 | |
| 0.12 | 18.0 | 19.68 |
| 0.3 | 49.2 | |
| 0.6 | 98.4 | |
| 1.2 | 196.8 | |
| 2.4 | 393.6 | |

RESULTS

The rate of metabolism of MBN and 4-MeMBN in vivo was investigated by determining the disappearance of the parent carcinogen from serum following a single i.v. injection. Experiments were carried out at doses of 0.12 and 0.017 mmol per kg, equivalent to 18 mg MBN per kg (the LD50 for MBN) and 2.5 mg MBN per kg, i.e., the dose of MBN which when given weekly s.c. induces a high incidence of esophageal carcinomas in rats (13). We found that at both doses the rate of disappearance of 4-MeMBN from rat serum was faster than that of MBN.

and 0.088 mCi/mmol for 4-Me[methyl-14C]MBN. Animals were killed at time intervals ranging from 10 min to 5 hr after the injection. Blood was collected from the aorta, and serum was separated by centrifugation (2000 × g, 10 min, 4°C). Serum proteins were precipitated by addition of 4 ml of 1 M NaOH. Samples (0.5 ml) were analyzed on Sephasorb HP columns as described above.
Chart 2. Serum concentrations of MBN and 4-MeMBN at various time intervals after a single i.v. injection. Both carcinogens were administered at dose levels of 0.017 mmol/kg (corresponding to 2.5 mg/kg for MBN (•) and 2.74 mg/kg for 4-MeMBN (Δ)) and 0.12 mmol/kg (corresponding to 18 mg/kg for MBN (○) and 19.7 mg/kg for 4-MeMBN (▲)).

Chart 2. Excretion of [methyl-14C]MBN- and 4-Me[methyl-14C]MBN-derived radioactivity as exhaled 14CO2 and in the urine. Data are plotted as cumulative percentages of the total radioactivity administered. Animals received a single i.v. 0.017 mmol/kg dose of [methyl-14C]MBN (•) or 4-Me[methyl-14C]MBN (Δ). In addition, 4-MeMBN was administered s.c. at a dose of 2.4 mmol/kg ( ■).

Chart 3. Chromatographic profiles of esophageal DNA hydrolysates from animals which received a single i.v. 0.017 mmol/kg dose of [methyl-14C]MBN (top) or 4-Me[methyl-14C]MBN (bottom). After a survival time of 4 hr, DNA was isolated from the pooled esophagi of 8 (top) and 12 (bottom) rats. Acid hydrolysates were analyzed on Sephasorb columns as described in "Materials and Methods." Upper chromatogram redrawn from Ref. 4. To allow direct graphic comparison, the radioactivity scales have been adjusted to compensate for differences in the specific radioactivity of the injected carcinogens. A, adenine; G, guanine; 7-meG, 7-methylguanine; O6-meG, O6-methylguanine; Pyr. Nucl., pyridine nucleotides; ▲, E260; ◦, dpm.

Using both carcinogens labeled in the methyl group, the extent of DNA methylation was determined in target and non-target tissues using a single i.v. dose of 0.017 mmol/kg and a survival time of 4 hr (Table 2). MBN caused the highest extent of methylation in esophagus, followed by liver, lung, and forestomach. Methylation of purine bases by 4-MeMBN was generally much less extensive, with highest values in liver, followed by esophagus and lung. In forestomach, DNA methylation by 4-MeMBN was not detectable. In DNA of the esophagus, i.e., the principal target tissue, MBN produced concentrations of 7-methylguanine 15 times higher than did 4-MeMBN.
sensitive chromatograms of DNA hydrolysates are shown in Chart 4. Methylation of nucleic acids (DNA plus RNA) by both carcinogens is shown in Chart 5 for a wider range of dose levels. MBN produces a considerably higher extent of methylation than equimolar doses of 4-MeMBN. In esophagus, similar concentrations of 7-methylguanine were produced by 0.12 mmol MBN per kg and 2.4 mmol 4-MeMBN per kg, corresponding to 18 and 395 μmol per kg, respectively, i.e., the LD₅₀ for each carcinogen. After administration of 4-Me[methylene-¹⁴C]MBN in the drinking water (15 ppm; 1.5 mg per kg), DNA methylation in the esophagus (187 μmol 7-methylguanine per mmol guanine) was 16 times higher than in liver and forestomach (11.1 and 10.6 μmol 7-methylguanine per mmol guanine, respectively). When animals were given i.v. injections (0.017 mmol of [methylene-¹⁴C]MBN or 4-Me[methylene-¹⁴C]MBN per kg), no measurable amounts of radioactivity were present in DNA hydrolysates from any of the tissues investigated. The limit of detection was in the order of 0.5 to 1 benzylated base in 10⁶ bases.

**DISCUSSION**

This study was undertaken to elucidate the biochemical basis of the selective induction of esophageal tumors in rats by MBN and its ring-methylated analog, 4-MeMBN. Chronic administration of either compound in the drinking water has been shown to produce a very high incidence of carcinomas of the pharynx and esophagus after a mean survival time of 310 (5 ppm 4-MeMBN), 250 (10 ppm MBN), and 220 (15 ppm 4-MeMBN) days. However, the acute toxicity following a single i.g. dose differs markedly for both compounds, the LD₅₀ being 18 mg/kg for MBN (1) and 400 mg/kg for 4-MeMBN (11). The results obtained in the present investigation largely resolve this apparent discrepancy.

Both compounds undergo rapid metabolism in vivo. At doses up to 0.12 mmol/kg, the parent carcinogens are cleared from rat serum within 5 hr (Chart 2). Data for MBN which at both dose levels disappeared from serum more slowly than did its ring-methylated analog are in good agreement with those reported by Kraft et al. (7). These authors calculated a half-life of 66 min for an i.p. dose of 4.7 mg/kg. We observed first-order kinetics only at the low dose level of 2.5 mg/kg (4), the apparent half-life being 35 min. Marked differences were found in the metabolic fate of MBN and 4-MeMBN. Following a single dose of [methyl-¹⁴C]MBN, 49% of the radioactivity administered was exhaled as ¹⁴CO₂ and less than 5% was recovered from the urine. These figures are in close agreement to those reported by Kraft et al. (7). Both the production of ¹⁴CO₂ and the metabolic labeling of purines via the C₁ pool have been observed after in vivo administration of various methyl-labeled nitrosamines and related carcinogens and are assumed to result from the intermediary formation of [¹⁴C]formaldehyde, the oxidation product of methanol which, in turn, is the reaction product of methylating agents with water. The observation (Chart 3) that only 13% of 4-Me[methyl-¹⁴C]MBN-derived radioactivity was converted into ¹⁴CO₂ may at first suggest that this pathway is impaired by ring methylation of MBN. However, a more likely explanation is that a competing metabolic route exists in which 4-MeMBN is efficiently oxidized to the respective benzoic acid, i.e., 4-[(N-methyl-N-nitrosoaminomethyl)benzoic acid (Chart 1). This urinary metabolite has been identified previously by Schweinsberg et al. (9) and was found to account for 65% of the total radioactivity administered as a single injection (0.017 mmol/kg) of 4-Me[methyl-¹⁴C]MBN (Chart 3). At higher doses, the proportion of 4-MeMBN oxidized at the 4-methyl group of the phenyl moiety was even higher. Of a single s.c. dose of 2.4 mmol/kg (394 mg/kg), only 9% was exhaled as ¹⁴CO₂, and 83% of the ¹⁴C radioactivity was excreted as benzolic acid derivative via the urine (Chart 3). The data on nucleic acid alkylation support this interpretation. The extent of DNA methylation by a single i.v. dose (0.017 mmol/kg) of MBN was considerably higher than by a similar dose of 4-MeMBN, with a 15-fold difference in the concentration of 7-methylguanine in esophageal DNA (Table 2). With the specific radioactivity of 4-Me[methyl-¹⁴C]MBN available (6.19 mCi/mmol), the promutagenic base O₆-methylguanine was not at detectable. The data contained in Table 2 also show that, after systemic administration, hydroxylation at the methylene bridge of MBN, leading to a methylating intermediate, occurs preferentially in the target organ (esophagus) followed by liver, lung, and forestomach. Ring methylation of MBN not only facilitates a very efficient detoxification pathway (with accordingly lower levels of methylation) but also changes the relative extent of metabolism in different tissues, with the highest concentrations of 7-methylguanine in liver, followed by esophagus and lung (Table 2). This indicates a remarkable organ-specific substrate specificity of cytochrome P-450 enzymes involved in the bioactivation of MBN and related nitrosamines. The data shown in Chart 5 further indicate that methylation of cellular macromolecules is likely to be responsible for the toxicity of both agents. When given s.c. as a single equitoxic dose, i.e., the LD₅₀, MBN and 4-MeMBN cause a similar extent of nucleic acid methylation in the esophagus. Systemic administration of equimolar doses will, on the other hand, result in markedly different biological effects. At doses up to 0.12 mmol/kg (the LD₅₀ for MBN), methylation of 7-methylguanine was produced by 0.12 mmol/kg, the parent carcinogens are cleared from rat serum within 5 hr (Chart 2).
cellular macromolecules by 4-MeMBN is too low to cause toxic effects and probably insufficient for the induction of tumors within the life span of experimental animals. Carcinogenicity studies currently performed by P. Kleihues have thus far shown that 20 weekly s.c. injections of 4-MeMBN (0.017 mmol/kg) induced no tumors within 15 months after the onset of treatment whereas a similar dose of MBN has been shown to induce esophageal carcinomas in all experimental animals within 45 weeks. The capacity of 4-MeMBN to induce esophageal neoplasms after p.o. administration to rats is probably due to direct uptake and metabolism of the parent carcinogen by the esophageal mucosa. When 4-Me[methyl-14C]MBN was added to the drinking water (15 ppm), methylation of DNA purines in the esophagus was more than 16 times higher than in any other rat tissue. By this route, a much lower proportion of 4-MeMBN is entering the systemic circulation and available for detoxification by oxidation of the 4-methyl group.

Although the extensive exhalation of 14CO2 from [methyl-14C]MBN (Chart 3) is likely to result from hydroxylation at the methylene bridge, the possibility cannot be ruled out that [14C]formaldehyde is directly produced by hydroxylation of the methyl group of MBN which would lead to a benzylation species as the ultimate carcinogen (Chart 1). Following the administration of [methylene-14C]MBN, Kraft et al. have detected urinary excretion of [14C]hippuric acid. This would be compatible with hydroxylation of the methyl group of MBN. In the present study, benzylation of DNA bases was not detectable after i.v. 0.017-mmol/kg injections of [methylene-14C]MBN and 4-Me[methylene-14C]MBN at specific radioactivities of 5.24 and 3.23 mCi/mmol, respectively. However, this does not allow the conclusion that the formation of a benzylation intermediate does not occur in vivo, since the extent of DNA methylation and benzylation is unlikely to reflect the molar ratios at which the respective intermediates are formed.

REFERENCES

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