Nondetection of O\textsuperscript{6}-Methylguanine in Rat DNA following in Vivo Treatment with Large Doses of Cimetidine Alone or in Combination with Sodium Nitrite\textsuperscript{1}

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ABSTRACT

Cimetidine was administered by stomach tube to rats at 70 or 700 mg/kg, doses corresponding to 5 or 50 times, respectively, the typical daily dose of individuals on cimetidine treatment. In some cases, cimetidine (70 mg/kg) was administered in combination with a 2-fold molar excess of sodium nitrite. This treatment was carried out up to 6 times over a 3-day period, the pH of the rat stomach being maintained at 2.3 to 3.0 for about 1 hr after each treatment. The DNA of the stomach, liver, and intestines (large and small pooled together) was isolated 6 hr after cessation of treatment and analyzed for the presence of O\textsuperscript{6}-methylguanine using a sensitive and specific radioimmunoassay. No evidence could be obtained for the presence of this methylated base in any of the DNA samples examined, the limit of detection being 3 \textmu m mol O\textsuperscript{6}-methylguanine per mg guanine. We suggest that the observed lack of DNA methylation may be primarily due to the slow rate of nitrosation of cimetidine in combination with its rapid absorption into the blood stream.

INTRODUCTION

The drug cimetidine (Tagamet) is a histamine H-2 receptor antagonist widely used in the treatment of gastric disorders (3, 4). Some concern has been expressed recently over the use of cimetidine, following reports of the appearance of gastric cancer in patients previously treated with it (7, 8, 23, 28, 30, 37). The concern raised by these reports was underlined by the fact that cimetidine is a derivative of N-methyl-N'-cyanoguanidine (Chart 1) and has the potential of undergoing in vivo A/-nitrosation to give a W-nitroso derivative chemically similar to the mutagen in bacteria (26) and an inducer of sister chromatic exchanges (1). Further, the typical daily dose of individuals on cimetidine therapy, accompanied by 2-fold molar excess of sodium nitrite.

In order to examine whether damage to DNA can occur as a result of the formation of NC under the conditions prevailing in the human stomach during cimetidine treatment, we have analyzed the DNA of rats treated p.o. with large doses of cimetidine, either alone or in combination with sodium nitrite. For the analysis of DNA, we have used a sensitive radioimmunoassay specific for O\textsuperscript{6}-methyldeoxyguanosine (16). We report that no such base modification could be detected in the DNA of rats treated in vitro (15). Of particular interest is the finding that the spectrum of DNA base methylation observed in the latter case is similar to that found with methylating carcinogens such as MNNG, NMU, and dimethylnitrosamine (17, 18) and includes, among other products, O\textsuperscript{6}-methylguanine. The latter, although a secondary product of DNA base methylation, has been strongly implicated in the mechanism of mutagenic and carcinogenic action of methylating agents (18, 24). Clearly then, NC demonstrates in vitro test systems many of the characteristic properties of a carcinogen. Consequently, the possibility of its formation in the human stomach during cimetidine treatment is of particular interest.

The formation of N-nitroso compounds by in vivo nitrosation of suitable precursors has been clearly demonstrated in experimental animals (19–21), and it is likely that it also occurs in humans (10, 33). Apart from the direct detection of N-nitroso compounds in body fluids, evidence of their in vivo formation can be obtained through the detection of the characteristic products of their interaction with stable cell components such as DNA (9, 22). This approach is particularly useful in the case of DNA-modifying agents the in vivo stability of which is such as to preclude their convenient isolation from body fluids or tissues and subsequent characterization. Furthermore, this method, apart from giving an indication of the transient formation of the reactive species, allows a direct estimate of the resulting DNA damage which, from the point of view of carcinogenesis, is perhaps a more significant observation. Using such an approach, Montesano and Magee (22) have demonstrated that, following administration of methylurea and sodium nitrite by stomach tube to rats, 7-methylguanine appears in the DNA of rat tissues, probably arising from reaction with transient, in vivo-generated NMU.

In order to examine whether damage to DNA can occur as a result of the formation of NC under the conditions prevailing in the human stomach during cimetidine treatment, we have analyzed the DNA of rats treated p.o. with large doses of cimetidine, either alone or in combination with sodium nitrite. For the analysis of DNA, we have used a sensitive radioimmunoassay specific for O\textsuperscript{6}-methyldeoxyguanosine (16). We report that no such base modification could be detected in the DNA of rats treated in vitro (15). Of particular interest is the finding that the spectrum of DNA base methylation observed in the latter case is similar to that found with methylating carcinogens such as MNNG, NMU, and dimethylnitrosamine (17, 18) and includes, among other products, O\textsuperscript{6}-methylguanine. The latter, although a secondary product of DNA base methylation, has been strongly implicated in the mechanism of mutagenic and carcinogenic action of methylating agents (18, 24). Clearly then, NC demonstrates in vitro test systems many of the characteristic properties of a carcinogen. Consequently, the possibility of its formation in the human stomach during cimetidine treatment is of particular interest.

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absence of cimetidine or sodium nitrite. Furthermore, about 80% of the volume of the above mixture disappeared from the rat stomach within 30 min, thus permitting the administration of a further 1.5 ml CB and the subsequent maintenance of the stomach pH for a further 30 min. Consequently, the following procedure was adopted for the treatment of animals.

Tagamet tablets (obtained from a local drugstore) were crushed into fine powder and suspended in CB at concentrations corresponding to 7-, 14-, or 70-mg/ml cimetidine. Sodium nitrite was dissolved separately in CB at a concentration of 7.7 mg/ml, corresponding to twice the stoichiometric concentration of cimetidine in the second of the above solutions. Where necessary, the pH of the solutions was adjusted to 2.3 with concentrated HCl. The solutions were kept at 0° during use and stored frozen at -20° between animal treatments.

Six groups of 5 male Wistar rats (from our own colony), weighing 200 g each and which had been starved for 24 hr, were treated by stomach tube with one of the cimetidine solutions (either alone or followed immediately by sodium nitrite, in a total volume of 2 ml) and, 30 min later, by 1.5 ml of CB. Either each treatment was carried out only once or it was repeated twice daily (with a 6-hr interval between treatments) and for a total of 3 consecutive days. Negative control animals received plain CB, while positive control animals received 2 ml of a solution of MNNG (Serva, Heidelberg, Federal Republic of Germany), in CB (1 mg/ml), followed 30 min later by 1.5 ml CB. The treatment given to the various groups of animals is summarized in Table 1.

All animals were kept without food during the whole course of the experiment but were allowed free access to water. They were killed with ether 6 hr after the initiation of the final treatment, and the stomach, liver, and intestines (large and small pooled together) were rapidly removed, pooled separately for each group, and frozen in liquid nitrogen.

**DNA Preparation and Analysis.** After being allowed to thaw in cold PBS, the tissues were washed thoroughly with the same buffer prior to homogenization. DNA extraction was carried out as described previously (16), except that 2 phenol extractions were carried out instead of only one.

The DNA prepared was hydrolyzed to nucleosides by dissolving in 10 mA mammonium acetate (pH 6.0) (2 mg/ml) and digesting with DNase I (EC 3.1.4.5; Sigma type I; 500 units/ml) for 30 min at 37°. After addition of 0.1 volume 1 M Tris-HCl (pH 8.6), snake venom phosphodiesterase (EC 3.1.4.1; Sigma type VII; 0.15 unit/ml) and alkaline phosphatase (EC 3.1.3.1; Sigma type VII; 15 units/ml) were added, and digestion was continued for 16 hr.

The enzymic DNA hydrolysate thus obtained (usually corresponding to 2 mg DNA) was chromatographed on a column of Aminex A6 resin (170 x 10 mm; Bio-Rad, Richmond, Calif.) eluted with 0.4 M ammonium formate, pH 4.26, at 50°. Fractions of 1 ml were collected with an ISCO fraction collector (ISCO, Nebraska) equipped with a flow interrupt valve. The fractions corresponding to the position of O6-methyldeoxyguanosine (determined in separate experiments using authentic marker compound prepared as described previously (16)) were analyzed for the presence of this nucleoside by radioimmunoassay. The

### Chart 1. Chemical formulas of cimetidine, NC, and MNNG.

<table>
<thead>
<tr>
<th>R</th>
<th>R-H</th>
<th>R-NO</th>
<th>NO</th>
<th>CH₂-N-C-NH-NO₂</th>
<th>NH</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-C-NHCH₂-S-CH₂-CH₃</td>
<td>CIMETIDINE</td>
<td>N-NTROSOCIMETDINE (NC)</td>
<td>CH₂-N-C-NH-NO₂</td>
<td>MNQ (10 mg/kg)</td>
<td>MMNG (10 mg/kg)</td>
</tr>
</tbody>
</table>

### Table 1

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Dose of cimetidine/ treatment (mg/kg)</th>
<th>Dose of sodium nitrite/ treatment (mg/kg)</th>
<th>Other treatment</th>
<th>No. of treatments</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>700</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>38.5</td>
<td></td>
<td>1</td>
</tr>
<tr>
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<td>70</td>
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</tr>
<tr>
<td>5</td>
<td>70</td>
<td></td>
<td>CB</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td></td>
<td>MNNG (10 mg/kg)</td>
<td>1</td>
</tr>
</tbody>
</table>

* Negative control.
* Positive control.

A **UM** of the deoxyguanosine fractions was measured and used for the quantitation of the deoxyguanosine in the chromatographed sample using a molar absorption coefficient $e_{260} = 12,300$ mol$^{-1}$ cm$^2$.

**Radioimmunoassay.** Radioimmunoassay was carried out on the fractions collected from the chromatography column by a modification of the procedure already described (16). The assay mixtures consisted of 1 ml ice-cold 0.4 M ammonium formate (pH 4.26), tracer O6-methyl[1',2'-3H]deoxyguanosine [30.3 Ci/mmol; prepared by the methylation of [1',2'-3H]deoxyadenosine (Radiochemical Centre, Amersham, U.K.) as described previously (16)] (in 25 μl PBS; approximately 1000 dpm), unlabeled O6-methyldeoxyguanosine (0 to 65 pmol in 25 μl PBS) and, added last, rabbit anti-O6-methyldeoxyguanosine antiserum (25 μl in PBS) diluted to give 30% binding of tracer in the absence of added O6-methyldeoxyguanosine. After incubation at 0° for 30 min, the mixtures were passed rapidly through a Millipore type HA filter (25 mm diameter; 0.45 μm) (Millipore Intertech, Inc., Bedford, Mass.). The filters were then washed twice with 2-ml aliquots of cold ammonium formate buffer, dried at 80° for 30 min, and allowed to cool, and the bound radioactivity was counted in 5 ml scintillation cocktail (0.5% PPO-0.02% POPOP in toluene) using a Packard Model 3320 liquid scintillation counter (Packard Instrument Co., La Grange, Ill.).

### RESULTS

Chart 2 shows the chromatographic profiles obtained from the Aminex A6 column using various enzymic DNA hydrolysates. The results in Chart 2A were obtained with liver DNA hydrolysates (from rats not treated in any way) to which authentic O6-methyl[1',2'-3H]deoxyguanosine had been added. Clearly, good separation of the methylated nucleosides from the normal nucleosides was obtained, 96% of the added marker compound being recovered in Fractions 41 to 45. A radioimmunoassay carried out on the corresponding fractions of the eluates of DNA hydrolysates from the livers of untreated rats (Table 1) showed no inhibition of tracer binding, indicating the absence of detectable amounts of O6-methyldeoxyguanosine or of any other cross-reacting substance. On the other hand, if O6-methyldeoxyguanosine were added in known quantities to control DNA prior to hydrolysis, it could be clearly detected in the corresponding column eluates (Chart 2B). From radioimmunoassay of the appropriate chromatographic fractions and a standard curve (Chart 3), the amount of O6-methyldeoxyguanosine which had been loaded onto the

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* The position of 7-methyldeoxyguanosine, the major product of DNA methylation by NC in vitro (15), was not determined, because it was not expected to be present in DNA in amounts likely to interfere with the radioimmunoassay (Ref. 16; S. A. Kyrtopoulos, unpublished observations).
Chart 2. Analysis of DNA by column chromatography and radioimmunoassay. DNA (2 mg) was enzymically hydrolyzed to the nucleosides and chromatographed on a column of Aminex A6 eluted with 0.4 M ammonium formate buffer, pH 4.26, at 50°C. The 3 absorbance peaks correspond to the pyrimidine nucleosides deoxyguanosine and deoxyadenosine, respectively. Fractions 36 to 55 were analyzed for O6-methyldesoxycytosine by radioimmunoassay. A, control liver DNA containing about 5 x 10^4 dpm added O6-methyl[1',2'-3H]desoxycytosine; B, control liver DNA containing 26.2 pmol added O6-methyldesoxycytosine; C, stomach DNA from rats treated with MNNG (10 mg/kg); D, stomach DNA from rats treated 6 times with cimetidine (70 mg/kg) and sodium nitrite (38.5 mg/kg). --- (B to D), limits of significance of the inhibition of tracer binding in the radioimmunoassay (2 S.D.).

Chart 3. Inhibition of tracer antibody binding by O6-methyldesoxycytosine. For experimental details, see "Materials and Methods." Duplicate measurements agreed to within ±5% (S.D.).

Column could be calculated. The average recovery thus quantitated (for 10 to 50 pmol O6-methyldesoxycytosine) was 94 ± 10% (S.E.).

Chart 2C shows a typical result obtained with DNA hydrolysate from rats treated with MNNG. O6-Methyldesoxycytosine was clearly detectable, and the extents of DNA methylation in various tissues could be calculated (Table 2). It can be seen that maximal methylation is found in the DNA of the stomach, followed closely by liver and then by intestine DNA.

Chart 2D shows the results obtained with DNA from the stomachs of rats treated 6 times with cimetidine (70 mg/kg) and excess sodium nitrite (Table 1, Group 4). Clearly, no inhibition of tracer binding was observed with the fractions corresponding to the position of O6-methyldesoxycytosine, indicating the absence of detectable amounts of the latter. Similar results were obtained with the DNA from the tissues of rats of Groups 1 to 3 (Table 1). The results are summarized in Table 2.

DISCUSSION

We have found that p.o. treatment of rats with single or multiple doses of cimetidine, alone or in combination with sodium nitrite, did not result in the formation of detectable amounts of O6-methylguanine in DNA. The minimum quantity of O6-methyldesoxycytosine which could be detected by the radioimmunoassay used is 1 pmol (Chart 3). Because this nucleoside is eluted from the Aminex A6 column in 5 fractions, with each of the central 2 fractions containing about one-third of the total (Chart 2A), the minimum quantity which would need to have been loaded onto the column and still be detectable by radioimmunoassay on the eluate is 3 pmol. If this amount originated from 2 mg methylated DNA (the quantity of DNA routinely analyzed), it would correspond to about 3 pmol O6-methylguanine per mol guanine in the original DNA. This constitutes the lowest level of DNA methylation detectable under the conditions used in our experiments.

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Nondetection of O6-Methylguanine in DNA after Cimetidine

Table 2

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Stomach</th>
<th>Liver</th>
<th>Intestines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
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</tr>
<tr>
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<td>ND</td>
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<tr>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

ND, not detected. The limit of detection was 3 µmol O6-methylguanine per mol guanine.

The kinetics of cimetidine nitrosation in aqueous solutions has been shown by Bavin et al. (2) to be similar to those of the nitrosation of methyurea (19), both reactions showing first-order dependence on the concentrations of nitrosatable substrate, nitrite, and hydrogen ions. Administration of methyurea and nitrite to rats gives rise to NMU in their stomachs (20, 21) as well as to DNA methylation (22). Montesano and Magee (22) found 8 to 15 µmol N\(^-\)methylguanine per mol guanine (depending on the tissue) in the DNA of rats treated p.o. with a combination of methyurea and sodium nitrite (23.7 µmol/kg and 0.61 mmol/kg, respectively). Because the ratio of O6- to N\(^-\)methylguanine in DNA methylated by NMU is (in the absence of repair) 0.11 (17), the amounts of O6-methylguanine formed can be calculated to have been 0.7 to 1.4 µmol/mol guanine. Such levels of DNA methylation would have been too low to be detected by our assay. However, the dose of cimetidine which we administered in combination with sodium nitrite was over 10-fold higher than the above dose of methyurea (277.3 µmol/kg), while our dose of sodium nitrite was only slightly lower (0.56 mmol/kg). Furthermore, we administered this combination twice a day for 3 days, amounting to a total dose of cimetidine over 70-fold higher than that used with methyurea. It can be confidently anticipated that, if the in vitro DNA-methylating efficiency of the combination of cimetidine and sodium nitrite were similar to that of methyurea and sodium nitrite, our treatment schedule would have resulted in levels of DNA methylation comfortably detectable by our assay. Nevertheless, despite the use of such large doses, no O6-methylguanine was formed in the DNA above our limit of detection of 3 µmol/mol guanine. Ignoring any possible DNA repair effects, it may be calculated from this result that cimetidine, in combination with sodium nitrite, methylates DNA in vivo at least 30-fold less than methyurea does under the same conditions. The actual in vivo-methylating capacity of cimetidine plus sodium nitrite is probably even lower than indicated by this figure, because during our cimetidine experiments the acidity of the rat stomach was maintained at a level about 100-fold higher than that which probably prevailed during the methyurea experiments of Montesano and Magee (22), a condition significantly favoring cimetidine nitrosation.

While N\(^-\)methylguanine does not appear to be subject to repair in the rat, O6-methylguanine is well known to be removed from DNA by repair processes (18, 24). However, significantly rapid repair of O6-methylguanine occurs only in the liver of the rat (18, 24, 25) and may have contributed to the nondetection of this lesion in liver DNA. On the other hand, the intestine is known to be poor in this type of repair activity (25) and, while no relevant information appears to be available for the stomach, repair does not appear likely to be an important factor here either (18).

Apart from possible pharmacokinetic effects, 2 other factors appear likely to determine the reduced ability of cimetidine plus sodium nitrite to methylate DNA in vivo relative to methyurea plus sodium nitrite: (a) the rates of the respective nitrosation reactions; and (b) the rates and pathways of breakdown of the respective N-nitroso derivatives.

Although the nitrosations of cimetidine and methyurea obey similar kinetic equations (2, 19), the molecular rate constant for the former reaction is some 200 times smaller than for the latter (0.050 M\(^{-2}\)sec\(^{-1}\) and 10.5 M\(^{-2}\)sec\(^{-1}\), respectively, at 25°). This difference is expected to influence significantly the proportions of cimetidine and methyurea nitrosated prior to disappearance from the stomach of the administered reactants. In this context, it should be underlined that cimetidine is absorbed into the blood stream rapidly, its blood concentration becoming maximal in well under 1 hr (12). This rapid absorption, in combination with the relatively slow rate of nitrosation, is probably the major factor determining the observed lack of DNA methylation.

The in vitro stability and reactivity of NC have been studied in some detail by Jensen and Magee (15). These workers reported that, in the absence of sulfur nucleophiles, NC is remarkably stable, having a half-life of 135 hr at pH 7.4 and 23°. For comparison, NMU has a half-life of under 1.2 hr under the same conditions (8), while its in vivo lifetime is much shorter (36). This relative stability of NC, if extending to the conditions inside the cell, may protect cellular DNA from damage by permitting NC to undergo detoxifying reactions resulting in loss of its methylating capacity.

The breakdown of NC is efficiently catalyzed by sulfhydryl compounds such as cysteine and glutathione (15). However, even after complete breakdown in the presence of excess cysteine, NC methylates DNA in vitro to about one-half the extent to which NMU does. This difference, reflecting different pathways of breakdown in the 2 cases, is probably a further factor contributing to the differences observed in vivo.

Our results have some bearing on the question of the risk faced by humans taking cimetidine. A typical clinical dose taken by humans on cimetidine treatment is 1 g/day, i.e., 14 mg/kg/day for a 70-kg individual (12, 27). The daily doses of cimetidine administered in our experiments were 10- and 100-fold higher and were continued for 3 days. We also administered cimetidine at 5-fold the human daily dose, accompanied by excess sodium nitrite, up to 6 times over a 3-day period. In no case did we detect any O6-methylguanine in the DNA of the treated animals. The concentration of the administered nitrite (in the rat stomach) in our experiments was 48.6 µM, while the concentration of nitrite in the gastric juice of fasting healthy humans is reported to be about 4 µM (33, 38), more than 10\(^4\) fold lower. Assuming that cimetidine nitrosation in vivo follows kinetics similar to the in vitro reaction, our results suggest that the highest level of methylation of the DNA of the stomach, liver, and intestines following a daily dose of 1 g cimetidine in the human is likely to be at least 5 \times 10^4 times lower than the maximum which could have been present in the DNA of our experimental animals. Extrapolation to in vivo reactions at lower concentrations of reactants should be made with some caution,
however, because it has been shown that sometimes (31), although not always (14, 20), the kinetics of such reactions differs quite significantly from that of reactions in vitro or at high reactant concentrations.

Our results do not exclude the possibility of NC formation or of DNA methylation during the treatment of humans with cimetidine. Indeed, the occurrence of a nitrosated derivative of cimetidine in the stomach mucosa of humans who have received cimetidine has been reported very recently (29, 35). However, the work reported here does point to differences in the chemical behavior of cimetidine in vivo relative to that exhibited in highly artificial in vitro systems and suggests that any adverse effects predicted by the latter may be less important in vivo than anticipated.

REFERENCES


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