Mutagenic Activities of Oxidized Derivatives of N-Nitrosodipropylamine in the Liver Cell-mediated and Salmonella typhimurium Assays

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ABSTRACT

The mutagenic activity of N-nitrosobis(2-oxopropyl)amine (BOP), N-nitroso(2-hydroxypropyl) (2-oxopropyl)amine (HPOP), N-nitrosobis(2-hydroxypropyl)amine (BHP), N-nitrosomethyl-2-oxopropylamine (MOP), and N-nitrosomethyl-2-hydroxypropylamine (MHP) was examined in the Ames liquid incubation assay, using hamster liver homogenate for metabolic activation, and in the hamster liver cell-mediated V79 cell assay. At similar concentrations, the cell-mediated assay showed a greater mutagenic response over background to these nitrosamines than did the bacterial assay. Also, the relative mutagenic potency in the cell-mediated assay (MOP > MHP > BOP > HPOP > BHP) correlated better than that in the Ames assay (HPOP > MHP > BOP = BHP = MOP) with overall carcinogenic potency in the hamster (MOP > BOP > HPOP > BHP). The liver cell-mediated assay may be an important adjunct to the battery of short-term tests for carcinogenicity prescreening.

INTRODUCTION

It is now generally accepted that a battery of short-term assays will be needed to rapidly identify potential carcinogens. At present, the most widely used short-term system is the Salmonella typhimurium assay developed by Ames (1). While mutagenic activity in this system has been reported to correlate with carcinogenicity data (12), there are certain classes of carcinogens which are not readily detected in this mutagenesis assay. Some of the nitrosamines, for example, are potent carcinogens in animals but, as a class, are not consistently detected in the Ames test (1, 12, 19, 20). Although recent modifications of the Ames procedure have allowed certain nitrosamines to show mutagenic activity (3, 6, 20, 23), a greater number of mutants per plate was obtained with a liquid incubation than by the direct plate incorporation method. Because DMN was shown to produce a dose-dependent mutagenic response with preincubation, it was run as a positive control in all experiments.

Several years in this institute, we have examined the carcinogenicity in Syrian golden hamsters of a wide range of oxidized derivatives of N-nitrosodipropylamine. After chronic s.c. administration, many of these nitrosamines induce pancreatic ductular cancer, and a successful animal model for this human disease has been developed (14–18). However, at higher doses or by different routes of administration, a wide range of other tumors are also induced, including liver tumors. In the present study, we report the mutagenicity of several carcinogenic nitrosamines in the bacterial system, using hamster liver "S-9" for metabolic activation, and in the mammalian cell system, using intact hamster liver cells for activation. The correlation between mutagenic potencies in the 2 systems and their carcinogenic potency in hamsters is discussed.

MATERIALS AND METHODS

Chemicals. BOP (13), MOP (15), BHP (8), HPOP (5), and MHP (15) (see Chart 1) were prepared in this institute and determined to be greater than 98% pure. DMN was purchased from Eastman Organic Chemicals, Rochester, N. Y. Ouabain was purchased from Sigma Chemical Co., St. Louis, Mo., and dissolved in boiling water prior to dissolution in medium. NADP and glucose 6-phosphate were obtained from Sigma. Nutrient broth, tryptic soy broth, yeast extract, and agar were from Difco Laboratories, Inc., Detroit, Mich.

S-9 Preparation. Hepatic S-9 was prepared from 8-week-old male Syrian golden hamsters pretreated with Aroclor 1254 (500 mg/kg) 5 days earlier (2). The S-9 was used immediately after preparation or quick frozen and kept at −80° until use. Studies indicated that the S-9 fraction was stable for at least 2 weeks under these conditions, and all preparations were used within this time. The "S-9 mix" contained liver homogenate, NADP, and glucose 6-phosphate and was prepared immediately before use.

Bacterial Mutagenesis. S. typhimurium strains were generously supplied by Dr. Bruce Ames. In preliminary experiments, tester strain TA1535 showed the greatest sensitivity when treated with the S-9 mix and DMN in liquid suspension; therefore, this strain was used in all subsequent experiments. A liquid preincubation with DMN was used because, as reported by others (3, 20, 23), a greater number of mutants per plate was obtained with a liquid incubation than by the direct plate incorporation method. Because DMN was shown to produce a dose-dependent mutagenic response with preincubation, it was run as a positive control in all experiments.

The bacteria were grown as described by Ames et al. (2)
the viability by trypan blue dye exclusion was greater than verified.

were isolated, and their growth in the absence of histidine was determined by absorbance and adjusted with nutrient broth to approximately 2 x 10^8 bacteria/0.1 ml. Suspensions were maintained on ice until incubation was initiated. Experiments were performed with and without addition of S-9 mix.

In preliminary experiments, the preincubation time and amount of S-9 to produce the maximal number of revertants per plate using DMN were found to be 20 min and 0.25 ml, respectively. Glucose 6-phosphate concentration was 5 mm and NADP concentration was 4 mm in the S-9 mix. Briefly, the assay was carried out as follows. Four glass tubes (13 x 100 mm) for each sample were maintained at 4°, and the compounds were added in the following order: (a) 0.1 M phosphate buffer, pH 7.4, calculated to give a final volume of 1 ml; (b) the bacterial suspension in 0.1 ml; (c) the nitrosamine in distilled water; and (d) 0.5 ml S-9 mix containing 0.25 ml S-9 where indicated. The tubes were vortexed briefly and incubated at 37° for 20 min in a gyrorotary bath. The contents of each of 3 tubes were mixed with 2 ml top agar at 45° containing a trace of histidine and biotin and poured onto minimal-agar plates. The fourth tube was used to determine survivors after a 10^-6 dilution of the incubation mixture was mixed with top agar containing an excess of histidine and biotin. All plates were incubated inverted for approximately 48 hr before counting. The mutation frequency is expressed as the number of revertants per plate.

Liver Cell-mediated Mutagenesis. Primary hamster liver cells from 6- to 10-week-old male Syrian golden hamsters were prepared by the method of Williams et al. (21), as described previously (9). Liver cells (10^7/5 ml) were then seeded in complete medium into 25-sq cm T-flasks that had been seeded with 2.5 x 10^5 V79 cells 18 hr earlier. The plating efficiency of the liver cells was approximately 20%, and the viability by trypan blue dye exclusion was greater than 90%. The maximum number of viable liver cells was attached by 3 hr after seeding, and the medium was then changed to 8 ml fresh complete medium containing the nitrosamine.

Mutation for Resistance to Ouabain. The liver cells and V79 cells were cocultivated in the presence of the nitrosamine for 48 hr, and then the cells were dissociated with trypsin/EDTA (Grand Island Biological Co., Grand Island, N. Y.). Because of their smaller size, the V79 cells could be distinguished from the liver cells by counting with a hemocytometer. Determination of cloning efficiency and mutation frequency has been described previously (9). Briefly, for cloning efficiency, 200 V79 cells in 5 ml medium were seeded into 60-mm dishes (6 dishes), and the colonies were stained with Giemsa 6 to 7 days later. For determination of the number of mutants, 10^5 V79 cells in 4 ml medium were seeded into 60-mm dishes (20 dishes), and 2 days later ouabain in 1 ml medium was added to a final concentration of 1 mm. This expression time allowed an optimal number of mutants to be detected (9). These dishes were stained 14 to 17 days after cell seeding. The mutation frequency for resistance to ouabain was calculated per 10^6 survivors based on cloning efficiency and number of cells seeded for mutant selection. All results are based on 2 to 4 experiments in duplicate per point. The mutation frequency varied up to 30% for each compound among the different experiments. Periodically, ouabain-resistant colonies were isolated prior to staining, grown in the absence of ouabain for 1 month, and then tested for growth in the presence of ouabain. All clones grew in the presence of 1 mm ouabain after this period.

RESULTS

Bacterial Mutagenesis. The mutagenic activities of the nitrosamines in the bacterial assay are shown in Chart 2. Data are expressed as the number of revertants per plate. The liquid preincubation method was used for all chemicals because initial studies demonstrated that DMN (see also Refs. 3, 20,
and 23) and BOP were mutagenic when this procedure was used but not by the direct plate incorporation method. Of the compounds studied, only HPOP showed significant mutagenic activity in the direct plate assay (22). Preliminary experiments also indicated that the Aroclor-induced hamster S-9 fraction increased the mutagenic activity of DMN and BOP by about 20% over uninduced liver preparations (data not shown).

DMN was run as a positive control in the bacterial assay, and at 10 and 100 mM it induced 1750 and 4400 revertants/plate, respectively, or 3 and 9 revertants/10^6 survivors. With metabolic activation, HPOP was the most potent mutagen of the derivatives studied. MHP, MOP, BOP, and BHP were also mutagenic with metabolic activation but were negative or only slightly active without activation. In the absence of metabolic activation, MOP was toxic at the 10 and 100 mM level. In general, the relative mutagenic activities of these chemicals were comparable, whether expressed as the number of revertants per plate or as the number of revertants per 10^6 survivors. The relative mutagenic potency of the nitrosamines in this system is HPOP > MHP > BOP > BHP = MOP.

Liver Cell-mediated Mutagenesis. The mutagenic activities of the nitrosamines in the hamster liver cell-mediated system are given in Chart 3. Attempts were made to use Aroclor-induced hamster liver cells; however, these cells rapidly lost viability during the cocultivation period and yielded lower mutation frequencies than did uninduced cells. Therefore, uninduced cells were used for all cell-mediated studies.

DMN, which we have reported previously to be mutagenic in the rat liver cell-mediated system (9, 10), was also used as a positive control in the hamster liver cell-mediated mutagenesis system. DMN induced 98 and 330 ouabain-resistant mutants/10^6 survivors at concentrations of 0.3 and 1.4 mM, respectively. DMN was not directly mutagenic to V79 cells. MOP was a potent mutagen in the liver cell-mediated system. Concentrations of MOP above 0.7 mM in the presence of liver cells were cytotoxic and resulted in reduced mutation frequencies (data not shown). MHP, a metabolite of MOP (Ref. 15; Chart 1), showed about one-half the mutagenic activity of MOP at 0.7, 0.3, and 0.1 mM (Chart 3). BOP and HPOP both showed mutagenic activity at 2.0, 0.7, and 0.2 mM in the presence of metabolic activation, while BHP, the dihydroxy metabolite of BOP, was the weakest mutagen of the series in this system. Only MOP and BOP showed slight mutagenic activity to V79 cells in the absence of liver cell activation. The relative mutagenic potency of the oxopropynitrosamines in this system when expressed as number of revertants per 10^6 survivors is MOP > HHP > BHP > MOP.

DISCUSSION

In the present study, we have attempted to compare 2 short-term assays for reliability and sensitivity of detecting the mutagenic activity of a group of structurally related nitrosamines. To improve the sensitivity of the bacterial assay for nitrosamines, a liquid incubation was used and, in agreement with the findings of others for DMN (3, 20, 23), the mutagenic activity of all the nitrosamines studied was enhanced by this procedure. Furthermore, as reported by Rao et al. (19) for aliphatic nitrosamines, we found that S. typhimurium strain TA1535 was the most sensitive tester strain for the oxidized derivatives of N-nitrosodipropylamine. However, these authors reported BOP and BHP to be inactive with rat liver S-9 by both liquid incubation and direct plate assay. In our studies, using hamster liver S-9 and substantially higher concentrations of BOP and BHP, both chemicals were mutagenic. Camus et al. (4) have also reported BOP and BHP to be more mutagenic with hamster liver preparations than with rat liver.

We have reported previously the development of a rat liver cell-mediated mutagenesis system which could distinguish between carcinogenic and noncarcinogenic aflatoxins and nitrosamines (8, 9). DMN and N-nitrosodiethylamine were mutagenic in this system, while the noncarcinogenic nitrosamine methyl-t-butyl-nitrosamine was nonmutagenic (9). In the present studies, we have extended this system, using hamster liver cells for metabolic activation, and increased the number of nitrosamines examined by studying compounds for which we have extensive carcinogenicity data. Furthermore, some of the nitrosamines have been shown previously to be metabolically related (5, 15).

No studies have compared the carcinogenic potency of these compounds at a range of equimolar doses. However, in Table 1, we have summarized the available bioassay data relative to the carcinogenic potency of the nitrosamines in the Syrian golden hamster. For these data, the compounds were administered under identical conditions at a range of equitoxic doses determined from the acute 50% lethal dose. The results presented are at doses which allowed a mean survival of about 40 to 50 weeks, and accordingly overt toxicity is not likely to have interfered with expression of carcinogenicity. Although the data

Chart 3. Mutagenic activities of nitrosamines in the hepatocyte-mediated assay. The number of V79 cells per T-flask was 6 × 10^6 when 10^7 hamster liver cells were seeded. The cells were treated for 2 days with the nitrosamines and then trypsinized and reseeded to determine cloning efficiency and mutation frequency as in the text. At this time, the number of V79 cells per T-flask ranged from 1.4 × 10^6 to 3.6 × 10^6. Untreated cells had a cloning efficiency of 74% and a mutation frequency of 1 ouabain-resistant mutant /10^6 survivors in the presence of hepatocytes. All experiments were repeated 2 to 4 times in duplicate, and the data are the average of the results from all experiments. The standard deviations were no greater than about 30%.
The carcinogenic potency of nitrosamines in Syrian hamsters

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>LD_{50} (mg/kg)</th>
<th>LD_{50} (mg/kg)</th>
<th>Av. survival (wk)</th>
<th>Sites of tumors (with incidence of 50% or greater)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOP</td>
<td>35</td>
<td>1.75</td>
<td>39</td>
<td>Pancreas, nasal cavity, liver, kidney</td>
<td>15</td>
</tr>
<tr>
<td>BOP</td>
<td>120</td>
<td>2.5</td>
<td>42</td>
<td>Pancreas, lung</td>
<td>14</td>
</tr>
<tr>
<td>HPOP</td>
<td>380</td>
<td>19</td>
<td>48</td>
<td>Pancreas, lung, nasal cavity, larynx-trachea</td>
<td>17</td>
</tr>
<tr>
<td>BHP</td>
<td>500</td>
<td>25</td>
<td>45</td>
<td>Pancreas, nasal cavity</td>
<td>11</td>
</tr>
</tbody>
</table>

LD_{50}, 50% lethal dose.

are limited, we have estimated the relative carcinogenic potency to be MOP > BOP > HPOP > BHP. DMN is not included in Table 1 because it has not been tested by a similar protocol in this institute, although it is a known potent carcinogen. MHP is not included because it has not yet been tested (see below).

When we compare the results of the bacterial and mammalian cell-mediated assays and the carcinogetic data in hamsters for the various nitrosamines, 2 factors are observed: (a) at similar concentrations, the cell-mediated assay showed a greater mutagenic response over background to these nitrosamines than did the bacterial assay; (b) as illustrated in Chart 4, when carcinogenic potency is plotted against mutagenic potency, at least for the 4 nitrosamines assayed, the relative potency in the cell-mediated mutagenesis system (MOP > BOP > HPOP > BHP) parallels their relative carcinogenic potency (MOP > BOP > HPOP > BHP). Carcinogenic potency is expressed as the reciprocal of the lowest dose required to induce an overall tumor incidence of at least 60 to 70% (see legend to Table 1). However, while the bacterial assay detects all these carcinogens as mutagenic, the correlation of carcinogenic potency with mutagenicity (HPOP > BOP = BHP = MOP) does not exist. The carcinogenicity of MHP has not yet been examined, but we might predict from the mammalian cell-mediated mutagenesis data (Chart 3) that its carcinogenic potency will be similar to MOP and BOP.

In summary, the data presented indicate that, for the limited number of nitrosamines studied, the liver cell-mediated assay is a sensitive indicator of mutagenic activity and yields data that correlate with carcinogenic potency. We therefore suggest that the cell-mediated assay may be an important adjunct to the battery of short-term tests needed for carcinogenicity pre-screening.

ADDITIONAL

Since submission of this manuscript, other authors have reported a correlation between carcinogenic potency of several nitrosamines in the rat and a rat hepatocyte-mediated V79 mutagenicity assay (7).

REFERENCES


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