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**Sequence-specific DNA damage induced by reduced mitomycin C and 7-N-(*p*-hydroxyphenyl)mitomycin C**

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ABSTRACT

Mitomycin C reduced with sodium borohydride induced the DNA damage at deoxyguanosines preferentially in dinucleotide sequence G-T. The DNA damage produced strand breaks when subsequently heated. The DNA damage scarcely occurred when the end-labeled DNA was preincubated with ethidium bromide or actinomycin D before the addition of mitomycin C and the reducing agent. Fully reduced mitomycin C did not induce the DNA damage. The mitomycin C-inducing DNA damage seems to require the intercalation of the partially reduced mitomycin C of short life time, probably semiquinone radical, between DNA base pairs. The inhibitory effects of sodium chloride and radical scavengers suggested that the requirement of the covalent bond formation of mitomycin C to DNA and the involvement of oxygen radicals in the DNA damage. 7-N-(*p*-hydroxyphenyl)mitomycin C, which is reported to show a higher antitumor activity and a lower toxicity than mitomycin C, was readily reduced with dithiothreitol and induced the sequence-specific DNA damage, whereas mitomycin C was not.

INTRODUCTION

Mitomycin C is a potent anticarcinogenic antibiotic. Metabolically or chemically activated mitomycin C interacts with DNA, resulting in a covalent binding of the drug to DNA and partly in the formation of cross-links between the complementary strands of DNA (1,2). These DNA modifications have been believed to be essential for the cytotoxicity of mitomycin C (1,3).

We investigated the interaction of mitomycin C with DNA by using a sequencing technique, and reported that the end-labeled DNA fragments reacted with chemically reduced mitomycin C were cleaved when they were subsequently heated at 90°C for 5 min in Tris-HCl buffer (4). The breaks occur at the 3' side of deoxyguanosines and of some deoxyadenosines. This is well consistent with the preference of the binding sites of mitomycin C to DNA, which are the O-6 position or the 2-amino group of guanine residues or the 6-amino group of adenine residues (5,6).

The most striking feature of the mitomycin C-inducing DNA damage was the sequence specificity. The DNA damage was induced most preferentially at

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dinucleotide sequence G-T (especially PuG-T)(4). The DNA sequencing technique revealed the sequence-specific interaction between DNA and several antitumor agents, e.g., bleomycin (7,8), neocarzinostatin (9), hedamycin (10), actinomycin D, and netropsin (11). The sequence specificity of the mitomycin C-DNA interaction was remarkable compared to other agents, although mitomycin C is a rather small molecule.

Substitution at certain sites on bases in DNA, e.g., 7-alkylguanine, increases the rate of hydrolysis of the N-glycosidic bond to yield apurinic sites (12) and then produces strand breaks. Mitomycin C does not alkylate the N-7 position (13) but mainly the O-6 position or the 2-amino group of guanine residues in DNA (5,6). It is not reported that O<sup>6</sup>-alkylguanine produces apurinic sites. The detailed mechanism of the mitomycin C-inducing DNA damage needs further study.

In this paper, we examined the effect of several inhibitors on the mitomycin C-inducing DNA damage, and investigated the DNA damage induced by 7-N-(p-hydroxyphenyl)mitomycin C (M-83) which is expected to be more useful as a clinical antitumor agent. The results suggest that the intercalation of mitomycin C semiquinone radical is required for the induction of the DNA damage, and that M-83 is readily reduced with dithiothreitol and induces the sequence-specific DNA damage although mitomycin C does not induce the DNA damage in the presence of dithiothreitol.

### Materials and Methods

Chemicals and Enzymes. Mitomycin C and 7-N-(p-hydroxyphenyl)mitomycin C were kindly supplied by Kyowa Hakko Co. Ltd., Tokyo, Japan. Restriction enzymes HaeIII, TaqI and HinfI, and the Klenow fragment of DNA polymerase I of Escherichia coli were obtained from Takara Shuzo Co. Ltd. [ $\alpha$ -<sup>32</sup>P]dCTP (specific activity about 3000 Ci/mmol) was purchased from New England Nuclear.

DNA substrates. Three DNA fragments of defined sequence were obtained from bacteriophage  $\phi$ X174 replicative form DNA. Double-stranded  $\phi$ X174 replicative form DNA was prepared as previously described (14) and digested with HaeIII, and 310, 234 and 194 base pair fragments [Z<sub>5</sub>, Z<sub>7</sub> and Z<sub>8</sub> fragments in the map reported by Sanger (15)] were purified. Fragments Z<sub>5</sub> and Z<sub>7</sub> were digested with TaqI, and was labeled by extension of the 3' termini with Klenow polymerase in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP (16). Fragment Z<sub>8</sub> was digested with HinfI and labeled at the 3' termini in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP and unlabeled dATP. Resulting 3'-end-labeled 141, 178 and 257 base pair fragments (C<sub>980</sub>-C<sub>1120</sub>, C<sub>492</sub>-C<sub>669</sub> and C<sub>723</sub>-C<sub>979</sub> in the map reported by Sanger,

respectively) were purified by electrophoresis on a 4% polyacrylamide gel.

Reaction Conditions. The standard reaction mixture (100  $\mu$ l) contained 25 mM Tris-HCl buffer (pH 7.1), 0.1 mM mitomycin C, 0.2 mM sodium borohydride and 3'-<sup>32</sup>P-labeled DNA fragment [approximately 50 ng (specific activity  $2 \times 10^3$  cpm/ng)]. The reaction was started by the addition of freshly prepared sodium borohydride solution, carried out for 15 min at 37°C and terminated by the addition of 4  $\mu$ l of 0.5 M EDTA, 2  $\mu$ l of 1 mg/ml tRNA, 10  $\mu$ l of 3 M sodium acetate (pH 5.2) and followed by ethanol precipitation. The reaction of mitomycin C in the presence of 1 mM dithiothreitol was carried out for 3 h at 37°C. The pellet was rinsed in 70% ethanol, dried and resuspended in 40  $\mu$ l of 10 mM Tris-HCl buffer (pH 8.1), and the suspension was heated for 5 min at 90°C. When the inhibitory effect of intercalating agents was examined, the DNA was incubated with the 30  $\mu$ M intercalating agents at 37°C for 5 min before mitomycin C and the reducing agent were added.

The heat-treated DNA was reprecipitated with ethanol, rinsed in 70% ethanol, dried, and dissolved in 5  $\mu$ l of 80% (V/V) formamide-10 mM NaOH loading buffer for gel electrophoresis. DNA was heat denatured for 1 min at 90°C, and loaded on a 8% or 10% polyacrylamide slab gel for sequence analysis. Electrophoresis was at 25 mA. Autoradiography was done on a Fuji RX film at -70°C.

Quantitative Analysis. The relative amount of the produced oligonucleotides were determined by scanning the autoradiograms with a microdensitometer as described earlier (4).

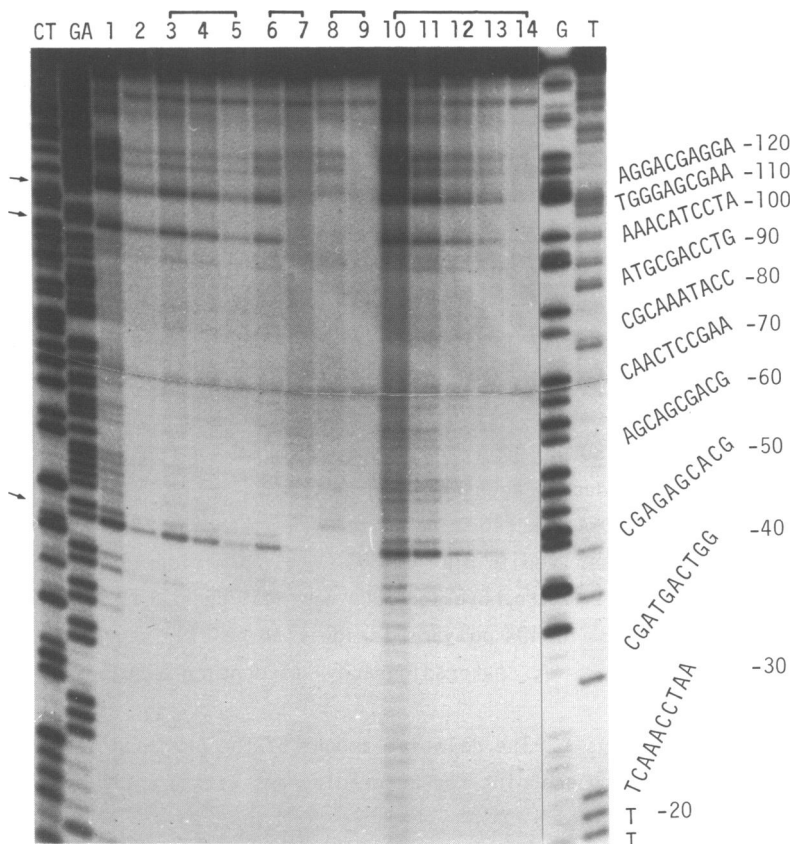
## RESULTS

### Sequence specificity of heat-labile sites induced by reduced mitomycin C

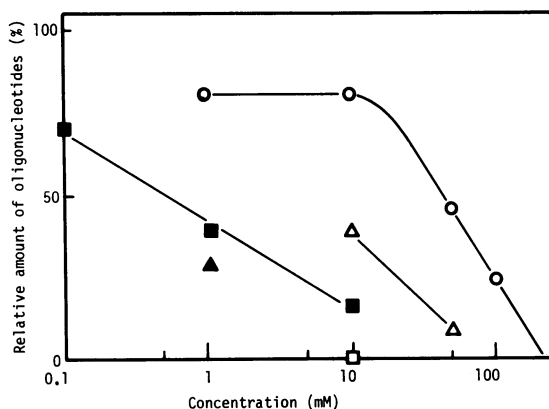
To investigate the interaction of mitomycin C with DNA, we used 3'-end-labeled 178 base pair restriction fragment of  $\phi$ X174 replicative form DNA. Oligonucleotides were detected on the autoradiogram as the products of the reaction with reduced mitomycin C and subsequent heat-treatment (Fig. 1, lane 1). Sequence specificity of mitomycin C-DNA interaction is clearly detected by the comparison with oligonucleotides produced by the chemical reactions of the Maxam-Gilbert procedure (17) and by thymidine-specific photo-reaction (18). The heat-labile sites were induced most extensively at the dinucleotide sequence G-T.

### Inhibition of mitomycin C-inducing DNA damage by sodium chloride, radical scavengers and metal-chelating agents

The inhibitory effects of sodium chloride, radical scavengers and metal-



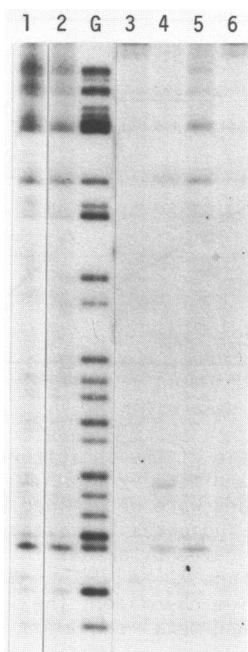
**Figure 1.** Induction of the DNA damage by reduced mitomycin C and the inhibitory effects of sodium chloride, metal-chelating agents and radical scavengers. The 178 base pair 3'-end <sup>32</sup>P-labeled DNA was incubated with 0.1 mM mitomycin C and 0.2 mM sodium borohydride for 15 min at 37°C. After the reaction, DNA was precipitated with ethanol, resuspended in 40 µl of 10mM Tris-HCl buffer (pH 8.1), and heated for 5 min at 90°C (lane 1). The reaction with mitomycin C was carried out in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA)(lane 2), diethylenetriaminepentaacetic acid (DETAPAC) at concentrations of 0.1 mM (lane 3), 1 mM (lane 4) and 10 mM (lane 5), sodium benzoate at concentrations of 10 mM (lane 6) and 50 mM (lane 7), 1,4-diazabicyclo[2,2,2]octane (DABCO) at concentrations of 10 mM (lane 8) and 50 mM (lane 9) and sodium chloride at concentrations of 1 mM (lane 10), 10 mM (lane 11), 50 mM (lane 12), 100 mM (lane 13) and 500 mM (lane 14). Products of base-specific chemical reactions of the Maxam-Gilbert procedure and the thymidine-specific photo-reaction were in lanes CT, GA, G and T, respectively. The sequence is indicated on the right side of the figure. Nucleotide numbers from the 3' end of the fragment are indicated.



**Figure 2.** Quantitative analysis of the inhibitory effects of sodium chloride, radical scavengers and metal-chelating agents. The relative amount of the produced oligonucleotides were determined by scanning the autoradiogram in figure 1. Each value indicates the average of the relative amounts of three oligonucleotides indicated by arrows in figure 1 to the corresponding oligonucleotides in lane 1. ▲, EDTA; ■, DETAPAC; △, sodium benzoate; □, DABCO; ○, sodium chloride. The inhibitory effects of sodium chloride, radical scavengers and metal-chelating agents were confirmed using another DNA fragments.

chelating agents were examined on the mitomycin C-inducing DNA damage (Fig. 1). The relative amount of produced oligonucleotides were measured as described in Materials and Methods to examine the inhibitory effect quantitatively (Fig. 2). Sodium chloride at the concentrations below 10 mM did not inhibit the production of oligonucleotides, but had an inhibitory effect above 10 mM, and 500 mM sodium chloride completely inhibited the mitomycin C-inducing DNA damage. 1,4-diazabicyclo[2,2,2]octane (DABCO) completely inhibited the production of oligonucleotides at a concentration of 10 mM, and 10 mM sodium benzoate also had an inhibitory effect. The metal-chelating agents had an inhibitory effect even below 10 mM.

Lipman et al. (19) reported that mitomycin C, at a moderate concentration, forms covalent bond with DNA preferentially at guanine residues, and that the covalent bond formation is inhibited by the high concentration of salts. The inhibitory effect of sodium chloride on the production of oligonucleotides in this study may result from the inhibition of binding of mitomycin C to DNA. The radical scavengers and metal-chelating agents used in this study are also salts, but the inhibitory effect of salts seems negligible when these inhibitors used below 10 mM, because 10 mM sodium chloride did not inhibit the mitomycin C-inducing DNA damage.



**Figure 3.** Inhibition by intercalating agents and effect of preincubation of mitomycin C with sodium borohydride. The 178 base pair 3'-end  $^{32}\text{P}$ -labeled DNA was incubated with 0.1 mM mitomycin C and 0.2 mM sodium borohydride (lane 1), in the presence of 30  $\mu\text{M}$  ethidium bromide (lane 3) and 30  $\mu\text{M}$  actinomycin D (lane 4). The DNA was added after mitomycin C was incubated for 1 min (lane 5) and for 5 min (lane 6) with sodium borohydride. Lane 2 contains products of reaction with 0.1 mM 7-N-(p-hydroxyphenyl)mitomycin C and sodium borohydride. The reacted DNAs were subsequently heated at 90°C for 5 min. Products of deoxyguanosine-specific chemical reaction was in lane G.

Inhibition by intercalating agents and effect of preincubation of mitomycin C with sodium borohydride

Covalent binding of mitomycin C to DNA is thought to be preceded by a noncovalent association, presumably of an intercalating type (20). The effect of intercalating agents on the interaction between DNA and mitomycin C reduced with sodium borohydride was examined. The production of oligonucleotides was inhibited when the DNA was preincubated for 5 min with ethidium bromide (Fig. 3, lane 3) and actinomycin D (Fig. 3, lane 4) before the reaction with mitomycin C. This result suggests that ethidium bromide and actinomycin D competed with reduced mitomycin C or some reductive intermediates for the intercalating sites, and that this type of association is prerequisite for the mitomycin C-inducing DNA damage.

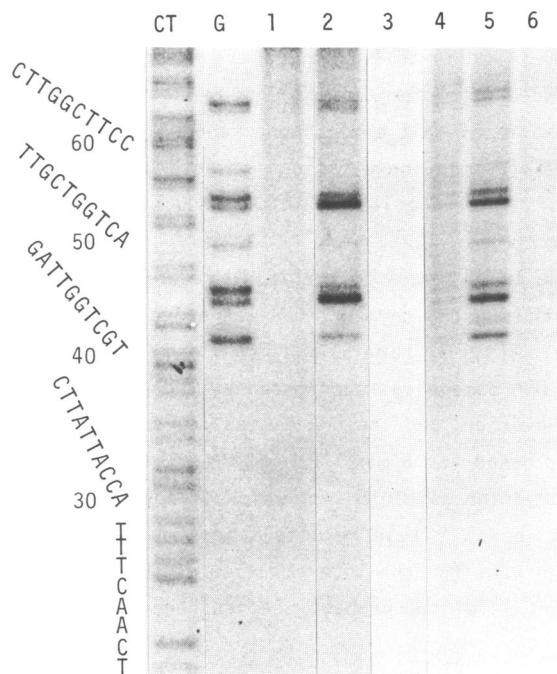
A partially reduced form of mitomycin C rather than the fully reduced hydroquinone is involved in the intercalative association with DNA (20), and the partially reduced form of mitomycin C is believed to be semiquinone radical. The lifetime of this semiquinone radical is about 10 seconds (21). The short lifetime of semiquinone radical is noteworthy, because DNA must be added within seconds after completion of the reduction for crosslinking to take place (22).

To examine the involvement of partially reduced form of mitomycin C in the mitomycin C-inducing DNA damage, DNA was added after mitomycin C was incubated for 1 min (Fig. 3, lane 5) and for 5 min (lane 6) with sodium borohydride. The DNA damage was suppressed by the preincubation of mitomycin C with sodium borohydride, and the DNA damage was scarcely induced by mitomycin C preincubated for 5 min. 0.1 mM mitomycin C is fully reduced by 0.2 mM sodium borohydride within 5 min, which is spectrophotometrically detected (data not shown). These results suggest that the mitomycin C-inducing DNA damage requires the intercalation of the partially reduced mitomycin C of short lifetime, probably semiquinone radical, between Watson-Crick base pairs.

#### Induction of DNA damage by 7-N-(p-hydroxyphenyl)mitomycin C reduced with sodium borohydride or dithiothreitol

7-N-(p-hydroxyphenyl)mitomycin C (M-83) has a higher antitumor activity than mitomycin C against lymphocytic leukemia P388 and fibrosarcoma Meth 1 (23,24), and a lower toxicity than mitomycin C with myelosuppression and leukopenia (25). We reported that M-83 has higher phage inactivating and DNA breaking activities than mitomycin C (26). The sequence specificity of DNA damage by M-83 was examined, as we expected that the difference of the sequence specificity between mitomycin C- and M-83-inducing DNA damage might contribute to the difference of their activities and toxicities. M-83 reduced with sodium borohydride induced heat-labile sites in the 3'-end-labeled DNA fragment, but the preferred sites of the DNA damage by M-83 were quite similar to those by mitomycin C (Fig. 3, lane 2).

Among chemical reducing agents, only three were reported to be suitable for reducing mitomycin C, which were sodium borohydride, sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) and hydrogen at atmospheric pressure in the presence of palladium catalyst (22). Dithiothreitol, thiol-containing reducing agent, is not reported to be effective for the reduction of mitomycin C, and mitomycin C did not induce heat-labile sites in DNA in the presence of dithiothreitol (Fig. 4, lane 4). M-83 reduced with dithiothreitol, however, induced heat-labile sites



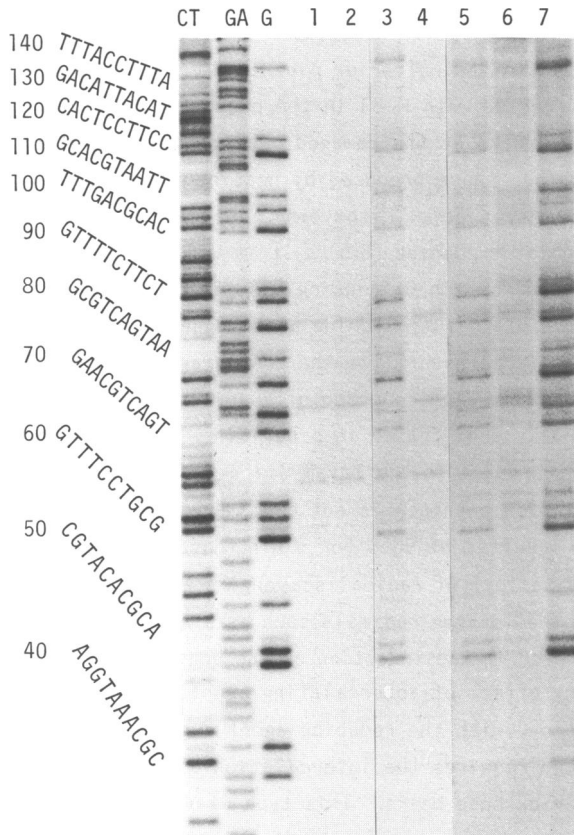
**Figure 4.** Induction of DNA damage by 7-N-(p-hydroxyphenyl)mitomycin C reduced with dithiothreitol. The 141 base pair 3'-end  $^{32}\text{P}$ -labeled DNA was incubated with 0.1 mM mitomycin C and 0.2 mM sodium borohydride (lanes 1 and 2), 0.2 mM sodium borohydride (lane 3), 0.25 mM mitomycin C and 1 mM dithiothreitol (lane 4), 0.25 mM 7-N-(p-hydroxyphenyl)mitomycin C and 1 mM dithiothreitol (lane 5) and 1 mM dithiothreitol (lane 6). After the reaction, DNAs (except lane 1) were heated at 90°C for 5 min in the buffer. Products without heat-treatment were in lane 1. Products of base-specific chemical reactions were in lanes CT and G.

in DNA (Fig. 4, lane 5). The preferred sites of the DNA damage by M-83 reduced with dithiothreitol were quite similar to those by mitomycin C reduced with sodium borohydride (Fig. 4, lane 2).

Piperidine-treatment of 3'-end-labeled DNA fragment reacted with M-83 and dithiothreitol

In the Maxam-Gilbert procedure, DNA fragments reacted with dimethyl sulfate or hydrazine are heated in 1 M piperidine to break the DNA chain at the modified bases (17). To investigate the chemical nature of the induced damage, 3'-end-labeled DNA fragment reacted with M-83 reduced with dithiothreitol was heated at 90°C for 30 min in 1 M piperidine (Fig. 5, lane 7). Piperidine-treatment increased the production of oligonucleotides, but did not change the pattern of oligonucleotide production compared with heat-treatment





**Figure 5.** Piperidine-treatment of the DNA reacted with 7-N-(*p*-hydroxyphenyl)mitomycin C and dithiothreitol. The 257 base pair 3'-end  $^{32}\text{P}$ -labeled DNA was incubated with 0.2 mM sodium borohydride (lane 2), 0.1 mM mitomycin C and 0.2 mM sodium borohydride (lane 3), 1 mM dithiothreitol (lanes 4 and 6) and 0.25 mM 7-N-(*p*-hydroxyphenyl)mitomycin C and 1 mM dithiothreitol (lanes 5 and 7). The reacted DNAs were subsequently heated at 90°C for 5 min in 10 mM Tris-HCl buffer (lanes 2-5), or for 30 min in 1 M piperidine (lanes 6 and 7). Lane 1 contains untreated DNA. Products of base-specific chemical reactions were in lanes CT, GA and G.

(90°C, 5 min in Tris-HCl buffer) of DNA fragment reacted with M-83 in the presence of sodium borohydride (lane 3) or with M-83 in the presence of dithiothreitol (lane 5).

#### DISCUSSION

We investigated the interaction of mitomycin C and 7-N-(*p*-hydroxyphenyl)-mitomycin C (M-83) with DNA by using a sequencing technique. The results

demonstrate that reduced mitomycins induces the DNA damage at deoxyguanosines preferentially in dinucleotide sequence G-T. The DNA damages produce strand breaks, when heated in the buffer or piperidine. Heating in 1 M piperidine of the DNA fragment reacted with M-83 in the presence of dithiothreitol stimulated strand breaks at the damaged nucleotides, but did not produce other oligonucleotides than those produced by heating in the buffer.

The first binding site of mitomycin C is reported to be the O-6 position or the 2-amino group of guanine (5,6). It is well consistent with the base specificity of the mitomycin C-inducing DNA damage. The inhibitory effect of high concentrations of sodium chloride also suggests the covalent binding of mitomycin C to DNA is necessary for the DNA damage induction. Alkylation at the O-6 position or the 2-amino group of guanine is not, however, reported to produce strand breaks when heated in piperidine. Furthermore, dimethyl sulfate-reaction's product in the Maxam-Gilbert procedure, which is alkylated at the N-7 position of guanine, is not cleaved at the modified bases even when heated in Tris-HCl buffer at 90°C for 5 min (data not shown). These results and the inhibitory effect of radical scavengers and metal-chelating agents strongly suggest that oxygen radicals such as singlet oxygen and hydroxyl radical are involved in the induction of the heat-labile DNA damage.

The inhibitory effect of intercalating agents and the effect of preincubation of mitomycin C with the reducing agent suggest that the mitomycin C-inducing DNA damage requires the intercalation of the semiquinone radical between Watson-Crick base pairs. This type of noncovalent association may contribute to the sequence specificity of the mitomycin C-inducing DNA damage. Daunomycin (27) and hedamycin (10) are reported to intercalate between DNA base pairs, and induce DNA damages preferentially at specific sequences.

The 257 base pair fragment used in Fig. 5 had interesting sequences such as GTGT (62-59) and GCGCG (53-49), and various sequences involving deoxyguanosine. Sequences GTGT and GCGCG were not the preferred sites of the DNA damage by mitomycins. Mitomycins interacted with DNA most preferentially at the trinucleotide sequence GGT.

The DNA damage induced by M-83 had the same sequence specificity as that induced by mitomycin C. M-83 is, however, readily reduced by dithiothreitol to induce the DNA damage, whereas mitomycin C is not. The readiness of being reduced to the active form may contribute to the high antitumor activity (23,24) and the low toxicity (25).

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