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Research Paper

Mechanism of UVA-dependent DNA Damage Induced by An Antitumor Drug Dacarbazine in Relation to its Photogenotoxicity

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Purpose. It has been reported that dacarbazine (DTIC) is photogenotoxic. The purpose of this study is to clarify the mechanism of photogenotoxicity induced by DTIC.
Materials and methods. We examined DNA damage induced by UVA-irradiated DTIC using ^{32}P-5′-end-labeled DNA fragments obtained from human genes. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in calf thymus DNA was measured by high performance liquid chromatograph with an electrochemical detector. Electron spin resonance (ESR) spin-trapping experiments were performed to detect radical species generated from UVA-irradiated DTIC.
Results. UVA-irradiated DTIC caused DNA damage at guanine residues, especially at the 5′-G*G*T-3′ sequence in the presence of Cu(II) and also induced 8-oxodG generation in calf thymus DNA. DTIC-induced photodamage to DNA fragments was partially inhibited by catalase, whereas 8-oxodG formation was significantly increased by catalase. NaN_3, a carbene scavenger, inhibited DNA damage and 8-oxodG formation in a dose-dependent manner, suggesting that carbene intermediates are involved. The ESR spin-trapping experiments demonstrated the generation of aryl radicals in the process of photodegradation of DTIC.
Conclusion. Photoactivated DTIC generates the carbene and aryl radicals, which may induce both DNA adduct and 8-oxodG formation, resulting in photogenotoxicity. This study could provide an insight into the safe usage of DTIC.

KEY WORDS: aryl radical; carbene; dacarbazine; DNA damage; photogenotoxicity.

INTRODUCTION

Dacarbazine, 5-(3,3-dimethyltriazen-1-yl) imidazole-4-carboxamide (DTIC), is clinically effective in the treatment of several malignant disorders, including metastatic malignant melanoma, Hodgkin's disease and soft tissue sarcomas, in combination with other anticancer drugs (1). DTIC is a prodrug that is activated in the liver to yield the highly reactive methyl diazonium cation. The formation of this cation is considered as the major mechanism for its antitumor effect. DTIC preparations are currently administered by intravenous injection or infusion. However, DTIC is unstable in aqueous solution. Previous study has demonstrated that this drug rapidly photodecomposes in sunlight, as shown in Fig. 1 (2).

Solar UV radiation is a well-known carcinogen to humans (3). Solar radiation can give rise to cellular DNA damage by direct excitation of DNA bases via short-wave UV radiation (UVB) and by indirect mechanisms observed at longer wavelength (UVA) (4). Such indirect mechanisms involve the excitation of endogenous photosensitizers, which may be transformed to reactive intermediates when they absorb UVA light (5–7). The potential to induce photogenotoxic effects is known for drugs of a few classes such as psoralenes, phenothiazines, and fluoroquinolones (8,9). These drugs have been reported to induce DNA photodamage (6,10,11), leading to mutations or chromosomal damage and eventually cause tumors (12). Recently, Kersten *et al.* reported that DTIC induced drastical photogenotoxic and photocytotoxic effects in Chinese hamster V79 cells, whereas the compound induced neither cytotoxic nor genotoxic effects without irradiation (13). However, the mechanism of DTIC-induced photogenotoxicity is still unclear.

In order to clarify the mechanism of photogenotoxicity induced by DTIC, we investigated the ability of DTIC plus UVA radiation to induce DNA damage using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor-suppressor genes and the human c-Ha-*ras*-1 protooncogene. We also measured the content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an oxidative product

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ABBREVIATIONS: CIP, calf intestine phosphatase; Diazo IC, 5′-diazoimidazole-4-carboxamide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide, MNP, 2-methyl-2-nitrosopropane; DTIC, Dacarbazine; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; ESR, electron spin resonance; HPLC-ECD, high performance liquid chromatograph with an electrochemical detector; SOD, superoxide dismutase; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

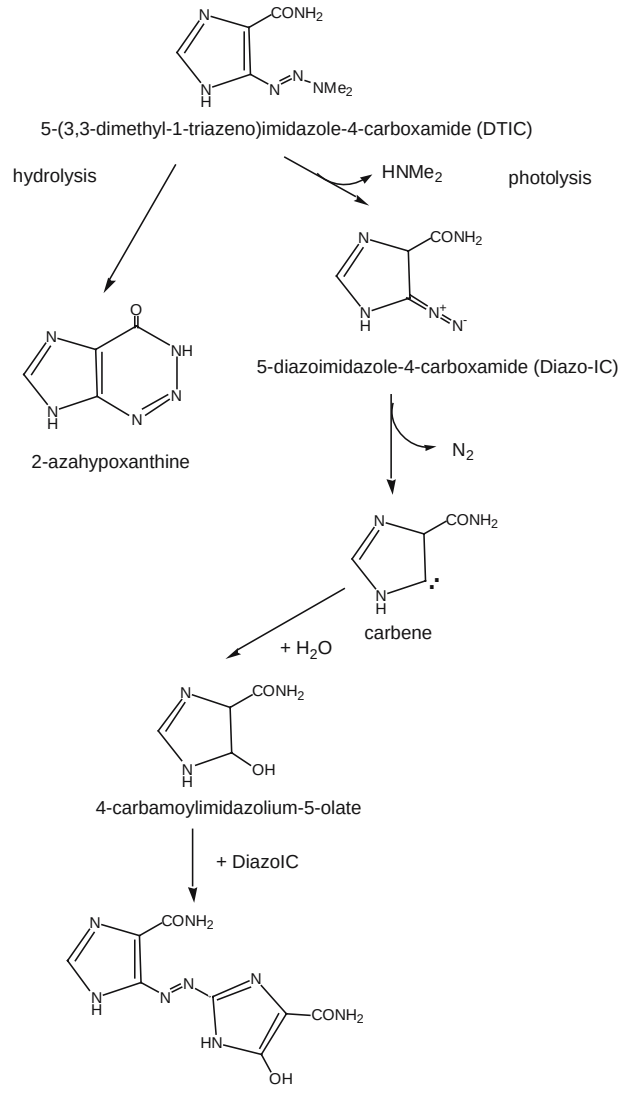

Fig. 1. Suggested scheme of dacarbazine degradation.

of guanine, formed by UVA-irradiated DTIC in calf thymus DNA. Furthermore, we analyzed the radicals generated from UVA-irradiated DTIC by electron spin resonance (ESR) spin-trapping experiments.

MATERIALS AND METHODS

Materials

Restriction enzymes (*Apa*I, *Eco*RI, *Bss*HII, *Xba*I, and *Pst*I) were purchased from Boehringer Mannheim GmbH (Germany). The restriction enzyme *Hin*dIII, *Ava*I and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was purchased from New England Nuclear (Boston, MA). DTIC, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Calf thymus DNA, calf intestine phosphatase (CIP), superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45000 units/mg from bovine liver) were acquired from Sigma Chemical Co. (St. Louis, MO). Nuclease P1 (400 units/mg) was obtained from Yamasa Shoyu Co. (Chiba, Japan).

Preparation of ^{32}P-5′-end-labeled DNA Fragments

DNA fragments were obtained from the human *p53* tumor suppressor gene (14). The ^{32}P-end-labeled 443-base pair (*Apa*I 14179-*Eco*RI* 14621) and 211-base pair (*Hin*dIII* 13972-*Apa*I 14182) fragments were generated as described previously (asterisks indicate ^{32}P-labeling) (15). DNA fragments obtained from the human *p16* tumor suppressor gene were subcloned into the pGEM®-T Easy Vector (Promega Corporation, Madison, WI) (16). The 460-base pair fragment was digested with *Bss*HII to obtain singly labeled 309-base pair (*Eco*RI* 9481-*Bss*HII 9789) and 147-base pair (*Bss*HII 9794-*Eco*RI* 9940) fragments. DNA fragments were also prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene (17). A singly labeled 261-base pair fragment (*Ava*I* 1645-*Xba*I 1905) and 337-base pair fragment (*Pst*I 2345-*Ava*I* 2681) were obtained according the method described previously (18,19). Nucleotide numbering starts with the *Bam*HI site (17).

Detection of DNA Damage Induced by UVA-irradiated DTIC

Standard reaction mixtures (in a 1.5 ml Eppendorf microtube) contained DTIC solution (containing the same concentration of HCl), ^{32}P-5′-end-labeled DNA fragments, non-labeled calf thymus DNA (5 μM/base), 20 μM $CuCl_2$ and 5 μM diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8). Calf thymus DNA was used to adjust the total concentration of DNA. The mixtures were exposed to UVA light using a 10 W UV lamp (λ=365 nm, UVP, Inc., CA) placed at a distance 20 cm from the sample. UVA dose exposed to samples was measured with a UVP Model UVX Digital Radiometer (UVP, Inc., CA), and we calculated the exposure time. To expose the sample to 10 J/cm^2 UVA, the sample was irradiated with UVA for approximately 90 min. After the irradiation, DNA fragments were treated with 1 M piperidine at 90°C for 20 min and treated as described previously (20,21). DNA fragments were subjected to electrophoresis on an 8 M urea/8 % polyacrylamide gel and visualized by autoradiography.

Preferred DNA damaging sites were determined by direct comparison of oligonucleotide position with the cleavage patterns produced by the Maxam–Gilbert procedure (21). The relative quantities of oligodeoxynucleotides from the treated DNA fragments were measured by laser densitometry (Personal Densitometer SI, Amarsham Biosciences).

Formation of 8-oxodG in Calf Thymus DNA by UVA-irradiated DTIC

The levels of 8-oxodG were measured by a modification of the method of Kasai *et al.* (22). Reaction mixtures containing DTIC solution (containing the same concentration of HCl), calf thymus DNA fragments (20 μM/base), 20 μM $CuCl_2$ and 5 μM DTPA in 200 μl of 4 mM sodium phosphate

buffer (pH 7.8) were exposed to 8 J/cm^2 UVA light. After ethanol precipitation, DNA fragments were digested into individual nucleosides with nuclease P1 and CIP. The nucleosides were then analyzed by using a high performance liquid chromatograph with an electrochemical detector (HPLC-ECD) as described previously (11). For the statistical analysis of the data, independent *t* test was used at a significant level of $p<0.05$.

Analysis of Free Radicals Derived from Photodegradation of DTIC

The reaction mixture containing 2 mM DTIC solution (containing the same concentration of HCl), 20 μM $CuCl_2$ and spin trapping agent (300 mM DMPO in phosphate buffer or 10 mM MNP in acetone) was exposed 2 J/cm^2 UVA light. ESR spectra were recorded at 25°C using a JES-TE100 spectrometer (JEOL, Tokyo. Japan) with 100 kHz field modulation, a microwave power of 16 mW and an amplitude of 0.1 mT.

RESULTS

UVA-mediated Damage to ^{32}P-labeled DNA Fragments Induced by DTIC

Figure 2 shows the autoradiogram of ^{32}P-labeled DNA fragments exposed to UVA light in the presence of DTIC. While UVA-irradiated DTIC induced DNA damage in the presence of Cu(II), UVA-irradiated DTIC alone did not (Fig. 2a). UVA-irradiated DTIC did not induce DNA damage in the presence of other metal ions, including Fe(II), Fe(III), Mn(II) or Mn(III) (Fig. 2a). DNA damage was observed only when the DNA fragment was treated with piperidine, suggesting that base modification is induced by UVA-irradiated DTIC in the presence of Cu(II). DNA damage was observed most prominently at 0.5 mM DTIC (Fig. 2b). UVA dose-dependently increased DNA damage by DTIC from 2 to 10 J/cm^2 (data not shown). UVA alone did not induce DNA damage.

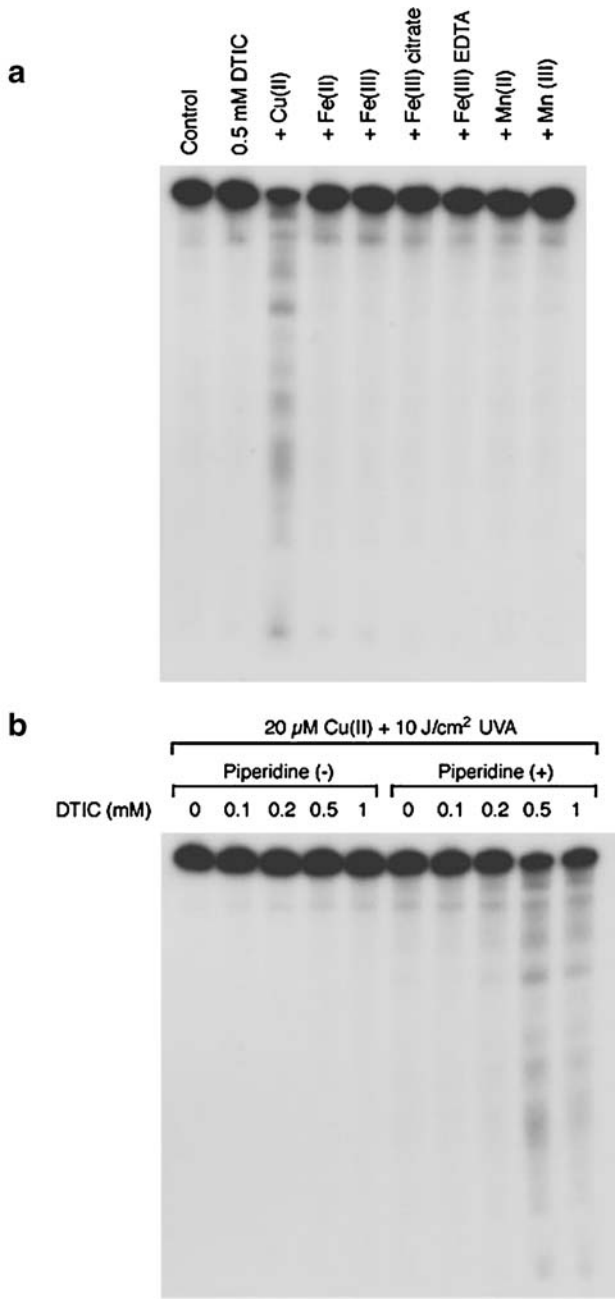

Fig. 2. Autoradiogram of ^{32}P-labeled DNA fragments incubated with UVA-irradiated DTIC in the presence of metal ion (**a**) and Cu(II) (**b**). Reaction mixtures contained DTIC solution (containing 0.5 mM HCl), ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (5 μM/base), 20 μM metal ion and 5 μM DTPA in 200 μl 10 mM sodium phosphate buffer (pH 7.8). The mixtures were exposed to 10 J/cm^2 UVA light placed at a distance 20 cm from the sample. After the irradiation, DNA fragments were treated with (**a, b**) or without (**b**) 1 M piperidine at 90°C for 20 min. The DNA fragments were then subjected to electrophoresis on an 8 M urea/8% polyacrylamide gel and visualized by autoradiography. The control contained no DTIC and was exposed to UVA light.

Effects of Scavengers and Bathocuproine on UVA-mediated DNA Damage Induced by DTIC

Figure 3a shows the effects of scavengers and bathocuproine on DNA damage induced by UVA-irradiated DTIC in the presence of Cu(II). Bathocuproine, a Cu(I)-specific chelator, inhibited DNA damage, suggesting that the DNA damage is Cu(I)-dependent. Catalase, methional, and SOD partially inhibited DTIC-induced DNA damage. Fig. 3b shows the inhibitory effect of NaN_3, which is reported to react with carbene intermediates (23), on DNA damage induced by UVA-irradiated DTIC in the presence of Cu(II). NaN_3 inhibited DNA damage induced in a dose-dependent manner, suggesting that carbene intermediates are involved. D_2O did not enhance the DNA damage induced by UVA-irradiated DTIC in the presence of Cu(II) (data not shown), defying the involvement of $^1O_2$.

Site Specificity of UVA-mediated DNA Damage Induced by DTIC

The pattern of DNA damage induced by UVA-irradiated DTIC in the presence of Cu(II) were determined by the Maxam–Gilbert procedure. An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensities of DNA damage in the human c-Ha-*ras* 1 protooncogene (Fig. 4). UVA-irradiated DTIC caused

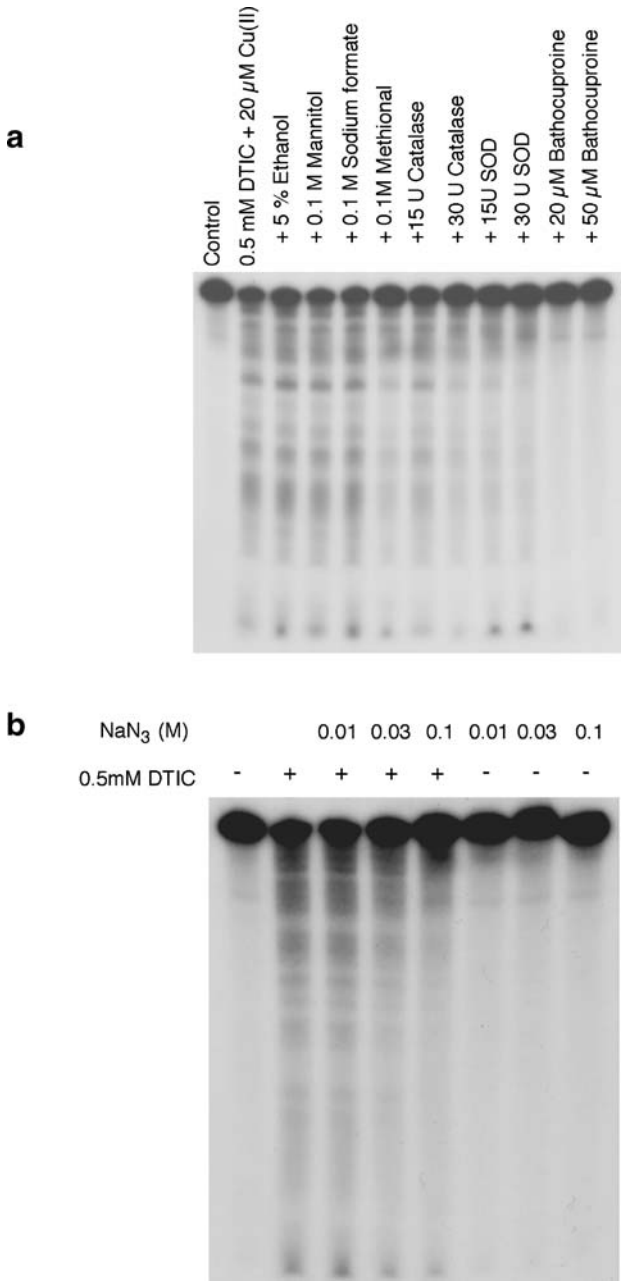

Fig. 3. Effects of scavengers and bathocuproine on DNA damage induced by UVA-irradiated DTIC in the presence of Cu(II). Reaction mixtures contained DTIC solution (containing 0.5 mM HCl), ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (5 μM/base), 20 μM $CuCl_2$ and 5 μM DTPA in 200 μl 10 mM sodium phosphate buffer (pH 7.8). The mixtures were exposed to 8 J/cm^2 UVA light placed at a distance 20 cm from the sample. Where indicated, 5% ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.1 M methional, catalase, SOD, bathocuproine (**a**) or NaN_3 at the indicated concentration (**b**) were added to the reaction mixture. Following subsequent piperidine treatment, the DNA fragments were analyzed as described in Fig. 2 legend. The control contained no DTIC and was exposed to UVA light.

DNA damage at guanine residues, especially at the 5′-G*G*T-3′ sequence in the presence of Cu(II) (Fig. 4a and b).

Formation of 8-oxodG in Calf Thymus DNA by DTIC in the Presence of UVA Irradiation

Figure 5 shows 8-oxodG formation induced by UVA-irradiated DTIC in the presence of Cu(II). The formation of 8-oxodG increased depending on DTIC concentrations. Upon the addition of catalase, 8-oxodG formation significantly increased following treatment with 0.5 and 1 mM DTIC. In contrast, NaN_3 significantly decreased the formation of 8-oxodG induced by UVA-irradiated DTIC. Without UVA irradiation, DTIC did not increase the formation of 8-oxodG in the presence of Cu(II). UVA alone induced little or no effect on 8-oxodG formation.

Generation of Free Radicals Derived from UVA-irradiated DTIC

ESR spectroscopic study using trapping agents, DMPO and MNP was performed (Fig. 6). The ESR spectrum using DMPO shows UVA-irradiated DTIC-derived radicals with the six-line signal, assigned to aryl radicals (a^N=16.0 mT, a^H=25.1 mT) (Fig. 6a). When using acetone as a solvent of MNP, we detected nine-line signal, assigned to acetone radical (a^N=15.7 mT, a^H=8.6 mT), which may be generated from dehydrogenation of acetone caused by UVA-irradiated DTIC. Assignment of the chemical structure of the free radical species was achieved by the close agreement of the hyperfine coupling constants with the published literature (24). The three-line signal, derived from the MNP decomposition product, di-*tert*-butyl nitroxide, was also

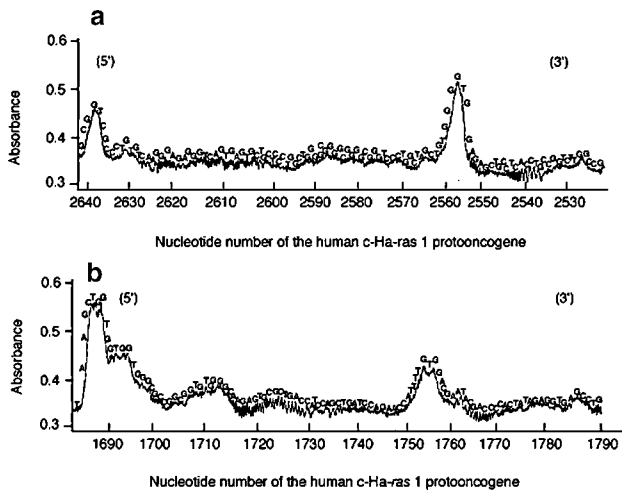

Fig. 4. a, b Site specificity of DNA damage induced by UVA-irradiated DTIC. Reaction mixtures contained 0.5 mM DTIC solution (containing 0.5 mM HCl), ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (5 μM/base), 20 μM $CuCl_2$ and 5 μM DTPA in 200 μl 10 mM sodium phosphate buffer (pH 7.8). The mixtures were exposed to 10 J/cm^2 UVA light placed at a distance 20 cm from the sample. After the irradiation, DNA fragments were treated with 1 M piperidine and electrophoresed as described in Fig. 2 legend. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (Personal Densitometer SI, Amarsham Biosciences).

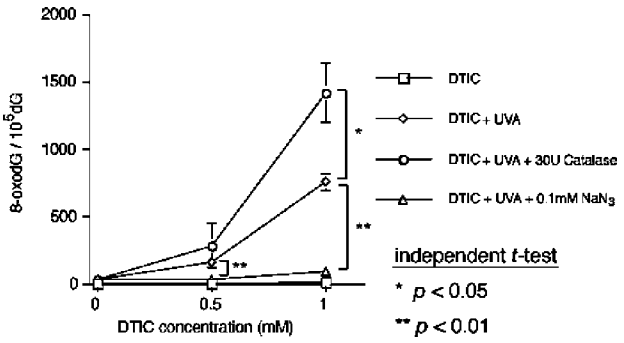

Fig. 5. Effects of catalase or NaN_3 on the formation of 8-oxodG induce by UVA-irradiated DTIC. Reaction mixtures containing DTIC solution (containing 0.5 mM HCl), calf thymus DNA fragments (20 μM/base), 20 μM $CuCl_2$ and 5 μM DTPA in 200 μl 4 mM sodium phosphate buffer (pH 7.8) were exposed to 8 J/cm^2 UVA light. Where indicated, 30 U catalase or 0.1 mM NaN_3 was added to the reaction mixture. After ethanol precipitation, DNA fragments were digested into individual nucleosides with nuclease P1 and CIP. The nucleosides were then analyzed by using a high performance liquid chromatograph with an electrochemical detector (HPLC-ECD). Results are expressed as means±SD of values obtained from four independent experiments. *Asterisks* indicating $P<0.05$, and *double asterisks* indicating $P<0.01$, significantly changed by the addition of catalase or NaN_3 (analyzed by independent *t* test).

observed (a^N=1.66 mT). Without UVA irradiation, DTIC did not produce free radical under the same condition (data not shown).

DISCUSSION

In this study, we demonstrated that UVA-irradiated DTIC induced DNA damage and 8-oxodG generation in the presence of Cu(II). The photodamage to ^{32}P-labeled DNA was partially inhibited by catalase, whereas 8-oxodG formation in calf thymus DNA was significantly increased by the addition of catalase. In the reaction mixture of UVA-irradiated DTIC and Cu(II), we could not detect the generation of H_2O_2 by the scopoletin method (25) (data not shown). These results suggest that UVA-irradiated DTIC-induced DNA damage involves two H_2O_2-independent mechanisms, that is, 8-oxodG generation and catalase-inhibited pathway. Firstly, we investigated the H_2O_2-independent mechanisms of 8-oxodG generation induced by DTIC. ESR spectroscopic study using a trapping agent MNP has shown the generation of acetone radical through dehydrogenation of acetone by UVA-irradiated DTIC. This result suggests that photodegraded product of DTIC can abstract hydrogen atom from deoxyguanine to give free radical. Triplet carbenes and aryl radicals are known to be able to abstract hydrogen atoms from the deoxyribose portion of DNA (26,27). Therefore, we consider that deoxyguanine radical at C8 is formed through the abstraction of hydrogen atom by photodegraded products of DTIC, such as carbene and aryl radical species, leading to 8-oxodG formation. It is noteworthy that 8-oxodG was generated via H_2O_2-independent pathway.

Secondly, we investigated the mechanism of DNA damage induced by UVA-irradiated DTIC, which is partially inhibited by catalase. DNA damage occurred after piperidine treatment, suggesting that DNA damage is due to base alteration with little or no strand breakage. The preferred site of DNA damage is guanine residues, especially at the 5′-G*G*T-3′ sequence. The previous study using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis showed that aryl radical formed an adduct with guanosine (28). In this study, we confirmed aryl radical formation from photolysis of DTIC by ESR spin trapping methods, although its precise structure remains to be clarified. NaN_3 inhibited DNA damage induced by UVA-irradiated DTIC and D_2O did not enhance it, suggesting that carbene intermediates, but not $^1O_2$ are involved in DNA damage. These facts indicate that aryl radical, which may be generated via 5′-diazoimidazole-4-carboxamide (Diazo-IC) and carbene formation, plays an important role in DNA photodamage induced by DTIC. Since there are few reports about DNA damage induced by carbene species (23), our results provide an insight into carbene-mediated DNA damage.

It has been reported that DTIC induced drastical photogenotoxic and photocytotoxic effects in Chinese hamster V79 cells with the significant increase of micronucleated cells (13). On the basis of our results, we proposed the mechanism of DNA damage induced by UVA-irradiated DTIC plus Cu(II) (Fig. 7). Photodegraded product of DTIC, Diazo-IC reduces Cu(II) to generate Cu(I) (29,30). Cu(I) can catalyze the release of the diazonium group from the aromatic ring of Diazo-IC to generate carbene and aryl radical (31). DTIC-induced DNA photodamage is considered to be induced via these two mechanisms, DNA adduct and 8-oxodG formation. DNA adduct formation was thought to be produced by the coupling reaction of aryl radical with deoxyguanine radical, which may be formed from dehydro-

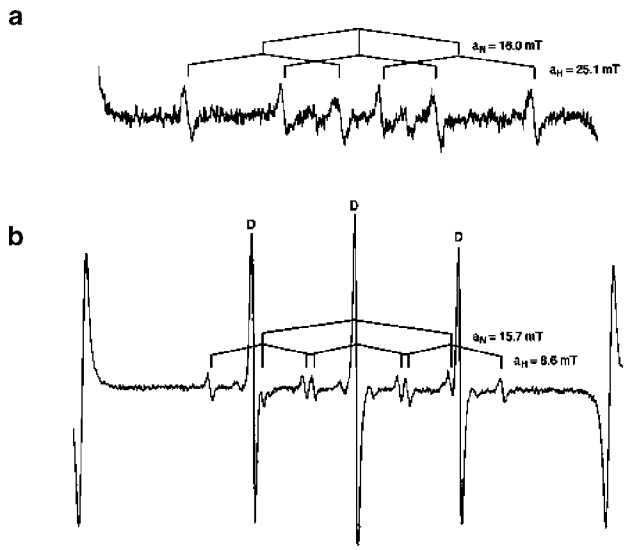

Fig. 6. Generation of free radicals derived from UVA-irradiated DTIC. The reaction mixture containing 2 mM DTIC solution (containing 2 mM HCl), 20 μM $CuCl_2$ and spin trapping agent, 300 mM DMPO in phosphate buffer (**a**) or 10 mM MNP in acetone (**b**), was exposed 2 J/cm^2 UVA light. ESR spectra were recorded at 25°C using a JES-TE100 spectrometer (JEOL, Tokyo. Japan) with 100 kHz field modulation, a microwave power of 16 mW and an amplitude of 0.1 mT. (**a**) The six-line signal consists of three 1:1 doublet spectra. (**b**) The nine-line signal consist of three 1:2:1 triplet spectra. *D*, di-*tert*-butyl nitroxide, an MNP decomposition product.

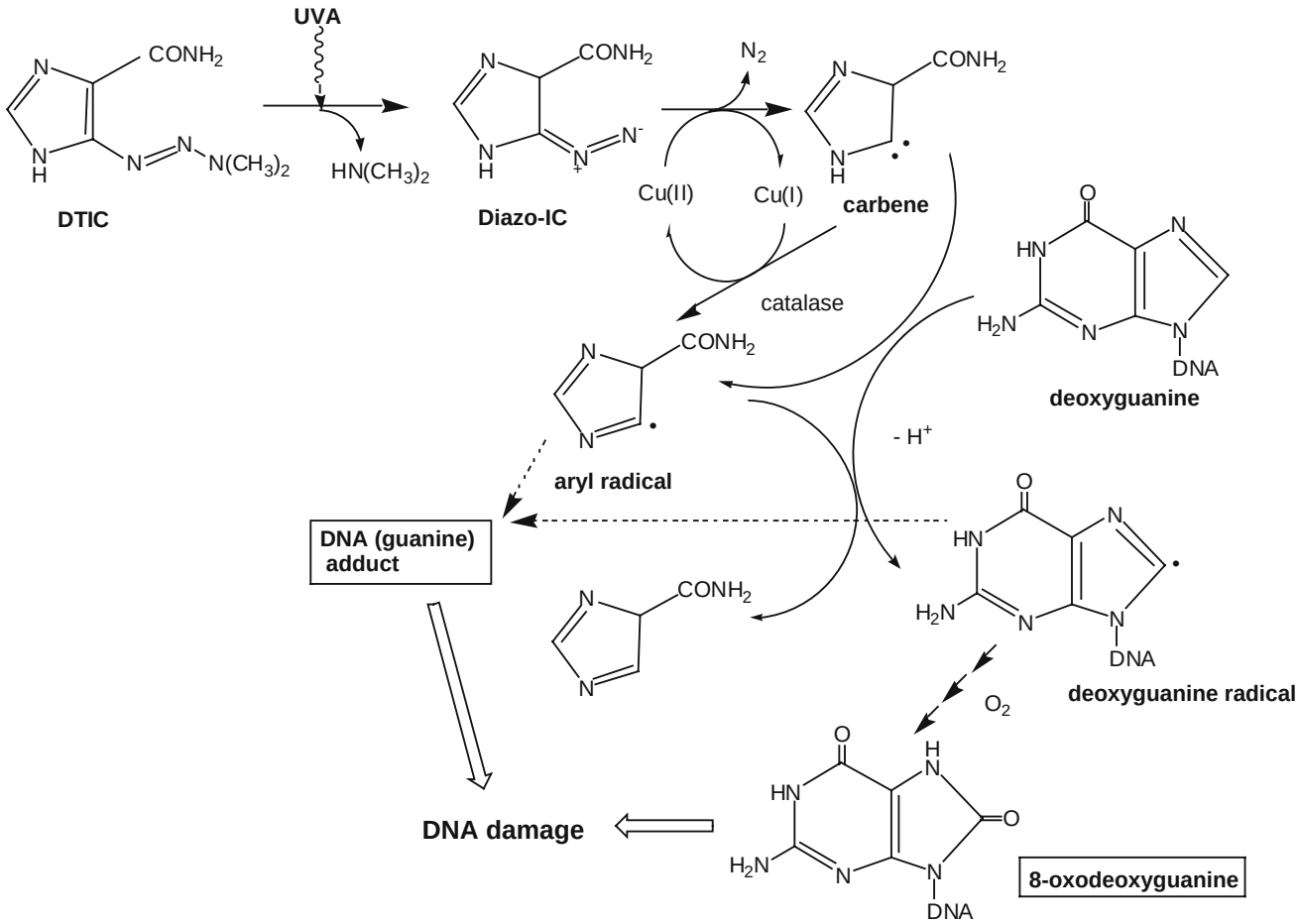

Fig. 7. A proposed mechanism of DNA damage induced by UVA-irradiated DTIC.

genation of deoxyguanine by carbene or aryl radical. Additionally, 8-oxodG may be oxidatively generated by deoxyguanine radical. Our results are reasonably explained by assuming that when catalase is added, the aryl radical generation, which is independent of the dehydrogenation of deoxyguanine by carbene, is accelerated. Because in this condition, a small amount of deoxyguanine radical is produced concurrently with aryl radical, DNA adduct formed via the coupling reaction of aryl radical and deoxyguanine radical is decreased. Then the remaining aryl radical may abstract hydrogen atom from deoxyguanine to produce deoxyguanine radical, resulting in the decrease of aryl radical and the enhancement of 8-oxodG formation. 8-OxodG is a highly mutagenic lesion causing GC → TA transversion (32). 8-OxodG is further oxidized to 2-aminoimidazolone (33), which may lead to GC → CG transversion (34). UVA-irradiated DTIC may induce the formation of these mutagenic DNA lesions. This is the first report to demonstrate the mechanism of DNA photodamage induced by DTIC.

At present, DTIC is employed in combination regimes for the treatment of malignant melanoma, Hodgkin's disease and soft tissue sarcomas (1). DTIC preparations are currently administrated by intravenous injection or short term infusion. It has been claimed that the photodegraded products of DTIC cause adverse reactions including local venous pain and phototoxic skin reaction when injected intravenously (35–37). Diazo-IC is considered to be a causative agent inducing local venous pain by DTIC (37). However, up to now, there is no study to show the distinct mechanism of DTIC-induced venous pain. It appears certain that the formation of free radicals plays a major role in acute and chronic inflammation and pain (38–40). In this study, we have demonstrated that carbene and aryl radical are generated from photoirradiated DTIC. Carbenes are highly reactive diradicals, which have been reported to be involved in the photodamage to DNA induced by fluoloquinolones (23). These findings suggest that DTIC induced harmful photoreaction, such as venous pain, could be caused by reactive species including carbenes or aryl radical. Therefore, it is essential to shield DTIC infusion solution from solar UV or fluorescent lights after its preparation to avoid the generation of reactive free radical species. Our results, indicating the possible mechanism of photogenotoxicity, could provide useful information to develop the prevention method for the phototoxicity induced by DTIC.

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