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Oxygen-independent DNA damage photosensitized by rhodamine-6G

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ABSTRACT

The mechanism of DNA damage induced by photoexcited rhodamine analogues was studied using ^{32}P-5'-end-labeled DNA fragments. As photosensitizers, rhodamine-6G, rhodamine-110, and rhodamine-123 were used. Rhodamine-6G photosensitized DNA damage under aerobic or anaerobic condition, whereas rhodamine-110 and rhodamine-123 induced no or very weak DNA photodamage. Photo-irradiated rhodamine-6G caused DNA cleavage specifically at every guanine residues, when the DNA fragments were treated with piperidine or *E. coli* formamidopyridine DNA glycosylase. These treatments can induce the excision of the 2,6-diamino-4-hydroxy-5-formamidopyrimidine residues of DNA. Trace amount of 8-oxo-7,8-dihydro-2'-deoxyguanosine, an oxidized product of 2'-deoxyguanosine, was generated by photoexcited rhodamine-6G. A possible mechanism of DNA photodamage by rhodamine-6G is the guanine modification via the electron transfer rather than the generation of reactive oxygen species. In conclusion, rhodamine-6G can photosensitize guanine-specific DNA damage through oxygen-independent mechanism.

KEYWORDS: rhodamine, DNA damage, photosensitization, electron transfer, guanine

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1. INTRODUCTION

Photosensitized DNA damage is a potentially important mechanism for photodynamic therapy (PDT), which is a promising treatment for cancer and other non-malignant conditions [1-3]. PDT involves the administration of a photosensitizer followed by exposure of the tissue to visible non-thermal light. When the photosensitizer is illuminated with light of appropriate wavelength, the photoexcited molecule induces photochemical damage to biomacromolecules, including DNA, resulting in cell death. In general, the important mechanism of biomacromolecules damage is the generation of reactive oxygen species such as singlet oxygen ($^1O_2$) [4]. Photosensitizers are often taken up by malignant or dysplastic tissues with some selectivity, and light delivery can be targeted to the appropriate tissue. The combination of drug uptake in malignant tissues and selective light delivery has the potential to provide an effective tumor therapy with efficient cytotoxicity and limited damage to the surrounding normal tissue. The mitochondrion has been shown to be a critical target in PDT. Lipophilic porphyrins have demonstrated intimate intracellular association with mitochondrial membranes, whilst cationic compounds such as rhodamine (RD) may accumulate in these organelles due to mitochondrial membrane potential. In addition, several reports have shown that DNA, including mitochondrial DNA, is an alternative potential target [5, 6].

Rhodamines are one of π-electron-delocalized, lipophilic, and cationic compounds that have been evaluated as selective anticarcinoma agents such as RD-123 and bromonated RD show selective toxicity towards carcinoma cells relative to normal epithelial cells [5, 6]. Rhodamine dyes, because of their low toxicity and rapid elimination, are potentially useful reagents for PDT. Furthermore, the possibility of DNA damage via oxygen-independent electron transfer mechanism by xanthene dyes, analogues of RDs, was reported [7]. Since the oxygen concentration in cancer cell is lower than that in normal cell [8], the oxygen-independent DNA damage should be advantageous for PDT. In this study, the mechanism of DNA damage by photosensitization of RDs (RD-6G, RD-110 and RD-123) (Figure 1) was examined.

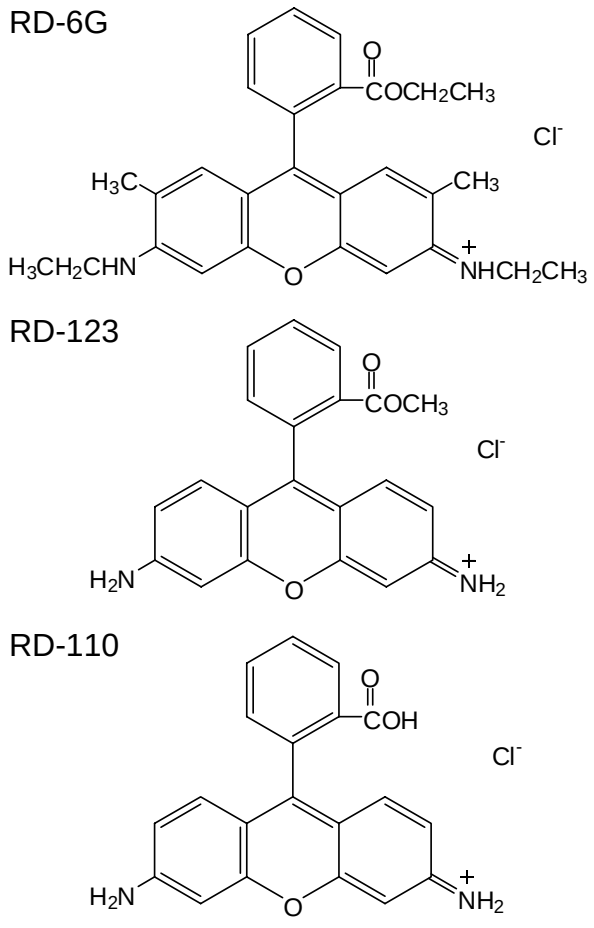

Figure 1. Structures of rhodamines.

2. MATERIALS AND METHODS

2.1. Materials

Restriction enzymes (*Ava*I, *Hind*III, *Mro*I, and *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). Restriction enzymes (*Eco*RI, *Apa*I, and *BssH*II) and calf intestine phosphatase were from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P]-ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA, USA). 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, USA). Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). *E. coli* formamidopyridine DNA glycosylase (Fpg) was from Trevigen Co. (Gaithersburg, MD, USA). RD-6G was from Tokyo Kasei (Tokyo, Japan). RD-110 was from Aldrich Chem Co. (Milwaukee, WI, USA) RD-123 was from ICN Biomedicals (Aurora, OH, USA). Riboflavin was from Nacalai Tesque. Inc (Kyoto, Japan).

2.2. Preparation of ^{32}P-5'-end-labeled DNA fragments

DNA damage photosensitized by RDs was examined using ^{32}P-labeled DNA fragments obtained from the human genes. The DNA fragment of the human *p53* tumor suppressor gene was prepared from pUC18 plasmid, ligated fragments containing exons of the *p53* gene [9]. A singly ^{32}P-5'-end-labeled double-stranded 211-base pair (bp) fragment (*Hind*III* 13972-*Apa*I* 14182) was obtained as described previously [10]. DNA fragments were also obtained from the human *p16* tumor suppressor gene [11]. A singly ^{32}P-5'-end-labeled double-stranded 158 bp *p16* fragment (*Mro*I 6137-*Eco*RI* 6330) was prepared from pGEM-T Easy Vector (Promega Corp., Madison, WI, USA) as described previously [12].

2.3. Detection of DNA damage induced by photosensitization of rhodamines

The standard reaction mixture in a microtube (1.5-mL Eppendorf) contained RDs, ^{32}P-labeled DNA fragment, and calf thymus DNA in 100 μL of 10 mM sodium phosphate buffer (pH 7.8). The mixtures were irradiated using 10 W UV lamp (λ_{max} = 365 nm, UVP, Inc., CA, USA, 1.6 mW cm^{-2}) placed at a

distance of 20 cm under air or nitrogen. The photosensitizers used in this study have absorption band around UVA and visible light region (~500 nm) and can be photoexcited with this lamp. Subsequently, the DNA was treated with 1 M piperidine for 20 min at 90°C or 6 units of Fpg in the reaction buffer (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/mL BSA) for 2 h at 37°C. The DNA fragments were subjected to electrophoresis on an 8 M urea/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 [13] using a DNA sequencing system (LKB2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). A laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

2.4. Measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine induced by photosensitization of rhodamine-6G

The content of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) [14, 15], an oxidized product of 2'-deoxyguanosine (dGuo), formed by photosensitization was measured with an electrochemical detector coupled to high-performance liquid chromatography (HPLC-ECD). The reaction mixtures containing 100 μM/bp calf thymus DNA and RD-6G or riboflavin in 100 μL of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were irradiated (λ_{max}= 365 nm, 6 Jcm^{-2}). 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 [16].

3. RESULTS

3.1. Damage to DNA fragments photosensitized by rhodamines

Figure 2 shows the autoradiogram of ^{32}P-5'-end-labeled DNA fragments photo-irradiated in the presence of RDs. RD-110 and RD-123 induced no or little DNA photodamage, whereas RD-6G photosensitized DNA damage clearly. RD-6G did not induce DNA damage without photo-irradiation. The extent of DNA photodamage increased in a dose-dependent manner of RD-6G. The DNA cleavage was observed when the DNA fragment was treated with piperidine, suggesting that the base modification was induced by photo-irradiated RD-6G, and the direct photo-break of DNA strand was not caused by RD-6G. Figure 3 shows the comparison of DNA photodamage induced by RD-6G under air or nitrogen. DNA damage was induced under anaerobic condition, indicating that DNA damage through oxygen-independent mechanism. In addition, sodium azide, physical scavenger of $^1O_2$, showed no inhibitory effect on DNA damage photosensitized by RD-6G (data not shown).

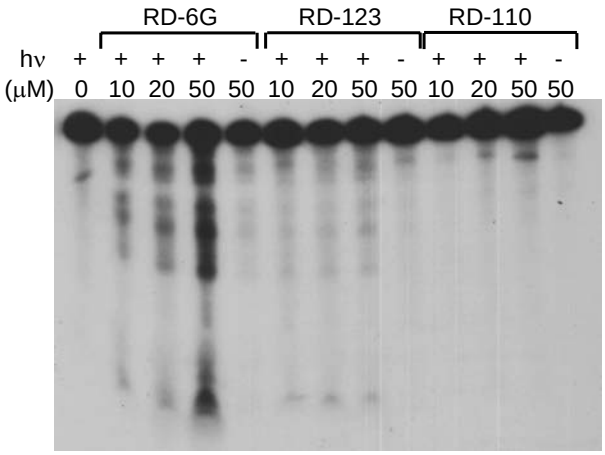

Figure 2. Autoradiogram of DNA fragments photo-irradiated in the presence of RD-6G, RD-123, or RD-110. The reaction mixtures containing ^{32}P-5'-end-labeled 211 bp DNA fragment (*p53* tumor suppressor gene), 10 μM/bp calf thymus DNA, RD-6G, RD-123 or RD-110, and 2.5%(vol) ethanol in 100 μL of 10 mM sodium phosphate buffer (pH 7.8) were irradiated (365 nm, 6 J/cm^2). Then, the DNA fragments were treated with piperidine and analyzed by electrophoresis.

3.2. Sequence–specificity of DNA damage photosensitized by rhodamine-6G

Figure 4 shows the sequence-specificity of DNA damage induced by photo-irradiated RD-6G. Fpg treatment and piperidine treatment demonstrated that photoexcited RD-6G damaged DNA at every guanine residue in double-stranded DNA. Fpg protein is known to recognize 8-oxodGuo and 2,6-diamino-4-hydroxy-5-formamidopyrimidine

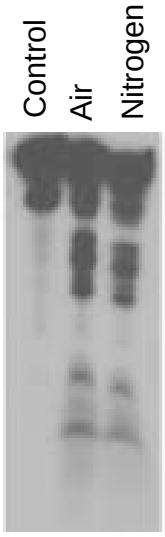

Figure 3. Comparison of DNA photodamage induced by RD-6G under air or nitrogen. The reaction mixture contained the ^{32}P-5'-end-labbeled 211 bp DNA fragment, 10 μM/bp of calf thymus DNA, 50 μM RD-6G, and 2.5%(vol) ethanol in 100 μL of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated (365 nm, 6 J/cm^2). Then, the DNA fragments were treated with piperidine and analyzed by electrophoresis.

(Fapy-G) residues and cleave DNA at these sites [17].

3.3. Formation of 8-oxodGuo induced by photosensitization of rhodamine-6G and riboflavin

Figure 5 shows 8-oxodGuo formation induced by photosensitization in the presence of RD-6G or riboflavin. Riboflavin can photosensitize DNA oxidation specifically at guanine residues [16]. In this experimental condition, the extent of DNA damage by RD-6G was comparable with that of riboflavin (data not shown). Photoexcited riboflavin generated 8-oxodGuo, an oxidized product of 2'-deoxyguanosine, in a dose-dependent manner, whereas only trace amount of 8-oxodGuo was generated by photoexcited RD-6G.

4. DISCUSSION

Photo-irradiated RD-6G induced DNA damage, whereas RD-123 and RD-110 caused no or little DNA photodamage. Since the values of absorbance of these dyes at the excitation wavelength are almost the same, the difference of their DNA damaging activities might be explained by the difference of interaction with DNA or photochemical property of RDs. DNA damage photosensitized by RD-6G was clearly observed under anaerobic condition similar to the aerobic condition. In general, photosensitized DNA damage is induced by the two mechanisms, electron transfer (Type I) and reactive oxygen generation (Type II) [18]. The major reactive species of Type II mechanism is $^1O_2$, and other reactive oxygen species, including superoxide and/or hydrogen peroxide, rarely mediate DNA damage. Since DNA damage was observed under anaerobic condition in this study, the Type II mechanism could be excluded. Indeed, the $^1O_2$ scavenger did not inhibit DNA photodamage by RD-6G. The analysis of the sequence-specificity of DNA damage with Fpg and piperidine treatments demonstrated that photoexcited RD-6G caused base modification at every guanine residue. Fpg catalyzes the excision of piperidine-resistant 8-oxodGuo and piperidine labile Fapy-G [17, 19, 20]. 8-oxodGuo is a major oxidized product of guanine by the Type I and Type II DNA damage. However, trace amount of 8-oxodGuo was produced by the photosensitization of RD-6G. This surprising result might be explained by the following mechanism (Figure 6). The photoexcited RD-6G induced base modification through photo-induced electron transfer from DNA (Type I mechanism). Indeed, the possibility of photo-induced electron transfer from guanine to xanthene dyes, analogues of rhodamines was reported [7]. Among them, guanine, which exhibits the lowest redox potential of one-electron oxidation (1.24 V vs. standard calomel electrode (SCE) in acetonitrile), is the preferential target over adenine (1.69 V) > thymine (1.90 V) and cytosine (1.90 V) [21]. The redox potential of guanine becomes smaller through interaction with other nucleobase in the duplex form [22]. Furthermore, aqueous media decrease the energy level of charge transfer state, resulting in the enhancement of electron transfer [23]. Although the redox potential of RD-6G (1.18 V vs. SCE) [24] is slightly smaller than that of free guanine, the electron transfer oxidation of guanine in the DNA duplex by photoexcited RD-6G might be possible. The formed guanine radical cation

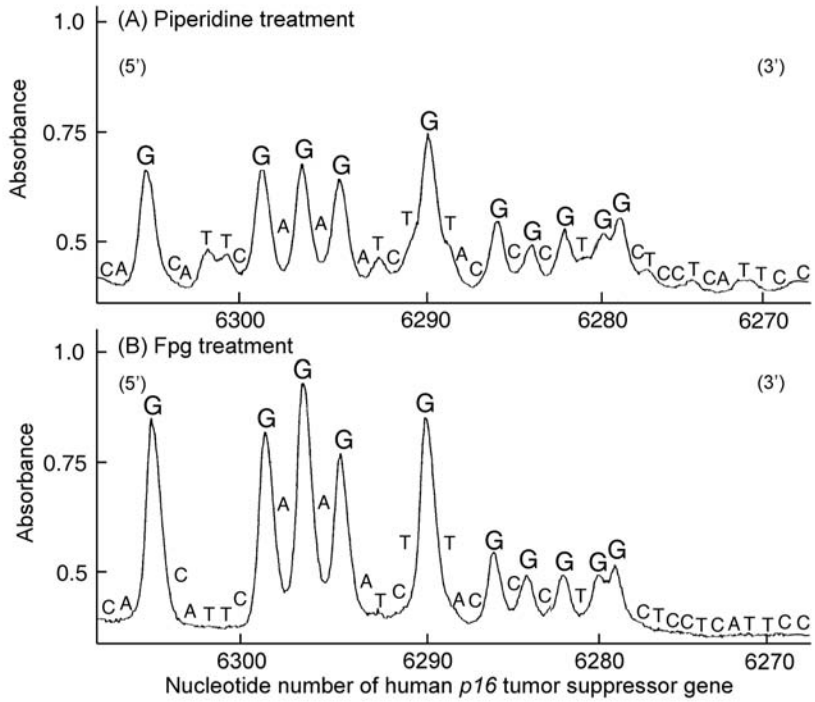

Figure 4. Sequence-specificity of DNA damage induced by photo-irradiated RD-6G. The reaction mixtures contained the ^{32}P-5'-end-labeled 158 bp DNA fragment (*p16* tumor suppressor gene), 10 µM/bp calf thymus DNA, 5 µM DTPA, 10 µM RD-6G, and 2.5%(vol) ethanol in 100 µL of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated (365 nm, 6 J/cm^2). Then, the DNA fragments were treated with piperidine or Fpg and analyzed by electrophoresis.

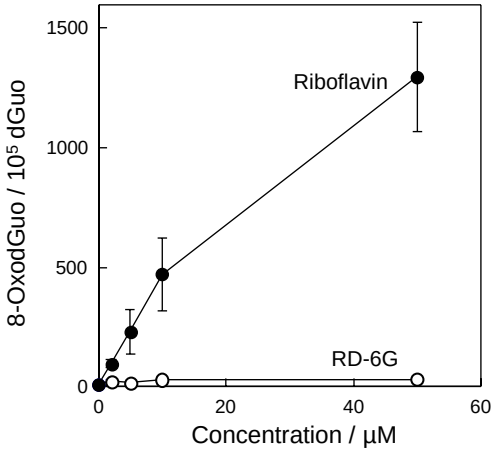

Figure 5. Amount of 8-oxodGuo generated by photosensitization of RD-6G or riboflavin. The reaction mixtures containing RD-6G or riboflavin, 100 µM/bp calf thymus DNA, and 2.5% (vol) ethanol in 100 µL of 4 mM sodium phosphate buffer (pH 7.8) were irradiated (365 nm, 6 J/cm^2). Then, the DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase, and analyzed with an HPLC-ECD.

(dGuo$^{\cdot+}$) by this electron transfer can undergo two competitive chemical reactions, including hydration and deprotonation. Hydration of the guanine radical cation gives rise to the reducing 8-oxo-7,8-dihydroguanyl radicals (dGuo-OH$^{\cdot}$). The latter radical is converted by a reducing process into Fapy-G. On the other hand, competitive oxidation, which may be achieved by molecular oxygen, give rise to 8-oxodGuo [25, 26]. Photoexcited RD-6G generated a trace amount of 8-oxodGuo, suggesting that this DNA damage is due to the formation of Fapy-G.

RD-6G caused DNA photodamage in vitro, whereas in the dark RD-6G showed no effect on isolated DNA fragment. Rhodamine dyes, because of their low toxicity and rapid elimination, are potentially useful reagents for PDT. However, because of their high quantum yields of fluorescence and consequently low quantum yields of triplet formation (< 0.01) [27] and $^1O_2$ formation [28], their usefulness as photosensitizers

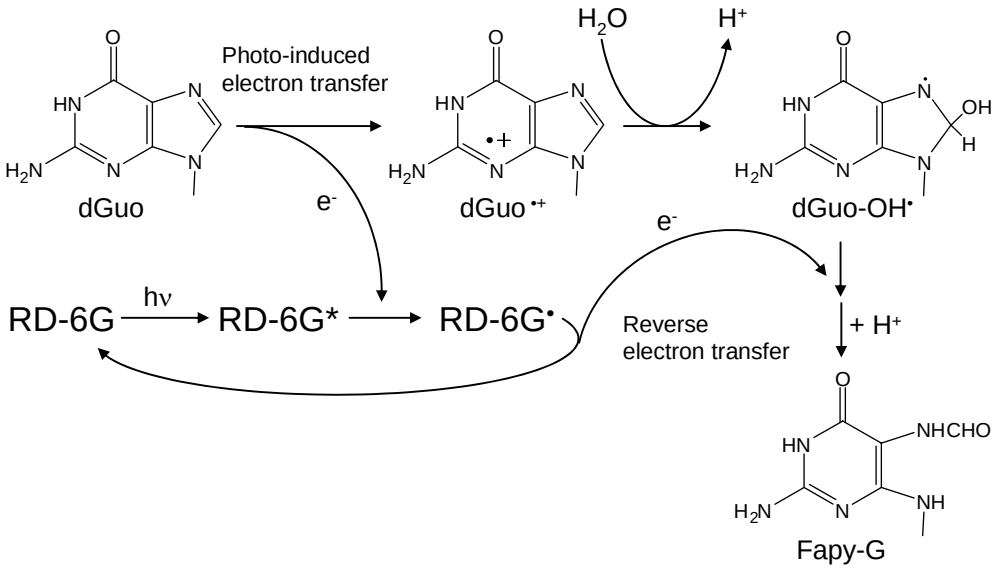

Figure 6. Proposed mechanism of DNA damage photosensitized by RD-6G.

is quite limited [6, 29]. Nevertheless, RD-6G could induce DNA damage under air and nitrogen. Since the oxygen concentration in human tissue is relatively low and that in cancer cell is lower than that in normal cell [8], the effect of PDT by traditional $^1O_2$ photosensitizer is limited. DNA damage might be induced by RD-6G via the oxygen-independent Type I mechanism. DNA is an alternative potential target of PDT. RD-6G is an efficient photosensitizer to damage DNA rather than RD-123 [30, 31], which is known to show phototoxicity via $^1O_2$ formation.

CONCLUSION

Photoexcited RD-6G induced DNA damage at every guanine residues via oxygen-independent mechanism. The possible mechanism of DNA damage is photoinduced electron transfer from guanine. These results suggest that DNA-damaging mechanism by RD-6G is advantageous for the photodamage of biomacromolecules in the condition of low oxygen concentration such as cancer cell.

ACKNOWLEDGMENTS

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