Use of 3,4-Dichlorobenzenethiol as a Trapping Agent for Alkylation Intermediates during in Vitro Metabolism of Nitrosamines

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ABSTRACT

Our studies using 3,4-dichlorobenzenethiol as a probe for methylating agent production during exposure of N-nitrosodimethylamine to rat liver preparations produced results different from those of an investigation reported in the literature. Methyl-3,4-dichlorophenyl thioether was detected, but the quantities found were not significantly different from the background levels of metabolism product detected in the absence of nitrosamine. Only about 10% of the thioether isolated after incubating N-nitrosodimethylamine as substrate was radioactive. The results indicate that the majority of the methyl groups transferred to the sulfur nucleophile in our experiments came from components of the incubation mixture other than the nitrosamine. Some artifactual methylation was also associated with the analytical procedure. We conclude that 3,4-dichlorobenzenethiol should be used with caution in studies of alkylation during the in vitro metabolism of carcinogenic nitrosamines.

INTRODUCTION

Successful use of 3,4-dichlorobenzenethiol as a trapping agent for electrophiles produced in vitro or in vivo has been described by several authors (2-4, 6, 7). Of particular interest to us was a 1976 report (7) that metabolic generation of alkylating species by exposure of various carcinogenic dialkylnitrosamines to rat liver S-9 preparations could be conveniently quantitated by using GC-MS to follow the production of the corresponding alkylthioether. In attempting to use this technique to establish the metabolic fate of unlabeled as well as 14C-substituted NDMA molecules, we have obtained results which are at variance with those of the 1976 work (7). We present below a summary of our findings.

MATERIALS AND METHODS

Chemicals. NDMA was prepared as previously described (5). Some preparations were column chromatographed on Fisher 923 silica gel (100 to 200 mesh) using dichloromethane:ethyl acetate (9:1) as mobile phase, and were distilled at aspirator pressure before use. [14C]NDMA, at a specific activity of 47 mCi/mmol was purchased from New England Nuclear (Boston, MA). The sample was found to be significantly contaminated after storage in the freezer for several months. It was purified by high-performance liquid chromatography (Waters Associates, Inc., Milford, MA) on a C18Bondapak column (3.9 mm x 30 cm; Waters Associates) using a mobile phase of 0.01 M potassium phosphate (pH 7.4) at a flow rate of 1.0 ml/min. Eluate fractions corresponding to the [14C]NDMA peak (retention time, 23.4 to 24.4 min) as detected by UV spectrophotometry at 254 nm were collected and diluted with cold NDMA to bring the solution to 0.05 M. One-ml aliquots (50 µmol at a specific activity of 0.16 mCi/mmol) of this solution were used for incubations.

3,4-Dichlorobenzenethiol was obtained from Aldrich Chemical Co., Milwaukee, WI and purified as follows before use to remove disulfide and methyl-3,4-dichlorophenyl thioether contaminants. The commercial thiophenol (25 g) was dissolved in 100 ml of 3 M sodium hydroxide under nitrogen in an inert atmosphere dry box, and extracted with three 100-ml portions of hexane. The aqueous layer was neutralized with hydrochloric acid, and the 3,4-dichlorobenzenethiol was extracted into hexane. The organic extracts were dried with anhydrous sodium sulfate, filtered, concentrated in vacuo to remove the hexane, and distilled (b.p. 80°C at 1 mm pressure).

Standard samples of methyl-3,4-dichlorophenyl thioether were prepared by mixing 8 g (45 mmol) of 3,4-dichlorobenzenethiol with 19.5 g (90 mmol) of 25% sodium methoxide in methanol (Aldrich Chemical Co.). Methyl iodide (12.8 g, 90 mmol) was added dropwise under ice bath cooling to control the exothermic reaction. The resulting 2-phase mixture was made homogeneous by adding enough peroxide-free ether to bring the total volume to 75 ml. After standing overnight, the solution was concentrated by distillation to 25 ml. The remaining liquid was decanted from the solid product using additional ether for rinsing, then distilled at aspirator pressure to isolate the desired product (b.p. 133°C; 17 mm pressure). The yield was 4.7 g (54%). [1H3]Methyl-3,4-dichlorophenyl thioether was prepared by similarly reacting [1H3]methyl iodide with 3,4-dichlorobenzenethiol; the mass spectrum of the product showed a molecular ion region with m/z (intensity) values of 192(0), 193(25), 194(2), 195(100), 196(9), 197(69), 198(6), 199(14), and 200(1), revealing that at least 98% of the methyl groups were CD3. For comparison, the corresponding ion cluster for the unlabeled analogue was m/z (intensity) 190(0), 191(4), 192(100), 193(11), 194(70), 195(6), 196(14), and 197(1).

Incubations. The in vitro metabolic studies were performed according to Preussmann et al. (7). Each "complete incubation mixture" contained either NDMA or [14C]NDMA at a concentration of 2.27 mm, 3,4-dichlorobenzenethiol at a level of 22.7 mm, and postmitochondrial S-9 rat liver fraction complemented with a NADPH-generating system in a total volume of 22 ml. Three other incubations which served as controls were carried out simultaneously, i.e., the "S-9-free control," the "nitrosamine-free control," and the "3,4-dichlorobenzenethiol-free control." In these incubations, an equal volume of buffer was added in place of the enzyme, the substrate, and the nucleophile, respectively.

Analysis by GC-MS. Incubations not involving labeled NDMA were each spiked with 100 µg of [1H3]methyl-3,4-dichlorophenyl thioether immediately after the 90-min incubation was complete. Ether extracts were then prepared as previously described (7), but they were concentrated to 0.5 ml instead of 10 ml for GC-MS analysis, using a Hewlett-Packard Model 5700 gas chromatograph interfaced with a JEOI JMS-01S-2 mass spectrometer. Aliquots of 4 to 8 µl each were injected...
directly onto a 1.8-m x 3.2-mm (outside diameter) stainless steel column of 3% OV-1 on 80 to 100 mesh Chromosorb W (AW-DMCS). Injector and detector temperatures were held at 250°C, and carrier gas (helium) flow was 30 ml/min. Chromatography was conducted either isothermally at 110°C or with a temperature program of 80–220°C at 8°C/min. Quantitation was done by comparing the summed total ion intensities of the molecular ion for unlabeled (m/z 192) thioether with those for the labeled internal standard (14H₃, m/z 195). The integral values being obtained from the mass spectral charts after manually scanning at regular intervals (4 to 7 s) during the GC-MS run. Appropriate corrections were made for the isotope contributions of the unlabeled material to the ion intensity at m/z 195.

The resistance of [14H₃]methyl-3,4-dichlorophenyl thioether to back-exchange of label with the medium was determined by incubating 2 µmol in the complete incubation mixture as described above, but with S-9 fraction that had been heated to 100°C for 3 min before addition to the mixture. The thioether detected when this mixture was analyzed by GC-MS had a deuteration pattern identical with that of the [14H₃]methyl starting material.

In some experiments, other extracts of the incubation mixture were, after concentration from 22 ml to 4 ml, extracted with 8 ml of 10% sodium carbonate solution. The ether layer was separated, concentrated to 0.4 ml, dried with anhydrous sodium sulfate, and then evaporated to 0.2 ml before analysis by GC-MS. These samples are referred to below as prepared "with base extraction." Analysis of 14C-containing incubation Mixture. Immediately after the incubations in which [14C]NDMA was used as substrate, 0.2 µmol of 37% formaldehyde solution and 2.00 g of pure methyl-3,4-dichlorophenyl thioether were added. The mixture was shaken vigorously for 1 to 2 min with the aim of equilibrating the cold carrier formaldehyde and thioether with any molecules of their respective 14C-labeled analogue produced during the incubation. Saturated barium hydroxide and 20% zinc sulfate (15 ml of each) were added. The mixture was shaken vigorously for 1 to 2 min and allowed to stand for 1 h to coagulate the proteins. The mixture was suction filtered using a 7-cm Büchner funnel, the filter cake was washed with 40 ml of water, and the combined aqueous filtrate was set aside for the formaldehyde analysis described below. The filter cake was then washed successively with methanol (40 ml) and ether (two 40-ml portions) to remove the relatively water-insoluble methyl-3,4-dichlorophenyl thioether. The combined organic filtrates were dried over approximately 1 g of anhydrous magnesium sulfate and distilled. After solvent had been substantially removed at atmospheric pressure (b.p. 20–74°C), aspirator pressure was applied and the remainder was distilled from Teflon boiling chips to minimize frothing. The cloudy distillate was fractionally redistilled until a relatively homogeneous fraction was obtained, b.p. 130–135°C (15 mm pressure), total 0.5 to 1.0 ml. An aliquot of this distillate was mixed with 10 ml of Aquasol-2 and subjected to scintillation counting. High-performance liquid chromatographic analysis of another aliquot showed a UV-active peak that corresponded to methyl-3,4-dichlorophenyl thioether. No NDMA was detected.

The [14C]formaldehyde concentration in the [14C]NDMA incubation mixture was determined by pouring about one-half of the approximately 80 ml of aqueous filtrate prepared above into a solution containing 0.76 g of 5,5-dimethyl-1,3-cyclohexanedione in 110 ml of 0.03 M sodium hydroxide. Glacial acetic acid (2 ml) was added, and the resulting precipitate was collected on a Hirsch funnel and washed with cold water. The precipitated dimerone-formaldehyde adduct was recrystallized twice from warm methanol [m.p. 188.5–189.5°C, literature m.p. 187–188°C (8)] and subjected to scintillation counting. The concentration of [14C]formaldehyde in the purified [14C]NDMA used as starting material was similarly determined.

RESULTS AND DISCUSSION

We have made many attempts over several years to use 3,4-dichlorobenzenthiole as a trapping agent for the quantitation of methyllating species generated in vivo during the incubation of NDMA with rat liver S-9 preparations. Experiments in which we followed as closely as possible the originally described (7) procedure (results shown in Table 1) have consistently led to the conclusion that the yields of methyl-3,4-dichlorophenyl thioether were always smaller and more variable than those previously reported (7). In addition, variations on this method using deuterated or ethylated substrates, micromolar instead of S-9 fractions, stored versus freshly prepared liver preparations, and more than one strain of rat (methods and results not given) have also supported this generalization. Moreover, in none of our experiments has a significant difference in thioether yield been observed between a given complete incubation mixture and its nitrosamine-free control. Table 1 summarizes the results of 3 representative experiments, as well as those of Preussmann et al. (7).

Incubations in which [14C]NDMA was used as substrate revealed that only a small fraction (approximately 10%) of the total methylation product was radiolabeled, suggesting that most of the alkylating activity was associated with some other component(s) of the S-9 rat liver fraction (possibly S-adenosylmethionine, which has been shown to be capable of methylyating a wide variety of substrates (1, 9)). Nevertheless, some methylation did seem to originate from metabolism of the nitrosamine. This was evidenced by isolation from complete incubation mixtures containing [14C]NDMA, not only of radiolabeled methyl thioether as mentioned above, but also of [14C]formaldehyde. As indicated in Table 1, the labeled substrate was converted during incubation for 90 min with S-9 Preparation 1 to roughly equimolar amounts of [14C]methyl-3,4-dichlorophenyl thioether and [14C]formaldehyde (0.13 and 0.10 µmol, respectively), as expected, based on the established mechanism (5) of in vitro NDMA metabolism.

In addition to the methylation that occurred during incubation, variable amounts of methyl-3,4-dichlorophenyl thioether were found to be generated during analysis. The artifactual methylation appeared to result from the presence in the gas chromatographic column of a nonvolatile 3,4-dichlorobenzenthiole derivative (e.g., the sodium salt) and/or methylyating agent from previous injections. Thus, either the volatile free thiol or the methylation components of subsequent injections combined with the reactive on-column contaminants to produce thioether. This was particularly extensive when we washed the extracts with sodium carbonate solution before injection, in an attempt to remove the acidic 3,4-dichlorobenzenthiole. Unexpectedly, the initial m/z 192 peak was followed by a large tail observable for up to 1 h. This tail integrated to as much as 10 µmol of thioether, suggesting the entrapment of some ionized (therefore more nucleophilic and less volatile) 3,4-dichlorobenzenthiole in the injected thioether. It is unlikely that the artifact problem resulted from our use of metal chromatographic columns instead of glass, as used in the previous work (7), because preliminary experiments performed using a glass column gave results similar to those of Table 1 (0.18 µmol found in the complete incubation mixture, 0.16 µmol in the nitrosamine-free control). It is possible that high-pressure liquid chromatography might be used to advantage for future analyses; a pilot experiment with [14C]NDMA led to isolation of 1 µmol of total thioether, of which 0.01 µmol contained a methyl group originating from the nitrosamine.

Chromatograms were easily corrected for on-column synthesis by curve resolution procedures. Methyl-3,4-dichlorophenyl...
IN VITRO METHYLATION OF 3,4-DICHLOROBENZENETHIOL

Table 1

Quantity of methyl-3,4-dichlorobenzenethiol thioether produced during incubation of NDMA with rat liver S-9 fractions and 3,4-dichlorobenzenethiol

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Value from Ref. 7</th>
<th>S-9 Preparation 1</th>
<th>S-9 Preparation 2</th>
<th>S-9 Preparation 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete incubation mixture</td>
<td>2</td>
<td>1.06 ± 0.35</td>
<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td>S-9-free control</td>
<td>N.D.</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Nitrosamine-free control</td>
<td>0.1–0.2</td>
<td>0.98 ± 0.06</td>
<td>0.66</td>
<td>0.08</td>
</tr>
<tr>
<td>3,4-Dichlorobenzenethiol-free control</td>
<td>*</td>
<td>0.02</td>
<td>N.D.</td>
<td>0.03</td>
</tr>
<tr>
<td>Complete incubation mixture with [14C]NDMA</td>
<td>—</td>
<td>0.13(12)*</td>
<td>0.01(8)*</td>
<td>0.01(11)*</td>
</tr>
</tbody>
</table>

* The theoretical yield is 50 μmol, assuming that each NDMA molecule should be converted to one molecule of thioether.
* Mean ± SD of 4 determinations (2 separate incubations, 2 replicate analyses each).
* N.D., not detected.
* Mean ± SD of 2 replicate analyses (1 incubation).
* _, no value reported.
* Detection limit was 0.0001 μmol. The OV-1 column used for this analysis had never been used before, and could not have contained 3,4-dichlorobenzenethiol residues from previous injections.
* Yield of [14C]methyl-3,4-dichlorophenyl thioether when [14C]NDMA was incubated with the same S-9 preparation indicated in the column heading. Yields were determined by isolation and scintillation counting, as described in "Materials and Methods."
* Number in parentheses, proportion of the total thioether that was radiolabeled; it was calculated by expressing the isotope dilution value on this line as a percentage of the total thioether yield (see "Complete incubation mixture" value above) found chromatographically when the same S-9 preparation was incubated separately but simultaneously with cold NDMA.
* Radiolabeled formaldehyde was also found, in amounts of 0.08 and 0.18 μmol, after 0 and 90 min of incubation, respectively. Since the former value corresponds to contamination of the starting material, net production of [14C]formaldehyde during incubation must have been 0.10 μmol in this experiment.
* Base extraction was not used in the [14C]NDMA experiments, but the incubation was performed simultaneously with and used the same S-9 preparation as the other 4 in this column.

In summary, our studies using 3,4-dichlorobenzenethiol to trap alkylating agents during in vitro metabolism of NDMA yielded small and variable levels of methylation by nitrosamine, as well as unexpectedly high background levels of methyl-3,4-dichlorophenyl thioether. The methylated thioether was produced not only as an artifact of the analytical procedure, but also through the involvement of some methylating agent(s) other than the nitrosamine during incubation. We conclude that 3,4-dichlorobenzenethiol and related compounds should be used with caution for measuring the extent of alkylation during the in vitro metabolism of indirect alkylating agents such as the nitrosamines.

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REFERENCES

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