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Photohydrolysis of Methotrexate Produces Pteridine, Which Induces Poly-G–specific DNA Damage Through Photoinduced Electron Transfer¶

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ABSTRACT

Methotrexate (MTX), an antineoplastic agent, demonstrates phototoxicity. The mechanism of damage to biomacromolecules induced by photoirradiated MTX was examined using ^{32}P-labeled DNA fragments obtained from a human gene. Photoirradiated MTX caused DNA cleavage specifically at the underlined G in 5′-GG and 5′-GGG sequences in double-stranded DNA only when the DNA fragments were treated with piperidine, which suggests that DNA cleavage was caused by base modification with little or no strand breakage. With denatured single-stranded DNA the damage occurred at most guanine residues. The amount of formation of 8-hydroxy-2′-deoxyguanosine (8-oxodGuo), an oxidative product of 2′-deoxyguanosine, in double-stranded DNA exceeded that in single-stranded DNA. These results suggest that photoirradiated MTX participates in 8-oxodGuo formation at the underlined G in 5′-GG and 5′-GGG sequences in double-stranded DNA through electron transfer, and then 8-oxodGuo undergoes further oxidation into piperidine-labile products. Fluorescence measurement, high-pressure liquid chromatography and mass spectrometry have demonstrated that photoexcited MTX is hydrolyzed into 2,4-diamino-6-(hydroxymethyl)pteridine (DHP). DNA damage induced by DHP was observed in a similar manner as was the damage induced by MTX. The extent of DNA damage and the formation of 8-oxodGuo by DHP were much larger than those induced by MTX. The kinetic analysis, based on the time course of DNA oxidation by photoirradiated MTX, suggests that DNA damage is caused by photoexcited DHP rather than by photoexcited MTX. In conclusion, photoexcited MTX undergoes hydrolysis through intramolecular electron transfer, resulting in the formation of DHP, which exhibits a phototoxic effect caused by oxidation of biomacromolecules through photoinduced electron transfer.

INTRODUCTION

The photochemical stability and the excited state property of pharmaceutical substances should be related to the occurrence of phototoxicity as an adverse side effect after drug administration to patients (1). Methotrexate (MTX), a strong inhibitor of dihydrofolate reductase, has been widely used for chemotherapy in many types of cancer (2). MTX also has been used in the treatment of psoriasis. However, the phototoxic effects observed after administration of MTX to patients have been reported as adverse side effects (3–6). Furthermore, after administration, MTX also causes phototoxic effects as adverse side effects of PUVA therapy (4–6). Because MTX has an absorption band in the UVA region, the UVA-induced damage to biomacromolecules may participate in the phototoxicity of MTX.

Photoinduced damage to biomacromolecules has been investigated using DNA as one of the target molecules (7–25). We have previously reported the mechanism of DNA damage induced by photoexcited drugs (20–25). In this study, to approach the clarification of the mechanism of biomacromolecules damage by photoirradiated MTX, we examined MTX-mediated photolesions of ^{32}P-labeled DNA fragments obtained from a human gene as a target molecule. We also measured the content of 8-hydroxy-2′-deoxyguanosine (8-oxodGuo) (26,27), an oxidative product of 2′-deoxyguanosine (dGuo), formed by photoirradiating MTX with an electrochemical detector coupled with high-performance liquid chromatography (HPLC-ECD). Photochemical degradation of MTX was investigated by fluorescence measurements. Furthermore, the photolysis product of MTX was analyzed by HPLC and mass spectrometry. The DNA-damaging ability of 2,4-diamino-6-(hydroxymethyl) pteridine (DHP), which is a photoproduct of MTX, also has been examined.

MATERIALS AND METHODS

Materials. Restriction enzymes (*Apa*I and *Hin*dIII) and T_4 polynucleotide kinase were purchased from New England Biolabs (Berverly, MA). A restriction enzyme (*Eco*RI) and calf intestine phosphatase were from Boehringer Mannheim GmbH (Mannheim, Germany). MTX was from Aldrich Chemical Co. (Milwaukee, WI). [γ-^{32}P]-ATP (222 TBq mmol^{-1}) was from New England Nuclear (Boston, MA). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) was from Dojin Chemicals Co. (Kumamoto, Japan). Calf thymus DNA was from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). DHP was from Acros Organics (New Jersey).

Preparation of ^{32}P-5′–end-labeled DNA fragments. The DNA fragment of the human *p53* tumor suppressor gene was prepared from pUC18

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Abbreviations: dGuo, 2′-deoxyguanosine; DHP, 2,4-diamino-6-(hydroxymethyl) pteridine; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; HPLC-ECD, high-pressure liquid chromatography equipped with an electrochemical detector; MTX, methotrexate; $^1O_2$, singlet oxygen; 8-oxodGuo, 8-hydroxy-2′-deoxyguanosine (also known as 8-oxo-7,8-dihydro-2′-deoxyguanosine).

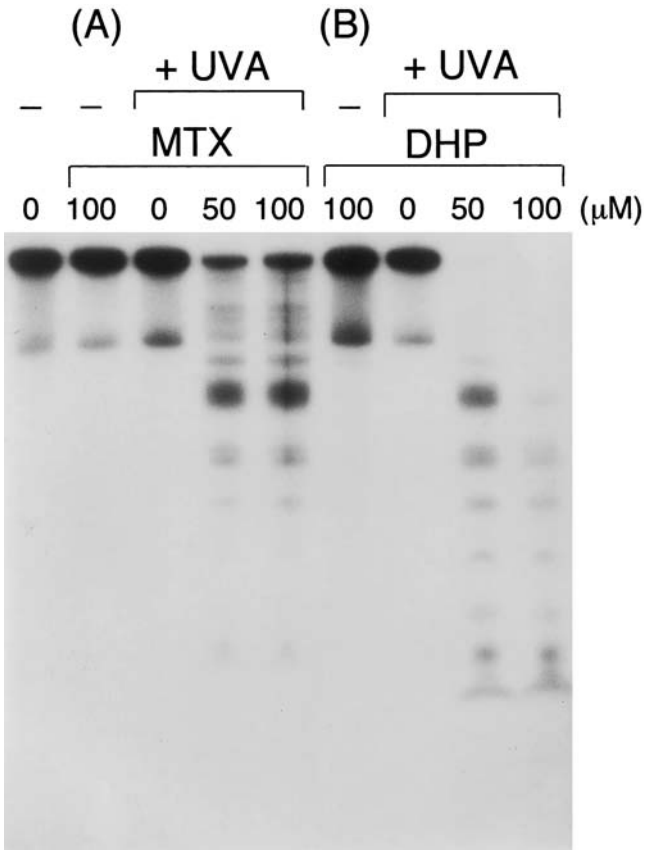

Figure 1. Autoradiogram of DNA fragments exposed to UVA light in the presence of MTX or DHP. The reaction mixture contained the ^{32}P-labeled 443-base pair (*Apa*I 14179–*Eco*RI*14621) fragment, 5 μ*M*/base of calf thymus DNA, and the indicated concentration of MTX (A) or DHP (B) in 100 μL of 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA. The reaction mixtures were exposed to 7 J cm^{-2} UVA light (λ_{max} = 365 nm). Then, the DNA fragments were treated with piperidine and analyzed by the method described in Materials and Methods.

plasmid, ligated fragments containing exons of the *p53* gene (28). The 5′–end-labeled 650-base pair (*Hin*dIII* 13972–*Eco*RI* 14621) fragment was obtained as described previously (29). This fragment was further digested with *Apa*I to obtain the singly labeled 443-base pair (*Apa*I 14179–*Eco*RI*14621) and 211-base pair (*Hin*dIII* 13972–*Apa*I 14182) fragments.

Detection of DNA damage induced by photoirradiated MTX or DHP. The standard reaction mixture in a microtube (1.5 mL Eppendorf) contained MTX or DHP, ^{32}P-labeled DNA fragment and calf thymus DNA in 100 μL of 10 m*M* sodium phosphate buffer (pH 7.8). Denatured single-stranded DNA fragments were prepared by heating DNA fragments at 90°C for 10 min, followed by quick chilling before exposure to UVA light. The mixtures were exposed to 6 or 7 J cm^{-2} UVA light by using 10 W UV lamps (λ_{max} = 365 nm, UVP, Inc., California) placed at a distance of 20 cm. After irradiation, the DNA fragments were treated with 1 *M* piperidine at 90°C for 20 min and treated as described previously (30). The DNA fragments were subjected to electrophoresis on an 8 *M* urea and 8% polyacrylamide gel. The autoradiogram was obtained by exposing an X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure (31) by using a DNA sequencing system (LKB2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). A laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech) was used for measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Measurement of 8-oxodGuo formation induced by photoirradiated MTX or DHP. The reaction mixtures containing 100 μ*M*/base calf thymus DNA and MTX or DHP in 4 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA were exposed to UVA light (λ_{max} = 365 nm). After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed with an HPLC-ECD as described previously (23).

Fluorescence measurements of MTX and DHP. The fluorescence spectra of MTX and DHP were measured with an RF-5300PC spectrophotometer (Shimadzu, Kyoto, Japan). All samples for the fluorescence spectra were measured in 10 m*M* sodium phosphate buffer (pH 7.8).

Assay of photolysis of MTX. MTX (200 μ*M*) in 10 m*M* phosphate buffer (pH 7.8) was irradiated with UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}) and analyzed by HPLC consisting of an LC-6A pump (Shimadzu) and equipped with a Wakopak ODS column (i.d. 4.6 × 150 mm, Wako, Osaka, Japan). The mobile phase consisted of 89.8% (vol/vol) of water, 10.0% (vol/vol) of acetonitrile and 0.2% (vol/vol) of acetic acid. The analysis was carried out at a column temperature of 25°C and a flow rate of 0.5 mL min^{-1}. The HPLC eluate was routed directly into a photodiode array UV–visible detector (SPD-M10A, Shimadzu), and the spectrum of the eluate was measured. The concentration of detected MTX and photoproducts were estimated by using absorbance at 340 nm.

Mass spectra measurements. Laser desorption mass spectrometry was performed on a Voyager MALDI-TOFMS (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser (337 nm, 3 ns pulse) to determine the molecular weight of photoproducts of MTX. The fraction isolated from the UVA-irradiated MTX solution by HPLC (retention time: 2.8 min) was air-dried on a stainless steel probe tip. No matrix solution was added to the sample. Mass spectrum was obtained to sum 50 laser shots.

RESULTS

Damage to DNA fragments induced by photoirradiated MTX or DHP

Figure 1 shows the autoradiogram of ^{32}P-5′–end-labeled DNA fragments exposed to UVA light (λ_{max} = 365 nm) in the presence of MTX or DHP. UVA irradiation caused DNA damage in the presence of MTX (Fig. 1A), whereas UVA irradiation alone caused little or no DNA damage under the conditions used. DHP also induced DNA photodamage (Fig. 1B), and the extent of DNA damage induced by DHP was greater than the extent of damage induced by MTX. DNA photodamage was observed when the DNA fragment was treated with piperidine, suggesting that the base modification was induced by photoirradiated MTX or DHP (data not shown).

Sequence specificity of DNA damage by photoirradiated MTX or DHP

Figure 2 shows the sequence specificity of DNA damage induced by photoirradiated MTX or DHP. Photoirradiated MTX caused damage to double-stranded DNA fragments at guanine residues, especially at the underlined G of 5′-G<u>G</u>-3′ and 5′-G<u>GG</u>-3′ (5′-T<u>GG</u>-3′) sequences (Fig. 2A). The difference in reactivity of GG doublets may be due to the difference in the oxidation potential of GG, depending on the neighboring sequences. When replacing MTX with DHP, the result was almost the same (Fig. 2A,C). When denatured single-stranded DNA fragments were used, MTX or DHP plus photoirradiation caused DNA damage at single guanines and consecutive guanine residues (Fig. 2B,D). The sequence specificity of DNA damage by photoirradiated MTX in double-stranded DNA was almost consistent with that of DNA damage by photoexcited riboflavin, a Type-I photosensitizer (data not shown).

Formation of 8-oxodGuo induced by photoirradiated MTX or DHP

Figure 3 shows 8-oxodGuo formation induced by photoirradiation in the presence of MTX or DHP. It has been reported that

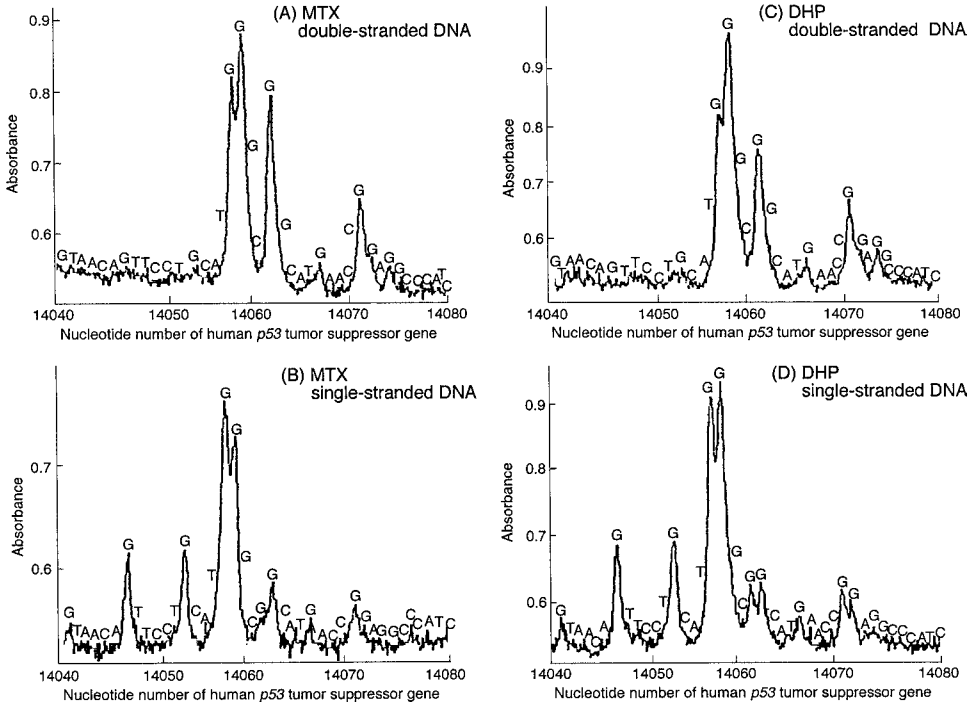

Figure 2. Sequence-specificity of DNA damage induced by photoirradiation in the presence of MTX or DHP. The reaction mixture contained the ^{32}P-labeled 211-base pair (*Hin*dIII* 13972–*Apa*I 14182) fragment, 5 μ*M*/base of calf thymus DNA, and 100 μ*M* MTX (A,B) or 50 μ*M* DHP (C,D) in 100 μL of 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA. Where indicated, the ^{32}P-labeled and calf thymus DNA fragments were denatured by heating at 90°C for 5 min, followed by quick chilling on ice (B,D). The reaction mixtures were exposed to 6 J cm^{-2} UVA light (λ_{max} = 365 nm). Subsequently, the DNA fragments were treated with piperidine. The DNA was analyzed, and the relative amounts of oligonucleotides were measured by the methods described in Materials and Methods. Horizontal axis shows the nucleotide numbers of the *p53* tumor suppressor gene.

formation of 8-oxodGuo can cause DNA misreplication that may lead to mutation or cancer (32,33). The amount of 8-oxodGuo formation increased in a dose-dependent manner up to 50 μ*M* of MTX or DHP and decreased at concentrations of over 50 μ*M*, suggesting that the 8-oxodGuo formed is converted into further oxidative products. The level of 8-oxodGuo in double-stranded DNA exceeded that in single-stranded DNA. The formation of 8-oxodGuo induced by DHP was about twice as much as that induced by MTX.

Photolysis of MTX into DHP

Fluorescence intensity of the pteridine moiety of MTX was very weak compared with that of DHP (Fig. 4), though pteridine is a strong fluorescent molecule. Relative fluorescence quantum yield (Φ_r) of MTX, estimated from the comparison with that of DHP, is 4.4×10^{-3}. When MTX was previously treated with photoirradiation, the fluorescence intensity increased depending on the extent of photoirradiation (Fig. 4), suggesting that UVA light can convert MTX into a stronger fluorescent molecule. The fluorescence spectrum of UVA-irradiated MTX was similar to that of DHP, suggesting that the photoexcited MTX is hydrolyzed into DHP.

Figure 5A shows the chromatogram of MTX. The peak height of MTX was decreased by photoirradiation, and the peak of a photolysis product was observed. The retention time and the absorption spectrum of the photolysis product were similar to those of DHP (data not shown). Furthermore, the photoproduct of MTX was analyzed by using a mass spectrometer. Mass spectrum with a molecular ion at *m/e* 192 (M^+) was obtained, confirming the formation of DHP. The concentration of MTX decreased, whereas that of DHP increased, depending on the photoirradiation dose (Fig. 5B). Another photohydrolysis product (absorption maximum at 279 nm, retention time of 6.4 min) was detected by HPLC analysis, and the intensity increased in a dose-dependent manner (data not shown). The absorption spectrum of this product was similar to that of a *p*-(*N*-methylamino)benzoyl compound. These findings indicate that photoirradiated MTX generates DHP and a *p*-(*N*-methylamino)benzoyl compound, possibly *p*-(*N*-methylamino)benzoyl-L-glutamic acid.

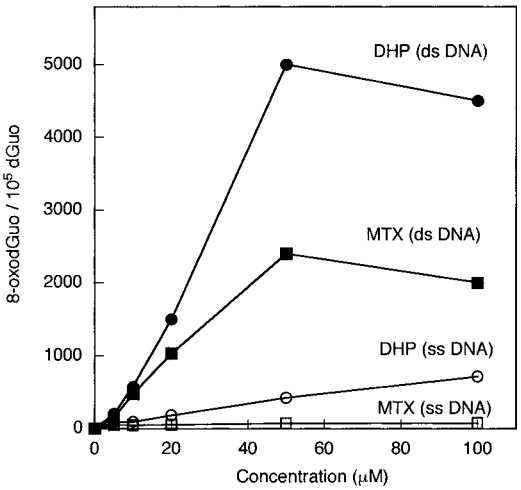

Figure 3. Formation of 8-oxodGuo induced by photoirradiated MTX or DHP. The reaction mixture containing 100 μ*M*/base double-stranded (ds) or single-stranded (ss) calf thymus DNA, and the indicated concentration of MTX or DHP in 100 μL of 4 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA was exposed to 6 J cm^{-2} UVA light (λ_{max} = 365 nm). Where indicated, the DNA fragment was denatured by heating for 5 min at 90°C followed by chilling on ice before photoirradiation. After irradiation, DNA was treated, and the amount of 8-oxodGuo was measured by the methods described in Materials and Methods.

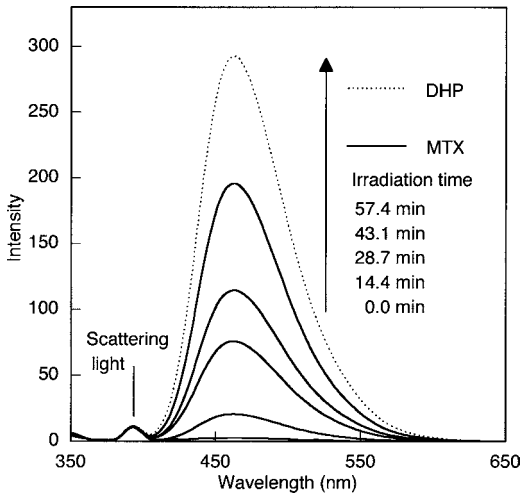

Figure 4. Fluorescence spectra of MTX previously treated with photoirradiation (solid line) and DHP (dotted line). MTX (200 μ*M*) in 10 m*M* phosphate buffer (pH 7.8) was previously irradiated with UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}) for the time indicated and diluted into 2 μ*M* solution for measurement. The concentration of DHP was 1 μ*M*. These spectra were measured in 10 m*M* sodium phosphate buffer (pH 7.8) at 340 nm excitation.

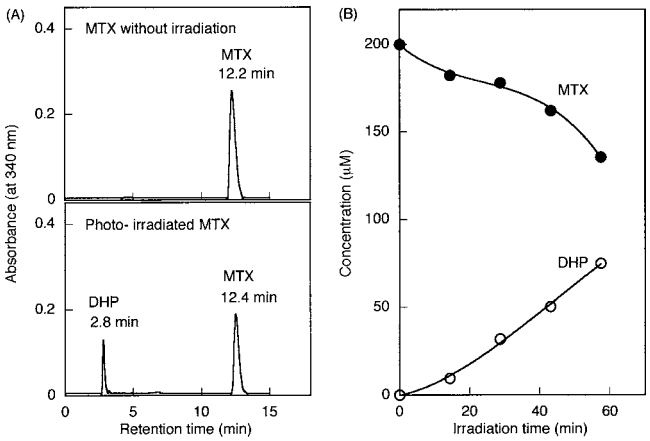

Figure 5. A: Chromatogram of MTX with or without photoirradiation. B: The time course of photolysis of MTX. MTX (200 μ*M*) in 10 m*M* phosphate buffer (pH 7.8) was irradiated with UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}) and analyzed by HPLC using a mobile phase consisting of 89.8% (vol/vol) of water, 10.0% (vol/vol) of acetonitrile and 0.2% (vol/vol) of acetic acid. A: The irradiation time was 57.4 min. B: The symbols are the concentrations of MTX (●) and DHP (○) detected by HPLC.

Measurement and simulation of the time course of the 8-oxodGuo formation by photoirradiated MTX or DHP

Formation of 8-oxodGuo by MTX or DHP increased depending on the photoirradiation time (Fig. 6). 8-oxodGuo formation by DHP was significantly larger than that by MTX. An induction period (approximately 15 min) was observed in the 8-oxodGuo formation by MTX, suggesting that MTX itself could cause little or no DNA oxidation and that reactive species generated from photoirradiated MTX induced DNA oxidation.

The curves in this figure were obtained by simulation according to the following procedure. The 8-oxodGuo formation is roughly expressed as

$$\mathrm{DHP} + \mathrm{h}\nu \longrightarrow \mathrm{DHP}^* \quad \text{and}$$
$$\mathrm{DHP}^* + \mathrm{dGuo} \xrightarrow{k_{ox1}} \mathrm{dGuo_{ox}} \xrightarrow[\mathrm{H_2O,-e^-}]{k_{ox2}} \text{8-oxodGuo} \qquad (1)$$

where DHP*, $\mathrm{dGuo_{ox}}$, and k_{ox1} and k_{ox2} are photoexcited DHP, oxidized dGuo (intermediate into 8-oxodGuo) and reaction rate constants, respectively. The rate equation can be practically expressed as follows:

$$[\mathrm{dGuo_{ox}}]/\mathrm{dt} = k_{ox1}[\mathrm{DHP}^*][\mathrm{dGuo}] = k_{ox1}a[\mathrm{DHP}][\mathrm{dGuo}] \quad \text{and}$$
$$\mathrm{d}[\text{8-oxodGuo}]/\mathrm{dt} = k_{ox2}[\mathrm{dGuo_{ox}}] \qquad (2)$$

where [dGuo], [$\mathrm{dGuo_{ox}}$], [8-oxodGuo], [DHP] and [DHP*] are concentrations of dGuo, $\mathrm{dGuo_{ox}}$, 8-oxodGuo, ground state of DHP and excited state of DHP, respectively. The nondimensional coefficient *a* depends on the number of photons absorbed by DHP. The fitting curve of time course of 8-oxodGuo formation has been obtained by numerical calculation. The values of $a \times k_{ox1}$ and k_{ox2} were determined by the time course of 8-oxodGuo formation by DHP as shown in Fig. 6 and were 0.12 M^{-1} s^{-1} and 7.4 × 10^6 s^{-1}, respectively. Based on the assumption that photoexcited DHP generated from MTX oxidizes dGuo, although photoexcited MTX does not, the time course of 8-oxodGuo formation was calculated from Eq. 2 using the $a \times k_{ox1}$ and k_{ox2} values mentioned above. The value of [DHP] formed from MTX was obtained from the curve fitting of the plots in Fig. 5. The curve obtained in Fig. 6 clearly fits with the experimental results, supporting the idea that DNA oxidation is caused by DHP generated from MTX.

DISCUSSION

Guanine-specific oxidation can be caused by excited photosensitizers through Type-I (electron transfer) mechanism, Type-II (singlet oxygen [$^1O_2$]) mechanism (7,8,20), or both. Cadet and co-workers (9,10) have reported that imidazolone and oxazolone are the major oxidation products of guanine formed by the Type-I mechanism, whereas 8-oxodGuo was found to be the main $^1O_2$-mediated guanine oxidation product (8). The present study has demonstrated that photoirradiated MTX and DHP induce base oxidation, especially at the underlined G in 5′-G<u>G</u>-3′ and 5′-G<u>GG</u>-3′ (5′-TG<u>G</u>G-3′) sequences in double-stranded DNA. We previously observed a similar DNA cleavage pattern when a Type-I photosensitizer, riboflavin, was used (23). The amount of 8-oxodGuo formed by photoirradiated MTX in double-stranded DNA was much larger than that in single-stranded DNA. We have previously reported that the sequence specificity of DNA damage by $^1O_2$ is quite different from that by the Type-I mechanism (20–22). Therefore, the present results can be reasonably explained by assuming that nucleobase oxidation is induced by the activated photosensitizer mainly through electron transfer (Type-I mechanism), although $^1O_2$ participates in DNA damage to some extent. Guanine is the most easily oxidized among the four DNA bases because the oxidation potential of guanine is lower than those of the other DNA bases (7,34). Molecular orbital calculations have revealed that stacking of two guanine bases in double-stranded DNA lowers the ionization potential significantly, and electron-loss centers are localized on the 5′-G of the 5′-GG-3′ sequence through charge transfer (16). Recently, it has been reported that the ionization potential of GGG is lower than that of GG and that the central G is the most reactive in the 5′-TG<u>G</u>G-3′ sequence (19). These reports support the proposed mechanism by which the photoirradiated photosensi-

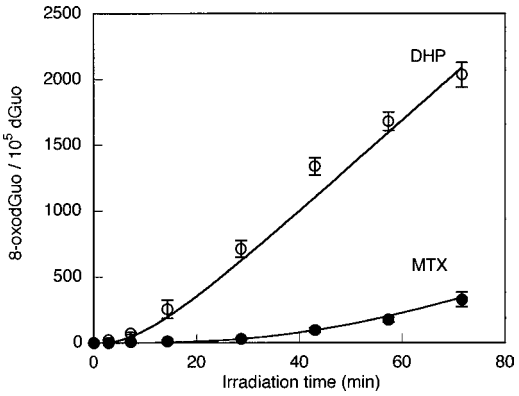

Figure 6. Time course of the formation of 8-oxodGuo induced by photoirradiated MTX or DHP. The reaction mixture containing 100 μ*M*/ base calf thymus DNA, 50 μ*M* MTX (•) or DHP (○) in 100 μL of 4 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA was exposed to UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}). After irradiation, DNA was treated, and the amount of 8-oxodGuo was measured by the methods described in Materials and Methods. Results represent means ± SD of four independent experiments. The curves in this figure were simulated as described in Results.

tizer causes the poly-G–specific oxidation in double-stranded DNA through electron transfer. Guanine cation radicals formed in DNA are finally localized on the underlined sites of the 5′-GG-3′ and 5′-GGG-3′ (5′-TGGG-3′) sequences and react with the water molecule to form the C-8 OH adduct radical, followed by oxidation, leading to the formation of 8-oxodGuo (7,8,13–15). Although the 8-oxodGuo site is not efficiently cleaved under piperidine treatment (15), 8-oxodGuo is more easily oxidized than dGuo is and can be converted into piperidine-labile products (*e.g.* imidazolone, oxazolone, or both [7–11]) through further oxidation (7,17,18,35,36).

Pteridine is a strong fluorescent molecule, but the pteridine moiety of MTX shows weak fluorescence, possibly due to an intramolecular electron transfer from the *p*-(*N*-methylamino)-benzoyl residue to the photoexcited pteridine moiety. This study showed that photoirradiation converted MTX into the fluorescent molecule, with the fluorescence spectrum similar to that of DHP. HPLC and mass spectrum measurements have also shown that photoexcited MTX generates DHP depending on the irradiation dose. We proposed the photolysis of MTX into DHP and a *p*-(*N*-methylamino)benzoyl compound, possibly *p*-(*N*-methylamino)benzoyl-L-glutamic acid, through an intramolecular electron transfer after hydrolysis (Fig. 7).

Sequence specificity of DNA damage induced by photoexcited DHP was similar to that of photoirradiated MTX, and the extent of DNA damage and 8-oxodGuo formation by DHP was much larger than in the case of MTX. The time course of 8-oxodGuo formation by photoirradiated MTX has been reasonably explained by assuming that DNA damage is caused by photoactivation of DHP generated from MTX and that MTX itself has little or no DNA-damaging ability. In the photoexcited MTX the intramolecular electron transfer leading to DHP formation competes with the intermolecular reaction with DNA. The intramolecular electron transfer rate coefficient (k_{et1}) has been roughly estimated from the following equation:

$$k_{et1} = (1/\Phi_r - 1) \times 1/\tau_0 \quad (3)$$

where τ_0 is the lifetime of the photoexcited pteridine moiety (ranges almost from 10^{-9} to 10^{-8} s^{-1}) (37). Because this study has

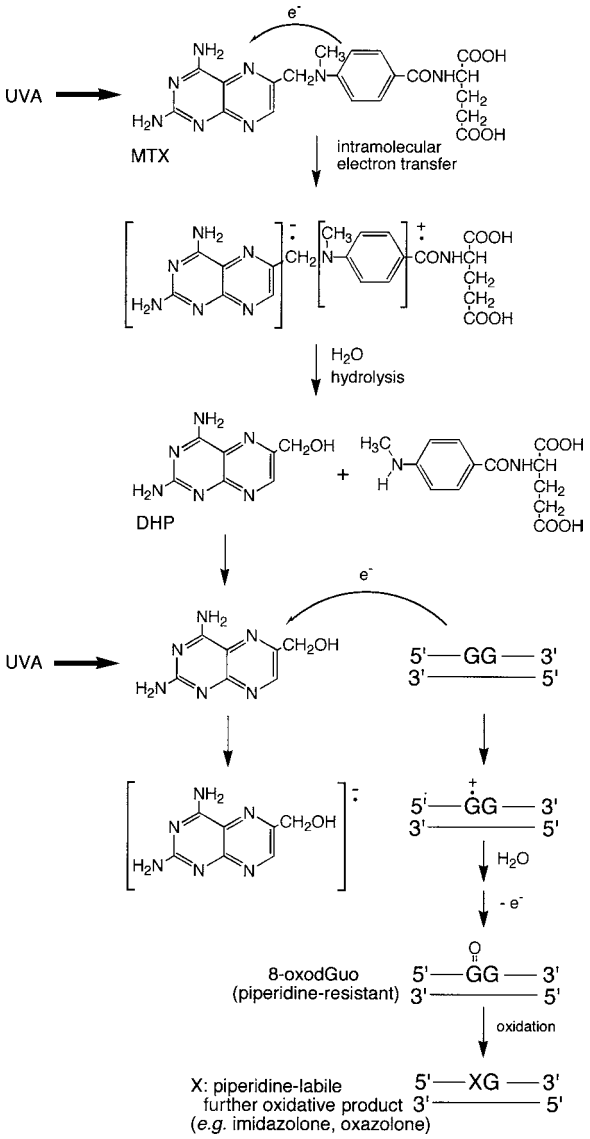

Figure 7. Proposed mechanism of DNA damage induced by photoirradiated MTX.

estimated that the value of Φ_r is 4.4×10^{-3}, the value of k_{et1} can be estimated to be almost 10^{10}–10^{11} s^{-1}. On the other hand, k_{et2}, the collision rate coefficient of one photoexcited MTX molecule with DNA, has been estimated from the equation of diffusion controlled reaction:

$$k_{et2} = 8RT[dGuo]/3\eta \quad (4)$$

where R is the gas constant, T is the absolute temperature, [dGuo] is 25 μ*M* and η is the viscosity of water (0.891×10^{-3} kg m^{-1} s^{-1}). The k_{et2} is calculated to be 1.8×10^5 s^{-1} and is markedly smaller than k_{et1}. Therefore, in the photoexcited MTX, intramolecular electron transfer preferentially occurs rather than DNA oxidation by photoexcited MTX itself.

In summary, the present study has demonstrated that photoirradiated MTX induces poly-G–specific DNA oxidation. The reactive species that induces DNA oxidation is photoexcited DHP, which is generated from photoexcited MTX through hydrolysis after intramolecular electron transfer. This study suggests that the

phototoxicity of MTX is due to the oxidation of biomacromolecules, mainly through electron transfer by photoexcited DHP.

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