Mutagenicity of N-Nitroso Bile Acid Conjugates in Salmonella typhimurium and Diploid Human Lymphoblasts

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ABSTRACT

Two N-nitroso bile acid conjugates, N-nitrosotaurocholic acid and N-nitroso-glycocholic acid, were tested for mutagenicity by forward mutation assay in Salmonella typhimurium TM 677 and in diploid human lymphoblasts, line TK6. N-Nitrosoglycocholic acid and N-nitrosotaurocholic acid showed similar concentration-response curves in the bacterial assay with statistically significant mutant fractions observed at about 0.12 mm. Non-nitrosated parent compounds were nonmutagenic. However, in the human cell assay, N-nitrosotaurocholic acid gave statistically significant mutant fractions only at 0.4 mm, but N-nitroso-glycocholic acid was mutagenic at 0.05 µM, some 9000 times more potent. Experiments with quantitative Ames' S. typhimurium reversion assays indicated mutagenesis via base substitution.

INTRODUCTION

Many N-nitroso compounds are carcinogens (4) with a wide range of organ specificity. In particular, N-nitrosamines and related compounds are associated with cancers of the stomach. N-Methyl-N-nitrosacetamide is a forestomach carcinogen in the rat (2), and N-methyl-N-nitrosoureia and MNNG produce tumors at many sites in the rat stomach (2).

Recently (7), we synthesized and characterized 2 model N-nitrosamines, NOTC and NOGC, which are derivatives of naturally occurring amides. Preliminary experiments (7) indicated that NOTC and NOGC were mutagenic in bacterial mutation assays, and we have undertaken a more extensive study of their mutagenicity in both bacteria and human cells.

MATERIALS AND METHODS

Bacterial Mutation Assays. Forward mutation to 8-azaguanine resistance in Salmonella typhimurium strain TM 677 (uvrB, rfa, pkM 101, gal-*, bio-, his*) was used as the principal means of measuring mutagenicity. Protocols for measurement of forward mutation have been described in detail (9). Briefly, exponentially growing bacteria were suspended in medium in the presence of test chemical for 2 hr and then resuspended in fresh medium. Aliquots were plated in the absence and presence of selective conditions (medium containing 50µg 8-azaguanine per ml). Two independent cultures were used for each treatment point. Colonies were counted at 48 hr, and the mutant fraction was determined as the number of colonies formed under selective conditions divided by the number of colonies formed under nonselective conditions multiplied by the dilution factor. If this ratio for a treated culture was greater than that found for untreated cultures used as simultaneous controls with greater than 99% confidence and if that ratio also exceeded the 99% upper confidence limit on the mutant fractions of all historical controls, the test was considered positive.

Human Lymphoblast Mutation Assays. Diploid human lymphoblasts, line TK6, were used to determine if NOGC and NOTC were mutagenic to human cells. Prior to its use in the mutation assays, TK6 was cultured in Roswell Park Memorial Institute Medium 1640 with glutamine, supplemented with 10% horse serum. Cells were grown in spinner cultures at 37°C with daily dilution to 3 x 10^5/ml. The doubling time was 12 to 16 hr.

To reduce the spontaneous trifluorothymidine resistant fraction cells were then grown for 2 days in Roswell Park Memorial Institute Medium 1640 (supplemented with 10% horse serum) containing 2 x 10^{-4} M aminopterin, 2 x 10^{-4} M hypoxanthine, 10^{-5} M cytidine, and 1.74 x 10^{-5} M thymidine. To reverse the inhibitory effects on aminopterin, cells were then grown for one additional day in the same medium but without aminopterin. Cultures were diluted with regular medium for either 3 or 4 days prior to use in mutation assays.

The protocol for the mutation assay has been described (11). Briefly, all cultures were exposed to the test compound, dissolved in dimethyl sulfoxide, for 3 hr at 37°C. Samples of the cultures were distributed on microtiter plates to determine toxicity. The remaining cultures were maintained in exponential growth for an additional 6 to 8 days to permit phenotypic expression of any induced mutation after which time samples were plated in the absence or presence of selective conditions (2 µg trifluorothymidine per ml of medium). Colonies were enumerated after 14 days, and the mutant fraction was calculated as the ratio of colony-forming efficiency in the presence and absence of selective conditions.

Chemicals. Bile acids and bile acid conjugates were purchased from either Vega Biochemicals, Tucson, Ariz., or Sigma Chemical Co., St. Louis, Mo., and were used as supplied.

NOTC, sodium salt (CA Registry No. 76757-84-1), and NOGC (CA Registry No. 76757-85-2) were prepared from the corresponding bile acid conjugates, according to the method of Shuker et al. (7) and were homogeneous by thin-layer chromatography and high-pressure liquid chromatography (for conditions, see Ref. 7).

RESULTS

Bacterial Mutation. NOTC and NOGC were tested for mutagenic activity in TM 677 at concentrations ranging from 0.09 to 1.70 mm and 0.10 to 2.0 mm, respectively. Both compounds showed marked mutagenicity over these ranges, and the dose-response curves shown in Charts 1 and 2 were obtained. The error bars represent 99% confidence limits at each concentra-
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1.0
0.5
0.0
-0.5
-1.0
NOTC (mM)

Chart 1. Mutant fraction and survival as a function of the concentration of NOTC in the forward mutation assay (TM 677). , minimum value for the mutant fraction, above which the response is considered positive with 99% confidence. Bars, 99% confidence limits on each point. 8-AG, 8-azaguanine.

Chart 2. Mutant fraction and survival as a function of the concentration of NOGC in the forward mutation assay (TM 677). , minimum value for the mutant fraction above which the response is considered positive with 99% confidence. Bars, 99% confidence limits on each point. 8-AG, 8-azaguanine.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Strain</th>
<th>Relative survival</th>
<th>Mutant fraction x 10^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTC (1.18 mM)</td>
<td>TA 98</td>
<td>0.3</td>
<td>NS^c</td>
</tr>
<tr>
<td></td>
<td>TA 100</td>
<td>0.5</td>
<td>94 ± 14^b</td>
</tr>
<tr>
<td></td>
<td>TA 1535</td>
<td>0.7</td>
<td>58 ± 6^c</td>
</tr>
<tr>
<td></td>
<td>TA 1537</td>
<td>0.1</td>
<td>NS^b</td>
</tr>
<tr>
<td></td>
<td>TA 1539</td>
<td>0.1</td>
<td>NS^b</td>
</tr>
<tr>
<td>NOGC (1.14 mM)</td>
<td>TA 98</td>
<td>0.1</td>
<td>NS^b</td>
</tr>
<tr>
<td></td>
<td>TA 100</td>
<td>0.3</td>
<td>56 ± 9^c</td>
</tr>
</tbody>
</table>

^a NS, not significant.
^b Not greater than historical control with 99% confidence.
^c Greater than historical control with 99% confidence.

Both compounds exhibited significant toxicity over the range of concentrations tested (also shown on Charts 1 and 2), although at low concentrations they appeared to stimulate cell growth. The parent compounds, sodium taurocholate and glycocholic acid, were not mutagenic at relatively high concentrations (~1.0 mM), nor did they have any significant effect on the toxicity or mutant fraction when added in 10-fold molar excess over the corresponding N-nitroso bile acid conjugate (data not shown).

NOTC (1.18 mM) and NOGC (1.14 mM) were tested in several mutant strains of *S. typhimurium* devised by Ames et al. (1) using the protocol described by Skopek et al. (9). The results are shown in Table 1.

**Human Lymphoblasts.** Cultures of diploid human lymphoblasts, line TK6, were exposed to several different concentrations of NOTC and NOGC for 3 hr. The growth of the cell cultures was monitored for a period of 6 to 8 days after treatment, during which time phenotypic expression of induced mutation occurs. The growth curves for NOTC and NOGC are shown in Charts 3 and 4. NOTC (Chart 3) displayed detectable cell-killing ability at a concentration as low as 0.18 mmol, but within 48 hr posttreatment the surviving cells had returned to the doubling time of the untreated controls. NOGC was more toxic (Chart 4), being detected as such at 0.05 μM with some...
growth irregularity persisting after treatment. Concentration-mutation response curves for NOTC and NOGC were obtained (Charts 5 and 6). Both compounds gave significant induced mutant fractions (i.e., >99% confidence limit for all historical controls = $5.0 \times 10^{-6}$) at 0.05 μM for NOGC and 0.4 mM for NOTC. Data for 2 separate experiments each performed with duplicate cultures are shown.

In both bacterial and human cell assays, the results were obtained in the absence of any metabolizing system.

Inasmuch as bile acid conjugates are surface-active compounds capable of forming micelles (10), we considered that this may affect the mutant fraction. However, the addition of a large excess of parent bile acid conjugate to either NOTC or NOGC had no significant effect on the mutant fraction (data not shown).

In the human lymphoblast assay, both compounds were significantly toxic at the concentrations tested, but this did not affect the subsequent exponential growth of surviving cells to any great extent.

NOGC and NOTC exhibited a remarkably different potency as mutagens toward the human cells. NOTC showed a statistically significant mutant fraction at about 0.4 mM, whereas NOGC was active at about 0.05 μM; i.e., NOGC is approximately 9000 times more potent than NOTC to diploid human lymphoblasts. In view of their closely similar activity in bacterial
assay systems, the large difference was quite unexpected. MNNG is also detected at 0.05 μM in diploid human lymphoblasts (5).

Both NOTC and NOGC were detectable at 1.2 nm for strains in which base substitution (AT → GC) reversion results in histidine prototrophy (TA 1535, TA 100). The same concentration did not give significant responses in TA 98, TA 1537, and TA 1538, which are reverted by either of 2 different frame-shift mutations. Many alkylating agents cause base-pair substitutions, but not all are so apparently devoid of frame shift mutation activity (cf. ethyl methanesulfonate) (9); the MNNG profile of reversion of Ames’ strains is similar to those of NOGC and NOTC (9).

The results presented in this paper show that NOTC and NOGC possess mutagenic activity similar to that of mutagens such as MNNG and N-methyl-N-nitrosoacetamide, which are also potent gastrointestinal carcinogens. There is, however, a lack of direct evidence implicating N-nitroso bile acid conjugates as gastrointestinal carcinogens in humans, but several accounts in the literature lend some support to this hypothesis. The occurrence of “operation sequel carcinoma” in the stomachs of rats which have undergone gastric resection is strongly linked with bile reflux (3). In addition, elevated levels of nitrite and total N-nitroso compounds have been observed in the gastric juice of human patients who have undergone gastric surgery of the type associated with bile reflux (6).

One may reasonably ask if NOGC could in fact contribute to the probability of cancer in the gastrointestinal tract. A way to approach this problem is to ask if the concentration of NOGC and the duration of exposure would be expected to significantly increase the amount of mutation in a human cell population.

Spontaneous mutation in dividing human cells at the TK locus is about 1.7 × 10⁻⁷ mutant/cell. Exposure to NOGC for one generation at 0.05 μM increased mutation at the TK locus by 3 and 6 × 10⁻⁸ mutants/cell in 2 independent experiments. Using the average of 4.5, we can see that continuous exposure to 0.05 μM NOGC would be expected to increase the rate of mutation by at least 4.5 × 10⁻⁸/1.7 × 10⁻⁷ or about 25-fold. The “doubling dose” assuming linearity would be no more than 0.05 μM/25 = 2 nm NOGC. Limits on the actual exposure must be recognized in that NOGC concentrations would be expected to drop during our one-generation exposures, and thus the cell exposure would be less than 0.05 μM × 16 hr.

We do not know what the levels of NOGC or similar compounds are in humans.

There is a need, therefore, for an analytical technique which would facilitate the analysis of N-nitroso bile acid conjugates in biological media so that a quantitative appraisal of the occurrence of these compounds can be made in animal models designed to produce gastric bile reflux and in humans who have undergone gastric surgery.

REFERENCES

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